

Invited Review

Nitric oxide and nitric oxide synthase: biology, pathology, localization

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Summary. Nitric oxide (NO) has opened a new and vigorous field of biological and clinical experimentation as evidenced presently by about one hundred original publications every week. Being a biological signal under physiological conditions, NO may be «foe or friend» to pathologically affected tissues. Major insights into the biology and pathology of this unorthodox biomolecule have come from the histochemical analysis of NO synthase (NOS) and its molecular isoforms that are responsible for the formation of NO. Immunocytochemistry as well as NADPH-diaphorase histochemistry are most widely used to visualize NOS in various tissues. There are several constraints regarding specificity and sensitivity of the techniques used and, therefore, apparent discrepancies in the literature concerning the cellular and subcellular distribution of NOS and its isoforms. Despite such inconsistencies there is a wealth of data that has an important impact on further investigation of NO-mediated processes underlying a vast number of NO-mediated physiological and pathological mechanisms.

Key words: Nitric oxide, Nitric oxide synthase, Histochemistry, Electron microscopy

Introduction

Nitric oxide (NO) has recently expanded its status from an environmental pollutant to a biological signal molecule that mediates blood vessel relaxation and immune responses, kills pathogens, inhibits platelet aggregation and adhesion, and serves as a neuro-modulator in the central and peripheral nervous system. There is, moreover, evidence that NO may be a physiological regulator of mitochondrial respiration and gene activity, and that NO is implicated in a variety of human diseases being, in dependence on the particular circumstances, a «foe or friend» to pathologically

affected tissues (Knowles and Moncada, 1994; Moncada et al., 1994a,b; Schmidt and Walter, 1994; Body et al., 1995; Gross and Wolin, 1995; Kerwin et al., 1995; Moncada and Higgs, 1995; Schroeder and Kuo, 1995; Vincent, 1995).

Nitric oxide, an unorthodox biomolecule

NO is a gaseous radical produced in the atmosphere by lightning and the burning of fossil fuels. Since the late 1970s NO is known to activate guanylyl cyclase and to cause vascular smooth muscle relaxation (Arnold et al., 1977; Gruetter et al., 1979). But it took several years to discover that inorganic oxides of nitrogen are biologically produced (Green et al., 1981) and that NO is identical to the so-called «endothelium derived relaxing factor» (EDRF) (Furchgott, 1988; Ignarro et al., 1988). Since that time there has been, despite initially considerable scepticism, an explosion of research activity on this topic. Named the molecule of the year in 1992, NO has opened a new and vigorous field of biological and clinical experimentation as evidenced presently by nearly one hundred original publications every week.

Due to its unique ability to diffuse quickly in both aqueous and lipid environment, NO is assumed to spread rapidly to neighbouring tissue elements regardless of intervening membranes. Derived from theoretical considerations, Wood and Garthwaite (1994) have calculated that the physiological sphere of influence of a single point source of NO emitting for 1-10 sec has a diameter of about 200 μm . Contrary to conventional biosignals that act via specific receptor molecules, NO functions in a fairly specific manner by a wide range of chemical reactions controlling enzyme activities, ion channels, gene transcription, mitochondrial respiration, and can interact with oxygen-derived radicals to produce other highly reactive substances (Brown, 1995; Crow and Beckman, 1995; Garthwaite and Boulton, 1995; Gross and Wolin, 1995; Moncada and Higgs, 1995). There are compelling reasons to believe that NO mediates a variety of functions in the nervous system, such as synaptic transmission, plasticity, regulation of

cerebral blood flow, induction and regulation of the circadian rhythm, and hyperalgesia (for review, see Schuman and Madison, 1994; Garthwaite and Boulton, 1995; Paakari and Lindsberg, 1995; Bachneff, 1996). Furthermore, NO is involved in the development of tolerance to and withdrawal from morphine (Bhargava, 1995; Vaupel et al., 1995) and alcohol (Lancaster, 1995). At higher concentrations NO has been proposed to have antibacterial, antifungal, antiprotozoal and tumoricidal activity (Vallance and Collier, 1994; Kerwin et al., 1995; Schoedon et al., 1995). NO overproduction in mammalian systems may contribute to cell damage or cell death (Dawson, 1995; Gross and Wolin, 1995; Krönke et al., 1995) (Fig. 1). Thus, NO might be implicated in cerebral disorders induced by hypoxia and ischemia, migraine, Parkinson's disease and AIDS dementia (Moncada, 1994; Olesen et al., 1995). On the other hand, manipulation of the NO pathway may offer therapeutic benefit in selected human diseases

underscoring the importance of large-scale experiments on the NO-biology as well as clinical trials to develop the NO pharmacology and to assess undesirable side effects that might arise from pharmacotherapeutic interventions (Body et al., 1995; Moncada and Higgs, 1995; Schroeder and Kuo, 1995).

Nitric oxide synthase

Major insights into the biology and pathology of NO have come from the characterization of the enzyme that is responsible for its formation in living tissues, called NO synthase (the term «synthase» stands, in contrast to «synthases», for enzymes that do not utilize ATP). Catalyzed by NO synthase (NOS; EC 1.14.13.39), NO is produced in a NADPH-dependent manner by oxidation of the guanidino nitrogen of L-arginine in the presence of molecular oxygen and several cofactors (FMN, FAD, tetrahydrobiopterin, heme moiety). Three major

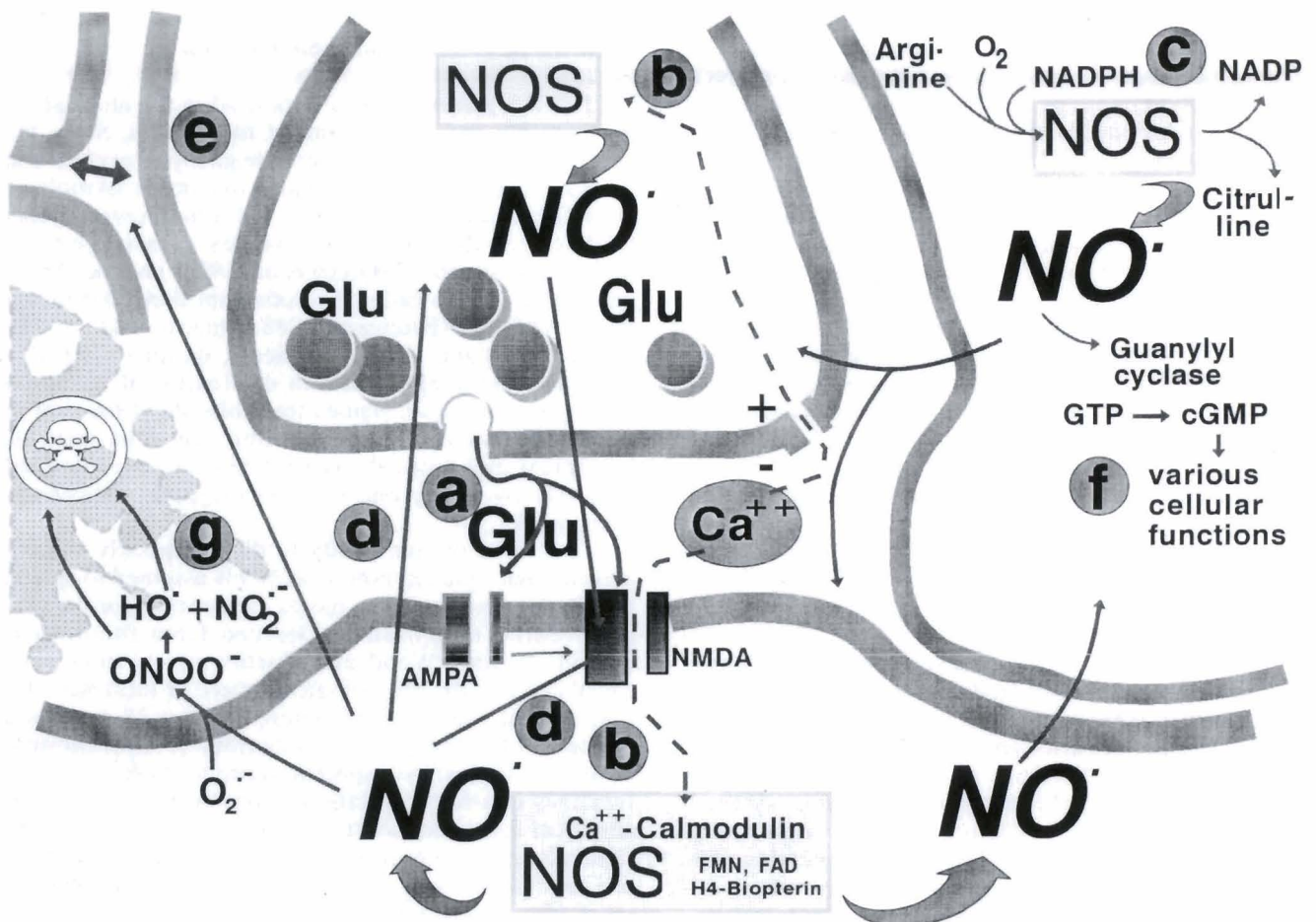


Fig. 1. Scheme of NO effects at a CNS glutamate synapse, partially hypothetical. Synaptic activity results in an increase in pre- and postsynaptic Ca^{++} levels via voltage- or transmitter(NMDA-receptor)-gated ion channels (a). Calcium binds to calmodulin and activates (b) nitric oxide synthase (NOS, neuronal isoform). NO generated by the conversion of arginine to citrulline (c) modulates synaptic activity (d), dilates blood vessels (e) and influences many other cellular functions by modification of cellular proteins, e. g. guanylyl cyclase (f). NO also reacts with superoxide anions (O_2^-) to peroxynitrite ($ONOO^-$) that may, in the case of a pathological overproduction, damage cells in the vicinity, possibly by initiating a cytotoxic cascade (g).

isoforms of NOS have been identified so far. Based on the historical order of purification and cDNA isolation, or on the cell type or organ in which the enzyme was originally discovered, the isoforms are termed: NOS I (brain or neuronal NOS, nNOS), NOS II (macrophage or hepatocyte NOS), and NOS III (endothelial NOS, eNOS) (Förstermann and Kleinert, 1995; Griffith and Stuehr, 1995). Whereas NOS I and NOS III were originally identified as constitutive in brain and vascular endothelium, type II is normally not expressed (or occurs at a very low level), but is inducible by cytokines and bacterial products (therefore also termed «inducible NOS», iNOS). After induction, there are apparently no regulatory mechanisms for NOS II. The activity of NOS I and III, on the other hand, is dependent on the availability of free calcium ions and calmodulin. Molecular cloning of the constitutive NOS isoforms has indicated a consensus sequence for phosphorylation sites, which can be modulated by protein kinase A, protein kinase C and Ca⁺⁺/calmodulin-dependent protein kinase as well (Nakane et al., 1991; Dinerman et al., 1994a). The functional significance of NOS phosphorylation, showing in vitro an inhibitory effect on the enzyme activity, is not yet clear.

As indicated by cloning studies, NOS isoforms are bi-domaine enzymes consisting of a reductase and a heme (oxygenase) moiety. NOS exhibits sequence similarities to both cytochrome P450 mono-oxygenases and the respective reductases (Knowles and Moncada, 1994; Griffith and Stuer, 1995; Mayer, 1995). Possibly, NOS has evolved phylogenetically from a fusion of both components.

The NOS catalyzed conversion of the substrate L-arginine into citrullin and NO is not yet fully understood. Basically, electrons are transferred from NADPH via the flavin cofactors FAD and FMN to the heme leading to the reduction of molecular oxygen. N^G-hydroxy-L-arginine is produced as an intermediate and, finally, oxidatively cleaved to L-citrulline and NO (Mayer, 1995). Substrate analogs have become the most commonly used inhibitors of NOS activity. They exhibit variable affinities for the NOS isoforms, although none is truly specific. N^G-monomethyl-L-arginine, N^G-nitro-L-arginine and its methyl ester, N-iminoethyl-L-ornithine and N^G-amino-L-arginine act as competitive and, in some cases, irreversible inhibitors of all the NOS-isoforms (Moncada and Higgs, 1995). Other compounds, such as 7-nitroindazole, are more specific. It inhibits neuronal NOS and exhibits an antinociceptive activity, but does not interfere with the protective actions of endothelial NOS (Moore et al., 1993; Southan and Szabo, 1996).

Localization techniques

Unlike conventional biosignals, NO is produced and released when required, instead of being stored in cellular compartments. Action sites of NO in tissue are therefore preferably localized by the identification of the

synthesizing enzyme NOS.

Several topochemical methods, above all immunocytochemistry, NADPH-diaphorase histochemistry and in-situ hybridization of NOS-mRNA, can be used to demonstrate NOS expression under in-situ conditions (Vincent, 1994; Beesley, 1995). Moreover, there are a few reports in which supplementary methods have been applied, such as the autoradiographic localization of the enzyme by demonstrating the binding of the irreversible NOS inhibitor [³H]L-nitroarginine to the enzyme molecule (Burazin and Gundloch, 1995), and, furthermore, single cell PCR of NOS-mRNA, by which the expression of nNOS was shown in single hippocampal neurons (Chiang et al., 1994).

Immunocytochemistry

Several antisera and monoclonal antibodies have been raised against the different NOS-isoform proteins by individual researchers, or have become commercially available, amongst others from Affiniti Bioreagents (UK), Transduction Laboratories (USA), Eurodiagnostica (Sweden), Biomol (Germany), Alexis Corporation, and Auspep (Australia). A critical factor in immunocytochemical studies is the specificity of the antibodies used as well as specimen preparation, especially fixation (Buwalda et al., 1995). There are several reports demonstrating NOS immunoreactivity at the electron microscopic level (Llewellyn-Smith et al., 1992; Valtschanoff et al., 1992; Aoki et al., 1993; Tomimoto et al., 1994; Loesch and Burnstock, 1995; Roufaïl et al., 1995). In most cases immunolabeling has not been specifically associated with any subcellular organelle or with endocellular membranes (but see "Electron microscopic NADPH-diaphorase cytochemistry"). For eNOS, however, immunoprecipitation was found to be to some extent concentrated to membranes of subcellular organelles such as mitochondria and endoplasmic reticulum (Tomimoto et al., 1994; Loesch and Burnstock, 1995; O'Brien et al., 1995). Biochemical studies indicate that eNOS is mainly particulate (Förstermann et al., 1991a) and may be translocated from the particulate to the cytosolic fraction (Michel et al., 1993; Robinson et al., 1995). Membrane binding of eNOS has been deduced from a consensus sequence for N-terminal myristoylation (Lamas et al., 1992).

NADPH-diaphorase (NADPH-d) histochemistry

The ability of the reductase domain located at the terminal sequence of NOS to transfer electrons from the coenzyme NADPH to other substrates, including tetrazolium salts, gives rise to the so-called NADPH-diaphorase activity (Dawson et al., 1991; Hope et al., 1991). The histochemical NADPH-diaphorase reaction by which soluble tetrazolium salts are converted to insoluble visible formazan is widely used as a robust method to localize NOS (Vincent, 1994). This

histochemical staining relies on a simple redox reaction, though several other enzymes, such as cytochrome C and cytochrom P 450 reductase, also display NADPH diaphorase activity. Fortunately, most of the staining activities that are not related to NOS can be suppressed by aldehyde fixation (Matsumoto et al., 1993; Tracey et al., 1993). Aldehyde-fixed tissue displays a distribution pattern of NADPH-d that appears to be largely identical to those of NOS immunolabeling (Dawson et al., 1991; Hashikawa et al., 1994; Schilling et al., 1994; Pullen and Humphreys, 1995; Roufail et al., 1995; Kugler and Drenckhahn, 1996). However, there are now several examples where NADPH-d staining is not due to NOS activity, such as in the olfactory epithelium and the vomeronasal organ (Kishimoto et al., 1993), in the cortex (Kharazia et al., 1994; Sobriela and Mufson, 1995), the olfactory bulb (Spessert and Layers, 1994), the spinal cord (Vizzard et al., 1994), or in the nervous system of the pulmonate mollusc *Helix* (Cooke et al., 1994). Therefore, care must be taken in the interpretation of NADPH-d staining results. Wörl et al. (1994) and Buwalda et al. (1995) have undertaken several experiments to further study the effect of aldehyde fixation on NADPH-d staining and NOS immunolabeling. Their results indicate that aldehydes differentially suppress or elicit NADPH-d activity in different groups of neurons. Also, the application of detergents for better penetration of medium constituents may influence the outcome regarding sensitivity (Fang et al., 1994) and localization (Würdig and Wolf, 1994). Knockout mice that lack the gene for respective NOS isoforms represent an ideal specificity control (Huang et al., 1993, 1995; Snyder, 1995). Thus, the disruption of the nNOS gene resulted, at light microscopical level, in an almost complete loss of NADPH-d staining in CNS neurons (Huang et al., 1993) and in retinal cells (Darius et al., 1995).

Electron microscopic NADPH-diaphorase cytochemistry

To adapt NADPH-d histochemistry to electron microscopic level, Hope and Vincent (1989) have proposed the use of 2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl) tetrazolium chloride (BSPT) in place of nitroblue tetrazolium chloride which is preferred for light microscopy. BSPT is a non-osmiophilic compound that yields an osmiophilic formazan on reduction (Kalina et al., 1972); therefore, it is particularly suitable for electron microscopic examination. The BSPT technique provides amazingly clear-cut pictures and has been further developed by Wolf et al. (1992, 1993, 1995), broadly used to localize NOS ultrastructurally in normal and excitotoxically lesioned nervous tissues (Calka et al., 1994, 1996; Faber-Zuschratter and Wolf, 1994; Darius et al., 1995; Schmidt et al., 1995). Abundantly BSPT-stained neurons were found to be rich in highly contrasted membranes of the endoplasmic reticulum, including the nuclear envelope. Portions of the outer membrane of mitochondria and

membranes of the Golgi apparatus also exhibited distinct BSPT-formazan deposits (Wolf et al. 1992, 1993; Calka et al., 1994) (Fig. 2). Many other neurons, as well as a few glial cells, were virtually unstained in the light microscope, yet showed labeled membrane portions at electron microscopic level, although to a far lesser extent. In contrast to these observations, several biochemical data suggest that nNOS is cytosolically located rather than membrane bound (Förstermann et al., 1991b). On the other hand, in cerebellar preparations Hecker et al. (1994) have found more than 60% of the total NOS in the particulate fraction and which, based on density gradient ultracentrifugation, are associated with the endoplasmic reticulum fraction. Hiki et al. (1992) have also reported an insoluble NOS in the rat brain. Concluding from these findings and with respect to the electron microscopic NADPH-d cytochemistry, the enzyme might be largely attached to endocellular membranes, but may become soluble during the homogenization procedure.

Since the electron microscopic BSPT-technique is by far more sensitive than the light microscopical NADPH-d histochemistry, the use of a powerful specificity control, such as NOS-knockout mice, is most important (Darius et al., 1995). Interestingly, iNOS as seen in activated microglial cells or macrophages exhibited a "sand-like" BSPT-formazan in cytosolic areas or in vacuoles without any accumulation at endocellular membranes (Schmidt et al., 1995; Calka et al., 1996). This particular precipitation form indicates that there is no tendency of BSPT-formazan to dislocate and to attach artifactually to lipophilic structures.

In-situ hybridization

Using radiolabeled probes, in-situ hybridization has been employed by several authors to study the distribution of NOS-mRNA within tissue sections. Interneurons containing nNOS-mRNA were detected in mice in parallel with the NOS protein in the plexiform layer of the main olfactory bulb and the granule cell layer of the main and accessory olfactory bulbs (Kishimoto et al., 1993). A quantitative analysis of NOS hybridization signal was performed in lumbar dorsal root ganglia after transection of the sciatic nerve in rats (Verge et al., 1992). Here, a dramatic increase in the numbers of NOS mRNA-positive neurons were found, indicating that even so-called constitutive NOS-isoforms can become inducible after exposure to a lesion stimulus. Endoh et al. (1994) have used riboprobes that have higher specific sensitivity than probes made by end labeling of oligonucleotides. Enabling to detect even low amounts of message, the authors succeeded in demonstrating NOS-mRNA in neurons of the hippocampal CA1 region where other authors failed to clearly see signs of NOS-staining (Dawson et al., 1991; Vincent and Kimura, 1992). After treatment with bacterial lipopolysaccharide or cytokines a widespread expression of the inducible isoform could be observed

(Kröncke et al., 1995), and has been demonstrated in many tissues by in-situ hybridization, e. g. in vascular smooth muscle and in cardiac myocytes (Luss et al., 1995), in synovial lining cells, endothelial cells and chondrocytes (Sakurai et al., 1995), and in epithelial cells in human paranasal sinuses (Lundberg et al., 1995).

Immunocytochemical localization of NO-targets

Due to the fact that NO activates the soluble isoform of guanylyl cyclase (Koesling et al., 1995) to generate cyclic GMP, NO production sites can be demonstrated immunocytochemically by using antisera raised against cGMP conjugated to protein carriers (de Vente et al., 1990). Consequently, in response to NO-donors there was a dramatic increase in cGMP-staining of cerebellar structures (de Vente and Steinbusch, 1992; Southam et al., 1992). A major disadvantage of the method, however, is that up to 80 % of the cGMP is lost during the immunocytochemical process (de Vente and Steinbusch, 1992).

Recently, immunostaining of nitrotyrosine residues in proteins has been used as a marker of peroxynitrite

formation which occurs by the reaction of NO with superoxide anions (Beckman et al., 1994; Kooy et al., 1995; Miller et al., 1995; Szabo et al., 1995). Consequently, nitrotyrosine immunostaining was found to be significantly reduced when the animal was treated with NO-inhibitors. Nitrotyrosine and iNOS were immunocytochemically colocalized in a guinea pig model of gut inflammation, and positive staining of both antigenic structures was most intense in epithelia and neurons of the myenteric and submucosal ganglia (Miller et al., 1995). Since antibodies against nitrotyrosine residues have become commercially available (e. g. from Upstate Biotechnology, USA), the immunocytochemistry of the NO-dependent tyrosine nitration will rapidly gain widespread acceptance in NO research.

Cellular NOS sources

NO, as well as its generating enzyme, occur in mammalian tissues almost ubiquitously. The constitutively expressed nNOS isoform was first purified from rat and porcine cerebellum (Bredt and Snyder, 1990; Mayer et al., 1990; Schmidt et al., 1991). Except

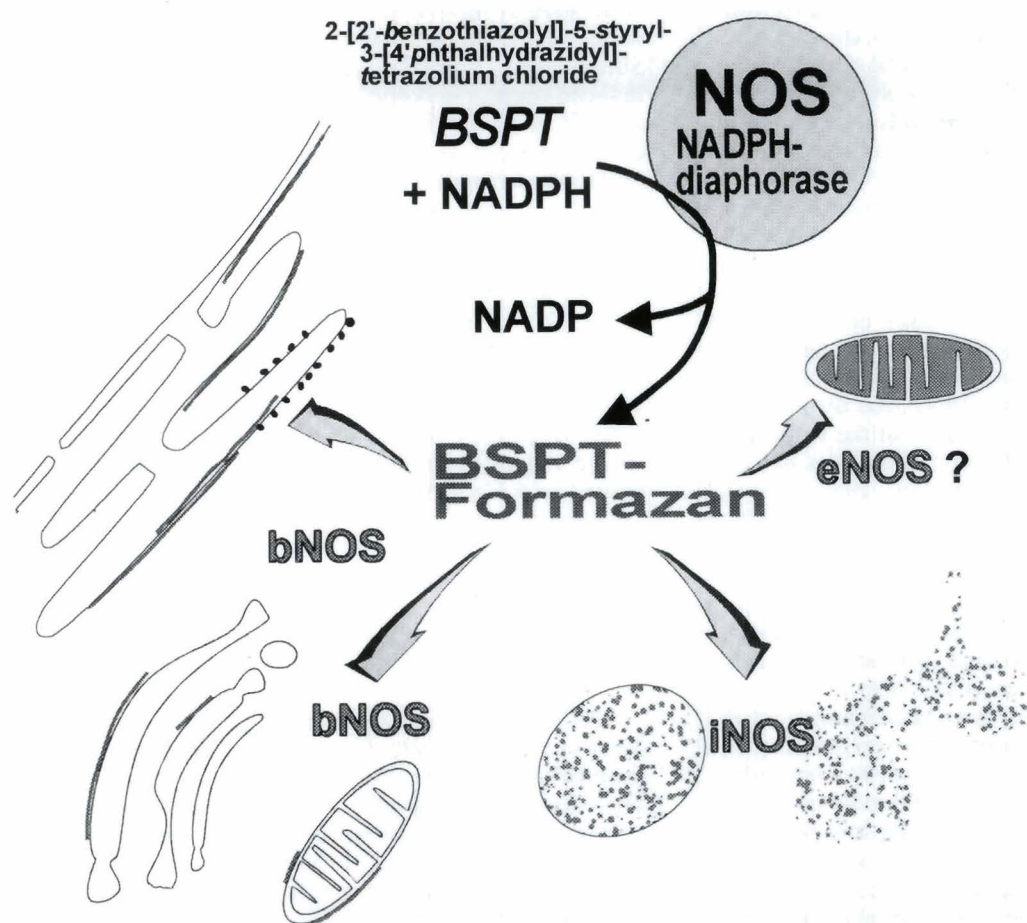


Fig. 2. Electron microscopic NADPH-diaphorase cytochemistry. BSPT-formazan deposits related to neuronal NOS have been found to be attached to distinct portions of endocellular membranes, predominantly to those of the endoplasmic reticulum and the nuclear envelope, and occasionally to membranes of mitochondria and the Golgi apparatus. The inducible NOS-isoform is located in the cytosol or in vacuoles forming "sand-like" formazan grains, whereas eNOS reaction product, based on preliminary results, appears to be confined to the mitochondrial matrix.

from prominently nNOS-positive solitary neurons, which are relatively evenly distributed throughout the brain (Egberongbe et al., 1994; Rodrigo et al., 1994; Vincent, 1994), evidence has been provided for small amounts of nNOS in other neurons and in astro- and microglial cells by means of the above mentioned electron microscopic BSPT technique for NADPH-d (Wolf et al., 1992, 1993, 1995; Darius et al., 1995; Calca et al., 1996) as well as by several other identification methods (Murphy et al., 1995; Gabott and Bacon, 1996). Contrary to theoretical expectations considering NO as a retrograde trans-synaptic transmitter (Garthwaite, 1991; Schuman and Madison, 1994), quantitative BSPT-EM studies revealed that mainly presynaptic areas (41% in hippocampus, 38% in neocortex) were NADPH-d labeled (Faber-Zuschratter and Wolf, 1994). Post-synaptic endings showed only exceptionally marked endomembranes.

As indicated by immunocytochemistry, abundantly nNOS-containing neurons can also be seen in the spinal cord (Valtschanoff et al., 1992; Saito et al., 1994; Vizzard et al., 1994) and in the peripheral neural system, such as myenteric and submucous ganglia of the gastrointestinal tract (Ekblad et al., 1994), subepicardial and interatrial ganglia (Klimaschewski et al., 1992; Tanaka et al., 1993), and in ganglion cells of the pancreas (Tay and Burnstock, 1994), the gallbladder (Siou et al., 1994), and the adrenal gland (Afework et al., 1994) as well as nerve fibers of the pineal gland (López-Figueroa and Müller, 1996). NO synthesized in peripheral nNOS-positive non-adrenergic non-cholinergic (NANC) nerves is known to mediate neurotransmission (Grozdanovic et al., 1994; Rand and Li, 1995), first demonstrated in anococcygeus muscles (Li and Rand, 1989; Ramagopal and Leighton, 1989). Later on, nitrenergic transmission by NANC nerve endings was postulated for many other organs, e. g. the corporal erectile tissue and the deep cavernous arteries in the penis (Burnett et al., 1993), different blood vessels (Yoshida et al., 1994), the pancreas (Wörl et al., 1994), the urinary bladder (Smet et al., 1996), the ovary (Jarret et al., 1994), and the principal bronchi of the respiratory tract (Fischer et al., 1993). The nNOS isoform has also been found in CNS tumors (Cobbs et al., 1995), visceral and somatic striated muscle fibers (Kobzik et al., 1994; Grozdanovic et al., 1995), mast cells (Bacci et al., 1994), mucosal cells of the colon (Torihashii et al., 1996), somatostatin producing cells of the stomach and pancreas (Burrell et al., 1996), the airway epithelium of the lung (Kobzik et al., 1993), and in boar spermatozoa (NADPH-d staining; Atanassov et al., 1990).

The eNOS-isoform was originally isolated from the endothelium of the bovine aorta (Förstermann et al., 1991b; Pollock et al., 1991). Using specific antibodies, eNOS has been localized in endothelial cells of various arteries and veins in many tissues (Pollock et al., 1993; Busconi and Michel, 1994; Fukuda et al., 1995; Miyawaki et al., 1995). There are apparently important phenotypic differences between endothelial cells of large

vessels and the microvasculature as well as among microvasculature endothelial cells isolated from different tissues and organs (Balligand et al., 1995). Apart from eNOS, both the neuronal (Loesch et al., 1994; Thomsen et al., 1995) and the inducible isoform (Balligand et al., 1995) have been found in endothelial cells. On the other hand, eNOS immunoreactivity has been detected in kidney tubular epithelial cells (Tracey et al., 1994), syncytiotrophoblasts of human placenta (Myatt et al., 1993), cardiac myocytes (Seki et al., 1996), interstitial cells of the canine colon (Xue et al., 1994), and in neurons of the rat brain, mainly those of the hippocampal formation (Dinerman et al., 1994b). The latter report remains questionable at present, as a re-investigation in our own and other laboratories (H.H.H.W. Schmidt, Würzburg, personal comm.) failed to demonstrate any detectable eNOS immunoreactivity in nerve cells of the rat brain.

The iNOS-isoform, first isolated from murine macrophages (Hevel et al., 1991; Stuehr et al., 1991), is constitutively expressed at a very low level, if at all, but can be induced ubiquitously upon exposure to inflammatory cytokines (interleukin-1, tumor necrosis factor, interferon γ) or bacterial lipopolysaccharide (Gross and Wolin, 1995; Schoedon et al., 1995; Sparrow, 1995; Shapiro and Hotchkiss, 1996). Recently, Kröncke et al. (1995) have listed several mammalian cell types which are so far known to express NOS on induction. Surprisingly, this NOS-isoform has been found to be induced even in endothelial cells (Balligand et al., 1995) as well as in neurons, as shown for cerebellar granule cells (Minc-Golomb et al., 1996), apart from the respective constitutive isoform. Neuronal iNOS might, according to the authors, contribute to the vulnerability of the brain to various insults besides other cellular sites of iNOS expression, such as microglia and astrocytes (Lee et al., 1993; Murphy et al., 1995; Schmidt et al., 1995; Chao et al., 1996). Long time expression of iNOS in infiltrating macrophages may be of pathogenetic significance in myocardial infarction (Dudeck et al., 1994) and in type I diabetes mellitus (Kröncke et al., 1993; Wu, 1995). Moreover, there are a wealth of reports on the induction of iNOS in various types of cells and tissues under conditions of inflammation, host defense, carcinogenesis, autoimmune disease, or transplant rejection (for review, see Moncada et al., 1994a,b; Gross and Wolin, 1995; Kerwin et al., 1995; Moncada and Higgs, 1995).

Conclusions

NO research has attracted great interest since the discovery in the late 1980s which showed that the endothelium-derived relaxing factor is identical to NO. The enormous multiplicity of NO actions as a biological messenger or a cytostatic/cytotoxic agent, and its ubiquitous distribution throughout the body deserve great efforts to further explore the physiology and pathology of this unconventional biomolecule. NO is,

instead of being stored in cellular compartments, formed and released on demand by the action of NOS. Methods for the localization of the enzyme and its molecular isoforms are, therefore, important tools to enhance our understanding of the functional interrelation between NO production sites and their cellular targets. A major challenge for the future is to develop highly specific topochemical techniques with an enhanced sensitivity that allow the identification even of traces of NOS. Low quantities of NOS may be of particular relevance to physiological aspects of NO effects as well as to NO-mediated processes of chronic inflammation and degeneration.

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