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NITRIC OXIDE AS A NEUROTRANSMITTER IN THE AUTONOMIC NERVOUS SYSTEM

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ABSTRACT

Nitric oxide has recently been appreciated as a normal biological messenger with a role in at least three areas: vasodilation, cytotoxicity and neurotransmission. Nitric oxide is generated from L-arginine by nitric oxide synthase: nicotinamide adenine dinucleotide phosphate- depedent and tetrahydrobiopterin- dependent hemeprotein. The primary signal transduction pathway of nitric oxide is the activation of soluble quanylate cyclase and subsequent production of cyclic guanosine 3', 5'- monophosphate. Studies on the immunocytochemical and histochemical localization of nitric oxide synthase point to the involvement of the neural L-arginine- nitric oxide pathway in the regulation of autonomic ganglia function and of several aspects of cadiovascular, respiratory, urinary and gastrointestinal tract functions. Pharmacological and electrophysiological data indicate that nitric oxide acts as a neurotransmitter in the autonomic nervous system. It fulfils most of the criteria for a neurotransmitter. Nitric oxide is released from nonadrenergic noncholinergic nerve terminals and mimic the effect of nerve stimulation. The changes in the mechanical and/or electrical activity of smooth muscle preparation in response to transmural stimulation of nonadrenergical noncholinergical nerves are antagonized by inhibitors of nitric oxide synthsis or oxyhemoglobin, an nitric oxide scavenger. Effects of arginine analogues can be restored by addition of excess L-arginine. Nitric

oxide also causes inhibitory junction potentials of gastrointestinal smooth muscle cells and relaxes muscle strips. Abnormalities of L-arginine- nitric oxide pathway in the autonomic nervous system may be the major cause of Hirschsprung's disease, infantile hypertrophic pyloric stenosis and achalasia as well as impotence. Abnormalities of peripheral nitrergic nerves may contribute to the pathogenesis of diabetes, asthma, cerebral ischemia and hypertension.

To Jiali and Bing, and to my parents.

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ABBREVIATIONS

ACh	acetylcholine
ADP	adenosine diphosphate
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
cG-PK	cGMP-dependent protein kinase
DMPP	1,1-dimethyl-4-phenyl-piperazinium
EDRF	endothelium-derived relaxing factor
EFS	electrical field stimulation
FAD	flavin dinucleotide
FMN	flavin mononucleotide
GABA	r-aminobutyric acid
GC	guanylate cyclase
GI	gastrointestine
GTP	guanosine triphosphate
IJP	inhibitory junction potential
LES	lower esophageal sphincter
LI	like-immunoreactivity
MPO	myenteric potential oscillation
NADPH	nicotinamide adenine dinucleotide phosphate
L-NAME	N-nitro-L-arginine methyl ester
NANC	nonadrenergic noncholinergic
L-NMA	N-methyl-L-arginine

NMDA N-methyl-D-aspartic acid

L-NMMA N-monomethyl-L-arginine

D-NNA D-nitro-L-arginine

L-NNA N-nitro-L-arginine

NOS nitric oxide synthase

bNOS brain nitric oxide synthase

eNOS endothelial nitric oxide synthase

macNOS macrophage nitric oxide synthase

nNOS neuronal nitric oxide synthase

PDE phosphodiesterase

PKC protein kinase C

SIN-1 3-morpholinosyndonimine hydrochloride

SCG superior cervical ganglia

SGC soluble guanylate cyclase

SOD superoxide dismutase

VIP vasoactive intestinal polypeptide

INTRODUCTION

One of the most significant discoveries in the past decade is that nitric oxide (NO) has an important biological role. It is a small, simple and free radical gas with only two constituent atoms, but it is highly chemically reactive and noxious because of its extra electron. Ten years ago, NO was regarded merely as a highly toxic gas. It is an atmospheric pollutant produced by cigarette smoke, car exhaust and lightning, and it is in smog. It is also suspected to be in acid rain, an ozone destroyer, carcinogen and involved in the etiology of lung diseases. NO has long been known to be generated by some bacteria, such as *Achromobacter cycloclastes*, *Alcaligenes faecalis* and *Bacillus halodenitrificans*. Few investigators ever thought that NO had any physiological role.

Most body functions are regulated by large and complex compounds that have sequence-dependent specificity. NO, however, has a molecular weight of only 30, and its high chemical reactivity makes it short lived. Also it has many disparate interactions. It is hard to believe that such a small and fleeting molecule has regulatory functions. Since 1987, however, many studies have shown that NO is involved in vasodilation, suppression of pathogens, cancer and neurotransmission. Results of these studies lead to reexamining how cells communicate and defend themselves. There is no doubt that in high

concentration, NO acts as a toxin and is involved in the pathogenesis of diseases, including hypertension, diabetes, and infantile hypertrophic pyloric stenosis, among others. At lower concentrations, however, it has important physiological functions.

NO is one of a novel class of unorthodox neurotransmitters and may be an important messenger molecule. Nerves containing NO synthase (NOS), which synthesize NO, are in the cerebellum, superior and inferior colliculi, granular cell layer of the olfactory bulb, in cerebral cortex, hippocampus, posterior pituitary, and in the retina. NO plays a role in synaptic transmission: when glutamate, an excitatory neurotransmitter, stimulates the N-methyl-D-aspartate (NMDA) receptor, NO is formed. NOS inhibitors like nitroarginine and methylarginine block this process.

Direct evidence for NO-specific neurotransmitter functions comes from studies in the peripheral autonomic nervous system. A nitrergic nervous system has been proposed because there are nerves staining for NOS in the cardiovascular system, bronchial tree, genitourinary system and gut. NO is released from many nonadrenergic noncholinergic (NANC) nerves and is the major NANC neurotransmitter.

While depolarization of myenteric plexus neurons produces relaxation of the gut smooth muscle, NOS inhibitors block this process. NO also plays a role in vasodilation cerebral arteries and those in the corpus cavernosum, as well as in bronchodilatation, and relaxation of smooth muscle in the urinary tract. NO is a neurotransmitter in reflexes that regulate penile erection. There is abundant evidence to support the concept that NO is a

neurotransmitter.

This thesis will focus on the neurotransmitter role of NO in the autonomic nervous system.

DISCOVERY OF THE BIOLOGICAL ROLE OF NITRIC OXIDE

Several lines of research reveal the physiological role of NO and its functions in biological signalling. More than 100 years ago, amyl nitrite was used as a vasodilator by Brunton for the treatment of angina (Brunton, 1867). Soon after glyceryl trinitrate was also used and these compounds are in use today. However, the mechanism of action of these nitrovasodilators has been shown only recently.

2.1. NO as an endothelium-derived relaxing factor (EDRF)

2.1.1. The discovery and nature of EDRF

The biological role of NO was first established in experiments on blood vessel relaxation. In 1980, Furchgott and Zawadzki (1980) demonstrated that the acetylcholine (ACh)-induced vascular relaxation depended on an intact vascular endothelium. The relaxation of blood vessels by acetylcholine was abolished when the endothelial layer was removed, but could be restored by reapplying endothelial cells to the smooth muscle layer. The EDRF (endothelium-derived relaxing factor) was labile and difficult to isolate despite efforts by several laboratories.

Since the late 1970s a chemically diverse set of compounds has been demonstrated to activate soluble guanylate cyclase (sGC) by means of

releasing NO. EDRF activates sGC and increases intracellular levels of cGMP. Endothelium-dependent relaxation is blocked when GC is inhibited by methylene blue. Hemoglobin and superoxide (O^{2-}) could inactivate EDRF. In 1986, Furchgott and Ignarro simultaneously proposed that EDRF is NO, or a related molecule (Furchgott, 1988; Ignarro et al., 1988). NO was later identified as EDRF by several groups (Ignarro et al., 1987; Palmer et al., 1987).

It has also been proposed that other molecules that either bind NO or are NO derivatives, may be responsible for some of the biological properties of EDRF. NO is involved in EDRF-elicicted vasorelaxation, indicating that EDRF is either NO or a molecule capable of releasing NO (Moncada et al., 1991).

2.1.2. Mechanism of action of nitrovasodilators

Nitrovasodilators are potent relaxers of smooth muscle and have been used to treat angina pectoris, achalasia and biliary spasm. Despite their use for over 100 years, their mechanism for producing vasodilation has been shown only recently. The smooth muscle-relaxing effects of nitroglycerine and other organic nitrate vasodilators involve an active metabolite, NO, whose properties are like those of EDRF. The smooth muscle relaxant activity of NO is a result of activation of sGC that increases intracellular cGMP (Rapoport and Murad, 1983; Ignarro and Kadowitz, 1985). The activation of GC by NO and nitrovasodilators is inhibited by methylene blue (Gruetter et al., 1981). The chemically detectable NO synthesized from arginine is released in

sufficient amounts from endothelial cells to account for all EDRF activity (Palmer et al., 1987).

2.2. NO role in the immune system

In a completely unrelated context, NO was found to be formed endogenously. This discovery came from studies of dietary nitrates as a source of carcinogenic nitrosamines. In the early 1980s, Tannenbaum and his colleagues were investigating nitrate metabolism. They noted that germ-free rats excrete more nitrate than they ingest (Green et al., 1981a). Humans and rats fed low-nitrate diets still excreted substantial amounts of nitrates (Green et al., 1981b). A marked increase in nitrates was detected in rats exposed to endotoxin (Wagner et al., 1983). Obviously, diet is not the only source of nitrates. The old hypothesis that nitrates are not normally formed in the body but are derived from intestinal bacteria was incorrect. Then which part of the body did the nitrates come from? A clue to this question was accidently provided during the study when a subject contracted an infectious diarrhea and excreted very high levels of nitrates. Certainly inflammatory processes associated with the diarrhea were related to the nitrate formation.

The definite source of nitrate formation was discovered by both Marletta and Hibbs research groups. Stuehr and Marletta (1985) found that mice with a genetically-determined lack of macrophages no longer excreted nitrates and that macrophages in tissue culture release nitrates when exposed to bacterial endotoxin. Also, they found macrophages could not produce nitrates when arginine was absent from the incubation medium.

When arginine was removed from the media, the formation of nitrites and nitrates was prevented. Therefore arginine was a precusor of the nitrites and nitrates.

Hibbs and his colleagues (Hibbs et al., 1987) also found macrophages need arginine to produce nitrates; furthermore, analogues of arginine, such as its methyl derivative, could block the above processes. The ability of activated macrophages to kill tumor cells and infected bacteria was greatly augmented by addition of arginine and disappeared when arginine was removed from the medium. It was demonstrated that NO was toxic to the tumor cells as were the activated macrophages. Without NO derived from arginine, the defense of macrophages to kill tumor and bacteria could not be executed. Thus, when macrophages are activated by endotoxin or other cytokines, they convert arginine to NO, then the toxic NO makes macrophages kill bacteria and tumor cells. Also, they found that the possible mechanism of NO to damage cells is binding to certain cellular respiratory enzymes and thus these cells starve to death.

2.3. First identification of NO's function in the brain

The wide existence of L-arginine-NO pathway in vascular endothelium and macrophage suggested that NO might be involved in other parts of the body. There is another vein of fascinating research that occured recently in the nervous system about the biological role of NO. The first hint of such involvement came in 1982. L-arginine was found to be the endogenous activator of the sGC in the brain (Deguchi and Yoshioka 1982). Previous

studies (Deguchi, 1977) showed that NO could stimulate the sGC in homogenates of mouse cerebral cortex and that a low-molecular-weight substance in the soluble fraction of rat forebrains could activate sGC. Hemoglobin inhibited the activation of the unidentified substance.

Garthwaite et al. (1988) obtained evidence that brain tissues can form a short-lived substance resembling NO. Furthermore, this short-lived substance could raise cGMP levels in neurons and endothelial cells and cause vasorelaxation. This substance could be protected by superoxide dismutase and inactivated by hemoglobin. Its properties strikingly resemble EDRF(NO). The release of the diffusible messenger is Ca^{2+} - dependent. The substance seems to be a glutamate mediator.

Parallel lines of investigation by Knowles and coworkers found that addition of L-arginine to the rat forebrain resulted in the NADPH-dependent formation of NO and citrulline accompanied by stimulation of sGC. The synthesis of NO is dependent on the free Ca^{2+} concentration and is inhibited by hemoglobin and L-NMMA. These data showed that the rat brain possesses the NO synthase (NOS) (Knowles et al., 1989). The enzymatic reaction is similar to that in the vascular endothelium and macrophages. The regulation of Ca^{2+} concentration appeared to be the physiological mechanism for stimulating the synthesis of NO in target cells and represented a mechanism of interneuronal communication.

Subsequently, Bredt and Snyder demonstrated that glutamate and related amino acids such as NMDA markedly stimulate NOS activity in cerebral slices via NMDA receptors. These enhance cGMP levels

stoichiometrically. L-NMMA completely prevented the stimulation of cGMP formation and production of citrulline with identical potencies. Arginine reversed the effects of L-NMMA. Superoxide dismutase stimulate NO and cGMP formation and hemoglobin prevented the stimulaton by NMDA (Bredt and Snyder, 1989). They monitored the activity of NOS by measuring the arginine-citrulline transfomation because it is almost impossible to monitor the labile NO directly. Arginine is stoichiometrically converted to citrulline as NO is formed.

2.4. First identification of NO as a neurotransmitter in the peripheral nervous system

There is compelling evidence that NO acts as a neurotransmitter in the autonomic nervous system. In the early 1980s, the properties of EDRF and the "inhibitory extract" obtained from bovine retractor penis (BRP) were known to have a number of similarities. Both EDRF and the inhibitory factor were labile and destroyed by borohydride (Gillespie et al., 1981). Superoxide generators, pyrogallol and hydroquinone, blocked both actions of EDRF and the inhibitory factor. Both were inhibited during anoxia and activated by acidic pH. EDRF and the inhibitory factor both acted via stimulation of cGMP production and could be inhibited by hemoglobin. There were also many similar properties between EDRF and the NANC relaxant transmitter.

Gillespie and colleagues worked on the rodent anococcygus and BPR muscles, two rather esoteric preparations. In 1989, NO was demonstrated to be involved in neurotransmission in anococcygeus muscles of the rat and mouse for the first time (Gillespie et al., 1989; Gibson et al., 1989). Strong

evidence showed that NG-substituted analogues of L-arginine such as L-NMMA and L-NNA blocked NANC-mediated relaxation of the anococcygus muscle. The blockade could be prevented by L-arginine but not D-arginine. The effects were concentration dependent and enantiomer-specific. Hemoglobin was a powerful antagonist for nerve stimulation-induced relaxation of the anococcygus muscle.

Histological studies of neuronal NOS revealed its localization in the peripheral and central nervous system (Bredt et al., 1990). NOS distribution indicated a neural role of NO. Using immunohistochemical method, NOS was found to be concentrated peripherally in cell bodies and nerve fibers in the myenteric plexus of the intestine, in autonomic nerve fibers in the retina, in the neural innervation of the posterior pituiary, in the adrenal mudulla, and in vascular endothelial cells. In the brain, NOS was exclusively associated with discrete neuronal populations. There was strong evidence that NO was associated with neurons. The histochemical localization of NOS in the peripheral neurons provided the morphological basis for nitrergic transmission in the peripheral autonomic nervous system.

It became clear that many of the pharmacological tools that had provided evidence about the nature of EDRF were also applicable in studies of the NANC inhibitory transmitter. NO synthesis inhibitors could inhibit NANC relaxations in the rat anococcygeus muscle which provided direct evidence for NO as a transmitter of NANC innervation of smooth muscle (Gillespie et al., 1989; Li and Rand, 1989). These results were soon confirmed in the mouse anococcygeus and the bovine penis retractor, and then in many other smooth muscle preparations of cardiovascular, urogenital, respiratory,

and digestive systems. Neurotransmission that involves NO was termed nitrergic transmission by Li and Rand (Li and Rand, 1989).

CELLULAR AND MOLECULAR ASPECTS OF NO SYSTEM

3.1. Nitric oxide synthase (NOS)

3.1.1. Characterization and molecular cloning

NOS, instead of NO itself, has been monitored to measure its disposition and activity since the early NO study, because NO is such a labile compound. NOS converts arginine and oxygen to NO and citrulline (Fig. 1). NOS could be more efficiently measured by measuring the stoichiometrically converted citrulline as NO is formed. It is tedious to measure the conversion of arginine to NO or derived nitrites and nitrates (Snyder and Bredt, 1991).

NOS can be generally grouped into two types: constitutive and inducible form. The constitutive NOS is cytosolic, a Ca2+/calmodulin-dependent enzyme. It exists in neuronal and endothelial cells. It is activated when intracellular Ca2+ levels increase. NOS releases picomoles quantities of NO for short periods, in response to receptor activation. The inducible NOS is present in macrophages, endothelial cells, hepatocytes and a number of other cells, although most of the enzymological work has been performed on it from macrophages. When these cells are activated by cytokines, NOS is induced. Inducible NOS is cytosolic, Ca2+ independent, releases nanomoles quantities of NO over long periods, and is inhibited by glucocorticoids. The constitutive

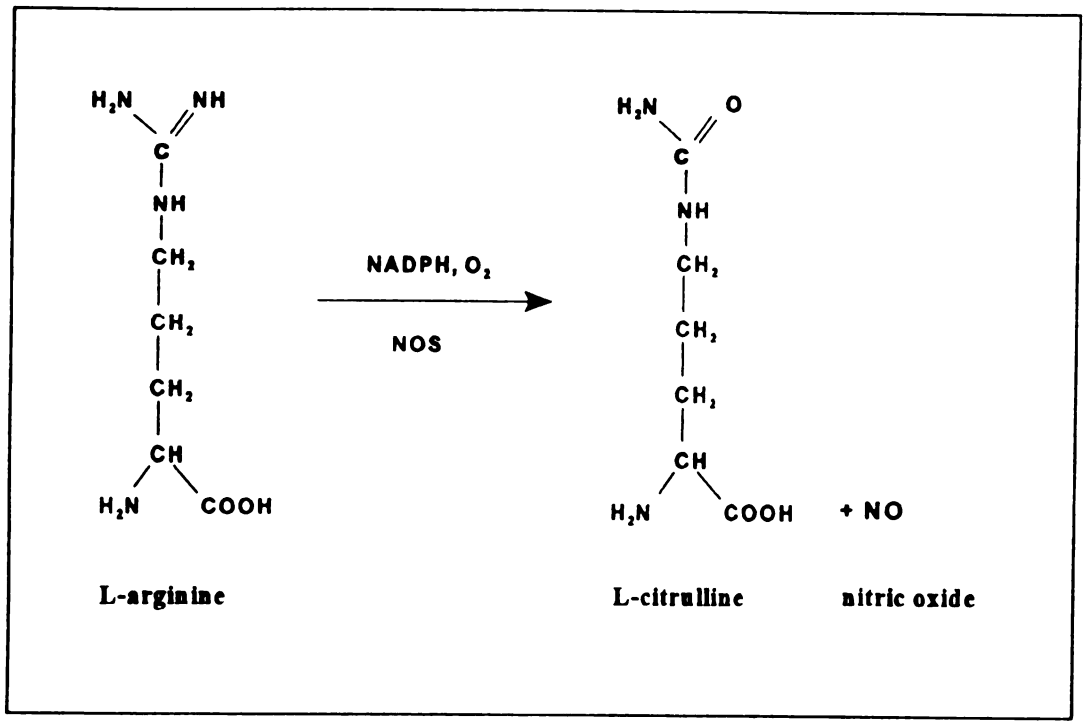

Figure 1. Synthesis of NO (nitric oxide)

NOS is important in nerves and endothelium while the inducible NOS seems to be primarily functioning in the immune response of various tissues (Moncada, 1992).

There is another classification of NOS based on the sources it comes from: brain NOS (type I: bNOS), macrophage NOS (type II: macNOS), and endothelial NOS (type III: eNOS). The properties of bNOS and eNOS are quite similar. Both isoforms belong to constitutive NOS. The properties of macNOS are quite different from those of bNOS and eNOS, and it is inducible. All forms of NOS use arginine as their substrate, tetrahydrobiopterin-dependent and require NADPH (nicotinamide adenine dinucleotide phosphate) as an electron donor (Fig. 2).

Additional insight into NO formation and its regulation has come from purification of the bNOS and its molecular cloning (Bredt et al., 1992). Via a two-phase polymerase chain reaction (PCR) cloning strategy, NOS complementary DNA (cDNA) was cloned to yield a product with an open reading frame of 4287 bases encoding a protein of 1429 amino acids of a relative molecular mass of approximately 160 Kd. The amino acid sequence of NOS has a number of recognition sites, which includes a α-helical, calmodulin-binding consensus sequence, a cyclic AMP-dependent protein kinase phosphorylation consensus sequence, a NADPH-binding domain, and consensus-binding sites for flavin mononucleotide (FMN) and flavine adenine dinucleotide (FAD) (Fig. 2). Thus, major second messenger systems regulate the formation of NO. The amino acid sequence of NOS closely resembles only one other mammalian, enzyme cytochrome P450 reductase (CPR). They share 50% of amino acid sequence homology. NOS and CPR are the only known

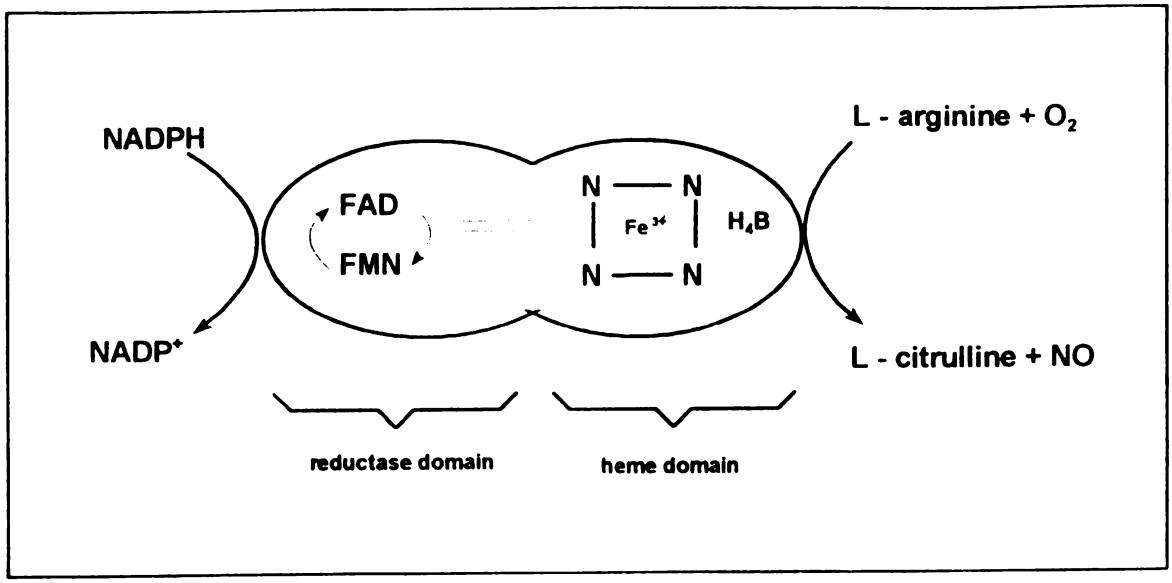

Figure 2. Oxidoreduction reaction for the synthesis of NO

mammalian enzymes with binding sites for two different flavins and NADPH. The electron transfer between the flavins probably has a role in the mechanism of NO synthesis. Part of NOS catalytic activity is the successive transfer of electrons between NADPH and the two flavins (Fig. 2).

NOS appears to be a highly regulated enzyme. Purified NOS is phosphorylated by cAMP-dependent protein kinase, protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase II (Bredt et al., 1991). All three enzymes phosphorylate distinct serine. Activation of PKC with phorbol ester leads to a more than 50% decrease in NOS activity. NOS phosphorylation may afford cross-talk between messenger systems.

Molecular cloning of macNOS reveals substantial homology with bNOS, especially in the C-terminal portion of the enzyme. The C-terminal portion of NOS is similar to CPR.

3.1.2. NOS inhibitors

The development of drugs that inhibit NOS have been the most important strategy in determining the physiological and pathophysiological roles of NO. L-arginine analogues block the formation of endogenous NO because they act as false substrates. This blockade can be overcome by adding back L-arginine but not by adding D-arginine. A number of arginine analogues have been developed, including NG-monomethyl-L-arginine (L-NMMA), NG-nitro-L-arginine methyl ester (L-NAME), N -nitro-L-arginine (L-NAA), NG-iminoethyl-L-ornithine (L-NIO). All of them are stereospecific, the corresponding analogues of D-arginine being inactive. They can be taken

up by cells and inhibit NO systhesis. The order of potency of their inhibition was L-NNA > L-NAME > L-NMMA > L-NIO (Knowles et al., 1989). The effectiveness of NOS inhibitors is dependent on the kinetics of their interaction with NOS, the efficiency of NO-Guanylate Cyclase coupling, the efficiency of NO synthesis and the release of a particular receptor system. L-NNA inhibition seems irreversible, it inhibited NOS in the brain when given systemically. L-NNA blocked the NMDA evoked activation of NOS at a very low concentration. The inhibition of NOS by L-NNA in cerebellar slices was biphasic. Other arginine analogues caused only low affinity monophasic inhibition. L-NMMA and L-NIO have been shown to inhibit L-arginine transport. Calmodulin inhibitors also inhibit enzyme activity with moderate potencies. Flavin moiety inhibitors provide another way to regulate NOS such as diphenyliodium. Nitro blue tetrazolium is the substrate of the NADPH-diaphorase histochemical reaction that strongly inhibited NOS (Table 1). L-NG-nitroarginine-p-nitroanilide (L-NAPNA) appears to be a selective inhibitor of NOS in the brain with little effect on endothelium. Hydroxycobalamin has a differential action in blocking responses to EDRF in rat aortic rings but not neurogenic NO in the rat anococcygeus muscle. Aminoguanidiane appears to be a selective inhibitor of inducible NOS compared with constitutive NOS. Selective NOS inhibitors may be the basis for potentially important therapeutic agents in the future.

3.1.3. NO and citrulline metabolism

Because NO is an uncharged molecule with an unpaired electron, it is highly reactive and readily diffusible. NO has a very short half-life, 3 to 5 seconds, in physiological salt solution. After transmitting a signal, NO

Table 1. Five Types of NOS Inhibitors

Class	cNOS	iNOS
1. L-arginine antagonists	N^G-monomethyl-L-arginine (L-NMMA)	N^G-monomethyl-L-arginine (L-NMMA)
	N^G-nitro-L-arginine methyl ester (L-NAME)	N^G-nitro-L-arginine methyl ester (L-NAME)
	N-nitro-L-arginine (L-NNA)	N-nitro-L-arginine (L-NNA)
	N^G-iminoethyl-L-ornithine (L-NIO)	N^G-iminoethyl-L-ornithine (L-NIO)
2. Flavin moiety inhibitors	Diphenylene iodonium Nitro blue tetrazolium	Diphenylene iodonium Nitro blue tetrazolium
3. Calmodulin inhibitors	Calcineurin Trifluroperazine	
4. Tetrahydrobiop-terin inhibitors		2,4-Diamino-6-hydroxypyrimidine
5. Heme binders	Carbon monoxide	Carbon monoxide

spontaneously decays into nitrite. The mechanisms of ending NO action are quite different from those of the usual transmitters, regarding uptake process and removing them by enzymatic methods. NO readily reacts with oxygen in aqueous solution to form nitrite (NO2-) and nitrate (NO3-). The reaction of NO and oxygen is very fast. Both nitrite and nitrate are inactive anions. NO readily diffuses into erythrocytes and binds with hemoglobin to form nitrosyl-hemoglobin (NO-Hb). NO-Hb is then oxidized primarly into nitrate and nitrite. Most metabolites are excreted in the kidney in the form of urine. NO interacts readily with non-heme, iron-sulphur coordinated complexes at the active sites of some enzymes, forming a nitrosyl-iron-sulphur complex (Stamler et al., 1992). This reaction may be the basis of cytotoxic effect of NO.

Recent enzyme kinetic studies show that NO conversion into nitrites and nitrates may be very slow under normal conditions. In contrast, the reaction of NO with superoxide anion to yield peroxynitrite anion (ONOO-) is very fast. Superoxide dismutase can prolong NO actions. Peroxynitrite may be the primary metabolite of NO during inflammatory reaction when superoxide anion and NO levels are dramatically increased. In activated alveolar macrophages, the majority of NO is converted to peroxynitrite with a small amount of nitrite and nitrate produced (Ischiropoulos et al., 1992). The peroxynitrite anion is highly reactive and mutagenic which accounts for much of the cytotoxic activity of NO. The reaction of NO with superoxide can produce a few highly toxic molecules and this is the basis of macrophages and activated neutrophils to exert their cytotoxic actions.

It has been known since the 1960s that the brain contains substantial amounts of citrulline and the urea cycle enzymes argininosuccinate synthase

(ASS) and argininosuccinate lyase (ASL). Using antibodies to citrulline, immunohistochemical studies localize citrulline in a subpopulation of NADPH-diaphorase stained neurons. It has been shown that the brain contains the metabolic pathway necessary to convert citrulline into arginine. Citrulline formed by NOS is recycled into arginine. This is the only pathway for citrulline metabolism in the brain. Recent immunohistochemical studies have identified ASS and ASL immunoreactivities in various neurons in the brain (Nakamura et al., 1990). In many regions ASS and NADPH-diaphorase coexist while ASS and NOS appear to be in separate neuronal populations. This implied a separate role for this urea cycle enzyme in some neurons. Citrulline may exit or enter these neurons by diffusion or via a neutral amino acid carrier.

3.2. Cellular and molecular targets of NO

The involvement of NO in neuromuscular transmission is based on numerous pharmacological observations. Transmural stimulation of NANC nerves caused membrane hyperpolarization and relaxation of smooth muscle. L-arginine analogues and oxyhemoglobin blocked and exogenous NO mimicked these reactions. NO appeared to have a direct postjunctional effect on the smooth muscle cell because the effects of exogenous application of NO were not affected by tetrodotoxin, NOS inhibitors or low extracellular Ca2+ concentration, but were blocked by oxyhemoglobin (Stark et al., 1991). Transmural stimulation of muscle cells from guinea pig ileum provided evidence that nitrergic axons were involved with other neurons. The tonic contractions were enhanced by L-NMMA and abolished by a substance P antagonist. The contractions evoked by exogenous substance P were not

modified by the presence of L-NMMA. NO appeared to modulate excitatory peptidergic neurotransmission.

Molecular targets of NO are diverse and include both low molecular weight species and macromolecules. The primary cellular responses to NO in the nervous system were the activation of the sGC and the subsequent production of cGMP. The sGC is the NO receptor. It has long been known that drug-derived NO activates sGC by binding to its heme. NO-induced reaction can be mimicked by cGMP, diminished by sGC inhibitors, and augmented by inhibitors of cGMP phosphodiesterase (Ignarro, 1990). The iron in a certain protein heme group or in an iron-sulfur complex is the best-characterized receptor of NO. NO exerts its effects by binding to iron-containing enzymes and either activating or inactivating the enzymes. sGC is activated by NO binding to its heme group; then it produces cGMP to ignite other cellular processes. sGC is purified from various tissues. It existed as a heterodimer with subunits of 70 and 82KDa. The enzyme contained 1 mole of heme per mole of holoenzyme. Nitrosyl-heme complex results in conformational change of sGC, causing its activation (Ignarro, 1991).

Another enzyme that can be activated by NO is an uncharacterized ADP-ribosyltransferase. NO can stimulate adenosine diphosphate (ADP) ribosylation via activation of ADP-ribosyltransferase. NO can cause auto-ADP ribosylation when it diffuses to a cell, resulting in ADP ribosylation of the target without enzyme catalysis. NO affects cells by facilitating the transfer of an ADP-ribose group to an accepting molecule. NO binding to non-heme iron containing proteins is thought to be a pathophysiological or cytotoxic action of NO (Brune et al., 1993).

A prominent action of macrophage-derived NO on tumor cells is inhibition of their synthesis of DNA. By inhibiting the tyrosyl radical or reaction with non-heme Fe, NO inhibits ribonucleotide reductase, a rate-limiting enzyme. Hydroxyurea, the prototypic ribonucleotide reductase inhibitor, can be oxidized to an NO-like compound. NO and NO-generating compounds can be oxidized to a mutagen that deaminates DNA. Superoxide anion is another important target of biologically derived NO. Depending on the situation, the reaction of superoxide with NO may present a detoxification of either molecule, or a route to the generation of peroxynitrite.

3.3. Actions of cGMP

There are three major cGMP receptor proteins: cGMP-dependent protein kinase, cGMP-regulated ion channels and cGMP-binding phosphodiesterases. Each receptor protein has been shown to mediate some of the physiological actions of cGMP in specific cell types. It is also possible that a specific cell type may have more than one type of cGMP receptor protein.

3.3.1. cGMP-dependent protein kinase

The enzyme has been found in various tissues. cG-PK1 and cG-PK2 are two basic types of the enzyme. Soluble cG-PK1 is a widely distributed enzyme composed of two identical 78KDa subunits. cG-PKIa is highly concentrated in the Purkinje cells of the cerebellum. It is scarce in other parts of the nervous system. cG-PKIa causes phosphorylation of the G-substate, a putative phosphatase inhibitor. cG-PKII is cloned from mouse brains and shares 50%

homology to cG-PKI. It is a 86KDa monomer found abundantly in the brain and the lungs. cGMP but not cAMP strongly activate cG-PK. DARPP-32 is a possible cG-PK target that can be phosphorylated by cG-PK in stantia nigra. DARPP-32 in its phosphorylated form is a strong protein phosphatase 1 inhibitor. It may be by this mechanism that NO maintains receptor proteins in their phosphoylated states (Schmidt, 1993).

3.3.2. cGMP-regulated ion channels

In retinal photoreceptor cells, cGMP-regulated ion channels are well known in that they cause the dark (inward) current. In the light, phosphodiesterases are activated to hydrolyze cGMP and thus the channels close and the membrane hyperpolarizes. cGMP-regulated channels are also found in the olfactory epithelium and retinal bipolar cells. Their properties are like those in rods (Schmidt et al., 1993). NO may be the stimulator of cGMP because nitroprusside, a NO donor, can mimic the effects of cGMP. Stimulators of GC can not produce similar results. PCR techniques detected DNA sequences specific for cGMP-opened channels in the heart but not in the brain. Patch-clamp recordings from isolated ganglion cells indicated a functional nonspecific cation channel that was activated by both cGMP and NO donors. cAMP had much less effect.

3.3.3. cGMP-binding phosphodiesterases

Phosphodiesterases function to catalyze cyclic nucleotides to their corresponding 5'-nucleotide monophosphates. Five classes of phosphodiesterases can be separated by their structure and pharmacological

properties. cGMP can activate or inhibit certain types of phosphodiesterases, such as cGMP-stimulated subtype (type II), cGMP-inhibited subtype (type III) and cGMP-specific subtype (type V). Type II phosphodiesterase is stimulated by low concentrations of cGMP and has a relatively low affinity for cyclic nucleotide substrates (Schmidt et al., 1993).

Type V enzyme is a major phosphodiesterase responsible for cGMP metabolism in the nervous system. This enzyme family includes the transducin-activated phosphodiesterase in rods and cones and an isozyme found in various tissues. There is evidence that Ca2+/calmodulin-dependent phosphodiesterase plays a role in regulation of cGMP levels. Dipyridamole and Zaprinast are two selective inhibitors of phosphodiesterases. Zaprinast has been shown to potentiate the NANC nerve innervated smooth muscle relaxations mediated by NO release, indicating that phosphodiesterases play a role of inhibiting the NO effect by affecting cGMP level in nervous system. Therefore, the major receptor proteins of cGMP have been demonstrated to mediate the biological effects of cGMP in these specific cell types.

LOCALIZATION AND FUNCTIONS OF NO IN AUTONOMIC GANGLIA

The autonomic nervous system has three parts: the sympathetic, the parasympathetic and the enteric nervous systems, with corresponding ganglia. It was originally thought that autonomic ganglia function as mainly relay stations. They induce, however, excitation and inhibition in viscera and provide integration. They are said to function as "little brains". NOS is in all autonomic ganglia, where it modulates transmission

4.1. Sympathetic ganglia

NOS is widely distributed among paravertebral, prevertebral and pelvic sympathetic ganglia (Santer & Symons, 1993). The presence and topographic distribution of NOS in the autonomic nervous system is investigated by using nitric oxide synthase (NOS) immunocytochemistry and nicotinamide dinucleotide phosphate (NADPH) diaphorase histochemistry. Both methods detect NOS. Neuronal NADPH-diaphorase has been shown to be NOS (Dawson et al., 1991; Hope et al., 1991). Now NADPH-diaphorase method has been widely used as a simple marker for NOS. It was found that NADPH-diaphorase staining was in neurons of prevertebral ganglia of rats,

such as those surrounding the coeliac-superior mesenteric ganglion complex and in interstitial nerve bundles and in nerve trunks. Many NADPH-diaphorase stained neurons were found in the inferior mesenteric ganglia and in pelvic (hypogastric) ganglia, as well as in a majority of sympathetic paravertebral ganglia (superior cervical and stellate).

NO was found in bovine superior cervical ganglion (SCG). It mediates the acetylcholine-induced stimulation of sGC. NG-methyl-L-arginine (L-NMMA) inhibited acetylcholine-induced increases of the cGMP levels. L-arginine revervsed L-NMMA inhibition. Soluble NOS was partially purified from the ganglia that required NADPH and tetrahydrobiopterin as cofactors. Oxyhemoglobin blocked and superoxide dismutase enhanced the action of NO formed by NOS (Sheng., 1993). Using an antibody generated against rat brain NOS, immunohistochemistry showed that NOS was located in postganglionic neuronal cell bodies of the ganglia. The results indicate that NO is produced in specific cells within superior cervical ganglia, and it modulates muscarinic receptor stimulation.

NO was demonstrated to mediate a potentiation of nicotinic synaptic transmission of rat superior cervical ganglion. It may increase neuronal excitability in sympathetic ganglia by activating the cGMP system. Briggs (1992) found that 8-Br-cGMP potentiated nicotinic transmission from 6±1% to 89±5% in a dose-dependent manner. 8-Br-cAMP only had less than 1/2 of this effect compared with 8-Br-cGMP. He measured the efficacy of neural transmission by recording the postganglionic compound action potential while stimulating the preganglionic nerves at a low rate (atropine was used to block muscarinic transmission). He also found that both sodium

nitroprusside and sodium azide potentiated nicotinic transmission. By contrast, ferricyanide did not affect this process. These findings indicated that NO, rather than the cyanide or feric moieties, contributed to the potentiation of nicotinic transmission in the rat superior cervical ganglia.

There are also data to show NO to mediate the cGMP response to synaptic activity in the rat superior cervical ganglion (Sheng., 1992). Preganglionic nerve stimulation of the ganglion increased the cGMP level in a Ca2+ -dependent manner. Oxyhemoglobin inhibited this response. Also, N-nitro-L-arginine, an inhibitor of NOS, stereospecifically blocked this process. These results indicate that NO or a similar substance mediates the neuronal cGMP response to synaptic activity.

NO may be a messenger molecule in preganglionic sympathetic neurons (Ceccatelli., 1994). NOS-like immunoreactivity (LI) was in dense fiber networks. These fiber networks disappeared after transection of the sympathetic trunk. Dominant cell bodies in the sympathetic lateral column of the spinal cord were shown to be NOS positive, indicating that majority of preganglionic fibers innervating sympathetic ganglia are NOS-positive.

4.2. Parasympathetic ganglia

NO may be a messenger in parasympathetic postganglionic neurons. Results of immunohistochemistry methods show that NOS-like immunoreactivity (LI) is present in most ganglion cells in parasympathetic ganglia, such as the sphenopalatine and submandibular ganglia. NOS appeared to coexist with vasoactive intestinal polypeptide (VIP) and other

peptides. A comparatively smaller proportion of neurons were NOS-positive in the pelvic parasympathetic ganglia where neurons frequently contained VIP-LI. In nasal mucosa and salivary glands innervated by parasympathetic ganglia, NOS-positive fibers were found around blood vessels and within the glandular parenchyma, although they were less abundent than VIP nerves. A closer correlation between NO and VIP was seen in the uterus, vas deferens and penis (Ceccatelli., 1994).

Histochemical methods revealed NADPH-diaphorase reactivity in cillary ganglia from the pigeon, cat, and monkey. About one-third of the cells in the cillary ganglia were highly NADPH-diaphorase positive neurons. These neurons were thought to control the lens, pupil and choroidal vasculature of the eye. Most other neurons were lightly stained. However, Sun et al. (1994) found that the cillary ganglia contained less than 2% of densely stained NADPH-diaphorase neurons whose function is known only to control the lens and the pupil. The data suggested that NO may act as a intercellular messenger in this ganglia. NADPH-diaphorase activity was also expressed in the guinea-pig intrinsic paratracheal ganglia. Many paratracheal neurons had moderate to high levels of NADPH-diaphorase activity and may be a major source of NO (Hassall., 1993). NADPH-diaphorase activity was also expressed in the guinea-pig intrinsic paratracheal ganglia. Many paratracheal neurons had moderate to high levels of NADPH-diaphorase activity, indicating that it may be a major source of NO (Hassall., 1993).

Axonal transport methods showed that NO-containing neurons were in the rat sphenopalatine ganglion that innervated the cerebral arteries

bilaterally. VIP often co-existed with NOS in the ganglia. (Minami., 1994). In another study, NOS-immunoreactivity (IR) was present in 70-80% of rat sphenopalatine ganglion cells . The major source of NOS-IR positive fibers in cerebral arteries came from the sphenopalatine ganglia (Nozaki., 1993). The histochemical data indicated that NO was involved in non-adrenergic, non-cholinergic neuronal transmission in the cerebral arteries.

NO affects the synaptic transmission process in chick ciliary ganglion. By measuring of the postganglionic compound action potential, sodium nitroprusside increased the synaptic efficacy by an average of 26%. Contrarily, potassium ferricyanide did not invoke a potentation of synaptic transmission. Sodium azide, shown to increase cGMP level in sympathetic ganglia, did not affect synaptic efficacy significantly. 8-Br-cGMP and 8-Br-cAMP increased synaptic efficacy by 61% and 46%, respectively. L-NAME reduced the long-term potentiation by an average of 47% (Scott., 1993). The results indicate that NO increases the efficacy of synaptic transmission in parasympathetic ganglia.

NO also affects calcium and calcium-activated potassium currents in postganglionic neurons of parasympathetic ganglia (Khurana., 1993; Cetiner., 1993). In cultured avian ciliary ganglia, sodium nitroprusside reduced both the voltage activated transient calcium current (ICa) and the sustained ICa during a test depolarization to +20 mV from a holding potential of -100 mV. L-Arginine reduced both the transient ICa and sustained ICa. This inhibition was prevented by the NOS competitive blocker L-NAME (Khurana., 1993). Sodium nitroprusside reduced the net outward potassium current during a test depolarization to +40 mV from a holding

potential of -40 mV. The outward current remained reduced for the duration of the recording after a single application of sodium nitroprusside. Much less reduction of the net outward current was observed by application of ferrocyanide. L-Arginine reduced the net outward current during a test depolarization to +40 mV. L-NAME reduced these inhibitions (Cetiner., 1993). The above effects indicated that NO modulate both calcium and calcium-activated potassium channels in postganglionic parasympathetic neurons.

4.3. Enteric ganglia

NOS is in enteric ganglia of many different species (Grozdanovic., 1992; Nichols., 1992; Ward., 1992; Giorgio., 1994). There is NOS-related diaphorase activity in numerous ganglia cells and in the myenteric plexus and submucosa plexus in the monkey and human digestive system, but labelled ganglion cells are less numerous in the submucosa plexus. NADPH-diaphorase labelled ganglion cells are also in the human gallbladder and in the monkey and human pancreatic intrapancreatic ganglia (Giorgio., 1994). A subpopulation of neurons in the myenteric and submucosal ganglia of canine proximal colon had NOS-like IR co-labled with NADPH-diaphorase. Labelled neurons had morphological characteristics similar to the Dogiel type I morphology (Ward., 1992). For the guinea-pig, NOS-containing terminals is extremely sparse in the myenteric ganglia of the esophagus, stomach and duodenum, moderate in the ileum and distal colon, and highly dense in the proximal colon and rectum. A sparse plexus of NOS-containing terminals is in the submucosal ganglia of the ileum and large intestine (Furness., 1994). Recent evidence indicates that NO and VIP are co-localized in enteric

neurons and are co-transmitters, released in parallel from enteric inhibitory nerves (Keef., 1994). These results indicate that NOS is localized in enteric neurons by using NOS or NADPH-related diaphorase activity labeling.

Direct evidence of nitric oxide production from enteric ganglia came from studies on the myenteric plexus of the guinea pig intestine (Grider., 1993). Grider and associates found that phenylpiperizinium (a nicotinic agonist) stimulated L-[3H] citrulline production (an index of nitric oxide production) and VIP release in isolated ganglia of the myenteric plexus. Tetrodotoxin, hexamethonium, and the NOS inhibitor L-NNA abolished both VIP release and L-[3H] citrulline production. The effect of NG-nitro-L-arginine was reversed by L-arginine but not by D-arginine. Exogenous NO stimulated VIP release whereas exogenous VIP had no effect on L-[3H] citrulline production, which implied that VIP release depends on and is facilitated by NO production. VIP release induced by 1,1-dimethyl-4-phenylpiperizinum or NO was blocked by KT 5823, an inhibitor of cyclic GMP-dependent protein kinase and by LY83583, an inhibitor of soluble guanylyl cyclase. These results indicate the existance of both NO production and NO-facilitated VIP production in enteric ganglia.

NO may serve as a neurotransmitter in enteric ganglia (Shuttleworth., 1993). In the presence of M&B 22948 and other phosphodiesterase inhibitors, exogenous NO and electrical field stimulation (EFS) caused an accumulation of cGMP like immunoreactivity in the enteric neurons of myenteric and submucosal ganglia and smooth muscle cells of the canine proximal colon. NADPH-diaphorase histochemistry showed that 94% of the neurons that responded to exogenous NO with an increase of cGMP-like IR were NADPH

diaphorase negative. Nitroarginine, an arginine anologue, abolished the EFS-induced cGMP-like IR. An increase in cGMP-like immunoreactivity was also observed in interstitial cells at the submucosal surface which may mediate neuromuscular transmission. The results support the neurotransmitter role of NO in both enteric ganglia and smooth muscle. The results also suggest that enteric inhibitory neurons may functionally innervate interstial cells. In summary, the distribution of NOS within enteric ganglia as well as sympathetic and parasympathetic ganglia coupled with growing eletrophysiological and pharmacological data support the role of NO as a neurotransmitter within autonomic ganglia. NO might mediate or modulate neurotransmission processes through cGMP mechanism within these ganglia.

PERIPHERAL TISSUES INNERVATED BY AUTONOMIC NITRERGIC NERVES

NANC nerves innervate several peripheral tissues, such as the gastrointestinal (GI) tract, cerebral arteries, some peripheral arteries, the respiratory tract and the urinary tract. The neurotransmitter for these tissues is still elusive, although there has been an intense search in the past two decades. Recent evidence suggests NO plays a role as a inhibitory neurotransmitter. This chapter presents evidence of nitrergic transmission to the heart, cerebral arteries, peripheral arteries, the respiratory tract and the urinary tract.

5.1. Cardiovascular system

5.1.1. Heart

Hassall first reported that both NOS immunoreactivity and NADPH-diaphorase activity were present in a subpopulation of intracardiac neurons in culture preparations from guinea-pig hearts (Hassall et al., 1992). NOS immunoreactivity was demonstrated in cardiac ganglia and nerve fibers innervating local neurons, blood vessels, nodes and myocardium in both rat and guinea pig hearts (Klimaschewski et al., 1992). Tanaka et al. (1993)

found many round intracardiac ganglia containing NOS-immunoreactive neurons *in vivo* by using immunoelectron microscopy. They demonstrated a direct synaptic contact between NOS-immunoreactive axons and intracardiac neurons and immunoreactive axons surround most non-immunoreactive neurons. The data suggest that NO might play a role in neural control of the heart and neurotransmission in cardiac ganglia.

5.1.2. Cerebral arteries

Dominant regulation of NANC vasodilator nerves and weak regulation of adrenergic vasoconstrictor nerves are unique characteristics of the cerebral circulation. There is substantial evidence to show that NO is the neurotransmitter in NANC vasodilator nerves innervating cerebral arteries.

5.1.2.1. Immunocytochemical & histochemical staining methods

Both immunocytochemical and histochemical staining methods were used to demonstrate NOS activity in cerebral arteries. It has been demonstrated that NOS-containing nerve fibers existed in NANC nerves innervating large and small cerebral arteries. Bredt et al. (1990) first found NOS in the perivascular nerve in large cerebral arteries in rats. He used the antiserum that was raised against NOS purified from the rat cerebellum. NOS-immunoreactive nerve fibers were also demonstrated in large cerebral arteries and distal cerebral arteries in dogs (Yoshida et al., 1993). They found that most fibers were distributed to the adventitia of blood vessels.

Recent studies show there are NO immunoreactive nerve fibers in the

dense network of perivascular nerves in human cerebral arteries (Nozaki et al., 1993). Positive staining of NADPH-diaphorase was found in perivascular nerves of large cerebral arteries and pial arteries and also in the sphenopalatine ganglion in rats. Sphenopalatine ganglia were demonstrated to be the main origin of NADPH-diaphorase-containing nerve fibers of cerebral arteries (Suzuki et al., 1993, 1994; Minami et al., 1994). Using the same method, Saito and Goto (1994) discovered that NADPH-diaphorase nerve fibers were present in the small cerebral arteries of guinea pigs as well. The above immunocytochemical and histochemical evidence supports the existance of NOS-containing nerve fibers and the neurotransmitter role of NO in different cerebral arteries.

5.1.2.2. Pharmacological methods

A number of pharmacological studies show that NO is a neurotransmitter in NANC vasodilator nerves innervating cerebral arteries. The first hint of nitrergic transmission of vasodilator nerves in cerebral arteries was that treatment with oxyhemoglobin and methylene blue abolished relaxation elicited by transmural electrical stimulation and nicotine (Toda, 1987; Linnik and Lee, 1989). Oxyhemoglobin is a NO scavenger and methylene blue is a sGC inhibitor. After the discovery that EDRF is NO or a closely related compound, it became clearer that nitroso-compounds mediate neurogenic vasodilation through producing cGMP.

L-NMMA and L-NNA suppressed NANC relaxation induced by transmural nerve stimulation or nicotine in isolated cerebral artery strips in dogs with or without endothelium. The effect of L-NNA is 20-30 times more

potent than the effect of L-NMMA (Toda et al., 1990; Toda and Okamura, 1990a,b,c, 1991). The effects of L-NMMA and L-NNA was reversed or prevented by L-arginine but not D-arginine. D-NMMA and D-NNA had no effect. L-NMMA and L-NNA did not affect NO- or nitroglycerin-induced vasodilation. Similar responses from L-NMMA, D- NMMA, L- arginine, D-arginine, NO and nitroglycerin were observed in EFS-induced and nicotine-induced relaxations in isolated cerebral artery strips in monkeys with or without endothelium (Toda and Okamura, 1990c; Yoshida et al., 1994). SOD did not have an effect on EFS-induced relaxations of cerebral arteries of both dogs and monkeys. These results suggest that NO is a inhibitory neurotransmitter for cerebral arteries of dogs and monkeys.

Immunoreactivity for VIP was localized in the perivascular nerves in isolated middle cerebral artery in sheep. L-NMMA suppressed both VIP-induced and stimulation-induced relaxation of the middle cerebral arteries. VIP-induced relaxation was also inhibited by hemoglobin and methylene blue and augmented by SOD and M & B 22948 (a cGMP phosphodiesterase inhibitor). The VIP-induced relaxation was not affected by removal of the endothelium. The results indicated that VIP acted indirectly by stimulating NOS, which was likely from nitrergic nerves (Gaw et al., 1991).

EFS-induced relaxation of cerebral artery ring in isolated bovine cerebral artery preparations was inhibited by L-NMMA and L-NNA.. L-arginine reversed the effects of L-NMMA and L-NNA (Gonzalez and Estrada, 1991). Superoxide dismutase (SOD) enhanced transmural nerve stimulation-induced relaxation. The inhibition did not occur when bovine cerebral arteries were denuded of endothelium, indicating that the mediator released

from the nerve fibers was endothelium-dependent. In another report, Ayajiki et al. (1993) found that L-NNA markedly reduced the stimulation-induced relaxation responses in helical strips denuded of endothelium of bovine basilar arteries. L-arginine reversed the inhibitory effect of L-NNA. L-NNA had no effect on exogenously applied NO-elicited relaxation. Both VIP and calcitonin gene-related peptides did not affect the neurally induced relaxation of basilar artery. These results indicate that NO coming from vasodilator nerves mediate neurogenic relaxation of the cerebral arteries, not VIP or calcitonin gene-related peptides. Pharmacological and physiological evidence of NO as a neurotransmitter of NANC vasodilator nerves was also found in porcine (Lee and Sarwinski, 1991; Chen and Lee, 1993, 1995), cat (Ayajiki et al., 1994) and human (Toda, 1994).

In summary, there is good evidence of a nitrergic mechanism in cerebral arteries of all mammal species. L-, but not D-, forms of NOS inhibitors (L-NMMA and L-NNA) abolished EFS- and nicotine-induced relaxations of cerebral arteries. Th effects of NOS inhibitors was reversed by L-arginine. Methylene blue and oxyhemoglobin abolished the neurally induced relaxation of cerebral arteries. There may be some different mechanisms among species. On one hand, EFS-induced relaxations of bovine cerebral artery rings were enhanced by SOD and were endothelium-dependent, suggesting that the substance released from the nerves was endothelium-dependent.On the other hand, with or without endothelium, EFS-induced relaxations in isolated cerebral artery preparations from dogs and monkeys were not affected by SOD. Meanwhile, VIP seems to be a necessary factor for nitrergic transmission in sheep isolated cerebral arteries.

5.1.3. Peripheral arteries

There is evidence to support that NO is a neurotransmitter in NANC vasodilator nerves innervating penile, mesenteric, temporal and pulmonary arteries.

The penile artery is innervated by excitatory sympathetic and inhibitory NANC nerves. Martin et al. (1991) found that L-NNA abolished stimulation-induced NANC relaxant responses of bovine penile artery. Liu et al. (1991) discovered that both L-NMMA and L-NNA abolished acetylcholine-induced relaxations of bovine penile arteries and increased the bovine penile arterial tone. L-NNA but not L-NMMA blocked NANC vasorelaxation of bovine penile arteries. These results suggested nitrergic innervation of the penile arteries.

L-NMMA increased the nerve stimulation-induced vasoconstriction in perfused dog mesenteric arteries in endothelium-denuded dog mesenteric arteries. The effect of L-NMMA was reversed by L-arginine (Toda and Okamura, 1990). L-NNA significantly reduced EFS-induced relaxation of bovine mesenteric arteries. Tetrodotoxin abolished the NANC relaxation (Leckstrom et al., 1993). Also, trypsin and chymotrypsin significantly reduced the stimulation-induced relaxation. These results indicated that NO and possible peptides mediated inhibitory NANC relaxation in bovine mesentic arteries. Ahlner et al. (1991) found that methylene blue and pyrogallol (a generator of superoxide anions) reduced, but zaprinast (a cGMP phosphodiesterase inhibitor) enhanced EFS-induced NANC relaxation after

using guanethidine and phenylephrine to raise the bovine mesenteric tone. Their results support that nitrergic transmission is involved in mesenteric arteries.

L-NNA enhanced contractions evoked by transmural electrical stimulation in temporal artery strips denuded of endothelium in dogs but had no effect on noradrenaline release and noradenaline-induced contractions (Toda et al., 1991). L-NNA decreased phentolamine-induced relaxation after electrically stimulation-induced constraction. Atropine did not affect the effect of L-NNA. L-arginine reversed the effect of L-NNA. L-NNA also inhibited NOx release from temporal arteries stimulated by electrical pulses. The results indicated that NO transmitted information from vasodilator nerves to the cerebro-arterial smooth muscle.

L-NMMA and L-NAME blocked EFS-induced relaxation in guinea-pig pulmonary arteries. EFS increased cGMP levels in tissues 3 fold. Zaprinast enhanced EFS-elicited relaxation. Methylene blue and pyrogallol inhibited the EFS-induced relaxation. The effects of methylene blue and pyrogallol were prevented by SOD. L- but not D- arginine completely reversed the inhibitory effect of L-NMMA. When pulmonary artery without endothelium is at resting tone, adrenergic contraction was enhanced by L-NMMA. The effect was also completely reversed by L-arginine. These results suggested that NO released from NANC nerve endings results in vasodilation by activating GC (Liu et al., 1992).

5.2. Respiratory tract

In addition to the classic cholinergic and noradrenergic innervations, there are also inhibitory and excitatory NANC nerves in the respiratory tract (Stretton, 1991). NOS is found to localize in inhibitory NANC nerves by immunocytochemical and histochemical staining methods and may be a inhibitory neurotransmitter of NANC nerves by using physiological and pharmacological methods.

5.2.1. Immunocytochemical & histochemical staining methods

Immunocytochemical or histochemical evidence has shown that NOS-containing nerve fibers are present in human and porcine (Diaz de Rada et al., 1993; kobzik et al., 1993), rat (Kobzik et al., 1993), guinea-pig, (Fischer et al., 1993) and ferret airways (Dey et al., 1993). For humans and pigs, NOS is immunocytochemically in a subpopulation of neurons of microganglia in the pulmonary brochi. It is also in the hilar portion of the lung along the blood vessels and in some nerve fibers in the lung (Diaz de Rada et al., 1993). Using NOS-immunoreactivity method got similar result as those observed by using NADPH-diaphorase methods (Kobzik et al.,1993). NOS-immunoreactive fibers were found in nerve structure of rat and human lung. Hassall et al. (1993) demonstrated histochemically that many paratracheal neurons were present in guinea-pig trachea. The paratracheal neurons is likely a source of NO in respiratory smooth muscle. Co-existance of NO and VIP have been detected in neurons of ferret trachea. (Dey et al., 1993). VIP was also detected in neurons of airway ganglia, suggesting that VIP nerves might originate from intrinsic neurons. These results support the possibity that NO and VIP

are co-transmitters in neurons of respiratory tract.

5.2.2. Pharmacological methods

Pharmacological evidence supports the concept that NO is a neurotransmitter in inhibitory NANC nerves innervating the respiratory tract. Tucker et al. (1990) first demonstrated that L-, but not D-, NNA reduced the NANC relaxations of guinea-pig tracheal smooth musclem in a concentration-dependent manner. L-arginine partially reversed this effect. L-NNA had no effect on VIP-induced relaxations or acetylcholine-induced constractions of the trachea. Li and Rand (1991) found similar results. L-NMMA and L-NAME decreased intramural stimulation-induced relaxations of guinea-pig tracheal smooth muscle. D-NMMA had no effect. L-NMMA and L-NAME did not affect VIP or sodium nitroprusside (a NO donor)-induced relaxations of the smooth muscle. L- but not D- arginine reversed the inhibitory effects of L-NMMA and L-NAME. VIP antibody and chymotrypsin reduced the relaxation elicited by VIP or electrical pulses. The results suggested that NO and VIP are co-transmitters of NANC relaxations of guinea-pig tracheal smooth muscle.

Kannan and Johnson (1992) found that there was an important inhibitory NANC relaxation in tracheal smooth muscle in pigs that was completely inhibited by NOS inhibitors and reversed by L-arginine (Kannan and Johnson, 1992a,b). L-NNA caused concentration-dependent inhibition of the electrical field stimulation (EFS)-induced relaxations. Prior treatment with L- but not D- arginine prevented the effect. L-NNA had no effect on relaxations of the tracheal smooth muscle induced by VIP and nicotine

agonist dimethylphenyl piperazinium chloride (DMPP). Similar results have been reported in cat airways (Fisher et al., 1993). Fisher et al. (1993) did not find VIP antagonists and VIP densentization to inhibit EFS-induced NANC relaxations of trachealis (the open or closed tracheal rings). These results indicate that NO was the principal inhibitory NANC neurotransmitter of these respiratory tract and that VIP was not likely the NANC mediator of cat trachea.

There is a prominent neural brochodilator mechanism in the human respiratory tract. NO was suggested to be a neurotransmmiter of NANC bronchodilation. L-NAME abolishes the neural brochodilation in human tracheal segments (Belvisi et al., 1992a). L-NNA almost completely blocked inhibitory NANC responses of human tracheal smooth muscle at all stimulation frequencies studied while L-NAME only inhibited low stimulation frequency. L-arginine partially reversed the inhibitory effect of L-NAME. D-arginine and D-NAME had no such effect. VIP-induced relaxations were antagonized by alpha-chymotrypsin. Inhibitory NANC responses were not affected by alpha-chymotrypsin. Also, phosphoramidon markedly enhanced low-dose VIP-induced relaxations, but had no effect on inhibitory NANC responses (Belvisi et al., 1992b). These results suggested that NO but not VIP mediated neural bronchodilator response.

Ellis and Undem (1992) found that L-NNA suppressed EFS-elicited NANC relaxations in both human central and peripheral bronchi, which were reversed by L-arginine. 3-morpholinosydnonimine (SIN-1) relaxed both human central and peripheral airways. Alpha-chymotrypsin, a peptidase, blocked VIP-induced relaxation, but had no effect on NANC relaxations in

the central bronchi.

These results suggest that nitrergic mechanism was involved in the NANC relaxation in respiratory tracts among all animals studied above. NO is the major inhibitory neurotransmitter of these tissues. VIP seems to be a co-factor or co-transmitter in the NANC relaxation in guinea-pig tracheal smooth muscle.

5.3. Urinary tract

The urinary tract is innervated by NANC motor and inhibitory nerves. There is substantial evidence to support that NO is a neurotransmitter in NANC inhibitory nerves innervating the urinary tract. The following are immunocytochemical, histochemical staining evidence and pharmacological and physiological evidence in urinary tract.

5.3.1. Immunocytochemical & histochemical staining methods

Immunocytochemical and histochemical data have revealed NOS localization in the urinary tract. In urinary tract of rats, NOS-immunoreactive nerve fibers were present in NADPH-diaphorase-positive fibers. These fibers were present in bladder wall, in the adjacent small ganglia, in the advential and muscular layers near to the urothelium and perivascular fibers. The major pelvic ganglia (MPG) also contained NADPH-diaphorase positive fibers (McNeill et al., 1992). NADPH-diaphorase positive fibers were demonstrated in afferent and postganglionic efferent pathways to the urinary bladder of the rats, although only a few bladder postganglionic

neurons in MPG were NADPH-diaphorase positive (Vizzard et al., 1993). These data indicate that NOS is in various regions of urinary tract.

NADPH-diaphorase activity and NOS-immunoreactivity were detected in nerve trunks and fine fibers in muscle bundles in the detrusor, trigone and urethra in pig urinary tract. The detrusor of pig was less innervated by NOS nerve fibers and trunks (Persson et al., 1993). In sheep urinary tract, NADPH-diaphorase positive fibers were demonstrated in the urethra and trigone, but not in detrusor and ureter. There was a direct regional correlation between EFS-elicited relaxations of precontracted urethra and trigone preparation and NADPH-diaphorase staining (Triguero et al., 1993). These results suggest the existance of NOS in urinary tract and the possibility of NO as a mediator in urinary tract.

5.3.2. Pharmacological methods

Pharmacological and physiological studies support that NO is a neurotransmitter of inhibitory NANC nerves innervating urinary tract. L-, but not D-, NMAE decreased bladder capacity and micturition volume in the rat urinary tracts. L-NMAE also increased spontaneous contraction of isolated rat urethra and detrusor muscle preparations. L-arginine prevented the effect of L-NAME. L-NAME inhibited the maximal relaxation of precontracted urethral preparations evoked by electrical stimulation. The effect of L-NAME was completely reversed by L-arginine. L-NAME had no effect on NO (present in acidified solution of NaNO2)-induced relaxations of urethral preparations (Persson et al., 1992). L-NNA inhibited the EFS-elicited relaxations of isolated external urethral sphincters in rats in dose-

dependent manner. The effect of L-NNA was reversed by L-, but not D-, arginine. L-NNA enhanced EFS-induced tonically contraction of the resting external urethral sphincter, whereas phentolamine suppressed the contraction. The results suggested that the NANC inhibitory nerves act by activating NO or an NO-containing compound (Parlani et al., 1993).

L- but not D- NNA inhibited the relaxation of isolated trigonal smooth muscle strips in pigs contracted by noradrenaline. The effect of L-NNA was concentration-dependent. At higher concentrations, L-NNA abolished all relaxations. L-NNA markedly reduced EFS-induced relaxations in trigonal preparations. L-arginine prevented the effects of L-NNA. NO induced concentration-dependent relaxations in trigonal strips contracted first by noradrenaline and carbachol. Methylene blue had no effect on EFS-induced relaxations in trigonal preparations (Persson and Andersson, 1992). L-NNA inhibited EFS-induced relaxation of pig isolated trigonal and urethral preparations. L-arginine inhibited electrically evoked detrusor contractions. The inhibition of L-arginine was reversed by L-NNA. L-NNA pretreatment caused a rightward shift of the concentration-response curves to acetylcholine (Persson et al., 1993). These results suggested that NO might be a inhibitory NANC neurotransmitter in these regions.

L-NNA suppressed the electrically induced relaxations of urethral smooth muscle strips in rabbit urinary tracts. L-arginine overcame the inhibitory effect of L-NNA. Methylene blue reduced but M & B 22948 enhanced the relaxation of the smooth muscle (Dokita et al., 1991). L-NNA and L-NMMA inhibited electrically induced relaxations of rabbit urethra in a concentration-dependent manner. At the highest concentration used,

contraction of urethra was observed. Methylene blue and SOD also affected EFS-induced relaxations. L-arginine pretreatment prevented the effect of low but not high concentrations of L-NNA. Also, L-arginine pretreatment significantly enhanced stimulation-induced relaxations of noradrenergic-precontracted rabbit urethra preparations. NO-induced relaxation was not affected by L-NNA or arginine, but was significantly reduced by methylene blue. Similar results were also observed in the isolated urethral preparations obtained from three patients (Andersson et al., 1992). James et al. (1993) found that L-NNA reduced EFS-elicited relaxations of human isolated detrusor smooth muscle. Methylene blue blocked the relaxation. These results indicated that NO is inhibitory neurotransmitter of urinary tracts of rabbit as well as of human being.

The data obtained by immunohistochemistry and NADPH diaphorase staining suggest that NOS is localized in nerve fibers of the urinary tracts. NANC-mediated relaxation, involving the L-arginine/ NO pathway, can also be demonstrated in the urinary tracts. Taken together of all evidnce in this chapter, these results indicate that NO acts as a neurotransmitter of inhibitory NANC nerves that innervate as widely as from heart, cerebral and peripheral arteries, respiratory tract as well as urinary tracts.

NITRERGIC AUTONOMIC NEUROEFFECTOR TRANSMISSION IN THE GASTROINTESTINAL SYSTEM

The gastrointestinal (GI) system is innervated by three kinds of nerves, which form a rich network of nerve supply to the system: (1) extrinsic cholinergic parasympathetic nerves coming from the cranial and pelvic regions to innervate upper and lower parts of the gut; (2) extrinsic noradrenergic sympathetic nerves coming from the thoracic and lumbar levels the spinal cord to innervate most parts of the GI tract; (3) complex neural network of the enteric nervous system subserving excitatory and inhibitory functions of the myenteric and submucous plexuses. The enteric nervous system is an intrinsic division, without direct connection with the central nervous system. The neurotransmitter for the enteric nervous system is noncholinergic nonadrenergic, with serotonin and other peptides as possible excitatory neurotransmitters and ATP, VIP and more recently NO as possible inhibitory neurotransmitters. There is immunocytochemical, histochemical, pharmacological and electrophysiological evidence to support NO as an inhibitory neurotransmitter in the gut.

6.1. Immunocytochemical and histochemical evidence for localization of NOS in the GI tract

At all levels along the GI tract, NOS activity has been detected in neurons and nerve fibers. Localization of NOS in the GI tract was first reported by Bredt et al. (1990), who found NOS in the myenteric plexus of the circular muscle in rat intestinal tracts by use of NOS immunocytochemistry method. Dawson et al. (1991) discovered that NADPH diaphorase staining neurons colocalized with NOS positive neurons in myenteric plexus of the rat intestine, suggesting the two methods of detecting NOS activity are fully consistent. Belai et al. (1992) also found colocalization of NOS and NADPH-diaphorase in the myenteric plexus of almost all regions of rat GI tract (antrum, duodenum, ileum, caecum, proximal colon and distal colon), with the circular muscle layer also staining for NADPH diaphorase in the stomach, duodenum and ileum. In the caecum and distal colon almost all the NOS-positive nerve fibers also stained for NADPH-diaphorase. The results implied that NO mediates GI tract neurotransmission.

Aimi et al. (1993) found that NADPH-positive stained neurons existed throughout the whole GI tract, from the esophagus to the rectum, of rats. Such neurons were also very common in the myenteric ganglia, in internodal strands, in the secondary and tertiary plexuses, and they were especially rich in the deep muscular plexus. Very few NADPH-positive stained neurons were found in the submucosal ganglia. The density of NOS neurons is higher in the small and large intestines than in the esophagus and stomach. The pattern of distribution of NOS neurons suggests the possibility that these positive neurons innervate muscles of the GI tract. VIP almost always colocalizes with NOS in the myenteric plexus. The results suggested that NO and VIP may be cotransmitters of NANC neurons of the enteric nervous system. Alm et al. (1993) demonstrated that NOS-immunoreactivity (NOS-IR) existed in

cytoplasm of many neurons in myenteric ganglia and in some neuron bodies in the submucosa. NOS-IR nerve fibers were found in circular muscle layer. Virtually all NOS-IR nervous structures were also NADPH diaphorase-positive. In rat gastric corpus, both the myenteric and submucosal plexuses contain NOS-positive neurons and fibers and are different from those containing VIP. The circular muscle layer is also innervated by NOS-positive fibers (Forster and Southam, 1993). They concluded that NO is a NANC transmitter associated with gastric function.

NOS-positive neurons and fibers were also verified by immunocytochemical and histochemical methods in guinea-pig GI tract. Costa et al. (1992) found many NOS immunoreactive neurons in the myenteric plexus of the guinea-pig small intestine, but few in submucous ganglia. NOS immunoreactivity was generally not present in non-neuronal cells. There are plentiful immunoreactive nerve fibers in myenteric ganglia, submucous ganglia and in the circular muscle innervated by myenteric neurons. VIP but not substance P imunoreactivity often coexisted with NOS immunoreactivity. VIP-like immunoreactivity (VIP-LI) was also found to coexist with NOS in the taenia of the guinea-pig caecum (Furness et al., 1992). In guinea-pig ileum enteric neurons, NOS immunoreactivity was localized in myenteric neurons but not associated with any subcellular organelles or membranes. NOS immunoreactive fibers have close contact with muscle cells (< 100nm) and have synaptic contacts with NOS immunoreactive and non-immunoreactive enteric neurons. Throughout the guinea-pig small and large intestine, intense NADPH diaphorase staining was found in a subpopulation of neurons in the myenteric plexus, deep muscular plexus and submucosa. Intensively stained nerve fibers were detected throughout the meshworks of the myenteric plexus,

Henle's plexus of submucosa, and circular muscle. Intensively stained neurons and nerve fibers were distributed in most myenteric ganglia but were rare in ganglia of Henle's plexus. NOS stained neural elements were more dense in distal parts than proximal parts of the intestine. In addition, submucosal blood vessels were also stained over their surface.

NOS was localized in many patch-shaped myenteric neurons with only one axon in the guinea-pig colon. In the submucosa, a few neurons were intensely stained, whereas many had only weak activity. Nerve fibers were detected in the longitudinal muscle, circular muscle, muscular mucosa as well as ganglia of two plexuses. Nerve fibers in the circular muscle and in more anally located myenteric ganglia originated from myenteric cells, and axons of myenteric neurons all extended in the anal direction. The results suggested that NOS is an inhibitory neurotransmitter to the muscle and of descending interneurons of the myenteric plexus (McConalogue and Furness, 1993). Young et al. (1992) found that there is a one-to-one correlation and similar staining intensities between NOS immunoreactivity and NADPH diaphorase staining in all neurons of the guinea-pig ileum and colon.

Throughout the porcine large intestine, NOS-immunoreactivity (IR) and NADPH-positive neurons were rich in the myenteric and outer submucous plexuses (Barbiers et al., 1993). A small number of positive neurons were found in the inner submucous plexus of the caecum and colon and mild activity was in the rectum. Nerve fibers were numerous in the circular muscle layer, scanty in the longitudinal muscle coat and negligible in the mucosal area. In all three plexuses, non-varicose and varicose NOS-IR and NADPH-positive nerve fibers were displayed in the ganglia and connecting filaments.

In porcine small intestine, many NOS-immunoreactive neurons were discovered in the external submucosal plexus (Krammer et al., 1993). NOS-immunoreactive neurons comprised 27% of total number of neurons in the myenteric plexus, whereas they were scarcely present in the internal submucosal plexus. The results indicate that NO is a messenger molecule in caecum, colon, and rectum as well as small intestine of pigs.

NOS positive neurons and fibers were also observed by histochemical methods in GI tract of other species. NADPH-diaphorase activity was discovered in GI and biliary duct systems as well as other organs innervated by peripheral autonomic nerves in mouse GI tract (Grozdanovic et al., 1992). A distinct subpopulation of neurons was labeled. The studies indicate NOS may be in the intrinsic neurons of the GI tract and other systems. In canine proximal colon, a subpopulation of neurons in myenteric and submucosal ganglia exhibited NOS-LI that also colabeled with NADPH diaphorase (Ward et al., 1992). NOS-positive nerve trunks were also found throughout the circular and longitudinal muscle layers and within the submucosal pacemaker area. Labeled neurons belong to Dogiel type I morphology. In human GI tracts, Springall et al. (1992) found that neural staining with antisera A and B was detected in the myenteric and submucous plexuses as well as in nerve fibers in smooth muscle of the GI tract. Faussone-Pellegrini et al. (1993) found that VIP-positive nerve fibers and cells were extremely scarce in human ileocecal junction, suggesting VIP only plays a minor role to regulate these junctions. All above immunocytochemical and histochemical evidence supports the neurotransmitter role of NO in the GI tracts.

6.2. Pharmacological evidence to support NO as inhibitory neurotransmitter in the GI tract

For the following discussion, the GI tract is divided into two parts: sphincters and segments between sphincters. For convenience, the pharmacological evidence for the role of NO is presented orally to anally, first for sphincters and then for segments between sphincters.

6.2.1. Sphincters

A sphincter is a circularly arranged muscle which exhibits reflex relaxation when the pressure is increased in the adjacent proximal segment and reflex contraction when the pressure is increased in adjacent distal segment.

6.2.1.1. Lower esophageal sphincter

Transmural field stimulation (TFS) elicited NANC relaxations in the isolated lower esophageal sphincter (LES) of the opossum. L-NNA reduced the relaxation in a concentration-dependent manner. TFS induced contraction in most preparations when L-NNA was used at concentration of 10 ($^{-4}$) M. This contraction was blocked by atropine. The concentration-response curve was shifted to the right by L-arginine. Neither L-NNA nor L-arginine had an effect on sodium nitroprusside (a NO donor)-induced or VIP-induced relaxation of LES. These findings suggest that arginine-derived NO is required for NANC inhibitory responses in LES (Tottrup et al., 1991a).

Electrical stimulation of the peripheral end of the right vagus nerve caused a frequency-dependent relaxation of LES, and also caused peristaltic and non-peristaltic contractions in the esophagus. L-NNA suppressed LES relaxation in a concentration-dependent manner. The effect of L-NNA was completely reversed by L-arginine. L-NNA had no effect on peristaltic contraction of the esophageal body or on resting LES pressure. Sodium nitroprusside decreased LES pressure to zero in both the control and the experimental animals (Tottrup et al., 1991b). These findings suggest that NO is a mediator of inhibitory reactions in LES.

In opossum LES, NANC nerves mediate relaxation of its circular muscle. The latencies between the cessation of the stimulus and the "off contraction" represent a gradient so that the latency is the longest in the cauded esophagus muscle. Electrical field stimulation (EFS) and NO induced LES muscle relaxation. EFS-elicited relaxation was suppressed by L-NNA and restored by L-arginine. The results suggested that NO or a NO-containing compound may be a neurotransmitter of NANC nerve-mediated relaxation of LES (Murry et al., 1991).

EFS-induced, tetrodotoxin-sensitive relaxations of canine and opossum LES were blocked by L-NAME and reversed by L-arginine. VIP-induced relaxations of both canine and opossum LES were not changed by L-NAME. Methylene blue enhanced the basal tension of the LES, but did not influence the relaxation to EFS or VIP. Methylene blue, however, blocked the NANC inhibition of opossum body circular muscle (Jury et al., 1992). The results suggested that arginine-derived NO contributed to the inhibitory neurotransmitter mediating the NANC relaxation of LES.

6.2.1.2. Pyloric sphincter

Allescher et al. (1992) discovered that intra-arterial L-NAME decreased the inhibition of pyloric activity in situ from antral field stimulation and electrical stimulation. L-arginine but not D-arginine reversed the effect of L-NAME. L-NAME also increased the pyloric contractions to intra-arterial acetycholine, and it also blocked substance P-induced NANC inhibition of the canine pylorus in vitro. Sodium nitroprusside was a potent relaxant in vitro. Bayguinov and Sanders (1993) found evidence to support NO as an inhibitory neurotransmitter in pyloric sphincter. L-NAME and L-NMMA suppressed the inhibitory junction potentials (IJPs) from cells near the circular muscle layer. L-arginine reversed this effect. Reduction in IJPs increased the amplitude of excitatory JPs (EJPs). Oxyhemoglobin reduced IJPs and totally obstructed IJPs in combination with L-NAME. Exogenous NO, 8-bromoguanosine 3', 5'-cyclic monophosphate (cGMP) and M & B 22948 (a specific cGMP phosphodiesterase inhibitor) hyperpolarized the membrane, suggesting that the NO-induced hyperpolarization response was mediated by augmented production of cGMP. The data indicated that NO or an NO-containing compound mediates enteric, inhibitory neurotransmission in the canine pyloric sphincter.

6.2.1.3. Sphincter of Oddi

The sphincter of Oddi has a typical NANC inhibitory innervation. In the sphincter of Oddi of the Australian possum, Baker et al. (1993) showed that EFS-elicited relaxation of the circular muscle strips was reduced by L-NAME. The effect of L-NAME was reversed by L-arginine but not D-arginine.

Oxyhemoglobin also decreased these relaxations. Sodium nitroprusside mimicked the relaxations in strips precontracted by erythromycin or carbachol. NADPH-diaphorase reactivity was shown in these preparations. After blocking muscarinic receptors with atropine, EFS evoked a potent inhibitory response (Allescher et al., 1993). L-NAME blocked and then reversed this inhibitory response, causing a potent stimulatory response. L-arginine abolished the excitatory response and reversed the excitatory response to inhibition. Methylene blue did not prohibit the EFS-evoked inhibitory effect in the presence of atropine and guanethidine. The results suggested that NO is a NANC inhibitory transmitter in the opossum sphincter of Oddi. Pauletzki et al. (1993) demonstrated that L-NAME markedly reduced the EFS-evoked relaxation of the sphincter of Oddi in the guinea pig in vitro. L-arginine restored the relaxation. Tetrodotoxin entirely abolished the relaxation. Sodium nitroprusside resulted in a concentration-dependent relaxation of the sphincter. The data implied that an endogenous NO is a major inhibitory NANC neurotransmitter.

6.2.1.4. Ileocolonic sphincter

The canine ileocolonic sphincter was one of the first tissues in which nitrergic transmission was studied intensively. EFS of circular strips of ileocolonic sphincter muscle caused a release of a NO-like substance (Bult et al., 1990; Boeckxstaens et al., 1990). This substance has chemical characteristics similar to NO. It is inactivated by hemoglobin, its release is inhibited by arginine analogues. Exogenous NO caused tetrodotoxin-resistant NANC relaxation from the ileocolonic sphincter (Boeckxstaens et al., 1990). L-NNA and L-NMMA inhibited the EFS-evoked NANC relaxation in a

concentration-dependent manner, and enhanced the basal tone of the ileocolonic sphincter. The effects of L-NNA and L-NMMA were partially restored by L-arginine but not D-arginine.

Bioassay (using the cascade superfusion technique) has been used to detect the EFS-induced release of NO from canine ileocolonic junction (Boeckxstaens et al., 1991b). EFS and activation of nicotinic receptors with 1,1-dimethyl-4-phenyl-piperazinium (DMPP) caused release of a labile factor. L-NNA abolished the release of the labile factor and hemoglobin eliminated the biological activity of the substance. Hexamethonium and tetrodotoxin blocked the release stimulated by DMPP. The effects of the labile factor were similar to the effects of authentic NO. ATP-, GABA-induced and electrically evoked relaxations were suppressed by L-NNA, L-NMMA and hemoglobin, which was restored by L-arginine (Boeckxstaens et al., 1991a). The results suggested that NO is the final inhibitory NANC neurotransmitter in the canine ileocolonic sphincter. There is evidence that indicates that serotonin (5-HT)-induced NANC relaxation is mediated by NO or a NO-containing substance (Bogers et al., 1991).

6.2.1.5. Internal anal sphincter

Nitrergic transmission has been verified in opossum internal anal sphincter (IAS) in vitro. Exogenous NO and neural stimulation resulted in a concentration-dependent decrease of the resting tension of IAS. L-NNA suppressed the neurally mediated decrease of the IAS tension in a dose-dependent manner; the suppression was restored by L-arginine but not D-arginine (Rattan and Chakder, 1992). Hydroquinone (a superoxide anion

generator) decreased neural stimulation-induced IAS relaxation and NO-induced fall in the IAS tension in a dose-dependent manner. Superoxide dismutase (superoxide anion scavenger) enhanced the NO-induced relaxation of IAS tension and antagonized the effect of hydroquinone (Chakder and Rattan, 1992). The direct release of NO in response to electrical stimulation of NANC inhibitory neurons of IAS was also detected (Chakder and Rattan, 1993). The data suggested that NO or NO-like substances are the inhibitory mediators of the IAS relaxation.

Nitrergic relaxation was demonstrated in the human IAS. Isolated muscle strips were mounted in a superfusion organ bath for recording isometric tension. L-NNA blocked EFS-evoked NANC relaxations of sphincter muscle strips. The effect of L-NNA was reversed by L-arginine (Burleigh, 1992; O'Kelly et al., 1993). Sodium nitroprusside caused relaxation of isolated muscle strips in a dose-dependent manner (O'Kelly et al., 1993). Oxyhemoglobin blocked the relaxations. Thus, nitrergic transmission was demonstrated in almost all kinds of sphincters in the GI tract. NO may be the inhibitory mediator in these sphincters.

6.2.2. Segments between sphincters

6.2.2.1. Esophagus

Transmural field stimulation caused the stimulation of NANC inhibitory nerves innervating the circular muscle of opossum esophageal body, but did not cause a mechanical response. A short period, called latency, also without mechanical response, came after the end of the stimulus. The latency

was followed by the "off contraction". L-NNA decreased the latency at concentration higher than 10^{-6} M in a dose-dependent manner. At concentration of 10^{-4} M, L-NNA abolished the "off contraction" but elicited a small contraction that was blocked by atropine. The effects of L-NNA were prevented by L-arginine. L-arginine had no effect on the latency. Atropine had no influence on the amplitude of the "off contraction" in control preparations (Knudsen et al., 1991). The data indicate that NANC nerves mediate the "off contraction". In opossum esophagus, the tone is low (compared to the LES muscle) and field stimulation resulted in little effect during its relaxation. L-NNA decreased the amplitude and latency of the "off contraction". L-arginine prohibited the effect of L-NNA. L-NNA weakened the gradient in the latency of the "off contraction" by narrowing latencies in distal esophageal muscle. Longitudinal muscles were not affected by L-NNA but were stimulated by cholinergic nerve activity (Murry et al., 1991).

6.2.2.2. Gastric fundus

The first evidence for a nitrergic transmission in the gastric fundus came from Li and Rand (1990). NANC relaxations in strips of rat gastric fundus were decreased by L- but not D-NMMA and restored by L- but not D-arginine. Residual responses to stimulation in the presence of the VIP antibody were further reduced by L-NMMA. Similar findings were obtained by others (Barbier and Lefebvre, 1992; Boeckxstaens et al., 1992; Lefebvre et al., 1992a). The release of an NO-like substance was observed during the nerve stimulation (Boeckxstaens et al., 1991). Both nitrergic VIP-mediated contributions are observed in the NANC relaxations in gastric fundus (Boeckxstaens et al., 1992; D'Amato et al., 1992). In the guinea-pig gastric

fundus, L-NNA reduced both long and short trains of stimulation-elicited relaxations, but it had no effect on VIP-induced relaxations (Lefebvre et al., 1992b). In dog gastric smooth muscle, L-arginine reduced the spontaneous contractions and L-NMMA while methylene blue as well as oxyhemoglobin enhanced it (Ozaki et al., 1992).

In vagally mediated relaxations of the gastric corpus, there are two components of the frequency dependent fall in intracorpus pressure to vagal stimulation: an initial fast fall followed by a slower decrease over the rest of the stimulation period. L-NAME significantly decreased the initial fast response at all frequencies of stimulation (Grundy et al., 1993). The data suggested that NO or an NO-containing compound may be inhibitory mediator of ferret stomach.

6.2.2.3. Duodenum

Nitrergic relaxation was demonstrated in dog duodenal longitudinal muscle. L-NNA inhibited EFS-evoked NANC relaxation, which was reversed by L-arginine (Toda et al., 1990; 1991). NO and nitroglycerin caused similar relaxations as EFS (Toda et al., 1990). Oxyhemoglobin blocked EFS-evoked NANC relaxation (Toda et al., 1991). NANC IJPs were recorded near the myenteric border in canine proximal duodenum (Bayguinov et al., 1992). Both IJPs and NO-induced hyperpolarization responses of membrane potential were sensitive to apamin.

6.2.2.4. Jejunum

NANC relaxations in jejunal circular muscle were suppressed by NOS inhibitors (Stark et al., 1991; 1993). Mechanical and intracellular electrical activities were recorded simultaneously from the jejunal circular layer. This was done in both human and canine circular muscle layers. Stimulation-elicited IJPs in human jejunum were composed of two components: an initial fast hyperpolarization followed by a late sustained hyperpolarization. NO suppressed the mechanical activity and elicited a hyperpolarization that mimicked the late hyperpolarization of IJPs. Only the fast hyperpolarization was observed in the canine jejunum. L-NNA and L-NAME decreased the fast hyperpolarization in canine jejunum and decreased only the late sustained hyperpolarization in human jejunum (Stark et al., 1993). The maximum IJP amplitude was similar to the maximum amplitude of NO-induced hyperpolarization. Oxyhemoglobin decreased both IJPs and NO-induced hyperpolarization in canine jejunum (Stark et al., 1991). These results indicate that NO may be a inhibitory mediator in both human and canine jejunum.

6.2.2.5. Ileum

Stimulation-evoked nitrergic responses of the ileum from human, dog and rat were composed of smooth muscle relaxation and formation of IJPs. In strips of canine terminal ileum, EFS-evoked NANC relaxations were significantly suppressed by NOS inhibitors that were restored by L-arginine. Hemoglobin blocked NO-elicited relaxations (Boeckxstaens et al., 1990; 1991). Nitrergic transmission contributed to EFS-evoked NANC relaxations

of human ileal circular muscle (Maggi et al., 1991). NO and cGMP were also demonstrated to play a key role in NANC inhibitory responses in circular and longitudinal muscles of the rat ileum in vitro (Kanada et al., 1992). EFS-produced NANC-mediated IJPs were markedly inhibited by L-NAME (Christinck et al., 1991).

6.2.2.6. Caecum

A nitrergic component of NANC relaxation was demonstrated in the circular muscle between the taenia of caecum (Shuttleworth et al., 1991). EFS-evoked relaxations were decreased by L-NNA and oxyhemoglobin up to 50%. However, Li and Rand (1989) and Knudsen and Tottrup (1992) found that L-NMMA had virtually no effect on neurogenic NANC relaxation.

6.2.2.7. Colon

The mediators of NANC relaxation of the longitudinal muscle of the rat colon depend on the portion of the colon. In the proximal portion inhibitory transmission is mainly nitrergic; in the distal portion VIP is the most likely inhibitory transmitter; the nature of the mediator between proximal and distal portion is not clear yet (Suthamnatpong et al., 1993). Nitrergic transmission is essential for inhibition in the rat circular muscle of proximal colon (Hata et al., 1990). In the circular muscle of rat distal colon, L-NMMA enhanced spontaneous contractions (Middleton et al., 1993). In the human colon, L-NNA suppressed only part of NANC relaxation (Burleigh, 1992). Nitrergic transmission mediated the EFS-elicited hyperpolarization of smooth muscle in the dog (Dalziel et al., 1991; Huizinga et al., 1992) and

human (Keef et al., 1993) colon. NOS inhibitors and oxyhemoglobin abolished and NO mimicked the hyperpolarization. Nevertheless, apamin (not L-NAME and oxyhemoglobin) inhibited the fast hyperpolarization of circular smooth muscle in the human colons (Keef et al., 1993). In the circular smooth muscle from guinea-pig (Maggi and Giuliani, 1993) and human (Boeckstanes et al., 1993) colon, NANC relaxations are also mediated by an apamin-sensitive transmitter (probably ATP) and also by an apamin-resistant transmitter, the effect of which was decreased or blocked by L-NNA.

In summary, nitrergic transmission has been demonstrated to exist in all regions of GI tract (in sphincters and in segments between sphincters), and to play an important role in NANC relaxations in these regions. There is immunocytochemical and histochemical evidence as well as pharmacological and physiological evidence for this conclusion.

6.3. NO-mediated IJPs support NO as a mediator of GI tract

Stimulation with single puslses elicits IJPs in the smooth muscle cells in tissues that exhibit nitrergic mechanism: esophagus, small intestine and colon.

6.3.1. NANC IJPs in esophagus

In the opossum esophagus, electrical field stimulation (EFS) of the circular smooth muscle caused hyperpolarization followed by depolarization of the membrane potential recorded with glass microelectrodes. L-NNA inhibited the amplitude of the hyperpolarization, the depolarization, and the

time to utmost depolarization. L-arginine counteracted the effect of L-NNA. The resting membrane potential was not influenced by L-NNA and L-arginine. Exogenous NO induced hyperpolarization of the membrane potential and decreased the hyperpolarization and depolarization evoked by EFS. The effect of NO was not changed by L-NNA and tetrodotoxin (Du et al., 1991). The results indicated that NO may be the mediator of NANC nerve-evoked hyperpolarization of the esophageal smooth muscle.

Electrophysiological studies using microelectrodes showed that NANC relaxation was related to IJPs (Jury et al., 1992). They were suppressed by L-NAME and reversed by L-arginine but not D-arginine. L-NAME also blocked EFS-evoked NANC relaxations. Sodium nitroprusside induced hyperpolarization of the muscle membrane that was not affected by L-NAME. VIP had no influence on the membrane potential. The results suggested that arginine-derived NO is responsible for the IJPs and the relaxations of canine and opossum LESs.

6.3.2. NANC IJPs in small intestine

IJPs with multple components occur in guinea-pig ileum (Lyster et al., 1992; He and Goyal, 1993), dog ileum (Christinck et al., 1991), and dog and human jejunum (Stark et al., 1991; 1993). There was a fast component that was abolished by apamin (Lyster et al., 1992) and therefore was probably ATP-mediated (He and Goyal, 1993) in circular muscle from guinea-pig. Another component of IJP was apamin-resistant and markedly decreased by L-NNA and restored by L-arginine (Lyster et al., 1992). Christinck et al. (1991) found that L-NAME markedly inhibited EFS-evoked IJPs in strips of

circular muscle from the dog ileum. EFS induced NANC IJPs and suppressed spontaneous contractions. Similarly, infusion of NO solutions induced transient hyperpolarization and inhibited spontaneous contractions that were unaffected by atropine, propranolol, phentolamine and tetrodotoxin (Stark et al., 1991). L-NMMA decreased NANC IJPs but not NO-induced responses that were reversed by L-arginine. Oxyhemoglobin decreased both NANC IJPs and NO-induced hyperpolarizations. Stark et al. (1993) also noted that EFS-evoked IJP in the human jejunum consists of two components: an initial fast hyperpolarization followed by a late sustained hyperpolarization. Only a fast hyperpolarization in the canine jejunum was observed by the field stimulation. L-NNA and L-NAME decreased the initial fast hyperpolarization in canine jejunum but decreased only the late sustained hyperpolarization in human jejunum, indicating different mechanisms were involved in NO-mediated neural inhibition in circular muscle of the two species.

6.3.3. NANC IJPs in colon

NANC IJPs were evoked by EFS in the myenteric border between the longitudinal and circular layers. Nitrergic transmission was demonstrated to play a role in production of NANC IJPs in the canine proximal colon. The IJPs were decreased by L-NAME and restored by L-arginine (Dalziel et al., 1991). Oxyhemoglobin abolished NO- and S-nitrosocysteine-induced hyperpolarization responses as well as NANC IJPs. NO increased the probability of Ca(2+)-activated K+ channels being open in isolated smooth muscle of canine proximal colon, which was the cause of NO-mediated IJPs and hyperpolarization (Thornbury et al., 1991). EFS-evoked NANC responses

were recorded from the submucosal border of the canine proximal colon, which consisted of hyperpolarization of membrane and reduction in slow-wave. Poststimulus excitatory (rebound) response was composed of depolarization and increased excitability. NO mimicked NANC responses, first hyperpolarization followed by rebound excitation. L-NAME and tetrodotoxin abolished neurogenic NANC responses while oxyhemoglobin abolished both NANC responses and NO-induced responses (Ward et al., 1992). These data indicate that NO may be a mediator of NANC IJPs in colon.

PATHOPHYSIOLOGICAL IMPLICATIONS OF NO IN THE AUTONOMIC NITRERGIC SYSTEM

Unraveling the pathophysiology of the NO and cGMP pathways has just begun. NO contributes to altered motility and sphincter function in the gut and urinary tract, and to impaired reactivity of airways and blood vessels. There is evidence that abnormalities of peripheral nitrergic nerves are the major reason for GI diseases, such as Hirschsprung's disease, infantile hypertrophic pyloric stenosis and achalasia, and for genitourinary disease such as impotence. The abnormalities include defect in or lack of nNOS of NANC nerves. NO is also involved in other diseases such as diabetes neuropathy, asthma, hypertension and cerebral ischemia. The pathophysiology of these diseases is related to the autonomic nervous system.

7.1. Hirschsprung's disease

Hirschsprung's disease is a congenital disease characterized by aganglionosis or hypoganglionosis in the distal bowel (usually colon) leading to non-propulsive nonrelaxing mechanical obstruction in the distal intestine. NANC nerves, which use NO as their neurotransmitter, are reponsible for relaxing smooth muscle in the the GI tract.

The lack of NO-producing nerve fibers in the aganglionic intestine has been found to be the cause of failure of the peristalsis and failure of the relaxation of the smooth muscle in Hirschsprung's disease. Vanderwinden et al. (1993) first suggested that NO was involved in the motility disorder and pathophysiology of Hirschsprung's disease. They studied the nNOS in the ganglionic and aganglionic human gut. NOS was missing in the myenteric plexus and musculature region of the aganglionated segments while mild staining was observed in the hypertrophied nerve bundles of the submucosa. NOS was strongly present in the ganglionated segment like those of the normal colon. Bealer et al. (1994a) found much less NOS activity in the aganglionic colon than in the ganglionated colon by using the [3H]arginine-to-[3H]citrulline conversion assay. This difference in NOS activity was especially obvious in the circular muscle layer.

An total absence of NOS activity was discovered in both the myenteric and submucosal plexuses in aganglionated gut and NOS localization presence in both areas of the ganglionic bowel (O'Kelly et al., 1994). Similar observations were made by Larsson et al. (1994, 1995) and Cuffari et al. (1993). Cuffari et al. (1993) suggested that NADPH-diaphorase staining could be used as a simple routine in the differential diagnosis of Hirschsprung's disease.

There is also pharmacological evidence to support that NO is involved in the pathophysiology of Hirschsprung's disease. Tomita et al. (1995) found that NANC inhibitory nerves functioned in normal human colons, but did not function in the aganglionated colon. Also, L-NNA reduced the EFS-induced relaxation in the normal colon in a concentration-dependent manner, but had

no effect on aganglionated colon. L-arginine reversed the inhibitory effect of L-NNA in normal but not aganglionated colon. These results support the hypothesis that lack of NOS in aganglionated colon is the major cause for Hirschsprung's disease.

The internal anal sphincter seems to have no relation with Hirschsprung's disease. Bealer et al. (1994b) demonstrated that the inability of the internal anal sphincter (IAS) to relax in Hirschsprung's disease is unrelated to NO and the NANC nervous system.

7.2. Infantile hypertrophic pyloric stenosis

Infantile hypertrophic pyloric stenosis is a disease with characteristics of an enlarged pyloric muscle and gastric-outlet obstruction. It often occurs in the first few weeks after birth. Pylorospasm (defect of pyloric relaxation) results in the gastric-outlet obstruction. Since NO is a neurotransmitter that produces relaxation of the GI tract, lack of or dysfunction of NOS may be responsible for pylorospasm leading to infantile hypertrophic pyloric stenosis.

Vanderwinden et al. (1992) suggested that the lack of NOS in pyloric tissue is the cause of pylorospasm. Using the NADPH-diaphorase histochemical method, they discovered that in the pyloric tissues from patients with infantile hypertrophic pyloric stenosis, the enteric nerve fibers in the hypertrophied circular musculature were distorted and enlarged. The NADPH-diaphorase activity in the longitudinal musculature and myenteric plexus was absent. In contrast, there was a strong NADPH-diaphorase activity in the nerve fibers of circular musculature, longitudinal musculature

and nerve bundles of myenteric plexus in the pyloric tissues from the control patients.

A "knock out" nNOS gene experiment by Huang et al. (1993) supported Vanderwinden's observations. Homologous recombination caused the production of mutant mice without the nNOS gene. nNOS immunoreactivity and NADPH-diaphorase staining were not present in the mutant mice. Although there is low activity of nNOS from other catalytic enzymes, these mice appeared to have normal central nervous system function and fertility. But, there were important changes including a grossly enlarged stomach, hypertrophy of the pyloric sphincter and circular musculature, similar to those findings in human infantile hypertrophic pyloric stenosis. In addition, gastric-outlet obstruction was observed and no NADPH-diaphorase neurons were found in the pyloric tissues. These results indicated that lack of NOS in the pylorus is the cause of infantile hypertrophic pyloric stenosis.

7.3. Achalasia

Achalasia is a disorder that causes incomplete relaxation of the lower esophageal sphincter leading to esophageal obstruction. Aperistalsis, enlargement of the esophagus and bleeding are other features that impair esophageal function. Abnormal inhibitory NANC nerve regulation of the lower esophageal sphincter is likely to be the cause of esophageal dysfunction. Because NO is a neurotransmitter of the inhibitory NANC nerves innervating the lower esophageal sphincter, lack of NO function was suggested to be the cause of achalasia.

Mearin et al. (1993) studied the change of NOS activity in the gastro-esophageal junction of achalasia invalids. Tissue samples at the gastro-esophageal junction were obtained from eight patients exhibiting achalasia and six non-achalasia control patients during esophagectomy. NOS activity was absent in samples from the achalasia patients, but was detected in samples from the control patients. NOS immunohistochemical studies confirmed this finding.

Sodium nitroprusside (SNP, a NO donor) relaxed precontracted muscle strips from both control and achalasia samples. These results support the hypothesis that NO is involved in the pathogenesis of achalasia (Guslandi, 1994). Using the NADPH-diaphorase staining method, Hirakawa et al. (1995) discovered the lack of NOS activity in the internal anal sphincter (IAS) muscle of patients with achalasia, but intensive staining of NADPH-diaphorase in IAS muscle of the normal controls. Acetylcholinesterase-positive nerve fibers were found to be more dense in the muscle of IAS achalasia patients than in the normal IAS muscle. The data suggested that the absence of nitrergic nerves is leading to IAS achalasia.

In another report, however, Mosley et al. (1994) did not find significant change of VIP- and NOS-containing fibers from a patient with a long history of achalasia compared with non-achalasia controls. In contrast, calcitonin gene-related peptide (CGRP)-containing nerve fibers were unusually rich. They suggested that CGRP is the cause of achalasia.

7.4. Impotence

Impotence is one of the major problems for adult men. Dysfunction of NOS may be the underlying mechanism of impotence. Low levels or absence of NO have been demonstrated in some forms of impotence (Rajfer et al., 1992; Patel et al., 1994; Truss et al., 1994; Burnett, 1995). Pickard et al. (1995) found that stimulation-induced relaxation of isolated human corpus cavernosum resulted in significant increased production of NO and cGMP, whereas the relaxation and NO formation were decreased in tissue from impotent patients. Brock et al. (1993) reported that NOS positive staining correlated significantly with a clinical history of cavernous nerve integrity. NADPH diaphorase may be a new tool to indicate the cavernous nerve integrity of patients and direct diagnosis of neurogenic impotence. Garban et al. (1995) found that aging-related impotence in old rats is due to diminishing penile NOS activity. Also, Carrier et al. (1995) found that radiation-induced reduction of NOS-containing nerves may be the cause for erectile dysfunction in rats. These results suggest that decreased NO production is responsible for the erectile dysfunction.

7.5. Diabetes

Autonomic neuropathy is a complication of diabetes. It causes dysfunction of cardiovascular, genitourinary or GI systems, leading to postural hypotension, impotence and diabetic diarrhoea. Changes in diabetic autonomic neuropathy include alterations of myenteric innervation and abnormal neuromuscular neurotransmission and abnormal myogenic reactivity to neurotransmitters. Butterfield (1993) noted autonomic pathways

are the first to be destroyed, leading to various abnormal vascular reactions. H523, a powerful hypoglycaemic substance, acts by affecting NO synthesis. Stevens et al. (1994) reported that both metabolic and vascular reasons are responsible for the pathogenesis of diabetic neuropathy. Vasodilators, aldose reductase inhibitors as well as (Na^+, K^+)-ATPase increase the nerve conduction velocity and blood flow in the streptozocin (STZ)-induced diabetic rat. NADPH is a cofactor for both NOS and aldose reductase. L-NAME reversed the increased nerve conduction velocity induced by aldose reductase inhibtor in acutely diabetic neuropathy rats. Extended use of L-NAME fully recovered the nerve conduction speed and (Na^+, K^+) ATPase activity Stenvens (1995). It is suggested that NO may provide a bridge linking divergent processes of diabetic neuropathy.

In early experimental diabetes, metabolic defects may cause an inhibition of NO synthesis in sympathetic ganglia and vascular endothelium with decreased nerve blood flow. NO may play a role in diabetic defects of somatic nerve metabolism, leading to reduced activity of the nerve Na/K-ATPase and thus affect nerve conduction speed. Kihara and Low (1995) found that inhibition of NOS and endothelin cause decreased the microvascular endothelium blood flow supplying the sciatic nerve in experimental diabetic neuropathy (EDN). L-NNA reduced inhibition of NBF in EDN, which was reversed by L-arginine. Hyperglycemia inhibits NOS. The results suggested that abnormal vasoreactivity resulted from impaired NOS activity and increased endothelin activity in EDN.

7.6. Asthma

Neural control of the airways and abnormalities of NANC neurogenic mechanisms may be responsible for the pathophysiology of asthma. Brochodilation is controlled by inhibitory NANC nerves. NO is demonstrated to be an inhibitory neurotransmitter of NANC nerves innervating the respiratory tract. There is also other evidence that lack of NOS is involved in the pathogenesis of asthma (Persson and Gustafsson, 1993; Hamid et al., 1993). Although NANC relaxation in tissues from mild asthma patients was not changed (Belvisi et al., 1993), NANC relaxation was markedly reduced in tissues from cystic fibrosis patients. That NO released from NANC nerves is degraded by inflammatory mediators may be the reason of impairment of inhibitory NANC function. Airway inflammation activates the release of superoxide anions from inflammation cells which block NO actions (Rubanyi et al., 1986; Lammers et al., 1993).These data suggested that lack of the NOS may be a mechanism involved in the brochoconstriction associated with asthma.

7.7. Hypertension

Hypertension is a vascular disease characterized by high blood pressure in which neural mechanisms may play a role. Toda et al. (1993) detected that L-NNA but not D-NNA resulted in sustained increase in systemic blood pressure and a decrease of heart rate in dogs. Hexamethonium (a ganglionic blocking agent) signifiantly reduced or abolished the L-NNA-induced hypertension. The results indicated that NOS inhibitor-induced hypertension is associated with an elimination of nitrergic

neural function rather than damage of the basal release of NO from the endothelium. There is evidence that NO produced in peripheral nerves may modulate vascular function by inhibiting noradrenaline release from sympathetic nerves (Shudo, 1994). Cunha et al. (1993) demonstrated that the sympathetic nervous system is a major factor for the L-NAME-induced chronic hypertension. These above results suggested that NOS inhibitor-induced hypertension may be directly related with blocking perivascular nitrergic nerve function rather than inhibiting the basal release of NO from the endothelium, possibly by suppressing sympathetic nervous function.

7.8. Cerebral ischemia

NO is demonstrated to be associated with NANC vasodilator nerves in the cerebral arteries. It mediates cerebral vasodilation with NOS activation. Activation of NO-mediated nerve fibers causes increases of cerebral blood flow (Faraci et al., 1994). NO has both beneficial and detrimental effects on cerebral ischemia (Choi, 1993). The beneficial effects of increased NO production include: maintenance of cerebral blood flow, inhibition of platelet aggregation and adherence. NO may mediate both NMDA- and glutamate-induced neurotoxicity by hypoxia (Dawson et al., 1993; Cazeville et al., 1993). Dalkara et al. (1994) suggested that neuronal NO overproduction may mediate neurotoxicity of focal cerebral ischemia. Enhanced NO formation blocks NMDA receptors whose excessive activation may mediate cellular damage. The detrimental effects of NO production include: NO is a cytotoxicity entity; its excessive production will damage the cells directly or form very toxic peroxynitrite with SOD. These data suggest the possibility

that NO may be involved in both recovery and pathogenesis of cerebral ischemia (Faraci et al., 1994).

In summary, abnormalities of peripheral nitrergic nerves within the autonomic nervous system have been shown in patients with Hirschsprung's disease, infantile hypertrophic pyloric stenosis and achalasia and may be the major cause of these diseases. Abnormalities of the arginine-nitric oxide pathway also may contribute to pathogenesis of impotence, diabetes, asthma, hypertension and cerebral ischemia.

CONCLUSIONS

A. NO:

1. is a gas with many biological roles.

2. is a neurotransmitter in nonadrenergic noncholinergic neurons of the autonomic nervous system and is a neurotransmitter or neuromodulator in autonomic ganglia.

3. Inhibits peristalsis, relaxes gastrointestinal sphincters and adaptively relaxes stomach.

4. Relaxes smooth muscle in the respiratory airways, causing them to dilate.

5. Relaxes cerebral vascular muscle.

6. Relaxes smooth muscle of the upper and lower urinary tract and causes penile erection.

7. Is the first of a new class of atypical neurotransmitters. It is not stored in synaptic vesicles, but is produced on demand. It does not act on receptors on synaptic membranes.

B. Defects of the arginine-NO pathway or abnormalities of peripheral nitrergic nerves might contribute to many diseases involving the autonomic nervous system. More research is needed to elucidate the role of NO in physiological and pathophysiological processes in the autonomic nervous system.

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GLOSSARY

ACh: abbreviation for acetylcholine. The acetic ester of choline, the neurotransmitter substance at cholinergic synapses, which causes cardiac inhibition, vasodilation, gastrointestinal peristalsis, and other parasympathetic effects; it is liberated from preganglionic and postganglionic endings of parasympathetic fibers and from preganglionic fibers of the sympathetic as a result of nerve injures, whereupon it acts as a transmitter on the effector organ; it is hydrolyzed into choline and acetic acid by acetylcholinesterase before a second impulse maybe transmitted.

ADP: adenosine 5'-diphosphate. A condensation product of adenosine with pyrophosphoric acid, formed from ATP by the hydrolysis of the terminal phosphate group of the latter compound.

cAMP: adenosine 3', 5'-cyclic monophosphate. An activator of phosphorylase kinase and an effector of other enzymes formed in muscle from ATP by adenylate cyclase and broken down to 5'-AMP by a phosphodiesterase; sometimes referred to as the "second messager". A related compound (2', 3') is also known.

cGMP: guanosine 3', 5'-cyclic monophosphate. An analog of cAMP, a second messenger for NO and atrial natriuretic factor.

DMPP: dimethylphenylpiperazinium. A highly selective stimulant of autonomic ganglionic cells; used experimentally.

EDRF: abbreviation for endothelium-derived relaxing factor.

EFS: abbreviation for electrical field stimulation.

FAD: flavin adenine dinucleotide. A condensation product of riboflavin and adenosine 5'-diphosphate; the coenzyme of various aerobic dehydrogenase, e.g., D-amino-acid oxidase and aldehyde dehydrogenase; strictly speaking, FAD is not a dinucleotide since it contains a sugar alcohol.

FMN: flavin mononucleotide; riboflavin 5'-phosphate. The coenzyme of a number of oxidation-reduction enzymes; e.g., NADH dehydrogenases and L-amino acid oxidase; strictly speaking, FMN is not a nucleotide since it contains a sugar alcohol instead of a sugar.

GABA: r-aminobutyric acid. A constituent of the central nervous system; quantitatively the pricipal inhibitory neurotransmitter. Used in the treatment of a number of disorders (e.g., epilepsy).

GC: guanylate cyclase. Analogous to adenylate cyclase, but cyclizing guanosine 5'-triphosphate to guanosine 3', 5'-cyclic monophasphate and also producing pyrophosphate; activated by nitric oxide.

GDP: guanosine 5'-diphosphate. Guanosine esterified at its 5' position with diphosphoric acid; bound tightly in microtubules.

GTP: guanosine 5'-triphosphate. An immediate precursor of guanine nucleotides in RNA; similar to ATP, has a crucial role in microtubule formation.

IJP: inhibitory junction potential. Hyperpolarization of smooth muscle produced by stimulation of inhibitory nerves.

LES: acronym for lower esophageal sphincter.

MPO: abbrivation for myenteric potential oscillation.

NADP: abbreviation for nicotinamide adenine dinucleotide phosphate.

NADP+: abbreviation for nicotinamide adenine dinucleotide phosphate.

NADPH: abbreviation for nicotinamide adenine dinucleotide phosphate (reduced form).

NAME: abbreviation for N-nitro-L-arginine methy ester, an arginine analog, a NOS inhibitor.

Neurotransmitter: Any specific chemical agent (including acetylcholine, 5 amines, 4 amino acids, 2 purines and more than 28 peptides) released by a presynaptic cell, upon excitation, that crosses the synapse to stimulate or

inhibit the postsynaptic cell. More than one maybe released at any gioven synapse. The neurotransmitters released by presynaptic cells may modulate transmitter release from presynaptic cells. NO may be a retrograde neurotransmitter, released from postsynaptic cells, to act on presynaptic cells.

NO: nitric oxide. A colorless, free-radical gas; it reacts rapidly with O_2 to form other nitrogen oxides (e.g., NO_2, N_2O_3, and N_2O_4) and ultimately is converted to nitrite (NO_2-) and nitrate (NO_3); physiologically, it is a naturally occuring vasodilator (endothelium-derived relaxing factor) derived from L-arginine in endothelial cells, macrophages, neurotrophils, platelets, etc. A gaseous mediator of cell-to-cell communication formed in bone, brain, endothelium, granulocytes, pancreaic b-cells and peripheral nerves by a constitutive nitric oxide synthase. In hepatocytes, kupffer cells, macrophages, and smooth muscle it is formed by an inducible nitric oxide synthase (e.g., induced by endotoxin). NO activates soluble guanylate cyclase. In endothelial cells it is an endothelium-derived relaxing factor (EDRF); it mediates penile erection, and may be the first known retrograde neurotransmitter. Physiologically, the short-lived NO molecule is manufactured by tissues, and plays a role in various processes, primarily by interacting between endothelium and smooth muscle cells. It is involved in dilation of blood vessels and penile erection, and possibly affects immune reactions and memory. Shortage or inactivation of NO may contribute to high blood pressure and formation of atherosclerotic plague. An excess of NO, which is a free radical, is toxic to brain cells, and NO is also responsible for the precipitate, often fatal, drop in blood pressure accompanying septic shock. The question of NO's medical importance represents a growing area of interest.

NO reductase: nitric oxide reductase. An enzyme oxidizing N_2 with some acceptor to 2NO; a first step in the fixing of atmospheric nitrogen by bacteria.

NOS: nitric oxide synthase. An enzyme that catalyzes the reaction of L-arginine with $2O_2$ AND 1.5 NADPH to form NO, L-citrulline, 1.5 NADP+, and $2H_2O$; there is both an inducible and a constitutive form of this enzyme, the latter requiring calmodulin. Both forms of enzyme play significant roles in vasodilation, renal function, vascular tone, etc. The constitutive form of the enzyme in bone, brain, endothelium, granulocytes, pancreatic b-cells, and peripheral nerves is calcium-calmodulin dependent. In brain the enzyme is cytosolic, in endothelium it is membrane-bound. The inducible form of the enzyme (e.g., by endotoxin) in hepatocytes, kupffer cells, macrophages, and smooth muscle is not calcium-calmodulin dependent.

NMDA: abbreviation for N-methy-D-aspartate; or excitatory amino acid used to identify a specific subset of glutamate (an excitatory amino acid) receptors.

PDE: phosphodiesterases. Enzymes cleaving phosphodiester bonds, such as those in cAMP or between nucleotides in nucleic acids, liberating smaller poly- or oligonucleotide units or mononucleotides but not inorganic phosphate.

PK: protein kinase. A class of enzymes that phosphorylates other proteins; many of these kinases are responsive to other effectors (e.g., cAMP, cGMP, insulin, epidermal growth factor, calcium and calmodulin, calcium and phospholipids, etc.)

PKC: protein kinase c. A multifunctional protein kinase phosphorylates serine and threonine residues in many target proteins. PKC is enzymatically active only in the presence of Ca^{2+} and phosphatidyl serine.

SOD: superoxide dismutase. An enzyme that the dismutation reaction; there are three isozymes of SOD; an extracellular form (ECSOD) that contains copper and zinc, a cytoplasma form that also contains copper and zinc, and a mitochodrial form that contains manganese; a deficiency of SOD is associated with amyotropic lateral sclerosis.

Vasoactive: Influencing the tone and caliber of blood vessels.