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The Pharmacology of Nitric Oxide in the Peripheral Nervous System of Blood Vessels

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***Abstract*——Unanticipated, novel hypothesis on nitric oxide (NO) radical, an inorganic, labile, gaseous molecule, as a neurotransmitter first appeared in late 1989 and into the early 1990s, and solid evidences supporting this idea have been accumulated during the last decade of the 20th century. The discovery of nitrergic innervation of vascular smooth muscle has led to a new understanding of the neurogenic control of vascular function. Physiological roles of the nitrergic nerve in vascular smooth muscle include the dominant vasodilator control of cerebral and ocular arteries, the reciprocal regulation with the adrenergic vasoconstrictor nerve in other arteries and veins, and in the initiation and maintenance of penile erection in association with smooth muscle relaxation of the corpus cavernosum. The discovery of autonomic efferent nerves in which NO plays key roles as a neurotransmitter in blood vessels, the physiological roles of this nerve in the control of smooth muscle tone of the artery, vein, and corpus cavernosum, and pharmacological and pathological implications of neurogenic NO have been reviewed. This nerve is a postganglionic parasympathetic nerve. Mechanical responses to stimulation of the nerve, mainly mediated by NO, clearly differ from those to cholinergic nerve stimulation. The naming "nitrergic or nitroxidergic" is therefore proposed to avoid confusion of the term "cholinergic nerve", from which acetylcholine is released as a major neurotransmitter. By establishing functional roles of nitrergic, cholinergic, adrenergic, and other autonomic efferent nerves in the regulation of vascular tone and the interactions of these nerves in vivo, especially in humans, progress in the understanding of cardiovascular dysfunctions and the development of pharmacotherapeutic strategies would be expected in the future.**

I. Introduction

The presence of nonadrenergic, noncholinergic (NANC[1]) inhibitory nerves was discovered in various smooth muscles in the1970s and 1980s. In some tissues, substance P, vasoactive intestinal peptide (VIP), calcitonin gene-related peptide (CGRP), ATP, and other endogenous relaxing substances were reported to be neurotransmitters (Burnstock, 1972; Lundberg, 1981; Owman, 1988). However, most of the NANC inhibitory neurotransmitters were not identified for many decades. The discovery of nitric oxide (NO) as a novel mediator of intercellular signal transmission (Ignarro et al., 1987; Furchgott, 1988; Moncada et al., 1988a), the determination of the NO-synthesizing process (Moncada et al., 1988b; Palmer et al., 1988a), discovery of NO synthase (NOS) inhibitors (Palmer et al., 1988b) and isolation of neuronal NOS (nNOS; Bredt and Snyder, 1990) that enabled the production of nNOS antiserum represented a major breakthrough toward understanding the mechanisms underlying inhibitory responses to NANC nerve activation. Putative neurotransmitters of peptides, amino acids, amines, acetylcholine, and nucleotides have been reevaluated, and some of them have been confirmed to be true, whereas others have required reconsideration. Despite the fact that there are still unidentified neurotransmitters, the discovery of nerves liberating NO that mediates the inhibitory response is an undoubtedly epoch-making discovery.

NANC vasodilator nerves were first discovered in dog cerebral arteries (Toda, 1975), and 15 years later, a hypothesis that NO acts as a neurotransmitter was raised (Toda and Okamura, 1990a,b). Similar conclusions were also reached by independent groups using the canine ileocolonic junction (Bult et al., 1990) and duodenum (Toda et al., 1990a), bovine retractor penis muscle (Gillepsie and Sheng, 1990), rat anococcygeus muscle (Gillepsie et al., 1989; Li and Rand, 1989) and guinea pig tracheal muscle (Tucker et al., 1990). These discoveries led us to consider ideas that defied conventional dogma that neurotransmitters are organic, relatively high molecular weight molecules and that the neurotransmission process involves neurotransmitters that are stored in nerve terminals, and upon their release would interact with membrane receptive sites. On the basis of long-term investigations in not only vasculature but also other smooth muscle on NO-mediated neurogenic response, the hypothesis of NO neurotransmission is now widely accepted as an important control mechanism in functions of autonomically innervated organs and tissues. The nerve whose transmitter function depends on the release of NO is called "nitroxidergic" (Toda et al., 1991b; Toda and Okamura, 1991a, 1992c) or "nitrergic" (Rand, 1992). However, the NO Nomenclature Committee of the International Union of Pharmacology (chairman: Paul M. Vanhoutte) has chosen "nitrergic" as the official name (Moncada et al., 1997).

Histological studies have revealed that vascular smooth muscle is innervated by neurons containing NOS immunoreactivity (Bredt et al., 1990), as well as by those containing norepinephrine/tyrosine hydroxylase and cholinesterase/choline acetyltransferase. Functionally, nitrergic nerves would be more important in vasculature than cholinergic nerves, which play only a role in modulating adrenergic and nitrergic nerve functions. The cerebral artery and corpus cavernosum have unique characteristics of an intense nitrergic dilatation with a minimal adrenergic contraction. Therefore, the role of an intercellular messenger NO in the peripheral nervous system in the vasculature is a crucial and current topic in Pharmacology, Toxicology, Clinical Pharmacology, Physiology, Pathophysiology, and Clinical Medicine.

This review article covers areas of research on vasodilatation mediated by NO from perivascular nerves in vitro and in vivo, on the pathophysiological implication of neurogenic NO, and possible development of new therapeutic strategies in reference to nerve-derived NO. A section on the corpus cavernosum and penile erection will be included in this review, because penile erection is controlled by blood inflow from the penile artery and by blood outflow to the penile vein, because endothelial cells covering cavernous smooth muscle have similar properties with those of arteries and veins, and because functional characteristics of the dilator nerve are quite similar to those in the cerebral artery.

II. Discovery of Nitrergic (Nitroxidergic) Nerve in Blood Vessels

A. From Nonadrenergic Noncholinergic Nerve to Nitrergic Nerve

NANC vasodilatation due to nerve stimulation was discovered in dog cerebral arteries by the use of nicotine (Toda, 1975). Supportive evidence was obtained in iso-

[1]Abbreviations: NANC, nonadrenergic, noncholinergic; EDRF, endothelium-derived relaxing factor; NO, nitric oxide; NOS, NO synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; oxyHb, oxyhemoglobin; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; BH_4, tetrahydrobiopterin; SOD, superoxide dismutase; PDE V, phosphodiesterase type 5; C_6, hexamethonium; TTX, tetrodotoxin; PTIO, 2-phenyl-4,4,5,5-tetra-methylimidazoline-1-oxy-3-oxide; KN62, [*S*]-5-isoquinoline sulfonic acid,4-[2[(5-isoquinolinyl-sulfonyl)methyl-amino]-3-oxo-(4-phenyl-1-piperazinyl)propyl]phenyl ester; L-NMMA; N^G-monomethyl-L-arginine; L-NA, N^G-nitro-L-arginine; D-NA, N^G-nitro-D-arginine; L-NAME, N^ω-nitro-L-arginine methyl ester; CGRP, calcitonin gene-related peptide; VIP, vasoactive intestinal peptide; ADMA, N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine); SDMA, N^G,N'^G-dimethyl-L-arginine (symmetric dimethylarginine); protein kinase G, cyclic GMP-dependent protein kinase; protein kinase A, cyclic AMP-dependent protein kinase; Ca/CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; IP_3, inositol trisphosphate; RVLM, rostral ventrolateral medulla; CVLM, caudal ventrolateral medulla; NTS, nucleus tractus solitarius; PVN, paraventricular nucleus; ED, erectile dysfunction; PIN, protein inhibitor of nNOS.

lated feline cerebral arteries applied transmural electrical stimulation (Lee et al., 1975). Transmural electrical stimulation and nicotine induced similar relaxations in human, monkey (Toda, 1981, 1982), and sheep cerebral arteries (Duckles et al., 1977). Pharmacological studies have suggested that substances, such as prostanoids, ATP and histamine, and electrogenic Na^+ pump do not participate in the response (Toda, 1975, 1978). There is considerable literature suggesting the involvement of acetylcholine (Vasquez and Purves, 1977; Bevan et al., 1982a,b), VIP (Lundberg et al., 1979; Lee et al., 1984; Brayden and Bevan, 1986), substance P (Edvinsson et al., 1981, 1982), and CGRP (Goodman and Iversen, 1986; Saito et al., 1989) in neurogenic cerebral vasodilatation (Lundberg, 1996). This conclusion was drawn on the basis of histological evidence demonstrating the presence of neurons containing acetylcholinesterase/choline acetyltransferase and the peptides in the vascular wall and of the ability of these compounds when applied exogenously to elicit vasodilatation in cerebral arteries. However, no evidence was shown that the molecules liberated from perivascular nerves dilated the arteries. The possible contribution of the peptides and acetylcholine synthesized and released from the nerve as mediators or neurotransmitters was excluded in dog and monkey cerebral arteries by the fact that the response to nerve stimulation was not influenced by capsaicin (Okamura and Toda, 1994c), by muscarinic receptor antagonists (Toda, 1975, 1982), by removal of the endothelium (Toda and Okamura, 1990b,c), or in arteries made tachyphylactic to the peptides under consideration (Toda, 1982; Okamura et al., 1989; Toda and Okamura, 1996a). In some studies with feline cerebral arteries, atropine depressed the neurogenic relaxation, suggesting that acetylcholine is one of the neurotransmitters (Bevan et al., 1982a,b). Acetylcholine-induced relaxations are mediated by endothelium-derived relaxing factor (EDRF) (Furchgott and Zawadzki, 1980). Since the relaxation to nerve stimulation is not influenced in feline arteries by endothelium denudation (Saito et al., 1989; Ayajiki et al., 1994), acetylcholine appears not to mediate the response in feline cerebral arteries. Substance P, an EDRF-mediated cerebral vasodilator (Conner and Feniuk, 1987; Onoue et al., 1988), can therefore be excluded as a transmitter candidate.

Attention was directed to discover endogenous cerebroarterial vasodilators as neurotransmitter candidates. In addition to VIP, CGRP, and substance P, ATP, γ-aminobutyric acid, pentagastrin (Toda, 1982), and atrial natriuretic peptide (Okamura et al., 1989) produced dilatation of dog and monkey cerebral arteries, but they were excluded as candidates either by experiments with receptor antagonists or by tachyphylaxis studies. Many other peptides and amino acids failed to produce relaxation (Toda, 1982). Nitroglycerin, although exerting strong vasodilator activity on cerebral arteries, could not be considered as an endogenous substance. Therefore, it was an important task to find inhibitors that selectively depressed this neurogenic relaxation. Oxyhemoglobin (oxyHb), an NO scavenger (Martin et al., 1985b), was such an inhibitor (Toda, 1988b; Linnik and Lee, 1989). Methylene blue, an inhibitor of soluble guanylyl cyclase (Gruetter et al., 1981), also inhibited the response (Toda, 1988b). However, no one, including ourselves, had the insight to regard EDRF as a neurotransmitter in the late 1980s. Garthwaite and his coworkers (1988) theorized that EDRF was released from neurons in the brain. Therefore, the hypothesis that NO was a neurotransmitter in vasodilator nerve innervating the vascular wall was not raised until 1990 (Toda and Okamura, 1990a,b).

B. Evidence for the Presence of Vasodilator Nerve-Releasing Nitric Oxide As a Neurotransmitter—Criteria for Transmitter

Abolition by N^G-monomethyl-L-arginine (L-NMMA), a NOS inhibitor (Palmer et al., 1988b), of vasodilatation caused by electrical nerve stimulation or by nicotine was obtained for the first time in isolated dog cerebral arteries (Toda and Okamura, 1990a–c). The inhibitory effect was reversed by L-arginine, a substrate of NOS, but not D-arginine. D-NMMA was without effect. Other NOS inhibitors, such as N^G-nitro-L-arginine (L-NA) (Mulsch and Busse, 1990; Rees et al., 1990; Toda et al., 1990c) (Fig. 1), L-NA methylester (L-NAME) and *N*-iminoethyl-L-ornithine (Rees et al., 1990), were also effective. Removal of the endothelium did not inhibit the neurogenic relaxation (Toda and Okamura, 1990b, 1991b). On the other hand, nerve stimulation-induced relaxation of dog coronary arteries was not influenced at all by the NOS inhibitors (Toda et al.,1990c) but was abolished by β-adrenoceptor antagonists (Toda and Hayashi, 1982). Exogenously applied NO (acidified $NaNO_2$) (Furchgott 1988) and NO donors, including nitroglycerin and sodium nitroprusside, induced dose-dependent dilatation of cerebral arteries, which was not affected by treatment with NOS inhibitors but was abolished by oxyHb and methylene blue. As described in the previous section, the latter two compounds abolished the response to nerve stimulation. ODQ (1*H*[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), a soluble guanylyl cyclase inhibitor (Garthwaite et al., 1995), was also effective in depressing the response to nerve stimulation (Toda et al., 1999).

NO is synthesized from L-arginine by nNOS, which is activated by Ca^{2+} in the presence of calmodulin and other cofactors (Bredt and Snyder, 1990). Responses to electrical stimulation were abolished by removal of extracellular Ca^{2+} and also by ω-conotoxin GVIA, an N-type Ca^{2+} channel blocker (Toda et al., 1995c), but not by blockers of the L-type Ca^{2+} channel (Toda et al., 1992). The Ca^{2+} entry into nerve terminals upon arrival of generated action potentials or via Ca^{2+} channel opening due to nicotinic receptor stimulation would be responsible for activation of nNOS and NO synthesis.

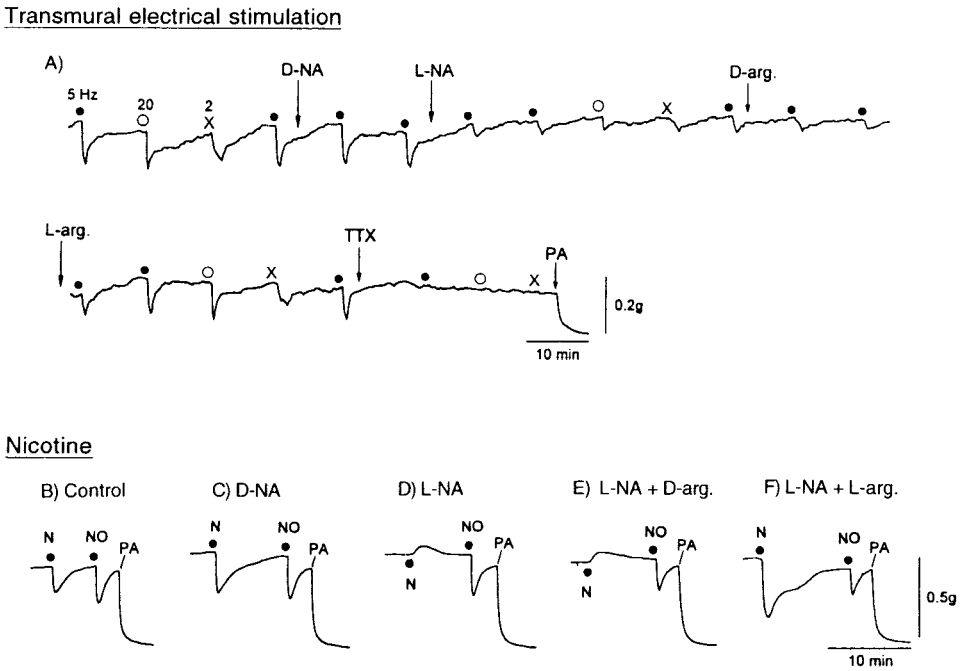

FIG. 1. Typical tracings of the response of monkey middle cerebral arterial strips to transmural electrical stimulation (A) at frequencies of 2 (crosses), 5 (solid circles), and 20 (open circles) Hz, and nicotine (10^{-4} M, N) and nitric oxide (10^{-7} M, NO) (B–F). The right end of the top tracing continues to the left end of the middle tracing. Two strips from different monkeys, used for studies on the action of electrical stimulation and nicotine/NO, were partially contracted with prostaglandin $F_{2\alpha}$. A, neurogenic relaxation was not influenced by D-NA (10^{-6} M) but was diminished by L-NA (10^{-6} M); D-arginine (D-Arg, 10^{-3} M) did not affect but L-arginine (L-Arg, 10^{-3} M) completely restored the responses. Abolishment of the responses by TTX (3×10^{-7} M) supports the view that the induced relaxation is derived from nerve stimulation. PA represents 10^{-4} M papaverine, which produced the maximal relaxation. B–F, the relaxation induced by nicotine was not affected by D-NA (C) but was reversed to a slight contraction by L-NA (D); the L-NA-induced inhibition was not reversed by D-arginine (E) but by L-arginine (F). The relaxation induced by NO was not affected by either treatment. After the papaverine-induced relaxation was stabilized, the strip was repeatedly washed by drug-free media and was equilibrated for the next trial.

In superfused cerebral arterial strips without the endothelium, the release of NO, measured as nitrite and nitrate (NOx), into the superfusate was increased during nerve stimulation by electrical pulses and nicotine (Toda and Okamura, 1990b). Tetrodotoxin (TTX) abolished the effect of electrical nerve stimulation, and hexamethonium (C_6) abolished the nicotine action. Increased release of NOx by electrical stimulation was also abolished by L-NA in superfused dog temporal arteries (Toda et al., 1991c). It may be possible in the cerebral artery to demonstrate the release of NO by bioassay as shown in the dog intestine (Bult et al., 1990), visualization by a reaction with luminol and hydrogen peroxide to generate photons as shown in the guinea pig ileum (Wiklund et al., 1997), or using a novel diaminofluorescein (Kojima et al., 1998) as shown in the porcine coronary artery (Itoh et al., 2000). The content of cyclic GMP in the tissue was also increased by electrical stimulation and nicotine, and L-NA abolished the stimulating effect. The nucleotide increment produced by electrical stimulation and nicotine was abolished by TTX and C_6, respectively (Toda and Okamura, 1991b).

Histochemical studies have demonstrated that guanylyl cyclase and phosphodiesterase, an enzyme that degrades cyclic GMP, are located in cells surrounding the rat brain vasculature (Poeggel et al., 1992). Perivascular nerve fibers containing NOS immunoreactivity have been demonstrated in dog, monkey (Yoshida et al., 1993, 1994a), and rat cerebral arteries (Bredt et al., 1990) by the use of antiserum raised against NOS purified from the rat cerebellum (Bredt et al., 1990). NOS-immunoreactive neurons are reportedly identical to reduced NADPH-positive ones (Dawson et al., 1991); therefore, the presence of networks of NOS-containing nerve fibers and cells in the vascular wall and brain has been widely demonstrated by the NADPH-staining method (Vincent and Kimura, 1992; Minami et al., 1994).

Findings presented thus far support the hypothesis that NO or stable analog of NO (R-SNO) acts as a neurotransmitter of vasodilator nerves innervating the cerebral artery. The general lines of evidence to support the concept of neurotransmission as delineated in Goodman and Gilman's text book (Hoffman and Taylor, 2001) include 1) demonstration of the presence of a physiologically active compound and its biosynthetic enzymes at appropriate sites; 2) recovery of the compound from the perfusate of an innervated structure during periods of nerve stimulation, but not in the absence of stimulation; 3) demonstration that the compound is capable of producing responses identical with those to nerve stimulation; 4) demonstration that the responses to nerve stimulation and to the administered compound are modified in the same manner by various drugs, usually antagonists. Except for the first part of 1), NO or R-SNO, like

S-nitroso thiol, fulfills the criteria for neurotransmitter. Verification of the presence of the unstable molecule NO or its storage in vesicles would not necessarily be required. Substrate L-arginine in sufficient amounts (Forstermann et al., 1994) (100–800 μM of L-arginine in endothelial cells) and nNOS activated by Ca^{2+} locally produce NO that easily crosses cell membrane and diffuses out of nerve terminals. This represents a new idea for this gaseous and labile transmitter substance with low molecular weight. Therefore, the criteria for defining a neurotransmitter may in the future need to be rewritten along these lines. Hypothetical scheme of the neurotransmission process from the nitrergic nerve to vascular smooth muscle is summarized in Fig. 2.

Many arguments against this quite unexpected hypothesis have arisen. For instance, it was suggested that NO may be liberated from extraneuronal tissues. Answers to this comment are: the presence of constitutive NOS is histochemically demonstrated in the neuron, nerve cell, and endothelium, but not in smooth muscle and other adjacent tissues (Bredt and Snyder, 1990); blockers of L-type Ca^{2+} channels that suppress Ca^{2+} entry in smooth muscle fail to interfere with the response to nerve stimulation (Toda and Okamura, 1992a); and NOS in tissues other than neurons and endothelium is inducible only when incubated for long hours in lipopolysaccharides and endotoxin. In addition, the fact that damage to the dog pterygopalatine ganglion for 1 week histologically causes the NOS-containing neurons to disappear in the cerebral arterial wall and abolishes the dilator response to nerve stimulation of the isolated artery (Toda et al., 1993d) excludes the possibility of NO release from extraneuronal sites. It has also been suggested that neurally induced vasodilatation in isolated sheep middle cerebral arteries may be mediated largely by VIP and that possibly VIP acts to stimulate the formation of NO within smooth muscle, macrophage, or other tissues in the vascular wall (Gaw et al., 1991). In accordance with the reasons presented above, this cannot be the case, at least in dog and monkey cerebral arteries.

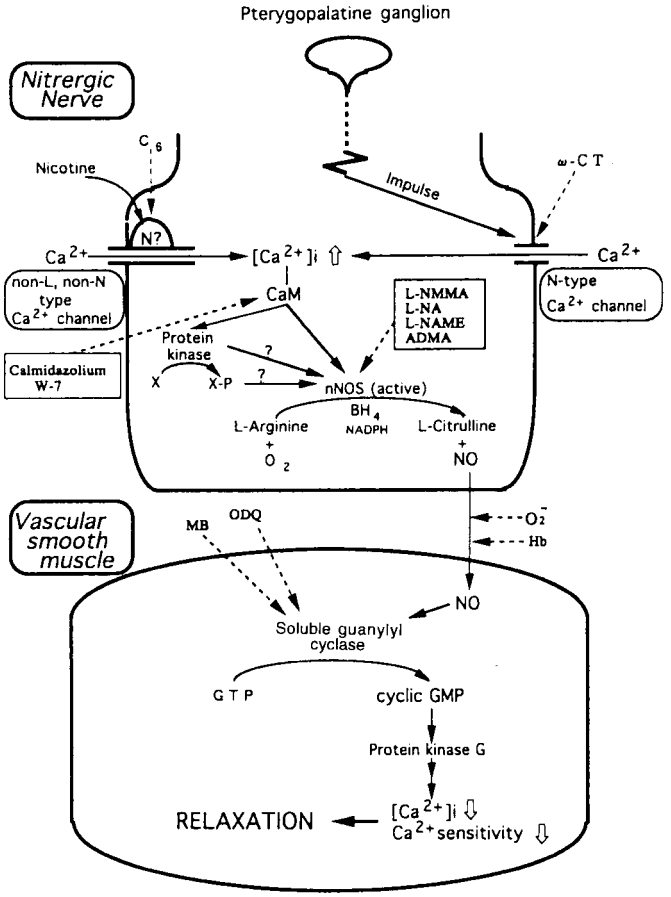

FIG. 2. Summary scheme of neurotransmission from the nitrergic nerve to smooth muscle. Solid line, stimulation; dotted line, inhibition. ω-CT, ω-conotoxin GVIA; N at non-L, non-N Ca^{2+} channel, nicotinic receptor; X, unknown protein that contributes to nNOS activation upon phosphorylation (X-P); O_2^-, superoxide anion; MB, methylene blue.

The other comment raised is that NO may act as a modulator of a vasodilator neurotransmitter. However, in cerebral arteries in which the neurogenic relaxation was abolished by NOS inhibitors, the addition of NO or NO donors did not restore the response (Toda and Okamura, 1990c). In bovine cerebral arteries, it is suggested that NO produced by perivascular nerves fully accounts for the experimental neurogenic relaxation, and VIP, present in the same nerves, acts as a neuromodulator by acting on nNOS (Gonzalez et al., 1997).

Whether the transmitter released is NO or R-SNO is still debatable in the perivascular nerve as well as in the endothelium (Myers et al., 1990; Feelisch et al., 1994). The reasons are as follows: the response to nerve stimulation was not inhibited by antioxidants, such as pyrogallol and hydroquinone, or augmented by superoxide dismutase (SOD) (Gillespie and Sheng, 1990; Toda and Okamura, 1990b), whereas actions of free radical NO were abolished by antioxidants (Moncada et al., 1986) and enhanced by SOD (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986). From studies showing that the response to NANC nerve stimulation in the rat anococcygeus muscle and gastric fundus was insensitive to carboxy PTIO [2-(4-carboxyphenyl)-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide], a scavenger of free radical NO (Akaike et al., 1993), in concentrations sufficient to depress the relaxation induced by endothelium-derived NO in the rat aorta, the authors concluded that the transmitter from nitrergic nerves does not appear to be identical to endothelium-derived NO and may not be the free radical NO (Rand and Li, 1995a). However, they did not examine the property of nitrergic nerve in vasculature. On the other hand, it was noted that treatment with diethyldithiocarbamate, a membrane-permeable inhibitor of Cu/Zn SOD, allowed pyrogallol, hydroquinone, and duroquinone to inhibit the NO-mediated neurogenic response in the bovine retractor penis muscle and mouse anococcygeus muscle (Martin et al., 1994; Lilley and Gibson, 1995). The inhibition was reversed by

SOD. Similar results were also obtained in monkey (Okamura et al., 1998c) and porcine cerebral arteries (Tanaka et al., 1999), suggesting that endogenous SOD protects neurons from functional impairment by superoxide anions and that the free radical NO, rather than R-SNO, acts as a neurotransmitter in these vascular and nonvascular tissues.

C. Mechanism of Nitric Oxide Formation and Action

The peripheral neurotransmitter of the NANC vasodilator nerve is presumed to be NO in intracranial vasculature in mammals so far investigated and in peripheral arteries of those other than rodents. Neuronal NOS, now designated as nNOSα (Eliasson et al., 1997), was originally purified and cloned from the brain (Bredt and Snyder, 1990; Bredt et al., 1991). The distribution and functional role of nNOS have been elucidated in tissues other than the brain, such as the skeletal muscle (Brenman et al., 1995; Stamler and Meissner, 2001) and macula densa in the kidney (Wilcox et al., 1998). In contrast to endothelial NOS (eNOS), nNOS is regulated posttranscriptionally and has many alternative splice variants (Wang et al., 1999b; Alderton et al., 2001). However, whether or not the nNOSs in brain, peripheral nitrergic nerve, and extraneuronal tissues are identical is not yet clear. Neuronal NOSs in brain and skeletal muscle are located in and associated with an adapter protein PSD-95 in the postsynaptic cells (Brenman et al., 1996; Tochio et al., 2000), whereas nNOS in the peripheral nitrergic nerve is located mainly in presynaptic nerve terminals. Since biochemical and molecular biological information of nNOS in the nitrergic vasodilator nerve per se is largely incomplete, mechanisms of NO formation and action can only be speculated upon from an immunological analogy to nNOS in the brain and on the basis of functional and histological data obtained from physiological and pharmacological experiments with isolated vascular preparations, including susceptibility of biological responses to selective nNOS inhibitors.

1. Formation of Nitrergic Neurotransmitter.

a. L-Arginine. L-Arginine serves as a substrate of NOS to yield NO and L-citrulline by two steps: from L-arginine and O_2 to N^G-hydroxy-L-arginine, which is then converted to NO and L-citrulline. L-Arginine alone does not potentiate the neurogenic response but completely prevents the inhibition induced by NOS inhibitors. The inability of this NOS substrate to increase the neurogenic relaxation of isolated blood vessels may be explained in terms of there being sufficient amounts of L-arginine in the nerve cells (approximately 100 μM in the brain) (Barbul, 1986). The K_m values of the enzyme for L-arginine are 1.5 to 2.3 μM (Bredt and Snyder, 1990; Schmidt et al., 1991; Ohshima et al., 1992).

L-Arginine is supplied by uptake from the extracellular space through a cation amino acid transporter system (system y+) or by synthesis from L-citrulline intracellularly via argininosuccinate synthetase and argininosuccinate lyase in neuronal cells (Wiesinger, 2001) (Fig. 3). In isolated porcine cerebral arteries without the endothelium, the uptake of L-citrulline and the active conversion from L-citrulline to L-arginine were noted, but these were absent in denervated arteries (Chen and Lee, 1995a). Presence of argininosuccinate synthetase and argininosuccinate lyase has been demonstrated histologically in NADPH diaphorase-positive nerve fibers innervating the cerebral artery (Yu et al., 1997). Therefore, a recycling process to synthesize L-arginine from L-citrulline seems to function in the perivascular nitrergic nerve. In cultured macrophages under treatment with lipopolysaccharide (Baydoun and Mann, 1994; Closs et al., 1997), the cationic transporter system y+ mediates the uptake of L-arginine, L-NMMA, N^G,N^G-dimethyl-L-arginine (ADMA), and N^G-iminoethyl-L-ornithine, whereas a neutral transporter mediates the uptake of L-citrulline, L-NA and L-NAME. On the other hand, two other L-arginine analogs, N^G,N'^G-dimethyl-L-arginine (SDMA) and α-amino-δ-isothioureidovaleric acid that fail to inhibit NOS enzyme activity, compete for the L-arginine transport (Closs et al., 1997). At present, it is not known whether these differences in substrate recognition of the transporter systems are also operative in the nitrergic nerve.

Arginases I and II affect intracellular L-arginine concentrations by catalyzing L-arginine as a substrate. K_m values for these arginases are 2 to 20 mM and approximately 1000 times higher than those for NOS (Grody et al., 1987; Griffith and Stuehr, 1995); therefore, arginases are not likely to compete with NOS in the enzymatic reaction. However, once arginase is induced by cytokines, arginases and NOS may compete for the utilization of L-arginine, since the V_{max} of arginases is 1000 times greater than those for NOS (Griffith and Stuehr, 1995; Wu and Morris, 1998). NOS inhibitors of L-arginine analogs so far described are ineffective on the arginase activity (Hrabak et al., 1994), but a sufficient

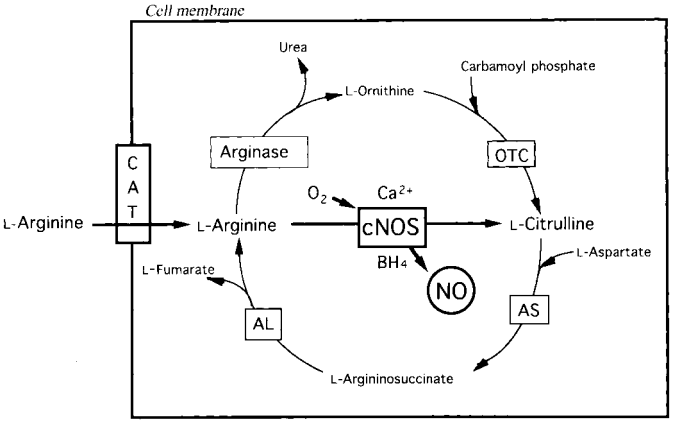

FIG. 3. Scheme of the formation of NO from L-arginine and its regulatory factors. cNOS, constitutive NOS; AS, argininosuccinate synthetase; AL, argininosuccinate lyase; OTC, ornithine transcarbamylase; CAT, cationic amino acid transporter in the cell membrane.

amount of N^G-hydroxy-L-arginine, an intermediate product of L-arginine by NOS, inhibits the arginase activity in iNOS-expressing macrophages (Hecker et al., 1995). These complex interactions between arginase and NOS may be important under pathological conditions. However, no data are available concerning the functional role of arginases in the nitrergic nerve.

b. Ca^{2+} and Calmodulin. Neuronal NOS is a constitutive type of NOS, and activity of the purified enzyme is dependent on Ca^{2+} (Mayer et al., 1992) and calmodulin (Bredt and Snyder, 1990) in the presence of cofactors such as FMN, FAD, NADPH, and tetrahydrobiopterin (BH_4). Generated action potentials evoked by a rapid rise of Na^+ influx in nerve terminals allow opening of slow channels, through which Ca^{2+} is introduced into the neuron along extra- and intracellular concentration gradients. Therefore, the increase in the intracellular Ca^{2+} mainly by increasing the transmembrane influx or by the release from intracellular storage sites facilitates its binding to calmodulin. The Ca^{2+}-calmodulin complex directly activates the enzyme, thus initiating electron flow from the reductase domain to the oxygenase domain to produce NO (Alderton et al., 2001). The Ca^{2+}-calmodulin complex may also activate Ca^{2+}/ calmodulin-dependent protein kinases. It has been reported that nNOS has a phosphorylation site and is phosphorylated at Ser^{847} by Ca^{2+}/calmodulin-dependent protein kinase II (Ca/CaM kinase II; Hayashi et al., 1999). This phosphorylation leads to a decrease in NOS activity (Nakane et al., 1991; Komeima et al., 2000). Neuronal NOS is also phosphorylated by cyclic AMP-dependent protein kinase (protein kinase A) and protein kinase C, with each kinase phosphorylating a different serine site on NOS (Bredt et al., 1992). Phosphorylation of nNOS by protein kinase C reportedly decreases (Bredt et al., 1992) or increases (Nakane et al., 1991) the enzyme activity. No change (Brune and Lapetina, 1991) or decrease (Dinerman et al., 1994) in nNOS activity was observed upon phosphorylation by protein kinase A. Phosphorylation by cyclic GMP-dependent protein kinase (protein kinase G) is reported to reduce the activity (Dinerman et al., 1994). Such diverse actions may be due to differences in the phosphorylation site(s) of the enzyme. The data discussed above are based on that obtained from nNOS protein purified from different species. In intact cells or in vivo systems, there may be other complex mechanisms of nNOS activation and deactivation by protein phosphorylation.

2. Action of Nerve-Derived Nitric Oxide on Vascular Smooth Muscle. The signal transduction system for smooth muscle relaxation produced by the nitrergic neurotransmitter involves the activation of soluble guanylyl cyclase and the production of the second messenger cyclic GMP (Schmidt et al., 1993) (Fig. 2). In isolated blood vessels, neurogenic relaxation is abolished by soluble guanylyl cyclase inhibitors, such as methylene blue and ODQ and is accompanied by an increase in intracellular cyclic GMP contents (Toda and Okamura, 1996b).

Soluble guanylyl cyclase expressed in vascular smooth muscle cells catalyzes the conversion of GTP to cyclic GMP (Papapetropoulos et al., 1996). The enzyme is a heterodimer of α and β subunits containing a heme prosthetic group (Lucas et al., 2000). Both subunits are required for basal and NO-stimulated catalytic activity (Harteneck et al., 1990; Nakane et al., 1990). NO activates soluble guanylyl cyclase by binding directly to heme to form a ferrous-nitrosyl-heme complex, breaking the bond between iron and His^{105}, and displacing the iron from the plane of the porphyrin ring. Termination of activation probably results from dissociation of NO from the heme and return of the iron core to the plane of the porphyrin ring (Lucas et al., 2000). NO also decreases stability of mRNAs encoding soluble guanylyl cyclase subunits in rat pulmonary artery smooth muscle cells (Filippov et al., 1997). Gene transfer of soluble guanylyl cyclase subunits to balloon injured blood vessels increases the responsiveness to NO in rats (Sinnaeve et al., 2001).

Although important roles of cyclic GMP in vascular relaxation are well recognized (Waldman and Murad, 1987), intracellular mechanisms and signal transduction pathways may vary in cells, tissue types or animal species. The final common step is a reduction in the intracellular free Ca^{2+} concentration or Ca^{2+} sensitivity (Fig. 2), which prevents the Ca^{2+}-dependent activation of myosin light-chain kinase and muscle contraction. A number of hypotheses on biosignaling processes from cyclic GMP production to decreased intracellular Ca^{2+} concentrations have been proposed. Cyclic GMP has been shown to inhibit Ca^{2+} entry into cells by inhibiting L-type Ca^{2+} channels (Lincoln, 1989). Cyclic GMP-mediated activation of protein kinase G may be involved (Lincoln and Cornwell, 1993), which is suggested to inhibit voltage-gated Ca^{2+} channels directly (Clapp and Gurney, 1991) or indirectly by activating Ca^{2+}-sensitive K^+ channels and hyperpolarizing the cell membrane (Archer et al., 1994). Protein kinase G has also been shown to activate the Na^+/Ca^{2+} exchanger (Furukawa et al., 1991), the Ca^{2+}-ATPase on the plasma membrane (Yoshida et al., 1991), or the Ca^{2+}-ATPase on the endoplasmic reticulum indirectly by phosphorylation of phospholamban, an endoplasmic reticulum Ca^{2+}-ATPase regulatory protein (Cornwell et al., 1991). Protein kinase G has been noted to inhibit the agonist-induced production of inositol trisphosphate (IP_3) (Hirata et al., 1990) or the effect of IP_3 by phosphorylation of IP_3 receptors on the endoplasmic reticulum membrane (Komalavilas and Lincoln, 1994).

Neuronal NOS-derived NO produces several actions other than vascular smooth muscle relaxation. In the central nervous system, NO, produced in and released from the postsynaptic cells, controls the release of the neurotransmitter glutamate (Garthwaite and Boulton,

1995). The linking of NO to the release (Meffert et al., 1996) of other neurotransmitters and their effects has also been reported in the brain and nonvascular tissues; i.e., acetylcholine (Gustafsson et al., 1990), dopamine (Hanbauer et al., 1992), γ-aminobutyric acid (Kuriyama and Ohkuma, 1995), and bombesin (Beltran et al., 1999). The mechanisms responsible for these actions are not fully understood, but direct *S*-nitrosylation of receptors, activation of cyclic GMP-dependent protein phosphorylation cascades, regulation of neuronal energy dynamics, and modulating effect on transporters may be involved (Esplugeus, 2002).

S-Nitrosylation of cysteine residues in proteins by exogenous NO is thought to be involved in certain physiological and pathological events that are not readily explained by a mediation of cyclic GMP. Several proteins are endogenously *S*-nitrosylated by neuronally generated NO, since this modification is not observed in animals harboring a genomic deletion of nNOS (Jaffrey et al., 2001). Whether or not this pathway is involved in the events mediated by NO derived from perivascular nitrergic nerves remains to be clarified.

III. Nitrergic Innervation in Intra- and Extracranial Vasculature

A. Cerebral Artery

1. In Vitro Studies in Various Mammals. Electrical (1–20 Hz) and chemical stimulation (10^{-6}–10^{-4} M nicotine) elicited relaxations in a frequency- and concentration-dependent manner, respectively, in cerebral arteries previously contracted with vasoconstrictors (Fig. 1). Treatment with α-adrenoceptor antagonists or adrenergic neuron blockers did not alter or augment the response in cerebral arteries of various mammals (Toda and Okamura, 1996b). However, in sheep cerebral arteries, the stimulation induced contraction, which was reversed to a relaxation response by α-adrenoceptor blockers (Duckles et al., 1977). Therefore, to elucidate the mechanism underlying neurogenic relaxation, experiments have been conducted in vasculature with α-adrenoceptor blockade.

Neurogenic vasodilatation mediated by NO in isolated cerebral arteries has been demonstrated in a variety of mammals, including the human (Toda, 1993), monkey (Toda and Okamura, 1990c; Toda et al., 1997b), dog (Toda and Okamura, 1990a,b, 1991b; Toda et al., 1993d), pig (Lee and Sarwinski, 1991; Tanaka et al., 1999), cow (Gonzalez and Estrada, 1991; Ayajiki et al., 1993), cat (Ayajiki et al., 1994), sheep (Matthew and Wadsworth, 1997), guinea pig (Jiang et al., 1997), and rat (Ignacio et al., 1997). In small mammals, only the basilar artery is used, although the intracranial, extracerebral arteries of different regions can be utilized in mammals of medium or large size. In dogs, nicotine (10^{-4} M)-induced relaxations average 45.4 ± 4.4% (n = 11) in anterior cerebral, 50.2 ± 3.1% (n = 18) in middle cerebral, 31.6 ± 5.8% (n = 12) in posterior cerebral, and 28.5 ± 3.7 (n = 10) in basilar arteries. Greater responses were obtained in the anterior and middle cerebral arteries than in the other vessels. According to the histochemical study by Suzuki et al. (1994), the density of NADPH diaphorase (NOS)-containing nerve fibers was higher in the anterior half of the circle of Willis than its posterior half in the rat. Main trunks of the dog middle cerebral artery responded to nicotine with a greater relaxation than did the third branches of the artery (56.2 versus 22.6%; Toda and Miyazaki, 1984). Nicotine-induced relaxations did not significantly differ in cerebral arteries isolated from beagles of different ages (30 days, 3 months, 1 year, and 3 years) (Toda et al., 1986). In contrast, relaxations induced by electrical nerve stimulation of cerebral arteries varied directly with age in Japanese monkeys ranging from infancy (3–4 weeks) to adulthood (over 7 years) (Toda, 1991). The values do not seem to reflect nitrergic nerve functioning directly, since nicotinic receptors are not always distributed evenly in these arteries. Studies with electrical nerve stimulation also have problems, such as positioning of the stimulating electrodes including clearance between the tissue and electrodes, thickness of the vascular wall, etc. Although responsiveness to vasoactive agents, including NO, of intracerebral microvessels were noted (Dacey et al., 1988; Takayasu et al., 1994), the functional role of nitrergic nerve was not evaluated. Histological evidence for neurons containing NOS immunoreactivity or NADPH diaphorase in the microvascular wall (Kummer et al., 1992; Estrada et al., 1993; Iadecola et al., 1993) suggests that NO derived from the nerve contributes to regulate vascular size.

In most but not all of the cerebral arteries from different species, relaxant responses to electrical nerve stimulation at frequencies up to 20 Hz are abolished by high concentrations of NOS inhibitors, oxyHb, and guanylyl cyclase inhibitors, suggesting that the response is exclusively or at least largely mediated by NO. However, in some cerebral arteries, other mechanisms are also involved.

In feline cerebral arteries, neurogenic relaxation was partially reduced but not abolished by high concentrations of L-NA, and the remaining response was abolished by capsaicin or in the arteries made tachyphylactic to CGRP but not to VIP (Ayajiki et al., 1994). Another study noted that the arterial relaxation was mediated by CGRP, although the involvement of neurogenic NO was not examined (Saito et al., 1989).

In bovine cerebral arteries, we concluded that NO appeared to be the sole transmitter (Ayajiki et al., 1993). In arteries that were made unresponsive to VIP and CGRP by their successive applications, the neurogenic relaxation was unaffected, suggesting that these peptides do not participate in the response. However, other authors have reported that the main mechanism mediated by nerve-derived NO is regulated by VIP liberated

from perivascular nerves in the bovine cerebral artery (Gonzalez et al., 1997).

In sheep cerebral arteries, the major mediator is considered to be VIP, which releases NO from the vascular wall (Gaw et al., 1991). This idea is supported by studies with VIP antiserum in the same artery (Matthew et al., 1997). In the guinea pig uterine artery, relaxations to high frequency stimulation (>5 Hz) are mediated by peptides, possibly VIP (Morris, 1993). However, in the dog (Toda et al., 1990c), monkey (Toda et al., 1997b), and bovine cerebral arteries (Ayajiki et al., 1993), no evidence supporting the involvement of peptides was obtained even when the nerve was stimulated electrically at frequencies as high as 20 Hz.

Although 7-nitroindazole was introduced as a promising selective inhibitor of nNOS (Moore et al., 1993), controversial data have been reported (Reiner and Zagvazdin, 1998). In a study with monkey cerebral arteries, the selectivity on responses to nitrergic nerve stimulation by electrical pulses and nicotine and to histamine, an endothelium-derived NO-releasing substance (Toda, 1990), was examined (Ayajiki et al., 2001b). This inhibitor was about as potent as L-NMMA and 100 times less potent than L-NA in attenuating the neurogenic relaxation and was about 5 times more potent in inhibiting nNOS than eNOS. The conclusion is that 7-nitroindazole is a relatively selective nNOS inhibitor.

It has been reported that NO-mediated neurogenic dilatation in guinea pig basilar arteries is associated with increased formation of intracellular cyclic GMP and activation of large-conductance Ca^{2+}-activated K^+ channel (Jiang et al., 1998). Relaxation or hyperpolarization of smooth muscle cell membrane induced by endothelial NO or NO donors is reportedly ascribed to activation of ATP-sensitive K^+ channels in cerebral arteries and arterioles of the pig and piglet (Armstead, 1996; Bari et al., 1996) or Ca^{2+}-activated K^+ channels in rabbit cerebral arteries and arterioles (Robertson et al., 1993; Taguchi et al., 1995; Dong et al., 1998).

2. Nerve Stimulation by Electrical Pulses and by Nicotine and Related Compounds. As previously described, stimulation of the nerve with electrical square pulses and chemically with nicotine or other nicotinic agonists produce similar patterns of responses in cerebral and peripheral arteries (Toda, 1995), and the neurotransmitters involved are identical (Fig. 2). Isolated dog cerebral and coronary arteries respond to electrical and chemical stimulation with relaxation, and the other arteries, including mesenteric, renal, femoral, pulmonary, superficial temporal, etc., contract in response to these stimuli. Evidence supporting the idea that the response to either mode of stimulation is due to the release of neurotransmitters from the activated nerve terminals in blood vessels has been mounting (Su, 1982; Toda, 1982; Nedergaard, 1988). Not only C_6 and pentolinium but also neosurgatoxin (Ayajiki et al., 1998) abolish the action of nicotine on nitrergic nerves innervating the dog cerebral artery and ganglionic cells of the dog duodenum. The inhibitory potency of the toxin is greater in the ganglion than in the nerve and is about 3000 times greater than C_6 in abolishing the response of dog cerebral arteries.

There are some discrepancies in the susceptibility of these response to inhibitors. The response to electrical stimulation is abolished by TTX but is not influenced by C_6. This response is potentiated by inhibitors of the amine transporter, such as cocaine, desipramine, etc., only when contractions are induced by adrenergic nerve stimulation (Toda, 1972). In contrast, the response to nicotine is abolished by C_6 or other ganglionic blocking agents and also by amine transporter inhibitors (Su and Bevan, 1970; Toda, 1976) but is resistant to TTX. Studies on Ca^{2+} concentration changes in single nerve terminal varicosities of the mouse vas deferens demonstrated that the nicotine action was insensitive to TTX at a concentration that blocked action potential-evoked Ca^{2+} transients (Brain et al., 2001). These findings indicate that different mechanisms of action are involved in electrical and chemical stimulation responses. It is well known that the generation of nerve action potentials by electrical stimulation evokes the transmembrane influx of Na^+ in nerve terminals, and the induced depolarization opens voltage-dependent Ca^{2+} channels, leading to Ca^{2+} influx. This influx triggers exocytosis of transmitter vesicles in adrenergic and cholinergic nerves or evokes activation of nNOS in nitrergic nerves (Fig. 2).

In contrast, the mechanism of nicotine actions is less well understood. For example, it is not clear whether nicotine can generate action potentials by acting on nicotinic receptors in nerve terminals. The variable efficacy of TTX on the vascular response to nicotine is puzzling. The action of nicotine is abolished (Bell, 1968a) or partially attenuated in the rabbit ear artery (Furchgott et al., 1975) by TTX, but it is not influenced in the rabbit pulmonary artery (Su and Bevan, 1970), rabbit aorta (Ikushima et al., 1981), and dog basilar artery (Muramatsu et al., 1978) by TTX. In dog mesenteric, renal, and femoral arterial strips, nicotine-induced contractions mediated by norepinephrine are partially inhibited by TTX in concentrations ($1–3 \times 10^{-7}$ M) sufficient to abolish the contraction induced by electrical nerve stimulation (Toda et al., 1976). However, the nicotine-induced relaxation of dog and monkey cerebral arteries mediated by NANC neurotransmitter (later proven to be NO) was not inhibited (Toda et al., 1976; Toda, 1982). These findings may lead us to speculate that action potentials are generated by nicotine in adrenergic nerve terminals in some mammals, including rabbits and dogs, but this is not the sole mechanism. In dog and monkey cerebral arteries, nicotine (10^{-4} M) appear not to mediate the generation of action potentials but may have acted on nicotinic receptors responsible for opening the Ca^{2+}

channel to exert NO-mediated relaxation. Studies on brain neurons suggest that at least two populations of presynaptic nicotinic receptors (TTX-sensitive and -insensitive) participate in the nicotine-induced amine release (Marshall et al., 1996; Kaiser and Wonnacott, 1999).

In eNOS knockout mice, acetylcholine (10^{-5} M)-induced dilatation of pial arterioles is blunted by TTX and abolished by L-NA (Meng et al., 1998). Because the response was abolished by atropine, the authors suggested that muscarinic receptor activation evokes the release of NO from the endothelium in which nNOS is expressed. Another possibility is that acetylcholine stimulates nicotinic receptors in nitrergic nerve endings and releases NO, since atropine used (10^{-5} M) was sufficient to induce complete blockade of muscarinic receptors and partial inhibition of nicotinic receptors.

Another question regarding nicotine actions is how inhibitors of the amine transporter abolish vascular actions of nicotine, a problem that has not been answered over several decades. The inhibitors include cocaine, tricyclic antidepressants such as imipramine and desipramine, propranolol, phentolamine, etc.; therefore, interpretations based on the use of pharmacological blockers for the analysis of nicotine actions must be made with care. On the basis of accumulated data concerning nitrergic nerve stimulation by nicotine, we hypothesize that nicotine or other ganglionic stimulants are transported by a cocaine-sensitive active process to the inside of cells where they act on Ca^{2+} channels (Fig. 2). Acetylcholine at the high concentration of 10^{-4} M relaxes dog cerebral and retinal arteries and monkey ciliary arteries, and this response is resistant to atropine but blunted by cocaine (Toda, 1979) and abolished by C_6 (Toda, 1979; Toda et al., 1995e, 1998). On the other hand, acetylcholine and nicotine act on nicotinic receptors of ganglionic cell membranes to increase ion permeability and to depolarize the cell membrane to a level of firing generated action potentials. This action is not influenced by amine transporter inhibitors; thus, nicotinic receptor stimulants do not necessarily cross the cell membrane but are generally recognized to act on receptors located on the outside of the membrane. Future refined microanalysis and molecular biology methods would be required to directly verify our hypothesis on nicotine's mechanism of action that is not based on the generation of action potentials.

The possibility that norepinephrine released by nicotine from adrenergic nerve stimulated the release of NO from nitrergic nerve was proposed in a study using porcine basilar arteries treated with guanethidine, an adrenergic neuron blocker, and 6-hyroxydopamine (Zhang et al., 1998), which chemically denervates adrenergic neurons. The validity of this idea is incomplete until it is shown that these compounds do not impair the uptake of nicotine by the amine transporter. In dog cerebral arterial strips, nicotine-induced relaxations are suppressed by treatment with propranolol, phentolamine, and bretylium but unaffected by sotalol, timolol, prazosin, and yohimbine (Toda, 1975; Leckstrom et al., 1993; Toda et al., 1995c). The inhibition by the former three inhibitors is considered to result from a blockade of the amine transporter. In contrast to the data presented by Zhang et al. (1998), we found no evidence supporting the view that norepinephrine applied exogenously stimulates the release of NO from vasodilator nerves in porcine cerebral arteries (Tanaka et al., 1999).

Susceptibility to pharmacological interventions of the NO-mediated relaxation induced by electrical and chemical (nicotine) stimulation has been compared. The responses to these stimuli are depressed by Mg^{2+}, Cd^{2+}, a nonselective Ca^{2+} channel inhibitor, or calmodulin inhibitors, such as W-7 and calmidazolium, and removal of external Ca^{2+} (Toda and Okamura, 1992a; Okamura and Toda, 1994a; Toda et al., 1995c), whereas nicardipine and tetramethrin, L- and T-type Ca^{2+} channel inhibitors, respectively, are without effect (Toda and Okamura, 1992a; Toda, 1998). Treatment with ω-conotoxin GVIA, an N-type Ca^{2+} channel inhibitor, dose-dependently inhibits the response to electrical nerve stimulation but not to nicotine (Toda et al., 1995c), suggesting again that the action of nicotine is not associated with the generation of nerve action potentials. These findings strongly suggest that Ca^{2+} introduced from the extracellular fluids and cellular calmodulin are prerequisites for the synthesis of NO from L-arginine via nNOS in nerve terminals. N-Type Ca^{2+} channels are responsible for the transmembrane influx of Ca^{2+} due to nerve action potentials, whereas Ca^{2+} channels of the L-, N-, and T-types are not involved in the ion influx evoked by nicotine (Fig. 2).

Molecular biological studies reveal the presence of at least nine types of α subunits (α2–α10) and three types of β subunits (β2–β4) in the neuronal type of nicotinic receptors. Three α subunits (α2, α3, and α4) have been shown to form functional heterometric receptors in combination with a β subunit (β2 or β4) in a recombinant expression system (Boulter et al., 1987; Wada et al., 1988; Cooper et al., 1991). Three other α subunits (α7, α8, and α9) form functional, homometric receptors (Schoepfer et al., 1990; Seguela et al., 1993). The receptor subtype classification by functional studies has been carried out mainly in the central nervous system and autonomic ganglia. In the central nervous system, α7-, β2-, and α3β4-like nicotinic receptors have been determined (Kaiser and Wonnacott, 1999). It appears that α3β4 nicotinic receptors are involved in neurotransmission in ganglia of the rat stomach (Yokotani et al., 2000, 2001). Our pharmacological study revealed that nicotinic receptor subtype antagonists including C_6 (α3, α4, α6), mecamylamine (α1, α3, α4, α6), and neosurugatoxin (α1, α3), but not α-bungarotoxin (α1, α7, α8, α9) or α-conotoxin IMI (α7), abolished the nicotine-induced relaxation in the canine cerebral artery, and nicotinic re-

ceptor subtype agonists such as anatoxin-a ($\alpha3\beta4$, $\alpha4\beta2$, $\alpha7$), but not RJR2403 ($\alpha4\beta2$), produced relaxation that was sensitive to C_6 (Ayajiki et al., 1998; K. Ayajiki and T. Okamura, unpublished data). These findings may indicate that relaxations induced by nicotinic agonists are mediated by $\alpha3\beta4$-like nicotinic receptor subtypes in nitrergic nerve terminals innervating the canine cerebral arterial wall. It has been reported that nicotine stimulates $\alpha7$ receptors in adrenergic nerve terminals to release norepinephrine in the porcine basilar artery (Si and Lee, 2001).

3. Is Protein Phosphorylation Involved in Neuronal Nitric-Oxide Synthase Activation? It has been reported that phosphorylation by protein kinase C increases, but that by Ca/CaM kinase II decreases, the activity of nNOS isolated from the rat brain (Nakane et al., 1991). Protein kinase A, protein kinase C, and Ca/CaM kinase II phosphorylate different serine sites on nNOS in NOS-transfected cells, and the enzyme activity is reduced in association with the phosphorylation by protein kinase C (Bredt et al., 1992). In isolated dog cerebral arteries, NO-mediated relaxations induced by electrical nerve stimulation and nicotine were dose-dependently attenuated by treatment with KN62 ([S]-5-isoquinoline sulfonic acid,4-[2[(5-isoquinolinyl-sulfonyl)methylamino]-3-oxo-(4-phenyl-1-piperazinyl)propyl]phenyl ester) or HDBA (2-hydroxyl-5-[2,5-dihydroxybenzyl]aminobenzoic acid), inhibitors of Ca/CaM kinase II (Toda et al., 1997c). Nicotine-induced increase in the tissue content of cyclic GMP was also inhibited. Inhibitions by these agents of the transmembrane Ca^{2+} influx and the calmodulin action are excluded. Similar inhibitory actions of KN62 are also observed in the neurogenic relaxation in monkey cerebral arteries; mean values at 5 Hz for 40 s in control and treated (10^{-6} M) arteries are 24.1 ± 3.5 and 12.6 ± 2.5%, respectively ($n = 9$, $P < 0.05$; N. Toda, T. Okamura, and K. Ayajiki, unpublished data). Our preliminary study (Fu-H. Ma and N. Toda, unpublished data) with cultured neuronal cells from the monkey pterygopalatine ganglion indicated that the cells were histologically stained by antibodies of nNOS and Ca/CaM kinase II. Phosphorylation on Ser^{57} by Ca^{2+} ionophore in neurofilament-L purified from bovine spinal cords was blocked by KN62 (Hashimoto et al., 2000). This phosphorylation was observed during long-term potentiation in mouse hippocampal slices. It may be concluded that phosphorylation of nNOS itself or proteins responsible for enzyme activation increases the production of NO in perivascular nerves (Fig. 2). Trends of augmenting the response to nerve stimulation by FK506, a phosphatase inhibitor (N. Toda, unpublished data), may represent further support for this hypothesis.

It has been demonstrated that phosphorylation by Ca/CaM kinase II of nNOS on Ser^{847} inhibits the enzyme activity in NG108–15 neuronal cells (Komeima et al., 2000). The reason for such a discrepancy in the data from isolated nNOS preparations and nNOS present in our peripheral nerve preparations in situ remains to be ascertained. Agonist-induced phosphorylation in intact bovine endothelial cells evoked translocation of eNOS from membrane to cytosol and its activation (Michel et al., 1993). Phosphorylation of eNOS on Ser^{1179} (Fulton et al., 1999) or Ser^{1177} (Dimmeler et al., 1999) by serine/threonine protein kinase Akt increased the production of NO, possibly by increasing the sensitivity of the enzyme to Ca^{2+} intracellularly released from its storage sites. It is suggested that the dual phosphorylation of serine and threonine determines the activity of eNOS in agonist-stimulated endothelial cells; $Ser^{1177,1179}$ phosphorylation and $Thr^{495,497}$ dephosphorylation activate the enzyme (Fleming et al., 2001; Harris et al., 2001). KN93, a Ca/CaM kinase II inhibitor, abolished the phosphorylation of Ser^{1177} (Fleming et al., 2001). As far as the data obtained are concerned, phosphorylation of nNOS or eNOS may importantly participate in the modulation of enzyme activity. To identify the site of phosphorylation in nNOS and to determine its relationship to the enzyme activation or inactivation in intact neurons are important but difficult tasks left for future studies.

4. In Vivo Studies. It has been reported that intravenous or intracisternal injections of NOS inhibitors constrict pial arterioles in the cerebral cortex and arteries in the basal brain, such as anterior, middle and posterior cerebral arteries or decrease cortical cerebral blood flow in the mouse (Rosenblum et al., 1990), rat (Faraci, 1990, 1991; Prado et al., 1992; Kelly et al., 1995; Yang, 1996), dog (Suzuki et al., 1993b; Toda et al., 1993b, 2000a), pig (Armstead, 1995b; Rebich et al., 1995), goat (Fernandez et al., 1995), and monkey (Okamura et al., 1995a; Toda et al., 2000b). Intraperitoneal injections of 7-nitroindazole, a selective nNOS inhibitor, decreased cerebral blood flow without modifying the systemic blood pressure in awake rats (Montecot et al., 1997) and decreased cerebral capillary flow in anesthetized rats (Hudetz et al., 1998). The response was reversed by L-arginine. Basal release of NO from the endothelium and the perivascular nerve tonically receiving impulses from the brain is postulated to maintain vasodilatation of cerebral arteries and arterioles. From studies with ARR 17477, a selective nNOS inhibitor, and L-NA in anesthetized rats subjected to forebrain ischemia, Santizo et al. (2000) suggested that the contribution of nNOS to intra-ischemic vasodilatation in vulnerable regions is substantially greater than that of eNOS. L-NA injected intracisternally constricted the basilar artery in anesthetized dogs and monkeys (Toda et al., 1993b; Okamura et al., 1995a) and the response was blunted by treatment with C_6. That portion of the vasoconstrictor response sensitive to the ganglionic blocker (about 70% of vasoconstriction to L-NA, which is about 37% reduction of the arterial size compared with that before NOS blockade) seems to be associated with depressed synthesis of NO in nerve terminals (Toda et al., 1993b), whereas the remaining part (about 30% of total

vasoconstriction) would be due to NO from extraneuronal tissues, possibly the endothelium. Tonic electrical discharges through nitrergic nerves from the vasomotor center appear to contribute to the maintenance of cerebral vasodilatation in these mammals.

Electrical stimulation of the sphenopalatine ganglion or facial nerve increased cortical blood flow in anesthetized rats (Seylaz et al., 1988; Suzuki et al., 1990a) and cats (Goadsby, 1991), which was not mediated by neurogenic acetylcholine. Increased cortical blood flow in anesthetized rats by electrical stimulation of parasympathetic nerves around the sphenopalatine ganglion was reduced by L-NAME in a dose-related manner (Morita-Tsuzuki et al., 1993). Chronic parasympathetic sectioning for 10 to 14 days decreased cerebral blood flow of ipsilateral side and increased infarct size after middle cerebral artery occlusion in spontaneously hypertensive rats (Koketsu et al., 1992). A novel role for neurogenic vasodilatation to protect cerebral nerve cells from ischemic insult is suggested. From studies with anesthetized wild-type mice and those lacking the nNOS gene, Ma et al. (1996a) concluded that the increase in cerebral blood flow by vibrissal stimulation was not mediated by endothelial NO but was a consequence of nNOS inhibition in wild-type mice. Stimulation of the pterygopalatine or geniculate ganglion in anesthetized dogs and Japanese monkeys induced vasodilatation of ipsilateral anterior and middle cerebral arteries, and intravenous L-NA

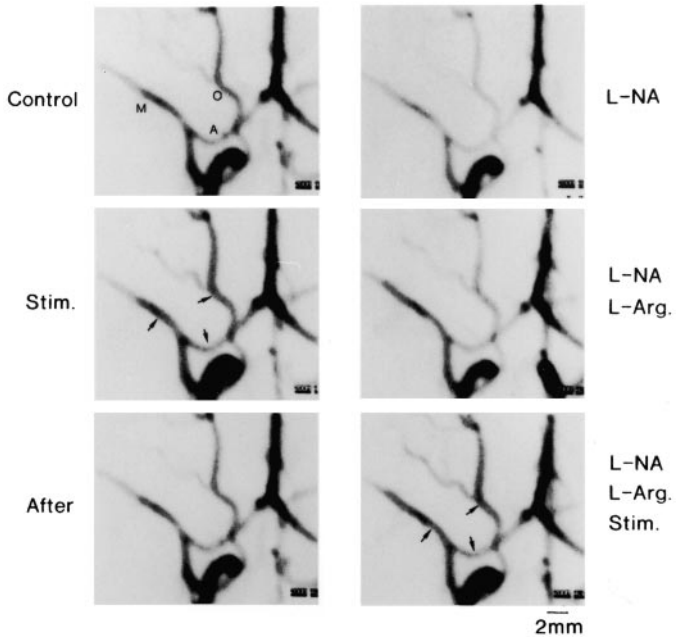

FIG. 4. Angiographic recordings of cerebral (A, anterior cerebral; M, middle cerebral) and ophthalmic (O) arteries before (control) and after (Stim.) electrical stimulation of the greater petrosal nerve (10 Hz for 15 s) (left column) and after treatment with L-NA (5 mg/kg i.v.) and additional treatment with L-arginine (L-Arg, 500 mg/kg i.v.) (top two of the right column) in an anesthetized monkey. As shown in the bottom right recording, electrical stimulation dilated the arteries under treatment with L-NA and L-arginine, whereas no vasodilatation was induced under treatment only with L-NA (data not shown). The recordings continue from the bottom left to the top right.

abolished, and L-arginine restored, the response (Toda et al., 2000a,b) (Fig. 4). Anesthetized dogs, in which unilateral damage to the pterygopalatine ganglion was induced by injections of absolute ethanol, were sacrificed 10 days later, and vasodilator responses to nerve stimulation and histology of NOS-containing neurons were compared in isolated middle cerebral arteries of the denervated and innervated sides (Toda et al., 1993d). In the denervated artery, the responses to electrical stimulation and nicotine were abolished, and positively stained neurons were absent, whereas the innervated artery was well innervated by positive neurons and responded to stimulation with moderate relaxations. Relaxations induced by exogenous NO were unchanged in the innervated and denervated arteries. Nicotine injected into the vertebral artery in anesthetized dogs elicited vasodilatation that was abolished by L-NA (Toda et al., 1993b). Histological examination using an axonal transport method, in which true blue, a retrograde transport tracer, was placed onto the unilateral middle cerebral artery, indicated that NOS-containing neurons in the sphenopalatine ganglion innervated the middle cerebral artery of rats (Minami et al., 1994). Findings so far presented reveal that the nitrergic neurons originate in the spheno- or pterygopalatine ganglion to vasodilate cerebral arteries.

In the study with anesthetized dogs (Toda et al., 2000a), denervation of nerves from the pterygopalatine ganglion to the artery constricted anterior and middle cerebral arteries. L-NA produces no or only a slight vasoconstriction in the denervated side, whereas clear vasoconstriction was observed with this inhibitor in the innervated side. These findings again support the hypothesis that the major mechanism underlying vasoconstriction by NOS inhibitors in dog cerebral arteries is a depressed functioning of the nitrergic nerve rather than of the endothelium.

5. Tracing the Origin of Nitrergic Nerve. To trace the origin of nitrergic nerves, pterygopalatine and geniculate ganglia, or adjacent greater petrosal nerve in anesthetized dogs were exposed for placement of stimulating electrodes (Toda et al., 2000a). Stimulation (2–20 Hz) of both ganglia vasodilated the anterior, middle, and posterior cerebral arteries and the posterior communicating artery of only ipsilateral side in a frequency-dependent manner. The response was quite sensitive to intravenous L-NA. C_6 abolished only the response to stimulation of the geniculate ganglion, suggesting that preganglionic neurons discontinue at the pterygopalatine ganglion. The origin of nerves through the geniculate ganglion to the petrosal nerve was histologically determined to be the superior salivatory nucleus, which is known as a source of cholinergic preganglionic nerves. Only postganglionic nerves contain nitrergic as well as cholinergic neurons. Similar findings have also been obtained in anesthetized monkeys (Toda et al., 2000b) (Fig. 4). In monkeys, a catheter inserted into the inter-

nal carotid artery enabled visualization of angiographic images in the cranial portion (anterior and middle cerebral arteries) of the circle of Willis of the injected side, whereas in dogs, when the catheter was placed only in the vertebral artery, angiographic images of both sides from caudal to cranial portions were visible. Unilateral stimulation of the greater petrosal cell group, a subgroup of the superior salivatory nucleus, by microinjection of L-glutamate reduced ipsilateral cerebrocortical vascular resistance in anesthetized rats (Nakai et al., 1993). Although the involvement of NO was not elucidated, they determined that pharmacological blockade of the pterygopalatine ganglion and removal of its postganglionic nerves abolished the response that was resistant to muscarinic receptor blockers. Electrical or chemical (by L-glutamate) stimulation of the rostral ventrolateral medullary neurons (Saeki et al., 1989; Golanov and Reis, 1994), basal forebrain (Raszkeiwicz et al., 1992), or nucleus basalis of Meynert (Adachi et al., 1992) also evoked increased cortical cerebral blood flow in the rat. Adachi et al. (1992) and Raszkiewicz et al. (1992) suggested that NO derived from the perivascular nerve has a critical role in the regulation of cortical blood flow. Therefore, in the mammals tested, the nerve action potential generated in the superior salivatory nucleus is expected to deliver central information through the geniculate and pterygopalatine ganglia to cerebral arteries and their branches in the circle of Willis to regulate vascular tone under resting and stimulated conditions. Figure 5 illustrates a scheme of the efferent neurons innervating the cerebral artery. The regulation of functions of this nucleus by the upstream cell groups or nuclei in the brain would be an important and intriguing project left for future studies.

Evidence suggesting that the ophthalmic and retinal arteries of monkeys also receive parasympathetic nitrergic nerves from the same route has been obtained (Ayajiki et al., 2000) (Fig. 5). Studies on the actions of L-NA and atropine on lacrimation and nasal secretion induced by ganglionic stimulation in dogs suggest that exocrine secretion from these glands is under control of cholinergic, but not nitrergic, nerves (Toda et al., 2000a).

6. Hypercapnic and Hypoxic Cerebroarterial Dilation and Hypothermia.

a. Hypercapnia. Cerebral blood flow is increased by hypercapnia. Involvement of NO in this phenomenon has been discussed by Iadecola et al. (1994) and Toda and Okamura (1998). In anesthetized rats (Iadecola, 1992; Wang et al., 1992a) and cats (Sandor et al., 1994), the hypercapnia-induced increase in cerebral blood flow was suppressed by intravenous or topical applications of NOS inhibitors, and L-arginine reversed the effect. However, no or only slight inhibition by NOS inhibitors has been reported in the goat (Dieguez et al., 1993), cat (Goadsby, 1994), pig (Parfenova et al., 1994), and monkey (McPherson et al., 1995). In the rat, NO-independent mechanisms underlying hypercapnic vasodilatation are also suggested (Iadecola and Zhang, 1994; Wang et al., 1994b; Estevez and Phillis, 1997), in which prostaglandins and adenosine are importantly involved. The

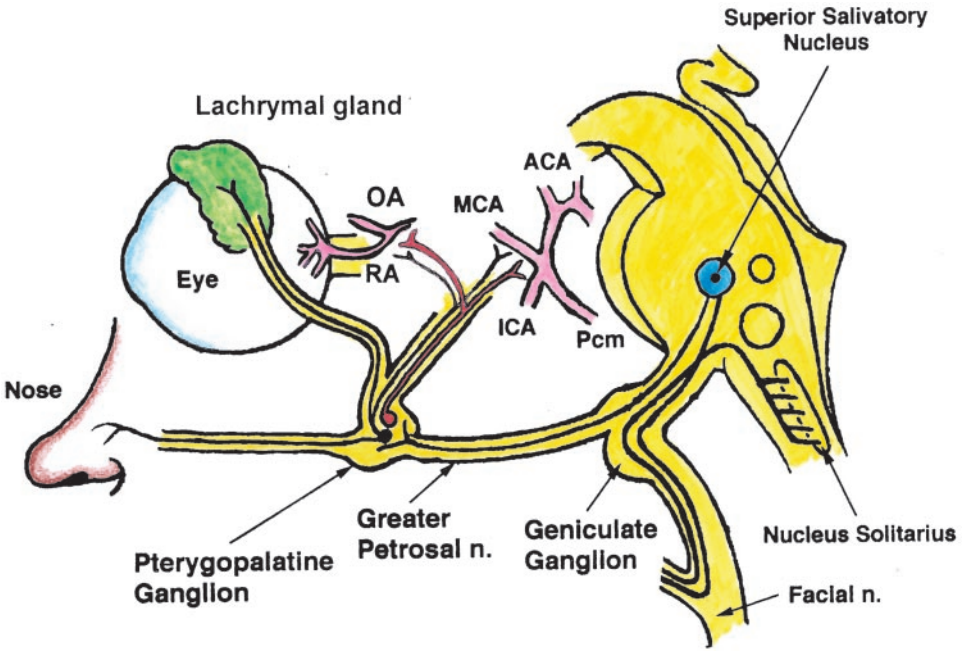

FIG. 5. Hypothetical scheme of innervation of nitrergic (red lines) and cholinergic nerves (black lines) originating from the superior salivatory nucleus through the geniculate ganglion, greater petrosal nerve, and pterygopalatine ganglion in cerebral and ocular arteries. Nitrergic nerves control the diameter of cerebral (ACA, anterior cerebral; MCA, middle cerebral; ICA, intracranial internal carotid; Pcm, posterior communicating; and also posterior and basilar) and ocular (OA, ophthalmic; RA, retinal) arteries. Postganglionic cholinergic neurons regulate the function of cerebral and ocular arteries and lachryimal and nasal glands.

synergistic action of NO-cyclic GMP and prostacyclin-cyclic AMP systems and the cross-talk between these systems in the hypercapnia-induced increase in cerebral blood flow have been postulated (Wang et al., 1998, 1999a; Heinert et al., 1999; Son and Zuckerman, 2002).

In spite of the papers supporting a role of NO in the vascular response to hypercapnia, the origin of NO is not fully understood. NO released from the endothelium is assumed to participate in the response (Iadecola, 1992), and failure to inhibit the vascular response to hypercapnia by topical application of TTX suggests the importance of eNOS-derived NO (Fabricius and Lauritzen, 1994). On the other hand, the blood flow response to hypercapnia was observed in endothelium-damaged arteries of rats (Wang et al., 1994a) and eNOS-knockout mice (Ma et al., 1996b), suggesting that NO derived from nNOS plays a crucial role in the response. The view has been supported by experiments with a relatively selective nNOS inhibitor, 7-nitroindazole (Harada et al., 1997; Okamoto et al., 1997).

In isolated canine and monkey cerebral arteries, hypercapnia evoked moderate persistent relaxation that is not suppressed by removal of the endothelium (Toda et al., 1989, 1993c). Treatment with ouabain moderately inhibits the response. These results indicate that hypercapnia fails to stimulate the release of endothelium-derived NO, but acts directly on arterial smooth muscle and induces vasodilatation, possibly by lowering extracellular pH and activation of the Na^+ pump. A further study has demonstrated that the cerebroarterial relaxation induced by electrical stimulation, nicotine, substance P, and exogenous NO is potentiated in the hypercapnic media, possibly due to a decreased degradation of NO (Toda et al., 1996a). Taken together, under resting conditions in vivo, the vasodilator response to NO continuously released from the endothelium and perivascular nitrergic nerves may be enhanced by hypercapnic acidosis.

Effects of L-NMMA on the hypercapnia-induced increase in middle cerebral blood flow measured with laser Doppler ultrasound were independently examined in conscious humans in two different studies. Schmetterer et al. (1997) observed that the hemodynamic change in response to hypercapnia, but not to hyperemia, was blunted by the NOS inhibitor and suggested the importance of NO in the hypercapnia-induced vasodilation in humans. On the other hand, White et al. (1998) noted that L-NMMA constricted the middle cerebral artery and failed to influence the hemodynamic response caused by hypercapnia. Therefore, the role of NO in this response is still a controversial issue, since the discrepant results were obtained in humans under quite similar experimental conditions.

b. Hypoxia. Hypoxia increases cerebral blood flow by dilating the arteries and arterioles. The mechanism underlying hypoxic cerebral vasodilation has been briefly reviewed by Pearce (1995). In general, hypoxia is considered to activate multiple mechanisms that influence cerebrovascular tone. Through actions on nonvascular cerebral elements, hypoxia stimulates the production of a variety of vasodilating metabolites such as potassium, hydrogen ions, prostaglandins, adenosine, and NO. Hypoxia also promotes the neuronal release of excitatory amino acids, which stimulate overall cerebral metabolism and further enhance the release of vasodilator metabolites. The remaining vasodilatation is attributable to direct effects of hypoxia on cerebral arteries. Components involving the vascular effect include the endothelium, smooth muscle, and perivascular nerves.

In isolated cerebral arteries, in which the active tone was produced by vasoconstrictors, hypoxia produced relaxation. Hypoxic inhibition of Ca^{2+} influx is reported in rabbit basilar arteries (Pearce et al., 1992). In isolated rat cerebral arteries, relaxation in response to reduced partial oxygen pressure is mediated by an endothelium-derived cyclooxygenase product that activates ATP-sensitive K^+ channels (Fredricks et al., 1994). In cerebral arteries isolated from sheep exposed to chronic hypoxia, both smooth muscle and endothelial functions are depressed, and the depression is greater in the fetus than in adults (Longo et al., 1993).

Hypoxia dilates pial arterioles and/or increases cerebral blood flow in anesthetized rats (Buchanan and Phillis, 1993), dogs (McPherson et al., 1994), pigs (Pourcyrous et al., 1990; Wilderman and Armstead, 1997), lamb (van Bel et al., 1995), cats (Ishimura et al., 1996), and rabbits (Todd et al., 1997), as well as in conscious dogs (Audibert et al., 1995) and humans (Van Mil et al., 2002). Involvement of NO has been suggested in the response (Audibert et al., 1995; van Bel et al., 1995; Ishimura et al., 1996; Todd et al., 1997; Wilderman and Armstead, 1997; Van Mil et al., 2002). Prostanoids (Pourcyrous et al., 1990; Leffler and Parfenova, 1997), methionine enkephalin released by NO and/or cyclic GMP (Armstead, 1995a), adenosine (Armstead, 1997a), and cytochrome P450 epoxygenase metabolites (Fredricks et al., 1994) are also involved in the response in rats and pigs. Hypoxia-induced pial arteriolar dilatation is mediated via ATP-sensitive (Reid et al., 1995; Armstead, 1997a) and Ca^{2+}-sensitive K^+ channels (Armstead, 1997b), which are activated by substances other than NO.

On the other hand, hypoxia produced contractions of isolated canine basilar (Elliott et al., 1989) and sheep middle cerebral arteries (Klaas et al., 1989). Vasoconstrictor substances derived from the endothelium may be involved in the response (Klaas et al., 1989). Chronic hypoxia increases sensitivity of the guinea pig middle cerebral artery to vasoconstrictors, most likely from a reduced production and/or activity of NO (Sillau et al., 2002).

In the isolated canine and monkey cerebral artery, neurogenic relaxation mediated by NO is inhibited by hypoxia. Since this inhibition is prevented or reversed

by treatment with Na^+/H^+ exchange inhibitors, restoration of normal intracellular pH by Na^+/H^+ exchanger in nerve terminals under hypoxia may impair nerve function (Okamura et al., 1997). Hypoxia reportedly decreases intracerebral NO concentrations in anesthetized pigs (Kirkeby et al., 2000) and chronic hypoxia reduces nitrergic function in the sheep (Buchholz et al., 1999). The contribution of nitrergic nerve activity to hypoxia-induced changes in the cerebral circulation requires further study.

c. Hypothermia. Body temperature is an important determinant of oxygen consumption and energy metabolism. Hyperthermia is deleterious in hypoxic animals, particularly for oxygen-sensitive organs, e.g., heart and brain. Conversely, a moderate degree of hypothermia could be beneficial during hypoxia (Wood and Gonzales, 1996). Forced hypothermia is sometimes used in surgical procedures, particularly for heart and brain surgery. In anesthetized rats, selective brain cooling to about 30°C increases cortical cerebral blood flow. During rewarming, as brain temperature increases, cortical cerebral blood flow decreases. Therefore, the cortical vasodilatory response to hypothermia may explain its protective effects during and after cerebral ischemia (Kuluz et al., 1993).

The vasodilatory mechanism is not clearly understood, but attenuation of responses of cerebral arterioles to vasoconstrictor agents such as potassium and prostaglandin $F_{2\alpha}$ has been reported in perfusion experiments in isolated rat intracerebral arterioles (Ogura et al., 1991). The effects of hypothermia on neurogenic relaxation under normoxia and hypoxia were studied in isolated dog (Okamura et al., 2001a) and porcine (Tanaka et al., 2003) cerebral arteries, in which neurogenic relaxation was mediated by NO liberated from perivascular nerves (Toda and Okamura, 1990; Tanaka et al., 1999). Hypoxia impairs vasodilator nerve function in canine cerebral arteries (Okamura et al., 1997), whereas hypercapnea potentiates this function (Toda et al., 1996a). The hypoxia-induced inhibition of vasodilator nerve function is prevented by hypothermia (Okamura et al., 2001a). The detailed mechanism of this response is not known, but amiloride, an inhibitor of Na^+/H^+ exchanger, or extracellular acidosis also attenuated the hypoxia-induced inhibition of neurogenic relaxation (Okamura et al., 1997), and hypothermia generally causes intracellular acidosis (Feher and Rebeyka, 1994). Intracellular pH may participate in the preventive action of hypothermia. These findings imply that prevention of the hypoxia-induced inhibition of vasodilator neurogenic relaxation by lowering body temperature would be beneficial by increasing the blood supply to ischemic brain regions.

7. Autoregulation. In vital organs such as the brain, heart, and kidney, blood flow is constantly maintained even when blood pressure changes rapidly. The mechanism of this phenomenon, termed the autoregulation, although intensively investigated, has not yet been determined. Although a myogenic theory that smooth muscle cells alone sense and evoke the response has been favored, the possible roles of the endothelium and perivascular nerve in autoregulation are also hypotheses of interest. Since NO regulates vascular tone as an EDRF and as a neurotransmitter of vasodilator nerves, the possible involvement of NO in autoregulation has been investigated.

In anesthetized rats, intra-arterial administration of 7.5 mg/kg L-NA did not affect the cerebral autoregulatory curve nor the lower limit of autoregulation. Elevation of the dose up to 30 mg/kg raised the upper limit of autoregulation, but cerebral blood flow remained constant, indicating that endogenous NO does not participate in cerebral autoregulation (Wang et al., 1992b). Similar findings were also obtained in anesthetized rats (Buchanan and Phillis, 1993), dogs (Saito et al., 1994), and cynomolgus monkeys (Thompson et al., 1996), and in conscious rats (Kelly et al., 1994; Takahashi et al., 1995). However, Tanaka et al. (1993) reported a significant role of NO in the autoregulation of cerebral blood flow in anesthetized rats. These authors noted that cerebral blood flow in various superficial and deep brain regions was decreased by lowering of systemic blood pressure in rats treated with 30 mg/kg L-NMMA but not in untreated rats. Similar results were also reported in anesthetized rats (Preckel et al., 1996; Jones et al., 1999) and cats (Kobari et al., 1994), in which the role of NO in maintaining the lower limit of cerebral autoregulation was emphasized. Hardy et al. (1999) have suggested that NO curtails the upper limit of cerebral autoregulation in anesthetized newborn pigs. The possible involvement of NO in the cerebral autoregulation has been reported in humans (White et al., 2000). Therefore, whether or not NO participates in the physiological autoregulation of cerebral blood flow is still controversial.

In the isolated perfused intraparenchymal arteriole of rats cannulated with micropipette, arteriolar NO from the endothelium appeared to participate in the autoregulatory vasodilation in response to hypotension (Kajita et al., 1998), whereas importance of nNOS in cerebral autoregulation was postulated in anesthetized pigs (Hardy et al., 1999). This apparent difference in the source of NO between endothelium and perivascular nerve may be a reflection of different animal species and experimental conditions.

8. Prejunctional Modulation of Nitrergic Nerve Function by Cholinergic and Adrenergic Neurotransmitters. Histochemical studies of the cerebral artery have demonstrated that there are networks of neurons containing immunoreactivity of NOS, acetylcholinesterase or choline acetyltransferase, VIP and tyrosine hydroxylase (Owman, 1988; Bredt et al., 1990), as well as NADPH diaphorase (Vincent and Kimura, 1992) that is reportedly identical to NOS in neurons (Dawson et al., 1991). NOS and VIP (Nozaki et al., 1993; Minami et al., 1994; Toda et al., 1997b) or VIP and

cholinesterase/choline acetyltransferase (Hara et al., 1985; Saito et al., 1985; Suzuki et al., 1990b) coexist in parasympathetic ganglionic cells and major cerebral arteries. The nitrergic nerve plays a major role in the control of cerebral arterial tone, and various other substances are undoubtedly involved in the modulation of NO synthesis in or NO release from the nerve.

Treatment with acetylcholine or other muscarinic agonists attenuated the inhibitory response to nitrergic nerve stimulation in cerebral arteries from the dog (Toda et al., 1995c), cow (Toda and Ayajiki, 1990; Ayajiki et al., 1993), pig (Liu and Lee, 1999; Tanaka et al., 1999), monkey (Toda et al., 1997b; Okamura et al., 1998c), and guinea pig (Jiang et al., 1999). Relaxations induced by exogenous NO or NO donors were not influenced by the muscarinic agonists. Muscarinic receptor antagonists abolished the inhibitory effect, suggesting that prejunctional muscarinic receptor activation inhibits the synthesis or release of NO. Based on studies with the antagonists of muscarinic receptor subtypes (M_1, M_2, and M_3), pirenzepine, AF-DX-116 and 4-DAMP, it was concluded that the M_2 receptor subtype is mainly involved in the inhibition in monkey (Toda et al., 1997b) and porcine cerebral arteries (Liu and Lee, 1999).

The response to neurogenic NO was also inhibited by physostigmine in concentrations that were sufficient to inhibit cholinesterase activity but did not induce actions directly on muscarinic receptors in monkey and porcine cerebral arteries (Toda et al., 1997b; Tanaka et al., 1999). However, this is not the case in the dog cerebral artery (Toda et al., 1995c). On the other hand, atropine potentiated responses of monkey and porcine arteries. Therefore, neurogenic acetylcholine acts on prejunctional sites to inhibit the function of nitrergic nerves in monkey and porcine, but not canine, cerebral arteries. It is suggested that neurogenic acetylcholine also inhibits the nitrergic nerve function in anesthetized monkeys but not in dogs in vivo (Toda et al., 2000a,b).

Although there is no direct evidence, it is possible that interference with Ca^{2+} influx into nerve terminals may be a mechanism underlying the action of acetylcholine (Liu and Lee, 1999). Cyclic GMP may not be involved, since 8-bromo-cyclic GMP is without effect (Toda et al., 1997c). By using the K^+ channel blockers, Jiang et al. (1999) suggested that opening of Ca^{2+}-activated K^+ channel in nerve terminals might participate in the acetylcholine action in the guinea pig cerebral artery. However, K^+ channels do not seem to be involved in the canine cerebral artery, in which the response to nerve stimulation is not attenuated in high K^+ bathing media (Ayajiki et al., 2001a).

Relaxant responses mediated by NO from nerves were not influenced by exogenous VIP in concentrations of 10^{-9}–10^{-7} M in canine, porcine, and monkey cerebral arteries; these concentrations produced slight to moderate relaxations by acting directly on smooth muscle (Ayajiki et al., 1993; Toda et al., 1995c, 1997a; Tanaka et al., 1999). In the mammals investigated, a prejunctional action of VIP could be excluded.

Treatment with clonidine or yohimbine failed to alter significantly the response to nitrergic nerve stimulation in dog and monkey cerebral arteries (Toda et al., 1995c; Okamura et al., 1998c). Although prejunctional α_2 adrenoceptor stimulation appears to inhibit the synthesis or release of NO from nerves in extracerebral arteries (Simonsen et al., 1997), evidence for an action mediated by prejunctional α_2-adrenoceptors in cerebral arteries has yet to be found. β-Adrenoceptor antagonists did not modify nerve function in the mammals used (Toda, 1975, 1981; Toda et al., 1990c; Ayajiki et al., 1993). 8-Bromocyclic AMP did not alter the neurogenic response in the canine (Toda et al., 1997c) and porcine cerebral artery (Liu and Lee, 1999).

9. Histochemical Studies of Neurons Containing Nitric-Oxide Synthase. Bredt et al. (1990) were the first to demonstrate the localization of nNOS by an immunohistochemical method in the rat brain and postulate a role of NO in neural function. Perivascular nerves innervating the rat cerebral artery were also intensely stained. The localization pattern did not coincide with any known neurotransmitters. NOS-positive neurons also stain for NADPH diaphorase. This enzyme has primarily been characterized histochemically, since its activity gives a blue color in brain sections incubated with nitro blue tetrazolium in the presence of NADPH, but not NADH. Since nNOS activity requires NADPH (Bredt and Snyder, 1990), the oxidative activity accounts for the diaphorase staining of these cells. Therefore, NADPH diaphorase is reportedly identical to nNOS in the brain and peripheral tissues (Dawson et al., 1991), and the staining has frequently been used to demonstrate the localization of nNOS. However, positively stained cells with NADPH diaphorase are not always those containing nNOS. Inducible NOS and eNOS also possess NADPH diaphorase activity. Neurons innervating adrenal cortical cells and the submucosal plexus of the gastrointestinal tract are positively stained with the NADPH diaphorase method but not by nNOS antibody (Snyder and Bredt, 1991). Furthermore, neuropeptide Y and somatostatin largely colocalize with NADPH diaphorase (Vincent et al., 1983), whereas the overlap between neurons containing nNOS and these peptide neurotransmitters is limited (Snyder and Bredt, 1991).

In the dog, the presence of nNOS-immunoreactive nerve fibers was demonstrated in the proximal (about 400 mm outside diameter) and distal portions (about 100 mm) of the middle cerebral artery (Yoshida et al., 1993). The fibers run irregularly along the arterial walls, and they sometimes form fiber bundles. From these bundles consisting of two to three thick axons, thin fibers ramify repeatedly. Thick fibers are generally situated in the outer layer of the adventitia. In a few cases, very thick fibers are seen in the outermost region of blood vessels, suggesting the site of entry of the nerve supply. On the

other hand, thin fibers running toward the lumen are distributed mainly in the adventitia close to the adventitio-medial border, which is identified by clearly different arrangements of the two layers. Much thinner fibers, if any, are observed in a narrow space just beneath the adventitio-medial border. Similar observations were also made in the basilar artery.

The nNOS immunoreactivity in neurons was abolished in the middle cerebral artery isolated from dogs receiving an ethanol injection into the unilateral pterygopalatine ganglion a week previously. This effect was seen only in the artery of the treated side (Toda et al., 1993d). In the ganglion excised from the treated side, the positive staining of cell bodies and nerve fibers were markedly decreased, compared with that from the untreated side. These findings strongly suggest that nitrergic nerve fibers to the middle cerebral artery arise from the ipsilateral pterygopalatine ganglion in the dog. A similar distribution of perivascular nerves containing nNOS immunoreactivity and/or NADPH diaphorase in the cerebral arteries and its origin have also been reported in the rat (Iadecola et al., 1993; Suzuki et al., 1993a). Nitrergic nerve fibers innervating middle cerebral arteries are originated not only from the pterygopalatine but also from the otic ganglion in rats (Edvinsson et al., 2001). The pterygopalatine ganglion may send nitrergic nerve fibers to the choroid plexus (Lin et al., 1996), suggesting a role of the nerve in the regulation of choroidal blood flow (Szmydynger-Chodobska et al., 1996). In ultrastructural studies with the rat cerebral artery, NADPH diaphorase deposits are observed on distinct membrane portions of the endoplasmic reticulum in axons of perivascular nerves (Aoyama, 1996), and positive nerve terminals are located within 250 nm from the basal lamina of arterial smooth muscle cells in the circle of Willis, indicating that nitrergic nerve fibers truly innervate the smooth muscle cells (Tsuchida et al., 2001).

Numerous histochemical and immunohistochemical studies have demonstrated that innervation of nitrergic nerves in the cerebral arteries are also observed in the guinea pig (Saito and Goto, 1994; Barroso et al., 1996), cat (Ayajiki et al., 1994; Kimura et al., 1997), monkey (Yoshida et al., 1994a; Toda et al., 1997b), pig (Sienkiewicz et al., 1995; Tanaka et al., 1999), cow (Toda and Okamura, 1996b; Gonzalez et al., 1997), sheep (Matthew, 1997), and human (Nozaki et al., 1993; Gorelova et al., 1996). Porcine pial veins are also innervated by neurons containing NADPH diaphorase (Ishine et al., 1999).

B. Ocular Vasculature

1. Retinal Artery and Arteriole. Central retinal arteries isolated from dogs and monkeys partially contracted with vasoconstrictors responded to transmural electrical stimulation with relaxations that were abolished by TTX (Toda et al., 1994b, 1996b). Nicotine and acetylcholine (high concentrations; Toda et al., 1995b) also relaxed the arteries, and the response was sensitive to C_6 but was unaffected by β-adrenoceptor antagonists and endothelium denudation. The neurogenic relaxation was abolished by treatment with L-NA, and L- but not D-arginine restored the response, whereas relaxations induced by NO donors were not influenced. OxyHb and methylene blue abolished the dilator response to nerve stimulation and exogenous NO or NO donors. Involvement of VIP and CGRP is excluded in the neurogenic NO-mediated relaxation of monkey retinal arteries. Histochemical studies have demonstrated a network of neurons containing NOS or NADPH diaphorase in retinal arteries and arterioles (Flugel et al., 1994; Toda et al., 1994b; Roufail et al., 1995). Denervation of the unilateral pterygopalatine ganglion in dogs abolished the NOS-positive neurons in the wall of ipsilateral retinal artery and also the response to nerve stimulation but not to exogenous NO (Toda et al., 1993d). As in the case of cerebral arteries, retinal arteries are supposed to be innervated by efferent nerves from which NO is released as a neurotransmitter.

In the anesthetized dog, intravenous injections of L-NA vasoconstricted arterioles of the ocular fundus, and L-arginine reversed the effect (Toda et al., 1994a). Under treatment with C_6, the vasoconstrictor action was markedly attenuated, suggesting that a depressed synthesis of neurogenic NO is mainly involved. Preretinal juxta-arteriolar microinjection of L-NA induces a segmental and reversible arteriolar vasoconstriction in the miniature pig (Donati et al., 1995) and newborn pig (Gidday and Zhu, 1995). Intravenous L-NAME decreased retinal blood flow in anesthetized (Seligsohn and Bill, 1993) and conscious rabbits (Sugiyama et al., 2000). In these studies, basal release of NO from the nerve and endothelium may be involved in the arteriolar dilatation.

Nicotine injected into the unilateral carotid artery in the dog induced dilatation of ipsilateral retinal arterioles that was abolished by L-NA or C_6 (Toda et al., 1994b). In the rabbit, retinal blood flow was increased by facial nerve stimulation with electrical pulses, and the response was abolished by treatment with L-NAME (Nilsson, 1996). Nitrergic vasodilator nerves appear to regulate the diameter of retinal arterioles and the retinal blood flow by receiving electrical impulses from the vasomotor center via the pterygopalatine ganglion. The depletion of nNOS-containing neurons in the diabetic rat retina that was prevented by aminoguanidine and NN0028, inhibitors of advanced glycation end products, was suggested to contribute to alterations in the autoregulation of blood flow (Roufail et al., 1998). On the other hand, autoregulatory vasodilator responses to hypoxia, hypotension, and hypercapnia occurred independently of NO participation in anesthetized newborn pigs (Gidday and Zhu, 1995).

2. Ciliary Artery. Electrical nerve stimulation or nicotine elicits relaxations of isolated monkey, porcine, bovine, and canine posterior ciliary arteries (Su et al., 1994; Wiencke et al., 1994; Toda et al., 1997a, 1998, 1999). Treatment with L-NA or other NOS inhibitors abolishes the relaxation or reverses it to a slight contraction that is abolished by α-adrenoceptor antagonists. L-Arginine restores the neurogenic relaxation. Similar findings have also been obtained in human ciliary arteries (Nyborg and Nielsen, 1994). In the bovine artery, CGRP also plays a role in neurogenic relaxation (Wiencke et al., 1994). Inability of antagonists of CGRP and VIP to inhibit the response to nerve stimulation in isolated porcine ciliary arteries indicates that these peptides are not involved (Toda et al., 1997a). Although the presence of VIP has histochemically been demonstrated in perivascular nerves (Stone et al., 1987; Flugel et al., 1994; Toda et al., 1997a), there is no evidence supporting a functional role of neurogenic VIP in ciliary arteries. NADPH diaphorase-positive neurons are histologically seen in monkey and dog ciliary arteries (Toda et al., 1998, 1999).

Intravenous infusion of NOS inhibitors decreased blood flow in the iris-ciliary body in cats (Ostwald et al., 1995) and choroidal blood flow in dogs (Deussen et al., 1993), cats (Mann et al., 1995), rabbits (Astin et al., 1994), and pigs (Jacot et al., 1998). 7-Nitroindazole, a putative nNOS inhibitor, depressed the ocular blood flow in unanesthetized rats (Kelly et al., 1998). On the other hand, Koss (1998) has reported that L-NAME is effective in reducing the blood flow in anesthetized rats but 7-nitroindazole is ineffective. This author used a dose (intraperitoneal, 50 mg/kg) of 7-nitroindazole five times higher than that of L-NAME (intravenous, 10 mg/kg). The contrasting results may depend on the use of anesthetics. Modifications by NOS inhibitors of basal ocular blood flow have been summarized by Koss (1999).

In anesthetized rabbits, electrical stimulation of the facial nerve resulted in an increase of uveal blood flow that was resistant to muscarinic blockade (Stjernschantz and Bill, 1980), and NOS inhibition suppressed a decrease in uveal vascular resistance by the nerve stimulation (Nilsson, 1996). Electrical stimulation of the short ciliary nerve increased choroidal blood flow in anesthetized cats, the effect being unaffected by atropine (Nakanome et al., 1994).

3. Ophthalmic Artery. In isolated canine internal and external ophthalmic arteries, stimulation of nerves with electrical pulses or nicotine produced relaxation. L-NA, but not D-NA, reversed the response to a contraction, and L-arginine restored the relaxation (Toda et al., 1995b, 1999). The contraction under NOS inhibition was greater in the external artery than in the internal artery. α-Adrenoceptor antagonists abolished the contractile response. It appears that the external arterial tone is controlled by nitrergic vasodilator nerves and also by adrenergic vasoconstrictor nerves, whereas the internal ophthalmic artery is innervated predominantly by nitrergic nerves. Monkey ophthalmic arteries also responded to nerve stimulation at 2 to 20 Hz with frequency-dependent relaxations, which were abolished or reversed to slight contractions by L-NA (Ayajiki et al., 2000). L-Arginine restored the relaxation. ODQ also abolished the relaxation. Neurogenic NO appears to increase the formation of cellular cyclic GMP, resulting in the relaxation of monkey ophthalmic artery smooth muscle.

In anesthetized monkeys, unilateral electrical stimulation of the pterygopalatine ganglion dilated only the ipsilateral ophthalmic artery in a frequency-related manner (Ayajiki et al., 2000). Intravenous injections of L-NA or pterygopalatine denervation constricted the artery, suggesting tonic nitrergic vasodilator innervation. Treatment with L-NA depressed the vasodilator response to the nerve stimulation, and L-arginine restored the response. As postulated in an earlier section on cerebral arteries, the pterygopalatine ganglion, receiving cholinergic neurons from the superior salivatory nucleus, seems to send off nitrergic neurons to the ophthalmic artery and possibly the retinal artery and arteriole (Fig. 5).

C. Lingual Artery

Deep lingual arteries isolated from the Japanese monkey responded to electrical nerve stimulation with contractions that were abolished by treatment with TTX. In the arterial strips contracted with prostaglandins, the response was attenuated by prazosin and reversed to a relaxation by combined treatment with α,β-methylene ATP (Toda et al., 1997d). The relaxation was abolished by L- but not D-NA and restored only by the L-enantiomer of arginine. Nicotine-induced relaxation in the strips treated with prazosin and α,β-methylene ATP was also abolished by L-NA, C_6, methylene blue, and oxyHb. Timolol and atropine were without effect. In the strips made unresponsive to VIP and CGRP by repeated applications of these peptides, the response is unaltered. Neurons containing nNOS immunoreactivity or NADPH diaphorase are present in the adventitia-medial border in monkey, porcine, and canine lingual arteries (Toda et al., 1997d; Yoshida and Toda, 1997). It appears that NO, but not VIP and CGRP, liberated from nerves upon stimulation acts on soluble guanylyl cyclase in the smooth muscle and produces cyclic GMP, resulting in the relaxation. Presence of cells containing NADPH diaphorase in the intralingual ganglia may indicate that nitrergic nerves are locally supplied from the ganglia (Yoshida and Toda, 1997).

Contractions to electrical nerve stimulation or nicotine of isolated canine lingual arteries were reversed to relaxations by α,β-methylene ATP that were also abolished by L-NA and TTX (for electrical stimulation) or C_6 (for nicotine) (Okamura et al., 1998b). The L-NA-induced inhibition was reversed by L-arginine. The mechanism

underlying the relaxation partially differed from that in monkeys. In canine arteries made tachyphylactic to VIP and CGRP, the neurogenic relaxation was blunted; however, antagonists of VIP and CGRP receptors failed to exert any inhibition. Beraprost, a stable analog of prostacyclin, that relaxes vascular smooth muscle possibly by increasing cellular cyclic AMP (Toda, 1988a) like VIP and CGRP, also attenuates the relaxation by nerve stimulation. Thus it can be concluded that neurogenic relaxations in monkey lingual arteries are mediated solely by NO, whereas NO as well as some substance(s) that increases the level of cellular cyclic AMP contribute to canine lingual artery relaxation; VIP and CGRP are not involved. The dilator response of isolated feline lingual artery in response to electrical nerve stimulation is suggested to be mediated by acetylcholine and also unidentified substance(s) (Rowan et al., 1984). In this study, involvement of NO could not be determined. Cholinergic and noncholinergic neurogenic vasodilatation were also reported in rabbit lingual artery (Brayden and Large, 1986). The mechanism underlying vasodilatation due to nerve-derived acetylcholine remains to be explained.

In anesthetized dogs, electrical stimulation of the brain stem elicited lingual artery dilatation and increment in blood flow in the tongue, which were not influenced by spinal cord transection and treatment with reserpine, α-adrenoceptor antagonists and atropine, but were abolished by C_6 (Nagai and Pleschka, 1981). Therefore, NANC innervation in canine lingual arteries was suggested. Increased blood flow in the tongue was also induced by intracranial facial nerve stimulation (Stjernschantz and Bill, 1980). Electrical stimulation of the parasympathetic chorda-lingual nerve caused biphasic vasodilatation of the cat tongue, with acetylcholine and VIP being suggested to mediate the response (Lundberg et al., 1982). The increase in lingual blood flow by stimulation of the peripheral and central ends of the chorda tympani nerve was depressed by C_6 treatment and abolished by the section of the lingual nerve (Izumi and Karita, 1994). Neurotransmitters involved were not determined. Seizures induced by bicuculline or pentylenetetrazole in anesthetized and sympathetically denervated rats evoked lingual vasodilatation that was resistant to cholinergic blockade but was blocked by C_6 (Faraci et al., 1986). Snuff applied to the buccal space increased plasma nicotine concentrations and blood flow in the tongue of anesthetized dogs (Huckabee et al., 1993). In these in vivo studies, whether neurogenic NO is involved has not been elucidated. On the other hand, local application of capsaicin onto the tongue produced L-NAME-sensitive vasodilatation in anesthetized rats (Fazekas et al., 1994).

D. Nasal Vasculature

Transmural electrical stimulation relaxed strips of canine mucosa, rich in small arteries, arterioles, and venules that had been treated with guanethidine and atropine and partially contracted with ergotamine (Watanabe et al., 1995). The response was sensitive to TTX, was abolished by L-NA but not by D-NA, and was partially restored by additional treatment with L-arginine, suggesting that NO released from perivascular nerves acts as a neurotransmitter. Capsaicin was ineffective. In strips of human nasal mucosa, the relaxation elicited by electrical nerve stimulation was suppressed by L-NAME and also by a CGRP receptor antagonist. NO and CGRP appear to mediate the response of human nasal vasculature (Okita and Ichimura, 1998). NADPH diaphorase-positive and nNOS-immunoreactive neurons have histologically been found in human nasal mucosa (Riederer et al., 1999). Cold exposure from 34 to 24°C augmented the NO-mediated relaxation induced by nerve stimulation without any change in the relaxation to sodium nitroprusside in canine nasal mucosa (Watanabe et al., 1998). An increase in the release of NO from the vasodilator nerve was suggested.

E. Temporal Vasculature

In strips of superficial temporal arteries from dogs and monkeys, transmural electrical stimulation or nicotine produced contraction that was abolished by phentolamine or guanethidine. In the prostaglandin-contracted strips with α-adrenoceptor blockade, the nerve stimulation elicited relaxation that was sensitive to TTX. L-NA abolished the response that was restored by L-arginine. The D-enantiomers were without effect (Toda et al., 1991c; Toda and Okamura, 1991a). Endothelial denudation and treatment with indomethacin, timolol, and atropine failed to inhibit the relaxation to nerve stimulation. The release of NOx during electrical nerve stimulation from superfused canine temporal artery strips denuded of the endothelium was depressed by treatment with L-NA (Toda et al., 1991). The content of cyclic GMP in monkey artery was increased by nerve stimulation with nicotine, and the effect was blocked by treatment with L-NA (Toda and Okamura, 1991a). There are neurons containing nNOS immunoreactivity innervating the canine and monkey temporal arteries (Yoshida et al., 1993, 1994a). Based on our observations, we were the first to hypothesize that in extracranial arteries, NO formed from L-arginine via nNOS in the nerve terminals plays a crucial role in transmitting information to smooth muscle. Neurogenic, NO-mediated vasodilatation by electrical stimulation and nicotine in canine temporal arteries was evidently greater in the distal portion (0.2–0.3 mm inside diameter) than in the proximal portion (0.5–0.6 mm), suggesting a contribution of neurogenic NO to the regulation of vascular resistance (Toda and Okamura, 1993). Relaxations in response to exogenous NO and nitroglycerin did not differ in the proximal and distal arteries. In monkey temporal arteries, relaxations induced by neurogenic and exogenous NO did not differ in the proximal and distal portions.

α-Adrenoceptor-mediated contractions of canine and monkey temporal artery strips were potentiated by L-NA but not D-NA, and L-arginine reversed the response (Toda et al., 1991c; Toda and Okamura, 1991a). Norepinephrine-induced contraction was not influenced by L-NA. The ^{3}H-overflow by electrical nerve stimulation from superfused temporal arteries previously exposed to the bathing media containing ^{3}H-norepinephrine was not altered by L-NA (Toda et al., 1991c). Prejunctional action of NO on adrenergic nerves may be ruled out. Therefore, the potentiation by L-NA of neurogenic contraction appears to be derived from the release of NO from nitrergic nerves that functionally counteracts the norepinephrine-induced contraction at the smooth muscle level.

Temporal veins from dogs and monkeys responded to nicotine with contractions that were abolished by C_6 and were suppressed or reversed to relaxations by prazosin (Toda et al., 1995d). The relaxation was partially inhibited by timolol and metoprolol and was abolished by additional treatment with L-NA, suggesting that NO and norepinephrine are vasodilator transmitters in the perivascular nerve. Butoxamine was without effect. β_1-Adrenoceptors may be involved in the amine-induced relaxation. A histochemical study has revealed that the dog and monkey venous wall contains nerve bundles and fibers stained by nNOS antiserum and NADPH diaphorase.

Seizures produced by bicuculline or pentylenetetrazole in anesthetized rats with bilateral cervical sympathetic denervation markedly increased the blood flow in masseter muscle. The effect was not affected by atropine but was blocked by C_6 (Faraci et al., 1986). Whether or not this noncholinergic neurogenic vasodilatation is mediated by NO has not been determined.

IV. Nitrergic Innervation in Blood Vessels of Viscera

A. Coronary Artery

In isolated canine and monkey epicardial coronary arteries contracted with prostaglandins and treated with α-adrenoceptor antagonists, transmural electrical stimulation, nicotine (Toda and Hayashi, 1982; Shiraishi et al., 1998), or norepinephrine (Toda and Okamura, 1990d) produced relaxations that were abolished by nonselective β- and selective β_1-adrenoceptor antagonists. The response to electrical stimulation is abolished by TTX, and nicotine-induced relaxation was C_6-sensitive. The neurogenic responses were not influenced by L-NA in concentrations sufficient to abolish the relaxation induced by nerve stimulation in canine and monkey cerebral arteries (Toda et al., 1990c; Toda and Okamura, 1996b; Shiraishi et al., 1998). There are nerve fibers and bundles containing nNOS immunoreactivity or NADPH diaphorase in the wall of canine and monkey coronary arteries; however, these fibers are located only in the adventitia but not in the adventitia-medial border and outer layer of the media as seen in canine and monkey cerebral, mesenteric, and temporal arteries (Yoshida et al., 1993, 1994a). Inability of nitrergic nerves to induce smooth muscle relaxation in large coronary arteries may be attributable to the sparser distribution of these nerves in areas close to smooth muscle. In the heart, the density of nNOS-containing neurons was shown to be much less than that of eNOS (Ursell and Mayes, 1995). Peptide-immunoreactive nerve fibers were histologically detected in human epicardial coronary arteries, suggesting that peptidergic (CGRP and substance P) nerves are predominantly involved in mediating vasodilatation in these vessels (Gulbenkian et al., 1993). Nerve fibers containing CGRP, substance P, and VIP also innervate the wall of human epicardial coronary veins (Saetrum et al., 1995). However, the functional role of neurogenic peptides as vasodilator neurotransmitters has not yet been elucidated. NO and neuropeptides were expressed in neurons by the late gestational period (19 days) in the developing rat heart (Shoba and Tay, 2000).

Histochemical study has demonstrated perivascular nerves containing nNOS immunoreactivity or NADPH diaphorase in guinea pig (Klimaschewski et al., 1992) and rat coronary arterioles (Sawada et al., 1997). Therefore, the role of nitrergic nerves in the regulation of coronary arteriolar tone and vascular resistance would not be excluded. Impaired formation and action of neuronal NO may be involved in the genesis of coronary small arterial vasospasm (Gutstein et al., 1987; Shimada et al., 1999). Neuronal NOS-immunoreactive or NADPH diaphorase-positive cardiac neurons exhibit choline acetyltransferase immunoreactivity in the human, monkey, dog (Yoshida and Toda, 1996) and guinea pig (Calupca et al., 2000). The nitrergic nerve is derived from the nodose ganglion. Neuronal NO was suggested to facilitate vagally induced bradycardia via a prejunctional modulation of neurotransmission (Herring et al., 2000). This hypothesis was supported by studies with nNOS knockout mice (Choate et al., 2001).

B. Pulmonary Vasculature

There is still some controversy about the mechanisms underlying the inhibitory response to NANC nerve stimulation in isolated pulmonary arteries. Endothelium-independent, TTX-sensitive neurogenic relaxations are mainly mediated by NO in guinea pig pulmonary arteries (Liu et al., 1992a; Scott and McCormack, 1999) and the endothelium-dependent, TTX-sensitive response is mediated by NO and additional neurotransmitters in rat pulmonary arteries (Gumusel et al., 2001). Nicotine caused relaxation in canine pulmonary arteries and veins that was abolished by C_6 and L-NA (Ayajiki et al., 2002). The venous relaxation was greater than the arterial one. Histological studies demonstrate perivascular neurons containing NADPH diaphorase or NOS immu-

noreactivity in guinea pig and rat pulmonary arteries and veins (Klimaschewski et al., 1992; Haberberger et al., 1997). Endogenous NO derived possibly from nerves seems to exert postjunctional inhibition of adrenergic neurotransmission in the guinea pig pulmonary artery (Cederqvist et al., 1991). On the other hand, sensory nerve-mediated relaxation was noted in isolated guinea pig pulmonary arteries; thus, involvement of CGRP was suggested (Maggi et al., 1990; Liu et al., 1992b; Butler et al., 1993). Neuropeptides including CGRP have histologically been demonstrated (reviewed by Widdicombe, 1990). Endothelium-dependent, NO-mediated relaxation by electrical field stimulation has also been shown in bovine pulmonary arteries (Buga and Ignarro, 1992). Since TTX is without effect, the response would be nonneuronal.

In intact dogs, intravenous injection of L-NA and methylene blue increased the pulmonary vascular tone under hypoxic conditions (Leeman et al., 1994). L-NA also decreased pulmonary blood flow and increased pulmonary arterial pressure in the ovine fetus and lamb (Abman et al., 1990; Fineman et al., 1991; Tiktinski et al., 1992). Endogenous NO production appears to be important for maintaining a low pulmonary vascular resistance (Fineman et al., 1991). Decrease in lobar pulmonary arterial pressure in response to vagal stimulation in intact-chest cats was abolished by atropine and was reduced by L-NAME, suggesting that NO released by neurogenic acetylcholine participates in the response. (McMahorn et al., 1992). Inhaled NO has been reported to be effective for treating patients with pulmonary hypertension and respiratory distress syndrome (Frostell et al., 1991, 1993; Pearl, 1993). NO if inhaled reaches pulmonary arterioles abluminally, which are closely associated with bronchioli and alveoli, and decreases pulmonary vascular resistance. Endogenous NO would also be supplied abluminally from perivascular nitrergic nerves. Impaired formation of NO in the nerves and endothelium may attenuate a reduction of pulmonary vascular resistance.

C. Digestive Tract Vasculature

In dog (Toda et al., 1991b), monkey (Toda and Okamura, 1992b), cow (Ahlner et al., 1991; Leckstrom et al., 1993), and guinea pig mesenteric arteries (Gyoda et al., 1995) partially contracted with vasoconstrictors, electrical nerve stimulation or nicotine caused a contraction that was reversed to a relaxation by treatment with α-adrenoceptor antagonists or adrenergic neuron blockers. The relaxation was independent of the endothelium and was abolished by the L-enantiomers of NOS inhibitors, and L-arginine restored the response. Neurogenic relaxation was inhibited by methylene blue but was potentiated by inhibitors of cyclic GMP phosphodiesterase (Ahlner et al., 1991). Tissue cyclic GMP was increased by nerve stimulation (Toda et al., 1991b; Toda and Okamura, 1992b). Histochemical studies demonstrated the network of NOS-immunoreactive neurons in the adventitia and adventitio-medial border of dog and monkey mesenteric arteries (Yoshida et al., 1993, 1994a). Nitrergic innervation would thus be inferred. Nitrergic nerves innervating the monkey mesenteric vein were also reported by functional and histochemical studies (Okamura et al., 1995c).

It was suggested that CGRP is also involved (Gyoda et al., 1995) or the sole mediator (Matsuda et al., 1995) in the neurogenic relaxation in guinea pig mesenteric artery. Endothelium-independent neurogenic relaxation was supposed to be mediated by CGRP from capsaicin-sensitive C fibers in rabbit mesenteric arteries (Kakuyama et al., 1998). In rat isolated mesenteric arteries and resistance vessels, CGRP (Kawasaki et al., 1988; Amerini et al., 1993), but not NO (Amerini et al., 1993), and opioid (Kannan and Seip, 1986) are involved in vasodilatation induced by nerve stimulation. Despite a significant role of neurogenic NO in mesenteric arteries from large mammals, including the monkey, dog, and cow, NO seems unlikely to be responsible for neurogenic vasodilatation in the rat mesentery. If this is the case in mouse peripheral vasculature, the inability of nNOS gene knockout to increase systemic blood pressure in mice would be explained in the same way (Ma et al., 1996a). In isolated, perfused rat mesentery, vasodilator responses to transmural electrical stimulation were augmented by L-NAME and methylene blue, suggesting that endogenous NO modulates the action of sensory nerves in a inhibitory fashion (Li et al., 1993). Mesenteric vasoconstrictor effects of NOS inhibitors in rats (Gardiner et al., 1990a; Adeagbo et al., 1993) may be due to a suppression of endothelial, but not neurogenic, NO synthesis.

In the longitudinal muscle of the rabbit portal vein, relaxations induced by inhibitory nerve stimulation are mediated solely by NO (Matsukado et al., 1997). Vasodilator action of endogenous NO in the portal vein was reported in studies on the isolated perfused rabbit liver (Browse et al., 1994). NO is also involved in the neurogenic relaxation in the posterior caval vein isolated from guinea pigs (Matsuda et al., 1995). Isolated endothelium-denuded canine hepatic arteries contracted with prostaglandins and treated with prazosin responded to nicotine with relaxations that were abolished by C_6 (Shiraishi et al., 1998). The induced response was reduced by timolol. Further attenuation was elicited by $CGRP_{8-37}$, a CGRP-receptor antagonist, but not by a VIP-receptor antagonist. The remaining relaxation was abolished by L-NA, and L-arginine reversed only the L-NA-induced inhibition. Norepinephrine, CGRP, and NO seem to act as vasodilatory neurotransmitters; contribution of NO is less than that of the others. CGRP was reportedly the sole transmitter for vasodilation in rat hepatic artery (Bratveit and Helle, 1991). In anesthetized cats, L-NAME raised basal vascular tone in the superior mesenteric artery but not in the hepatic artery, suggesting

that the role of endogenous NO is, if any minimal, in hepatic vasculature (Greenblatt et al., 1993; Macedo and Lautt, 1997).

In anesthetized rats, intravenous or topical application of NOS inhibitors induced a fall in the resting gastric mucosal blood flow and vasoconstriction of gastric arterioles (Pique et al., 1989; Tepperman and Whittle, 1992; Chen et al., 1993). NO seems to function as a local vasodilator in the gastric mucosal microvasculature, although the relative contributions of neurogenic and endothelial NO have not been determined. NOS inhibitors also decreased the vascular conductance in the mesenteric vascular bed (Greenblatt et al., 1993; Macedo and Lautt, 1997).

Contractions mediated by neurogenic norepinephrine in dog and monkey mesenteric arteries were augmented by L-NA, which failed to enhance the contraction by exogenous norepinephrine (Toda and Okamura, 1990a, 1992b; Toda et al., 1991b; Zhang et al., 1993). Contractions of isolated human and canine gastroepiploic arteries in response to nicotine were also significantly augmented by L-NA (61.3 ± 11.8% increase in human arteries, $n = 5$, and 111 ± 19.2%, in dog arteries, $n = 8$; N. Toda, K. Ayajiki, and T. Okamura, unpublished data). In endothelium-denuded superfused canine mesenteric arteries preincubated with ^{3}H-norepinephrine and then treated with atropine, the ^{3}H-overflow by electrical nerve stimulation was not influenced by EDRF, possibly NO, liberated by acetylcholine applied to the endothelium-intact canine femoral artery segment (Toda et al., 1990b). In addition, treatment with L-NA did not influence the ^{3}H-overflow in superfused monkey mesenteric arteries (Toda and Okamura, 1992b). As previously postulated in the section of "temporal vasculature", physiological antagonism by neurogenic NO of the contraction caused by nerve-derived norepinephrine in smooth muscle would be a mechanism underlying the decreased noradrenergic vasoconstriction in vasculature with reciprocal dual innervation.

D. Renal Vasculature

Dog and monkey renal artery strips contracted with vasoconstrictors and treated with prazosin responded to nicotine with relaxations that were abolished by L-NA and restored by L-arginine (Okamura et al., 1995d). The response was also abolished by C_6, oxyHb, and methylene blue. The NO-cyclic GMP pathway would be involved. In porcine isolated renal arteries, contractions induced by transmural stimulation were reversed to relaxations by prazosin or guanethidine that were resistant to β-adrenoceptor antagonists but were susceptible to TTX (Ferguson et al., 1985). Unidentified transmitters were suggested. Neuronal NOS- or NADPH diaphorase-positive nerves were detected in arcuate and interlobar arteries and preglomerular afferent arterioles in the rat and human kidney (Bachmann et al., 1995; Liu et al., 1996b) and dog and monkey kidney (Okamura et al., 1995d). In isolated perfused rabbit renal afferent arterioles contracted with norepinephrine, nicotine induced vasodilatation, and treatment with L-NA reversed the response to vasoconstriction (T. Tamaki, S. Kimura, and Y. Abe, unpublished observations), suggesting a release of NO from periarterial nerves. Isolated rabbit renal arteries without the endothelium constricted in response to transmural nerve stimulation, and the contraction is enhanced by L-NAME, as was the response to norepinephrine (Vials et al., 1997). L-Arginine reversed the effect of the NOS inhibitor. Prejunctional inhibitory action of NO was suggested. In anesthetized dogs, it was postulated that endogenous NO plays a role as an inhibitory modulator of renal noradrenergic neurotransmission (Egi et al., 1994). However, there was no prejunctional action on adrenergic nerves in isolated monkey mesenteric arteries (Toda and Okamura., 1992), canine temporal arteries (Toda et al., 1991) and rat tail and mesenteric arteries (Vo et al., 1991; Bucher et al., 1992; Yamamoto et al., 1997). To conclude the prejunctional inhibitory action of NO in renal vasculature in contrast to other vessels, modulation by NO, NO donors, or NOS inhibitors of the release of norepinephrine from adrenergic nerves in the isolated renal artery would have to be assessed.

Intravenous injections of NOS inhibitors reduced renal blood flow and vascular conductance in anesthetized rats and conscious rabbits and decreased renal plasma flow before elevating systemic blood pressure in conscious dogs (Pucci et al., 1992; Evans et al., 1994; Baylis and Qiu, 1996; Reinhart et al., 1997; Gabbai, 2001). L-NAME in the drinking fluid given to rats for 4 days decreased renal blood flow and increased renal vascular resistance (Hably et al., 2001). In spontaneously hypertensive rats, afferent glomerular arteriolar constriction by L-NAME was greater than that of efferent arterioles (Ono et al., 1995).

In the blood-perfused juxtamedullary nephron preparation of rats, *S*-methyl-L-thiocitrulline, a selective nNOS inhibitor (Furfine et al., 1994) decreased the afferent arteriolar diameter (Ichihara et al., 1998; Ichihara and Navar, 1999). Superoxide anions appear to inhibit the control of afferent arteriolar diameter by nNOS in spontaneously hypertensive rats but not Wistar-Kyoto rats (Ichihara et al., 2001). An nNOS inhibitor administered into the abdominal aorta also reduced renal plasma flow in streptozotocin-diabetic rats and nondiabetic rats (Komers et al., 2000). Infusion of 7-nitroindazole, an nNOS inhibitor, to conscious pregnant rats decreased renal plasma flow and glomerular filtration rate without affecting mean blood pressure, whereas in the virgin rat, no change was induced (Abram et al., 2001). Intravenous nNOS inhibitors, N^{ω}-propyl-L-arginine (Zhang et al., 1997a), and N^5-(1-imino-3-butenyl)-L-ornithine (Babu and Griffith, 1998), did not alter blood flow in the renal cortex and medulla, whereas L-NAME did decrease the blood flow (Kakoki et al.,

2001). At comparable levels of arterial blood pressure, renal blood flow was not different between nNOS knockout and wild-type mice (Vallon et al., 2001). The observations made so far are inconclusive, so the role of nNOS in the control of renal vascular resistance and blood flow in rats remains controversial. It would be interesting to know whether nNOS in tissues other than the perivascular nitrergic nerve in kidneys is responsible for renal vasodilatation and whether nNOS also contributes to the regulation of renal circulation in vivo in large mammals, including primates.

E. Uterine Vasculature

Noncholinergic vasodilatation induced by periarterial nerve stimulation was observed in the isolated, perfused uterine artery from pregnant guinea pigs (Bell, 1968b). In isolated nonpregnant human (Toda et al., 1994a), monkey (Okamura et al., 2000), dog (Okamura et al., 1995b), and guinea pig uterine arteries (Morris, 1993) treated with α-adrenoceptor antagonists or guanethidine and then precontracted with vasoconstrictors, transmural electrical stimulation or nicotine induced moderate relaxation that was determined to be mediated by NO from perivascular nerves by the use of NOS inhibitors and L-arginine. Atropine, β-adrenoceptor antagonists, indomethacin, and endothelium-denudation failed to inhibit the response, which was however abolished by oxyHb. The neurogenic, NO-mediated relaxation was greater in the uterine artery from pregnant patients undergoing hysterectomy than that from nonpregnant patients (Nelson et al., 1995b). In the guinea pig artery, high frequency stimulation (>5 Hz) also liberates vasodilator peptides (Morris, 1993), but this does not seem to be the case in the other mammals. There are networks of neurons containing nNOS immunoreactivity or NADPH diaphorase in the human (Toda et al., 1994a), canine (Okamura et al., 1995b), guinea pig (Kummer et al., 1992), and rat arteries (Natuzzi et al., 1993; Papka et al., 1995). Parasympathetic, NOS-imunoreactive nerves in the rat uterus originate from the pelvic paracervical ganglia. Histochemical study has also demonstrated the presence of NADPH diaphorase-reactive nerves and cell groups in or in close proximity to the uterus (Yoshida et al., 1995), suggesting that some nerve fibers distributing to the uterine arterial wall arise from this ganglia. It would be intriguing to know the physiological roles of these local ganglia in the control of arterial tone and possibly blood flow via mediation of neurogenic NO and also in the function of the uterus and other female genital organs.

Uterine veins obtained from nonpregnant myoma patients under α-adrenoceptor blockade also responded to nerve stimulation with relaxation, possibly through mediation by NO (Toda et al., 1995a). The neurogenic response was less evident than that seen in the human uterine artery.

Pregnancy and estradiol therapy increased the induction of Ca^{2+}-dependent NOS in guinea pig (Weiner et al., 1994), ovine (Li et al., 1996; Salhab et al., 2000), and human uterine arteries (Nelson et al., 2000). Increased expression of nNOS and eNOS or augmented release of NO from the nerve and endothelium were reported in the former three articles, whereas the authors of the last paper demonstrated only an upregulation of eNOS expression in the uterine artery from pregnant women.

F. Penile Artery and Vein

Functional roles of NANC vasodilator nerves have been reported in canine and bovine penile arteries (Bowman and Gillespie, 1983). Under α-adrenoceptor blockade, penile arteries isolated from the cow (Liu et al., 1991), horse (Simonsen et al., 1995), and dog (Hayashida et al., 1996) relax in response to nerve stimulation by electrical pulses and nicotine. Evidence supporting the idea that NO mediates the response as a neurotransmitter has been presented. In the horse penile artery, it was suggested that NO increases the formation of cyclic GMP in smooth muscle that opens the charybdotoxin-sensitive Ca^{2+}-activated K^+ channels, hyperpolarizes the muscle membrane and results in muscle relaxation (Simonsen et al., 1995). NADPH diaphorase-reactive nerve plexuses are demonstrated in the adventitia of human deep cavernous arteries (Burnett et al., 1993). Corporal vasodilatation estimated by the increase in the ratio of corpora cavernosal pressure/systemic blood pressure following electrical stimulation of the sacral part of the spinal cord is mediated by NO in the pithed rat (Finberg et al., 1993).

The canine (Hayashida et al., 1996) and human penile vein (Segarra et al., 1998) also responded to electrical stimulation or nicotine with endothelium-independent relaxations that are mediated by NO. Segarra et al. suggested that the human vein is an active component of penile vascular resistance. On the basis of a comparative study concerning the inhibitory response to nitrergic nerve stimulation of the canine penile artery and vein and the corpus cavernosum, the rank order of neural action was corpus > artery > vein (Hayashida et al., 1996). Integrative investigation in vivo would be required to know how the nitrergic neurogenic dilatation of the arterial and venous sides and the counteracting noradrenergic vasoconstriction influence penile erection.

V. Nitrergic Innervation in Blood Vessels of Skin and Skeletal Muscle

A. Cutaneous Small Artery

In the small artery isolated from the abdominal subcutaneous plexus of dogs that was denuded of the endothelium and then treated with prazosin and α,β-meth-

ylene ATP, relaxations were induced by electrical nerve stimulation or nicotine (Uchiyama et al., 1997). The response was partially attenuated by L-NA and was abolished by additional application of $CGRP_{8-37}$, a CGRP receptor antagonist. The L-NA-resistant neurogenic relaxation was also abolished in the artery made unresponsive to CGRP by its repeated applications, but it was unaffected by a VIP receptor antagonist. The response seems to be mediated by NO from efferent nerves and CGRP from sensory nerves. Canine labial small arteries obtained as branches of the maxillary artery close to the skin of the upper lip without the endothelium, in which neurogenic vasoconstriction was pharmacologically eliminated, relaxed in response to electrical nerve stimulation and nicotine (Okamura et al., 1999b). The response to electrical stimulation (5 and 20 Hz) was abolished by L-NA, and restored by L-arginine. However, the nicotine (2×10^{-5} and 10^{-4} M)-induced relaxation was reduced only partially by L-NA and was abolished by treatment with L-NA and the CGRP antagonist. The reason for the selective stimulation by electrical pulses of the nitrergic nerve, and the nonselective stimulation by nicotine of nitrergic and CGRP-mediated sensory nerves in the labial arteries remains to be solved. It may be postulated that sensory nerve endings are sensitive to chemical stimuli, not only nicotine but also chemical mediators liberated upon acute inflammation, which induce antidromic vasodilatation and flare of the inflammatory reaction (Okamura et al., 1999b). Blood vessels in the feline lower lip are innervated by parasympathetic postganglionic fibers originating in the otic ganglion (Izumi, 1995). Microvascular blood flow increase by intradermal capsaicin in rabbit skin was mediated by CGRP (Hughes et al., 1992), and the release of this peptide was NO-dependent (Hughes and Brain, 1994). CGRP-containing nerve fibers around blood vessels of the human (Gibbins et al., 1987; Dalsgaard et al., 1989; Schulze et al., 1997) and canine skin (Uchiyama et al., 1997) have histochemically been demonstrated. There are NADPH diaphorase-containing perivascular nerves in the human (Schultze et al., 1997) and dog skin (Uchiyama et al., 1997).

Chronic inhibition of NO production by L-NAME reduced the resting blood flow in the rabbit ear skin (Khan et al., 1993). Infusion of L-NMMA via a brachial artery catheter decreased forearm skin blood flow in normal subjects (Coffman, 1994). Tonic release of NO from the nerve and endothelium under resting conditions appears to contribute to lower cutaneous vascular resistance and increase skin blood flow. In the rat hind footpad blister model, vasodilatation induced by antidromic electrical stimulation of the sciatic nerve was inhibited by nNOS inhibitors and $CGRP_{8-37}$, suggesting that CGRP and NO of neuronal origin participate in neurogenic vasodilatation (Merhi et al., 1998). Since the nNOS inhibitors are without effect in capsaicin-treated rats, the authors have postulated that the source of NO is the sensory nerve. It was suggested that cutaneous vasodilatation by cholinergic nerve stimulation in humans is not mediated by acetylcholine but mediated by an unknown cotransmitter (Kellogg et al., 1995). Involvement of NO, peptides or other molecules in the response was not investigated. Low frequency transcutaneous nerve stimulation increased the skin temperature due to increased cutaneous microcirculation in ischemic limbs of patients with Raynaud's disease and diabetic polyneuropathy (Kaada and Eiesen, 1983). The response was not antagonized by β-adrenoceptor, cholinergic, histaminergic, purinergic or dopaminergic antagonists. Mechanisms contributing to the rise in skin blood flow during local heating in humans are a vasodilator system mediated by axon reflexes and a vasodilatation that relies on local production of NO (Minson et al., 2001).

Neovasculature in adenocarcinoma and melanoma in mice constricted in response to NOS inhibitors to a greater extent than that in non-neoplastic tissues, suggesting that flow in tumor vessels is modulated by NO, which maintains the dilator tone in neoplastic tissue (Andrade et al., 1992). In anesthetized BD9 rats bearing subcutaneous P22 carcinosarcoma, NO is important for maintaining the vasodilator tone, and the NOS inhibition may provide a means for enhancing therapeutic regimens that benefit from a selective decrease in tumor blood flow (Tozer et al., 1995, 1997). Whether or not the tumor vasculature is innervated by nitrergic nerves has not been determined.

B. Skeletal Muscle Vasculature

Endothelium-denuded, precontracted canine saphenous arteries treated with α-blockers and α,β-methylene ATP responded to nicotine with relaxations (Okamura and Toda, 1994b). L-NA attenuated the response only partially, and the remaining relaxation was abolished by a CGRP receptor antagonist and CGRP tachyphylaxis, suggesting a mediation of NO and CGRP from autonomic efferent and sensory nerves. In the isolated rat tail artery, nicotine activated capsaicin-sensitive sensory nerves via nicotinic receptors (Li and Duckles, 1993). NO has either no effect or enhances the release of norepinephrine from nerves, suggesting that augmentation by NOS inhibitors of the adrenergic neurogenic contraction is due not to the prejunctional actions of NO, but due to its postjunctional ones (Vo et al., 1991; Bucher et al., 1992).

Arterial infusions of NOS inhibitors decreased finger blood flow, forearm blood flow, and vascular conductance in humans (Calver et al., 1992; Coffman, 1994) and blood flow in the rabbit knee (Najafipour and Ferrell, 1993). Continuous generation of NO appears to be important in regulating basal vascular resistance in skeletal muscle (Du et al., 1992). Sympathetic cholinergic vasodilatation of skeletal muscle small arteries induced by electrical stimulation of the hypothalamic defense area in anes-

thetized cats (Matsukawa et al., 2002) seems to be mediated by NO (Matsukawa et al., 1993). The reflex vasodilatation in the rat hindlimb produced by electrical stimulation of the superior laryngeal nerve involves the release of newly synthesized NO from NOS-positive postganglionic lumbar sympathetic nerves (Possas and Lewis, 1997). In the hindquarter of conscious rat, neurogenic vasodilatation by air-jet stress was abolished by intravenous injections of bretylium or L-NAME (Davisson et al., 1994). Lumbar sympathetic fibers projecting to the hindquarter vasculature contain NADPH diaphorase. The authors have concluded that the release of NO from postganglionic sympathetic nerves accounts for the response. It is suggested that the hindlimb vasodilatation elicited during a conditioned emotional response in conscious rats is mediated by neural NANC factors (Alper and Zink, 1994). However, the role of NO or peptides has not been analyzed. Neurogenic vasodilatation in the rabbit hindlimb is mediated by NO and tachykinins, and NO is produced subsequent to neural release of tachykinins (Gustafsson et al., 1994). Studies on cremaster muscle from nNOS knockout mice and those lacking eNOS suggest that increases in cyclic GMP and NO-dependent arteriolar dilatation in contracting fast-twitch skeletal muscle may require both nNOS and eNOS (Lau et al., 2000). Exercise enhanced blood flow to working skeletal muscle. Hindquarter blood flow and conductance were reduced by L-NAME to a greater extent in swim-trained rats than in sedentary rats (Tatchum-Talom et al., 2000). Expression of nNOS in hindquarter skeletal muscle was increased in the trained rats. Physiological adaptation to swim training may be characterized by hindquarter vasodilatation with the up-regulation of nNOS. Whereas blockade of NOS reduces muscle blood flow at rest and during recovery from exercise, there is no exercise-induced increase in the muscle perfusion in humans and experimental animals (Radegran and Hellsten, 2000). The increase in forearm blood flow induced by mental stress was reduced by intra-arterial infusion of L-NMMA in humans (Cardillo et al., 1998). Whether NO is derived from the nerve, the endothelium or both has not been determined.

VI. Interaction of Nitrergic, Cholinergic, and Adrenergic Nerves in Peripheral Vasculature

It would be intriguing to know whether the nitrergic nerve function in blood vessels is modulated by cholinergic and adrenergic neurotransmitters and whether the adrenergic nerve activity is influenced by neurogenic NO. Histological studies with antibodies against cholinesterase/choline acetyltransferase and tyrosine hydroxylase have determined the presence of neurons containing these immunoreactivities (Owman 1990), together with that of NOS (Nozaki et al., 1993; Yoshida et al., 1993). There may be cross-talk between these neurons of close proximity.

Acetylcholine dose-dependently inhibited the neurogenic relaxation mediated by NO in isolated porcine (Toda et al., 1997a) and monkey ciliary arteries (Toda et al., 1998), dog external ophthalmic arteries (Toda et al., 1999), and monkey ophthalmic arteries in situ and in vitro (Ayajiki et al., 2000). Relaxations elicited by nitrergic nerve stimulation are also blunted by physostigmine, an acetylcholinesterase inhibitor that does not act directly on muscarinic receptors, and they are potentiated by atropine in monkey, porcine, and dog ciliary and ophthalmic arteries. Therefore, neurogenic acetylcholine and prejunctional muscarinic receptors appear to participate in the inhibition of NO release or synthesis. As described earlier (*Section III.A.8.*) on cerebral arteries (Toda et al., 1997b) and in the literature on cat cerebral artery (Alonso et al., 1991), dog mesenteric artery (Zhang et al., 1997b), saphenous vein (O'Rourke and Vanhoutte, 1987), and guinea pig carotid artery (Casado et al., 1994), M_2 muscarinic receptors would be responsible for the inhibitory action (Fig. 6).

Relaxations mediated by NO released from perivascular nerves were not influenced by clonidine and yohimbine in dog mesenteric and temporal arteries (N. Toda and T. Okamura, unpublished observation), as seen in cerebral arteries (Okamura et al., 1998c). On the other hand, BHT920, an α_2-adrenoceptor agonist, inhibited the response to nitrergic nerve stimulation in horse penile arteries treated with guanethidine and atropine, and rauwolscine antagonized the inhibitory effect (Simonsen et al., 1997). Prejunctional α_2-adrenoceptors appear to be involved in the interference with the release or synthesis of NO in this artery.

The release of norepinephrine from stimulated adrenergic nerves was augmented by β-adrenoceptor agonists in various blood vessels (Borkowski, 1988). β-Adrenoceptor antagonists, such as timolol and propranolol, did not inhibit the relaxation mediated by NO from vasodilator nerves in dog and monkey temporal (Toda et al., 1991c; Toda and Okamura, 1991a; Okamura et al., 1993), mesenteric (Toda and Okamura, 1992b), renal (Okamura et al., 1995d), lingual (Toda et al., 1997d), uterine (Okamura et al., 1995b), and skin arteries (Uchiyama et al., 1997); human uterine arteries and veins (Toda et al., 1994a, 1995a), and dog and monkey ophthalmic and retinal arteries (Toda et al., 1994b, 1995b, 1996b). It was reported that cyclic AMP does not seem to modulate the release of NO from the nerve (Toda et al., 1997b). It seems that as far as the blood vessels tested to date are concerned, the β-adrenoceptor-cyclic AMP system in nitrergic nerve terminals does not participate in the regulation of release and synthesis of NO.

In peripheral arteries and veins, contractions induced by transmural electrical stimulation are potentiated by NOS inhibition, and this effect is reversed by L- but not D-arginine. Since the induced contraction is suppressed

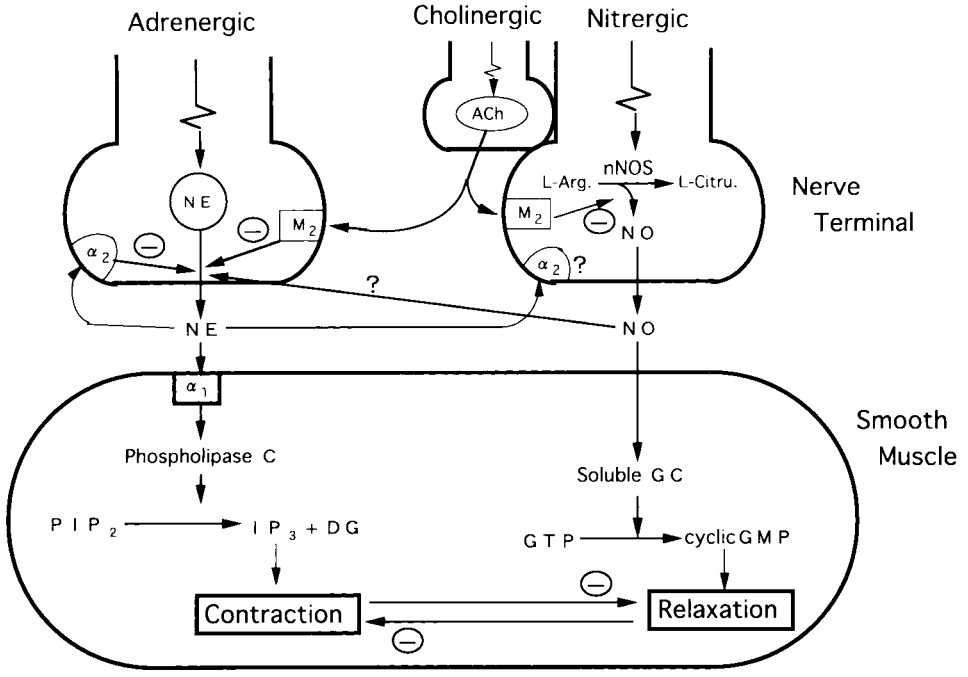

FIG. 6. Interaction between nitrergic, cholinergic and adrenergic nerves in pre- and postjunctional sites. Minus denotes "inhibition". NE, norepinephrine; L-Arg, L-arginine; L-Citru, L-citrulline; M_2, M_2 muscarinic receptor; α_1 and α_2, α_1- and α_2-adrenoceptors, respectively; GC, guanylyl cyclase; DG, diacyl glycerol.

by α_1-adrenoceptor antagonists and adrenergic neuron blockers, neurogenic norepinephrine is expected to be involved. The ^{3}H-overflow by perivascular nerve stimulation from superfused dog mesenteric arteries, which were previously exposed to bathing media containing ^{3}H-norepinephrine, was not influenced by the addition of NO donors (nitroglycerin or sodium nitroprusside) in dog mesenteric arteries (N. Toda and K. Yoshida, unpublished observation) and also unaffected in rat tail and rabbit mesenteric arteries (Bucher et al., 1992; Yamamoto et al., 1997). On the basis of electrophysiological studies on isolated guinea pig and porcine mesenteric arteries, 2-nicotinamidoethyl nitrate enhanced, nitroglycerin did not affect, and sodium nitroprusside suppressed the amplitude of excitatory junction potentials generated by nerve stimulation (Itoh et al., 1981). The reason for such a discrepancy of the effects of NO donors has not been determined. Endothelium-derived relaxing factor, possibly NO, released from the dog mesenteric artery (Toda et al., 1991b) and vasodilators liberated from the endothelium of rabbit carotid arteries (Cohen and Weisbrod, 1988) also failed to influence the overflow of norepinephrine evoked by nerve stimulation. In contrast, endothelium denudation augmented the contractile response and the release of ^{14}C-norepinephrine in dog pulmonary arteries and veins (Greenberg et al., 1989). Since the efflux of ^{14}C-norepinephrine by transmural electrical stimulation in canine mesenteric arteries is blunted by NO donors, such as SIN-1, nitroglycerin, and sodium nitroprusside, and also by inhibitors of cyclic GMP phosphodiesterase, cyclic GMP is suggested to be an inhibitory modulator of norepinephrine release from adrenergic nerves (Greenberg et al., 1990). The interaction of efferent nerves in blood vessels is summarized in Fig. 6.

Treatment with NOS inhibitors did not affect the amine release in the dog mesenteric and temporal arteries (Toda et al., 1991b,c) and rat caudal and tail arteries (Bucher et al., 1992; Pa et al., 1992). In the rat tail artery, the release of ^{3}H-norepinephrine was slightly increased by 10 μM L-NA or unchanged at 100 μM, and it was enhanced by sodium nitroprusside (Vo et al., 1992). In the sheep middle cerebral artery, the stimulation-evoked amine release was inhibited by L-NAME but augmented by NO donors (Mbaku et al., 2000). Therefore, NO does not seem to impair the release of adrenergic neurotransmitters in these arteries, but rather enhances the release in some arteries. On the other hand, L-NAME enhanced the vasoconstriction evoked by transmural electrical nerve stimulation and norepinephrine in the rabbit renal artery with no endothelium (Vials et al., 1997). The authors postulated the prejunctional inhibitory action of NO, possibly from nitrergic nerves, on adrenergic nerves without any data on the release of adrenergic neurotransmitter. Our idea for the mechanism underlying the potentiating action of NOS inhibitors on adrenergic vasoconstriction is that NO released from the nerve opposes the contracting action of norepinephrine at the postjunctional smooth muscle, as a physiological antagonist (Fig. 6). NO may also prejunctionally modulate the adrenergic nerve function in some mammalian vasculature.

VII. Nitrergic Innervation of Corpus Cavernosum and Penile Erection

Many central and peripheral neurotransmitters and signal transduction systems participate in the regulation of penile erection. In this regard, several physiological and pharmacological aspects have been presented in the reviews (Andersson and Wagner, 1995; Giuliano and Rampin, 2000; Steers, 2000; Andersson, 2001). The balance of contracting (anti-erectile) and dilating (erectile) factors that depend on the penile vascular tone and the smooth muscle contractility of the corpora cavernosa is a determinant of the functional state of the penis. Although norepinephrine and adrenergic cotransmitters have long been recognized as anti-erectile factors, the hypothesis that NO is a major erectile neurotransmitter was proposed only about 10 years ago.

In response to sexual (tactile, visual, auditory, olfactory, and imaginary) stimuli via the sensory and/or central nervous system, discharges of efferent inhibitory nerves, now postulated mainly to be nitrergic, innervating the penile artery and corpus cavernosum are increased, thus causing smooth muscle relaxation in these tissues. Decreased tone of the arterial and cavernosal smooth muscle elicits pooling of blood in the corpus cavernosum. Expansion of the cavernous tissues compresses the plexus of subtunical venules against the tunica albuginea and causes a decrease in venous outflow. The increased arterial inflow and cavernosal capacitance and the decreased venous outflow lead to an elevation of the intracavernous pressure and penile erection.

A. In Vitro Studies

In isolated human corpus cavernosum pretreated with guanethidine, transmural electrical stimulation evoked a relaxation, which was atropine-resistant and TTX-sensitive, suggesting the involvement of NANC nerves (Pickard et al., 1991; Rajifer, 1992). The relaxation caused by electrical stimulation was concentration-dependently inhibited by NOS inhibitors, but not by their D-enantiomers, and was restored by the addition of L- but not D-arginine. Similar findings were also reported in rats (Burnett et al., 1992), rabbits (Ignarro et al., 1990), dogs (Trigo-Rocha, 1993; Hayashida et al., 1996), horses (Recio et al., 1998), monkeys (Okamura et al., 1998a), and cows (Simonsen et al., 2001). The soluble guanylyl cyclase inhibitor methylene blue also inhibited the relaxation induced by electrical nerve stimulation in a concentration-dependent manner in humans (Pickard et al., 1991), rabbits (Ignarro et al., 1990), and dogs (Hayashida et al., 1996). The more specific inhibitor of guanylyl cyclase, ODQ, was also effective in inhibiting the neurogenic relaxation in dogs (Okamura et al., 2001b) and rabbits (Cellek and Moncada, 1998). Formation of NO metabolites and cyclic GMP in the human corpus cavernosum was increased in response to nerve stimulation, and the formation and neurogenic relaxation were decreased in the tissue obtained from patients with vascular impotence (Pickard et al., 1995). Selective phosphodiesterase type 5 (PDE V) inhibitors potentiated the relaxation induced by electrical stimulation in humans (Ballard et al., 1998), dogs (Noto et al., 2000), and cows (Simonsen et al., 2001). The neurogenic relaxation was endothelium-independent (Okamura et al., 1999a) and was inhibited by Ca^{2+} channel antagonists of the L- plus N-type, such as amlodipine and cilnidipine, but not of the L-type, like nifedipine and nicardipine (Okamura et al., 2001b). These findings indicate that NANC nerves responsible for neurogenic relaxation of the cavernosal smooth muscle appear to be nitrergic in nature. The nerve stimulation facilitates the Ca^{2+} influx through N-type Ca^{2+} channels, which is followed by NOS activation to produce NO in nerve terminals. Neurogenic NO activates soluble guanylyl cyclase and increases the generation of cyclic GMP in cavernosal muscle cells, resulting in the relaxation.

Therefore, impairment of the nitrergic nerve function leads to erectile dysfunction (ED). In fact, nitrergic relaxations were shown to be deficient in the corpus cavernosum of diabetic impotent men (Saenz de Tejada et al., 1989). Since cyclic GMP is selectively metabolized by PDE V in the corpus cavernosum (Taher et al., 1997; Wallis et al., 1999), inhibitors of the enzyme have been used in the therapy for ED (Boolell et al., 1996a; Gibson, 2001). The orally active inhibitor sildenafil is recognized as useful for the treatment of ED (Goldstein et al., 1998; Maytom et al., 1999; Eardley et al., 2002).

Under treatment with prazosin, magnitudes of the neurogenic relaxation mediated by NO were in the order of corpus cavernosum > penile artery > penile vein from dogs (Hayashida et al., 1996). Responses to exogenous NO did not differ between these tissues. Nitrergic nerves have a greater ability to increase the cavernous cavity and arterial dilatation compared with their ability to increase venous dilatation, so this may be why they function to increase cavernous pressure with minimal influence on the vein. However, there is no evidence for the integral regulation by inhibitory and excitatory nerves of inflow, pooling, and outflow of blood in the penis.

Prejunctional inhibition by nitrergic inhibitory nerves of the excitatory, adrenergic nerve function is suggested in humans, monkeys, and rabbits (Cellek and Moncada, 1997). Reduction of adrenergic nerve activities may therefore be one of the strategies for treatment of ED (Bivalacqua et al., 2000; Saenz de Tejada et al., 2000). Antioxidants and aldose reductase inhibitors showed beneficial effects on the nitrergic nerve function in diabetic rats (Keegan et al., 1999, 2000).

B. In Vivo Studies and Penile Erection

The penis receives three different neuron groups from thoracolumbar sympathetic, lumbosacral parasympa-

thetic and lumbosacral somatic pathways (Dail, 1993). Among the nerves directly innervating the cavernous tissue, electrical stimulation of parasympathetic nerves (cavernous or pelvic nerve) elicited an elevation of intracavernous pressure and a penile erection (Burnett et al., 1992; Ayajiki et al., 1997a). Phentolamine and atropine did not significantly alter the pressor response to pelvic nerve stimulation. Intravenous or intracavernous injection of L-NMMA, L-NA, L-NAME, asymmetrical dimethylarginine or 7-nitroindazole inhibited the pressor response to electrical stimulation of cavernous or pelvic nerves in rabbits (Holmquist et al., 1991), rats (Burnett et al., 1992; Spiess et al., 1996), and dogs (Ayajiki et al., 1997a). The inhibition was reversed by L-arginine, but not by D-arginine. Methylene blue also suppressed the erection evoked by the nerve stimulation (Trigo-Rocha et al., 1993), and sildenafil potentiated the response in dogs (Carter et al., 1998). The erectile response to nerve stimulation was accompanied by an elevation of NO levels in the corpus cavernosum (Escrig et al., 1999). Therefore, NO released from efferent inhibitory nerves in vivo appears to be responsible for penile erection. In the dog, an increase in the intracavernous pressure by electrical stimulation of pelvic nerve, distal to the pelvic plexus, was abolished by a ganglion-blocking dose of C_6. Therefore, the ganglion sending postganglionic nitrergic nerves to the corpus cavernosum seems to be located in the vicinity of the cavernosum (Ayajiki et al., 1997a). Histochemical studies with denervation support this idea (Ayajiki et al., 1999).

In rodents, the erectile function seems to be dependent on androgens. Castration or administration of androgen antagonists suppressed the penile erection in response to nerve stimulation and also the NOS activity in the cavernous tissue in rats (Lugg et al., 1995; Penson et al., 1996). Treatment of castrated rats with androgens recovered the pressor response and the enzyme activity in rats and rabbits (Traish et al., 1999). Decreased histochemical staining for NADPH diaphorase in the cavernous tissue was also recovered by androgens (Zvara et al., 1995; Baba et al., 2000). Androgen receptors are present in about 40% of the neurons of the pelvic ganglion innervating the rat corpus cavernosum (Schirar et al., 1997). However, dependence of the penile erection on the hormone has not been clarified in humans.

Attenuation by aging of the erectile response was improved by long-term oral administration of L-arginine in the rats (Moody et al., 1997). In vivo gene transfer of eNOS, alone or in combination with a PDE V inhibitor, improved the erectile response to cavernous nerve stimulation (Champion et al., 1999; Bivalacqua et al., 2000).

Despite reported evidence supporting the view that neurogenic NO plays crucial roles as an erectile transmitter, mice lacking nNOS (deletion of exon 2) respond to electrical stimulation of the cavernous nerves with erection and show normal mating behavior (Burnett et al., 1996). These authors have suggested that the augmented eNOS activity in the nNOS knockout mice presumably indicates a compensatory mechanism, which may suggest that nNOS is principally involved in penile erection of wild-type animals. On the other hand, since nNOS has several splice variant forms (Eliasson et al., 1997), a form of nNOS lacking the PDZ domain (relating to postsynaptic density protein), which is encoded by exon 2, may be expressed in these knockout mice. The presence of the nNOS variant in the penis of rats and mice has been demonstrated (Magee et al., 1996; Gonzalez-Cadavid et al., 2000). In humans, the nNOS isoform in the penile tissue from patients with ED is reported to be different from that expressed in skeletal muscle and heart (Lin et al., 1998). However, functional roles of these splice variant forms of nNOS remains to be clarified.

C. Histochemical Studies of Neurons Containing Nitric-Oxide Synthase

Histological studies on NOS in the peripheral nervous system have been reviewed (Vincent, 2000). NOS-positive fibers densely innervate cavernous tissues, penile arteries, and veins in rats (Burnett, 1992; Alm et al., 1993; Vizzard et al., 1994a; Dail et al., 1995), dogs (Hedlund et al., 1995: Hayashida et al., 1996a), monkeys (Okamura et al., 1998a), and humans (Burnett et al., 1993; Ehmke et al., 1994; Tamura et al., 1995). Retrograde tracing studies revealed that these were postganglionic parasympathetic nerves mainly derived from the major pelvic ganglion in rats (Keast, 1992; Ding et al., 1993; Schirar, 1994). In dogs, ganglionic cells containing NADPH diaphorase activities were closely located to the penile tissues (Ayajiki et al., 1999). Most of these neurons express VIP in rats (Domoto and Tsumori, 1994; Ding et al., 1995), dogs (Hedlund et al., 1995), and humans (Ehmke et al., 1994; Tamura et al., 1995). Intracavernous injections of VIP evoked the penile erection in normal rats but not in diabetic rats (Maher et al., 1996), suggesting the role of VIP in the erectile response. However, it has not been clarified that VIP released from nerves is responsible for relaxation of cavernosal smooth muscle in vivo and in vitro (Andersson and Wagner, 1995; Hayashida et al., 1996b). On the other hand, tyrosine hydroxylase is not colocalized with NADPH diaphorase in postganglionic parasympathetic neurons, but colocalization of these enzymes is seen in postganglionic sympathetic neurons in the rat (Vanhatalo et al., 1996).

The number of NOS-immunoreactive or NADPH diaphorase-positive nerve fibers in the penile tissue alters with development, aging, hormones, and pathological conditions. In the young rat, penile tissues showed a significant increase of NADPH-positive granules in the corpus cavernosum and dorsal penile nerve when they became able to make an erection (Vachon et al., 2001). Aging caused a decrease in the response to nerve stimulation and also a reduction in NOS-containing nerve

fibers in the penile tissue (Carrier et al., 1997). Administration of growth hormone enhanced the regeneration of NOS-containing fibers in the dorsal and intracavernosal nerves after unilateral cavernous nerve injury (Jung et al., 1998). In streptozotocin-induced diabetic rats, the nNOS-positive nerve fibers were less than those in the controls (Xu et al., 2001). Insulin treatment increased the number of the fibers in penile tissues.

In addition to postganglionic parasympathetic nerves, NOS-positive nerves have been seen in preganglionic sympathetic and parasympathetic nerves (Ceccatelli, 1994) and in afferent nerves projecting to the sacral parasympathetic nucleus (McNeill, 1992). The afferent projections to the sacral cord alter with development and may be related to maturation of the visceral reflex pathway such as that involved in micturition (Vizzard et al., 1994b). Functional roles of these nerves in relation to penile function await further investigations.

VIII. Blood Pressure Control by Neurogenic Nitric Oxide

A. Involvement of Nitrergic Nerve Innervating Vasculature

Autonomic efferent nerves undoubtedly play important roles in the regulation of systemic blood pressure and vascular resistance via changes in the diameter of arteries and arterioles and the vascular resistance. Sympathetic nerves innervating most of the peripheral arteries are mainly involved in blood pressure regulation. Over-function of sympathetic nerves is one of the pathogenetic factors of hypertension; thus drugs that block sympathetic discharges and adrenoceptor functions are useful as anti-hypertensive therapeutics. However, the sympathetic nerve is not the sole vasomotor regulator. As discussed formerly, the vascular tone in mammals is reciprocally regulated by sympathetic vasoconstrictor and nitrergic vasodilator nerves (Toda and Okamura, 1992). Predominant innervation differs among the vasculatures in various organs and tissues. We will now discuss the evidence for the role of nitrergic nerves in the regulation of blood pressure.

Administration of NOS inhibitors, such as L-NMMA, L-NA and L-NAME, elevated blood pressure and vascular resistance in anesthetized rats (Fozard et al., 1991; Lacolley et al., 1991), rabbits (Rees et al., 1989), guinea pigs (Aisaka et al., 1989), dogs (Klabunde et al., 1991; Toda et al., 1993e), Japanese monkeys (Okamura et al., 1996), conscious rats (Gardiner et al., 1990b; Wang et al., 1991; Cunha et al., 1993), and healthy humans (Haynes et al., 1993). The elevation of blood pressure was reversed by L-arginine. The D-enantiomer of arginine analog NOS inhibitors did not elevate the blood pressure. Therefore, it is concluded that endogenous NO participates in lowering of systemic blood pressure. However, in most of the early studies, whether NO responsible for the control of blood pressure is derived from the endothelium, perivascular nerve or brain has not been determined.

In anesthetized dogs (Toda et al., 1993e) and monkeys (Okamura et al., 1996), elevation of blood pressure induced by intravenous injection of L-NA was unaffected by phentolamine treatment, but it was markedly suppressed by ganglion blocking agents, C_6 or pentolinium. Doses of the blocking agents employed were sufficient to lower blood pressure to an extent similar to that induced by the α-adrenoceptor antagonist and to reverse reflex bradycardia induced by exogenous norepinephrine to tachycardia (Toda et al., 1993e). Inhibition by C_6 of the pressor responses to the NOS inhibitor was also observed when the reduced blood pressure induced by the blocking agent was compensated to the control level by a continuous infusion of angiotensin II before the administration of L-NA (Okamura et al., 1996). These results suggest that perivascular neurogenic NO, in addition to endothelium-derived NO, plays important roles in lowering of the systemic blood pressure in primate and large subprimate mammals. On the other hand, impairment of nitrergic nerve function seems unlikely to participate in the pressor response to NOS inhibitors in rats, since neurogenic vasodilatation in the periphery is mainly mediated by CGRP but not NO (see *Section IV.C.*). Bredt et al. (1990) have clearly demonstrated that neurons containing NOS immunoreactivity are present in the wall of rat cerebral arteries but not in the peripheral vasculature. This may also be the case in another rodent, the mouse, although no information is available concerning the functional role of nitrergic nerves in the vasculature of this species. Selective nNOS inhibitors may be useful for determining whether nerve-derived NO is involved in blood pressure control. However, studies with these inhibitors have been performed only in rats.

One may speculate that NOS inhibitors act on certain brain regions and increase the sympathetic renal nerve discharges in the rat (Sakuma et al., 1992). Intracisternal L-NMMA evoked hypertension accompanied by increased sympathetic nerve discharges in the rat (Togashi et al., 1992). NOS inhibitors facilitate baroreceptor resettings in the anesthetized rat, suggesting that endogenous NO may participate in the homeostasis of baroreceptor function (da Silva et al., 1994). On the other hand, in anesthetized dogs and monkeys, the L-NA-induced pressor actions are not influenced by phentolamine (Toda et al., 1993e; Okamura et al., 1996), and hypertension in healthy human volunteers induced by L-NMMA is not associated with an increased discharge of muscle sympathetic nerves (Hansen et al., 1994), suggesting that the sympathetic nerve activation fails to contribute to the induced elevation of blood pressure. Furthermore, intracisternal injections of L-NA did not affect the systemic blood pressure in the dog (Toda et al., 1993b) and monkey (Okamura et al., 1995a). The ability of intravenous L-NA to elevate the blood pressure is

eliminated after treatment with ganglion blocking agents as described above. Monkey distal mesenteric arteries (150 to 200 μm outside diameter) are innervated by NADPH diaphorase-containing neurons and respond to nerve stimulation with relaxations (Okamura et al., 1996). Therefore, it may be concluded that NO continuously released from vasodilator nerves by tonic discharges delivered from the central nervous system contributes to the dilation of arteries and arterioles in various organs and tissues of primate and large subprimate mammals but not in those of rodents. Elimination of nitrergic neural function appears to be one of the mechanisms underlying the elevation of systemic blood pressure by NOS inhibitors.

B. Effect of Centrally Applied Nitric Oxide Donors and Nitric-Oxide Synthase Inhibitors

The presence of nNOS in the central nervous system focuses attention on finding its physiological significance in cardiovascular regulation. To examine the role of NO in the central regulation of blood pressure, microinjections of NO donors and/or NOS inhibitors into certain sites or nuclei in the brain have been performed.

Injection of NO donors such as sodium nitroprusside into the rostral ventrolateral medulla (RVLM), which is essential for the maintenance of arterial pressure (Dampney, 1994), attenuated the renal nerve sympathetic activity and lowered the systemic blood pressure in the anesthetized cat, whereas injections into the caudal ventrolateral medulla (CVLM) elicited the opposite effects (Shapoval et al., 1991). Similar findings were observed when L-arginine was applied to the RVLM and CVLM of anesthetized rats (Freda et al., 1999). Microinjection of L-arginine into RVLM in anesthetized rats (Tseng et al., 1996) and overexpression of eNOS in the RVLM of conscious rats (Kishi et al., 2001) caused hypotension and bradycardia. In contrast, in the anesthetized rabbit with denervated baroreceptors, pressor and sympathoexcitatory actions of NO donors injected into the RVLM were demonstrated (Hirooka et al., 1996). Administration of L-NAME to the RVLM and dorsomedial medulla did not alter resting blood pressure but inhibited the glutamate-induced pressor response in anesthetized cats (Chen et al., 2001). Whether or not these discrepancies are due to the differences in the materials and experimental conditions used is not known.

Another site responsible for the central regulation of blood pressure and sympathetic nerve activity is the nucleus tractus solitarius (NTS). Microinjection of L-NMMA into the NTS increased arterial pressure and renal sympathetic nerve activity in rabbits with intact as well as denervated sino-aortic baroreceptors and vagi (Harada et al., 1993). The NOS inhibitor increased the heart rate only in rabbits with sino-aortic denervation and vagotomy. L-Arginine microinjection into the NTS did not alter the baseline blood pressure, heart rate, and renal sympathetic nerve activity, but prevented the rise in blood pressure and nerve activity evoked by L-NMMA. Control of renal sympathetic nerve activity by the arterial baroreflex was not affected by L-NMMA. Microinjection of L-NMMA in the area postrema did not change these parameters. Therefore, endogenous NO synthesized in the NTS is considered to mediate tonic inhibition of renal sympathetic nerve activity in the rabbit (Harada et al., 1993). Stimulation of *N*-methyl-D-aspartate receptors in the NTS caused hypotension and bradycardia in anesthetized rats (Matsuo et al., 2001) and cats (Wu et al., 2000). This is mediated by the release of glutamate that stimulates NO production in the nucleus, since L-NAME abolishes the increased NOx level and cardiovascular response due to *N*-methyl-D-aspartate. The inhibitory role of NO via the NTS in the control of blood pressure and sympathetic nerve activity was also seen in the rat and cat. Conversely, in the rat, NO had a pressor action on the NTS, based on data obtained by microinjection of L-NAME, carboxy PTIO and NOC18 (Matsumura et al., 1998). Therefore, the discrepancy remains to be clarified.

NO within the paraventricular nucleus (PVN) was also demonstrated to have an inhibitory effect on the renal sympathetic nerve activity via mediation of GABA in the rat (Zhang and Patel, 1998). This mechanism may be one of the pathogenetic factors of heart failure, since the inhibitory effect is weaker in the rat with heart failure than in the control rat (Zhang et al., 2001).

The role of NO in the central regulation of blood pressure and sympathetic nerve activity is still controversial. Some reports do not support the importance of NO in the control of sympathetic nerve activity in rats (Habler et al., 1997) and humans (Hansen et al., 1994), whereas others indicate that it is an important regulator in humans (Owlya et al., 1997). This important issue awaits further investigation.

C. Nitric-Oxide Synthase Knockout Mice

To determine the roles of the constitutive types of NOS, nNOS, and eNOS, in the regulation of systemic blood pressure, disruption of either NOS isozyme gene has been accomplished with NOS knockout mice. Deletion of the gene encoding the endothelial form of NOS leads to the development of systemic and pulmonary hypertension in mice (Huang et al., 1995; Shesely et al., 1996), whereas those lacking the gene for nNOS (Nelson et al., 1995a) or inducible NOS (Laubach et al., 1995) isozyme are normotensive, suggesting that the eNOS is essential for the circulatory homeostasis. However, this does not simply mean that we should exclude the possible involvement of nNOS in the regulation of blood pressure. It is postulated that compensatory mechanisms in the nervous and peripheral tissues for the lack of nNOS diminish physiological manifestations of the biomedical dysfunction (Huang et al., 1993). For example, vasodilatation induced by acetylcholine in pial arterioles of

eNOS knockout mice was partially inhibited by L-NA and was abolished by L-NA plus TTX, indicating that nNOS is involved in the acetylcholine-induced vasodilatation in eNOS knockout mice (Meng et al., 1996). Furthermore, the acetylcholine-induced vasodilatation sensitive to L-NA in pial arterioles was enhanced in nNOS knockout mice (Meng et al., 1996). Neuronal NOS may constitutively be expressed and up-regulated in eNOS-deficient mutants and vice versa. Moreover, knockout animals may induce secondary alterations that also compensate for the induced change. In the case of eNOS knockout mice, other endothelial signal molecules such as prostacyclin and endothelium-derived hyperpolarizing factors may conserve the vasodilatory potential (Godecke and Schrader, 2000). Vasodilator nerves other than the nitrergic ones may mainly contribute to the regulation of vascular function in nNOS knockout mice (Huang et al., 1993). In addition, nNOS has several splice variant forms (Eliasson et al., 1997). As reported for the penile erection in nNOS-knockout mice (Burnett et al., 1996), the nNOS isozyme produced in the presynaptic nerves may not be identical to that in the postsynaptic cells such as those of the cerebellum and skeletal muscle.

IX. Acupuncture, Axon Reflex, and Neurogenic Inflammation

Electro-acupuncture stimulation of muscle afferent fibers has been used as an effective and adjunctive therapy in relieving symptoms such as pain in orthopedics. It is known that somatosensory inputs elicit hemodynamic responses (Sato et al., 1997). The acupuncture stimulation to the vastus medialis muscle of the rat elicited arteriolar dilatation in the knee joint capsule (Yamaguchi et al., 2001; Loaiza et al., 2002). This effect was reversed to a vasoconstriction after treatment with L-NAME. The axon reflex triggered by the stimulation vasodilates arterioles possibly by liberating NO from the axon terminal. CGRP-mediated arteriolar dilatation in the knee joint capsule is also evoked by acupuncture stimulation applied to a site adjacent to the observation area.

Stimulation of the peripheral ends of efferent and afferent neurons by axon reflexes in vivo or by electrical stimulation or nicotine in vitro evoked vasodilatation. This dilatation is prominent in the cutaneous vasculature, where it appears to be an integral part of the inflammatory response (Holzer, 1992). In the skin, the vasodilator response induced by axon reflexes or antidromic electrical stimulation seems to be mediated mostly by CGRP (Brain and Williams, 1989; Holzer, 1992). NO is unlikely to be involved in the rat skin microvasculature since NOS inhibitors do not affect the response (Ralevic et al., 1992; Holzer and Jocic, 1994). It is suggested that release of NO is involved in edema formation in rat paw skin induced by electrical stimulation of the saphenous nerve, but the vasodilator action of NO is unimportant in this context (Kajekar et al., 1995). On the other hand, in cutaneous small arteries isolated from the canine lip and abdomen, transmural electrical stimulation and nicotine exert relaxations that are mediated by NO from efferent vasodilator nerves and also by CGRP possibly from sensory nerve terminals, since both responses resistant to NOS inhibitor and CGRP-immunoreactive neurons are abolished by capsaicin (Uchiyama et al., 1997; Okamura et al., 1999b). It is suggested that NO may facilitate the release of CGRP from afferent neurons or be a secondary messenger of the peptide in the rat skin (Holzer et al., 1995). These nerves may be responsible for the axon reflex, acupuncture response, and inflammatory and immune reactions in the skin. As mentioned previously, CGRP, but not NO, seems to play a role as an efferent or sensory neurotransmitter in rat peripheral vasculature (Kawasaki et al., 1988). One might have to take species variations (rodents versus large mammals including primates) into consideration for the analysis of NO actions in blood vessels.

Neurogenic inflammation is defined as the phenomenon where products released from sensory nerves contribute to the inflammatory response, such as vasodilatation, edema formation, and cellular component of inflammation (Brain and Foster, 2000). There were evidences for the involvement of NO formed by nNOS in experimentally induced inflammation. Sensory saphenous nerve-induced neurogenic edema in the rat was inhibited by TRIM [1-(2-trifluoromethylphenyl)imidazole] and 7-nitroindazole, selective nNOS inhibitors, intraperitoneally, intravenously, or topically applied (Towler et al., 1998), but the vasodilator action of NO-induced vasodilation was not involved (Kajekar et al., 1995). Hindpaw edema induced by intraplantar injection of carrageenin in the rat is reduced by intraperitoneal 7-nitroindazole (Handy and Moore, 1998). Carrageenin increased concentrations of NO_2/NO_3 in the dialysate measured by a subcutaneously implanted microdialysis probe. Locally applied L-NMMA or denervation of the sciatic nerve suppressed the carrageenin-induced increase in NO_2/NO_3 (Omote et al., 2001).

X. Pathological Implications of Neurogenic Nitric Oxide

A. Cerebral Vasospasm after Subarachnoid Hemorrhage

Subarachnoid hemorrhage in humans is provoked mainly by rupture of aneurysms, resulting in cerebral vasospasm during the early period (2–3 days) and late period (1–2 weeks). The delayed vasospasm frequently elicits serious neurological symptoms and sometimes a fatal outcome.

Injections of homologous blood into the cisterna magna twice (second injection usually 2 days after the

first) evokes experimentally induced delayed vasospasm in dogs, rabbits, cats, monkeys, baboons, and rats (Echlin, 1965; Liszczak et al., 1983; Svendgaard et al., 1983; Sasaki et al., 1985; Baker et al., 1987; Otsuji et al., 1994). Mechanisms underlying the vasospasm have been analyzed intensively in these animal models to provide effective prophylactic maneuvers. Cisternally applied oxyHb constricted the basilar artery within 2 h, and the response persisted for 12 h or longer (Toda et al., 1991a). OxyHb possibly formed several days later by lysis of blood clots around the cerebral arteries is considered to play a major role in the genesis of delayed vasospasm after subarachnoid hemorrhage (reviewed by Macdonald et al., 1991; Cook, 1995).

Injections of L-NA into the cisterna magna produced intense persistent constriction of the basilar artery in anesthetized dogs (Toda et al., 1993b) and Japanese monkeys (Okamura et al., 1995a). Cisternal applications of L-arginine reversed the arterial diameter to the control level before the NOS inhibitor injection. In the dog treated with phentolamine, the basilar arterial diameter was reduced by about 35% compared with that before L-NA, whereas the decrease in the diameter by intracisternally injected oxyHb was about 50% (Toda et al., 1991a). In the monkey, the basilar artery diameter was reduced by 28 and 49% following cisternal injections of L-NA (Okamura et al., 1995a) and oxyHb (Toda et al., 1991a), respectively. The magnitude of the vasoconstriction in response to L-NA indicates that it is due to a depressed NO synthesis in the vasodilator nerves, located in the adventitia of cerebral arteries and also in the endothelium. OxyHb, a strong scavenger of NO (Martin et al., 1985a), induced endothelium-dependent and -independent contraction (Bowman et al., 1985; Martin et al., 1986) in canine and monkey cerebral arteries (Toda et al., 1991a, 1993a). Methemoglobin was much less potent in scavenging NO (Martin et al., 1985b). In isolated, perfused rabbit basilar and dog femoral arteries, it was noted that extraluminal application of oxyHb interfered with the relaxation mediated by NO liberated from the endothelium, although the extent was less than that induced by intraluminal application (Hongo et al., 1988; Toda et al., 1988; Tsuji et al., 1989). The difference in vasoconstrictions in response to oxyHb and L-NA in vivo would be due to non-NO compounds such as vasoconstrictor prostanoids (Toda et al., 1980, 1991a; Okamoto et al., 1984; Fujiwara et al., 1984), free radicals, such as superoxide anions (Katusic and Vanhoutte, 1989) and hydroxyl radicals (Steele et al., 1991), endothelin-1 (Cooks et al., 1991; Matsumura et al., 1991; Itoh et al., 1994), etc. Production of endothelin is inhibited by endogenous NO (Ahlborg and Lundberg, 1997; Thorin et al., 1998).

In in vivo studies, it is not easy to differentiate between the involvement of NO from the nerve and that of NO from the endothelium. One possible way to do it would be to compare the NO-mediated response in the presence and absence of pharmacological blockade or surgical excision of efferent nerves. Treatment with ganglionic blocking agents vasoconstricted cerebral arteries (10.3 and 5.7% decrease of basilar artery diameter in anesthetized dogs and monkeys treated with phentolamine) and clearly reduced the vasoconstrictor action of L-NA intracisternally applied to anesthetized dogs and monkeys (Toda et al., 1993b; Okamura et al., 1995a). L-NA-induced vasoconstriction under ganglionic blockade is thought to be due to non-neuronal mechanisms. In these studies on dogs and monkeys, neuronal portions (values obtained with L-NA in controls minus those under treatment with ganglionic blockers) are 25 and 18%, respectively, relative to the diameter before the application of L-NA, whereas the non-neuronal portions are 11 and 10%, respectively. The other way of differentiation is to carry out experiments in dogs with or without surgical denervation of pterygopalatine ganglion (Toda et al., 2000a). Again, denervation induced vasoconstriction (12% decrease in middle cerebral artery diameter) and minimized the vasoconstrictor effect (4% decrease from the value under denervation) of intravenously applied L-NA. In the nondenervated side, the mean value of reduced diameter by L-NA was 14%. L-Arginine reversed the inhibitory effect of L-NA. As far as the cerebral arteries in dogs and monkeys under the experimental conditions used are concerned, NO derived from the nerve appear to be predominant in vasodilating the arteries over NO from the endothelium.

Another possible way to ascertain whether neural and endothelial mechanisms are involved is to use inhibitors selective for nNOS. The available nNOS inhibitors are 7-nitroindazole (Moore et al., 1993), *S*-methyl-L-thiocitrulline, *S*-ethyl-L-thiocitrulline (Furfine et al., 1994), N^{ω}-propyl-L-arginine (Zhang et al., 1997a), and N^{5}-(1-imino-3-butenyl)-L-ornitine (Babu et al., 1998). As to 7-nitroindazole, which has a long history of use in research from its first introduction, there were discrepancies in the selectivity of this compound in isolated preparations and in in vivo studies (Reiner and Zagvazdin, 1998). Information so far reported was obtained from different tissues and different experimental conditions in each study, for instance, comparisons of effectiveness in blood vessels or blood pressure for eNOS and effects on anti-nociceptive activity or on purified brain NOS for nNOS from various animal species. Our study on the responses to transmural electrical stimulation or nicotine for nNOS and those to histamine for eNOS in monkey cerebral arteries have revealed that 7-nitroindazole is more potent in inhibiting the response to nerve-derived NO than that to endothelium-derived NO, and the potency ratio is approximately 5:1 (Ayajiki et al., 2001b). This property may be used to analyze predominant participation of nNOS in the response of isolated tissues over other NOS isozymes. In anesthetized rats, nNOS-selective actions have been reported (Yoshida et al., 1994b). However, on the basis of our experiments with

large mammals in vivo, it is difficult to find the dose of 7-nitroindazole that exerts a clear inhibition of responses related to nNOS without effect on the eNOS-dependent response.

B. Migraine and Cluster Headache

The etiology of migraine and cluster headache is controversial (Olesen et al., 2000). However, the clinical effectiveness in headache patients of ergotamine, sumatriptan, and other serotonergic agonists that constrict intra- and extracranial arteries has led to the hypothesis that vasodilatation of these arteries is involved in the vascular headache. It is suggested that NO donors, such as nitroglycerin, provokes migraine headache (Iversen et al., 1989). In addition, NOS inhibitors are effective in preventing the attack (Lassen et al., 1997). Histamine, one of the mediators that evokes migraine and cluster headaches, reportedly liberates NO from the endothelium in human cerebral arteries by stimulation of H_1-histamine receptors (Toda, 1990). Mepyramine, an H_1-histamine-receptor blocker, is effective in relieving the histamine-induced headache in sufferers of migraine (Lassen et al., 1995). These findings suggest that endogenous NO plays a role in the generation and persistence of migraine and cluster headaches (Olesen et al., 1994).

Intra- (anterior, middle, posterior cerebral arteries, etc.) and extracranial (retinal, ophthalmic, and superficial temporal) arteries dilate in response to stimulation of parasympathetic, nitrergic nerves and NO from the endothelium. In addition to NOS inhibitors, flunarizine, an anti-migraine Ca^{2+} channel inhibitor that passes the blood-brain barrier (Louis, 1981; Frenken and Nuijten 1984; Leone et al., 1991) and sumatriptan (Humphrey and Feniuk, 1991) antagonized the NO-mediated vasodilator response to nerve stimulation in dog cerebral arteries (Ayajiki et al., 1997b, 2001a). Flunarizine likely inhibits the synthesis and release of NO by reducing the influx of Ca^{2+}. Sumatriptan does not appear to blunt the release of NO by acting on prejunctional serotonin receptors, but it may oppose NO-induced vasodilatation by its contracting action on smooth muscle (Ayajiki et al., 2001). Therefore, NO derived from this nerve may also be involved in the vascular mechanism of headaches (Toda, 1997).

It is suggested that sensory disturbances during migraine auras can be ascribed to a disturbance in the cerebral cortex, probably the cortical spreading depression (Milner, 1959; Lauritzen, 1994). This disturbance results in complex changes in cerebrovascular tone, characterized by a transient period of intense arteriolar dilatation (Hansen et al., 1980; Lauritzen, 1987; Piper et al., 1991). The cortical spreading depression is used as an animal model of migraine (Lauritzen, 1994). In anesthetized rats and rabbits, the importance of the arginine-NO pathway in the regulation of arteriolar dilatation during cortical spreading depression is suggested by the use of NOS inhibitors and L-arginine (Wahl et al., 1994; Fabricius et al., 1995; Meng et al., 1995). However, whether NO is derived from perivascular neurons or nerve cells adjacent to the arterioles remains to be determined. The review article by Olesen and Jansen-Olesen (2000) summarizes their hypotheses that NO released from blood vessels, perivascular nerve endings or brain tissues triggers spontaneous migraine pain.

C. Impaired Ocular Circulation: Relation to Glaucoma

NO derived from the endothelium and nerve plays important roles in the regulation of the ocular circulation (Flugel et al., 1994; Donati et al., 1995; Koss, 1999) and prevention of atherosclerosis and thrombosis. In human eyes with primary open-angle glaucoma (Rankin et al., 1994) and in monkey eyes with laser-induced glaucoma (May et al., 1997), decreased retinal blood flow velocity or reduced diameter of retinal arterioles, together with changes in the histological NOS staining pattern of retinal vasculature, have been reported. Microcirculatory hypoperfusion and autoregulatory dysfunction in glaucomatous human eyes were observed (Sponsel et al., 1997). Whether the impaired retinal circulation is the cause or result of glaucoma is not known. However, the increased vascular resistance in the eye distal to the ophthalmic artery in normal-tension glaucoma patients (Harris et al., 1994) does not support the idea that the vascular change is ascribable to persistent ocular hypertension. The findings that even eyes with normal visual fields in patients with asymmetric glaucoma had decreased blood velocity in the retrobulbar vessels suggested that circulatory changes probably precede detectable glaucomatous damage and may therefore be involved in the pathogenesis of the disease in some patients (Nicolela et al., 1996).

Topical application of nitroglycerin and other NO donors lowered intraocular pressure in rabbits and monkeys (Nathanson, 1988, 1992; Schuman et al., 1994; Behar-Cohen et al., 1996). A new drug for glaucoma therapy, nipradilol, was developed, which has a chemical structure liberating NO, like nitroglycerin, together with β- and α-adrenoceptor blocking actions. Topical application of this drug decreased intraocular pressure, increased ocular blood flow, and protected retinal neurons from death due to ischemic insults (Mizuno et al., 2001). Released NO would contribute to these effects, in particular neuroprotection. The important goal of glaucoma therapy is to protect the ocular fundus from neuronal damage. This is a strategy similar to the therapy for systemic hypertensive patients in which the final goal is protection of vital organs, such as the heart, brain, and kidney, from fatal damage. Unimpaired blood flow and direct neuroprotective actions if achievable through the mediation of neurogenic and endothelial NO may contribute to preventing the genesis of neural damage in glaucomatous eyes and delaying the development of this disease.

D. Pre-Eclampsia (Pregnant Intoxication)

Pre-eclampsia complicates approximately 6 to 8% of all gestations and is the leading cause of fetal growth retardation, infant morbidity and mortality, and premature birth (Buhimschi et al., 1998). Hypertension, proteinuria, thrombocytopenia, and renal damage frequently accompany the disease. From studies on experimental animals, long-term NOS inhibition is noted to mimic these syndromes, smaller litter size and decreased fetal weight, and these responses are reduced by administration of L-arginine in high doses (Salas, 1998). Therefore, inhibitions of the NOS activity and/or the NO function would be considered as causative factors in the pathogenesis of pre-eclampsia. The initiating event in pre-eclampsia has been postulated to derive from reduced uteroplacental perfusion as a result of abnormal cytotrophoblast invasion of spiral arterioles (Granger et al., 2001).

Placental ischemia leads to widespread dysfunction of the maternal vascular endothelium. In isolated resistant arteries from women with pre-eclampsia, endothelium-dependent NO-mediated relaxations were less than those from normotensive pregnant women (McCarthy et al., 1993; Knock and Poston, 1996; Suzuki et al., 2000a,b), whereas endothelium-independent relaxations did not differ (McCarthy et al., 1993). It was demonstrated that responses to atrial natriuretic peptide (Suzuki et al., 2000a) and a cyclic GMP analog (Suzuki et al., 2000b) were also attenuated in pre-eclampsia, suggesting that lower sensitivity of smooth muscle cells to cyclic GMP may be involved. Agonist-induced contractions were augmented in small omental arteries from individuals with pre-eclampsia (Pascoal et al., 1998). In perfused isolated arteries, shear stress-mediated dilatation was absent in the myometrial arteries from gravid women with pre-eclampsia (Kublickiene et al., 2000). Furthermore, based on the analysis of Doppler waveforms in the uterine arteries, uteroplacental blood flow was decreased in parallel with diminished NOS activity in the uterine placental vasculature (Beinder et al., 1999). Reduction of NOS in the umbilical artery endothelium in pre-eclampsia was also demonstrated by an in vitro autoradiography study with ^{3}H-labeled L-NA (Rutherford et al., 1995). These alterations in the vascular function and hemodynamics may contribute to the placental ischemia in patients with pre-eclampsia (Kublickiene et al., 2000).

Oxidative stress via formation of peroxynitrite is one of the causal factors for vascular dysfunction in the placenta (Myatt et al., 2000). Immunostainings showing increased eNOS, decreased SOD, and increased nitrotyrosine suggest the increased peroxynitrite formation in the maternal vasculature in pre-eclampsia (Roggensack et al., 1999). Regulation of the NOS activity may be impaired in the placenta of pre-eclampsia patients. In the patients, regulation by BH_4 of the NOS activity was also impaired (Kukor et al., 2000). Increase in the plasma level of ADMA, an endogenous NOS inhibitor, was increased in pre-eclampsia (Pettersson et al., 1998). SDMA that inhibits the L-arginine transporter was also increased (Ellis et al., 2001), but the activity of the placental L-arginine transport systems was not altered (Ayuk et al., 2002).

On the other hand, despite the circulatory disorder, the NOS activity and the amount of NO metabolites (nitrate and nitrite) in the placenta and plasma were increased in pre-eclampsia and eclampsia compared with those in normal pregnancy (Norris et al., 1999; Benedetto et al., 2000; Shaamash et al., 2000, 2001; Yanik et al., 2001). The increases were directly related to the severity of this disorder; thus they are considered to be diagnostic indicators (Benedetto et al., 2000; Shaamash et al., 2000). Such an increase in the formation of NO possibly represents a physiologic adaptive response to overcome the increased placental vascular resistance and to minimize platelet and leukocyte adhesion to the surface of placental villi (Shaamash et al., 2001).

During pregnancy, maternal adaptation is required in many biological functions. The L-arginine-NO pathway is one of the important factors that act in concert to maintain a symbiotic relationship between mother and fetus. Therefore, pre-eclampsia may be a maladapting state of the pathway, in which the placenta inadequately responds to demands for altered blood flow during pregnancy. As described formerly, NO derived from vascular endothelium is not the sole endogenous NO participating in circulatory homeostasis. The presence of nitrergic nerves in human uterine arteries and female genital organs has been histologically determined (Toda et al., 1994a; Yoshida et al., 1995); and the intense neurogenic relaxation mediated by NO was observed in human (Toda et al., 1994a; Nelson et al., 1995b), monkey (Okamura et al., 2000), dog (Okamura et al., 1995), and guinea pig uterine arteries (Morris, 1993). It would be quite intriguing to clarify the physiological role of nerve-derived NO in the regulation of uterine circulation in women with normal pregnancies and in patients with pre-eclampsia.

E. Hypertension

Chronic administration of NOS inhibitors such as L-NA or L-NAME markedly elevated blood pressure (Arnal et al., 1992; Ribeiro et al., 1992), indicating the depressor role of endogenous NO. Constitutively synthesized NO by eNOS in vascular endothelial cells and also by nNOS in nerve terminals is released under resting conditions and in response to chemical, physical, or electrical stimuli. Since eNOS-knockout animals were hypertensive (Huang et al., 1995) and nNOS-knockout animals were normotensive (Nelson et al., 1995a), one may consider that nNOS is not involved in the regulation of blood pressure. As described previously, this may not be the case. However, this does not seem to be a fair as-

sessment for a number of reasons. During development in the gestation period or after birth, several adaptive or compensatory mechanisms may be induced if some genes are readily deleted (Huang et al., 1993; Godecke and Schrader, 2000). In the case of nNOS, deletion of a target gene available for knockout at present may not abolish all nNOS genes, such as the case in penile erection (Burnett et al., 1996). The inability of selective nNOS inhibitors such as 7-nitroindazole to raise systemic blood pressure (Moore et al., 1993) may be due to their selectivity toward NOS isozymes, doses used, animal species, and experimental conditions (Alderton et al., 2000). Perivascular nitrergic nerves are histologically found to be sparse in rats (Bredt et al., 1990), and their functional roles in blood vessels are not actually determined (Kawasaki et al., 1988). Therefore, findings obtained in rodents cannot be extrapolated to other mammals including primates, in which vasodilator nitrergic innervation has functionally and histologically been proved in peripheral blood vessels (Toda, 1995).

NOS-containing nerves are located in the afferent nerves, several nuclei in the brain stem, preganglionic parasympathetic and sympathetic nerves, and postganglionic parasympathetic nerves (Vincent, 2000). Neurogenic NO appears to act mainly as a neuromodulator in the central nervous system and as a neurotransmitter in the peripheral nervous system, particularly in postganglionic parasympathetic neurons. The sympathetic nerve function is mainly postjunctionally and also prejunctionally blunted by nerve-derived NO. The central role of NO in the regulation of blood pressure was previously described (see *Section VIII.B.*). NO is also involved in the baroreflex mechanism (Liu et al., 1996a; Zanzinger et al., 1996; Hironaga et al., 1998; Murakami et al., 1998) and in the control of vagal activity (Conlon et al., 1996, 1998; Yabe et al., 1998; Choate et al., 2001). Interactions of NO and angiotensin II in the sympathetic nervous system have also been reported (Liu et al., 1998; Moreau et al., 1998; Cervenka et al., 2001).

Impairment of nNOS function in extravascular tissues is reportedly involved in the development and maintenance of hypertension in certain types of hypertensive rats (Matsuoka et al., 1994; Wu et al., 1999; Eshima et al., 2000; Xavier et al., 2000; Yuasa et al., 2000), suggesting that NO formed by nNOS other than that in perivascular nerves also contributes to the lowering of systemic blood pressure. In the kidneys, basal release of nNOS-derived NO attenuated the proximal fluid uptake, which might be involved in the depressor action of NO (Wu et al., 1999). Since this attenuation was abolished by acute renal denervation, renal sympathetic nerves were considered to be involved. Such an inhibitory action of NO was not observed in stroke-prone spontaneous hypertensive rats (Wu et al., 1999). On the other hand, increased renal sympathetic nerve activity does not appear to contribute to NOS inhibitor-induced hypertension in dogs (Reinhart et al., 1997). In the rat chronically treated with NOS inhibitors, Na^+ repletion and elevation of urinary catecholamine excretion were observed (Yuasa et al., 2000), and induced hypertension was attenuated by renal denervation (Matsuoka et al., 1994), possibly because of an additional decrease in tubular Na^+ reabsorption (Xavier et al., 2000). Whether the tubular epithelia are innervated by nitrergic nerves has not been determined. However, in rodents, NO derived from nNOS seems to lower systemic blood pressure by increasing Na^+ excretion.

In chronic hypertensive animals or animals chronically treated with NOS inhibitors, adaptive and compensatory mechanisms for hypertension may be induced. In salt-sensitive Dahl rats, suppression by neuronal NO of sympathetic nerve discharges was enhanced by salt loading, suggesting that salt-sensitive hypertension is potentiated by nNOS inhibition, because the counteracting actions of neurogenic NO in tonic sympathetic activity that promote urinary Na^+ excretion have been removed (Nishida et al., 2001). In portal hypertensive rats, hyposensitivity of blood vessels to perivascular nerve stimulation was reversed by inhibition of NO formation (Sieber et al., 1997). Acute inhibition of nNOS by treatment with 7-nitroindazole stimulated the renin release in low salt-diet rats, but the stimulating effect was not observed in rats made hypertensive by chronic inhibition of nNOS (Ollerstam et al., 2001). How nNOS-derived NO is involved in the adaptive and compensatory mechanisms under chronic hypertension has not been elucidated.

F. Erectile Dysfunction

ED is common with advancing age (Schiavi and Rehman, 1995) and is associated with several systemic diseases (Benet and Melman, 1995; Fabbri et al., 1997), such as diabetes, atherosclerosis, hypertension, renal failure and endocrine disorders, and surgical procedures. Trauma, adverse effects of drugs, such as antidepressants, and psychological problems are also included in the causes of ED (Benet and Melman, 1995; Fabbri et al., 1997). Since psychological, humoral, neuronal, and vascular factors are involved in the control of erection, failure to coordinate any of these factors readily causes ED. Organic ED can be divided into vascular, neurogenic, and cavernosal EDs.

The following mechanisms have been suggested to underlie the impaired neurogenic relaxation of the corpus cavernosum in the pathological states: a) selective degeneration of nitrergic nerves (Cellek et al., 1999); b) disruption of the NO formation due to an alteration in the expression or the activity of NOS (Azadzoi et al., 1998; el-Sakka et al., 1999); c) less sensitivity to NO of cavernosal smooth muscle (Christ et al., 1995a); d) generation of superoxide anions and increased extracellular inactivation of NO in cavernosal smooth muscle (Cartledge et al., 2000; Khan et al., 2001); and e) expression of dysfunctional soluble guanylyl cyclase (Laber et al.,

2002). In addition to the prophylaxis and reliable treatment of the causal diseases, medication with PDE V inhibitors that are expected to accumulate neurogenic NO in cavernosal smooth muscle is undoubtedly important to patients with ED. However, development of new therapeutics with different mechanisms of action is also awaited.

XI. Pharmacological Implications of Neurogenic Nitric Oxide

A. Phosphodiesterase Type 5 Inhibitors

An inhibitor of PDE V, sildenafil (Viagra) (Boolell et al., 1996a,b), in male patients with ED or impotence has widely been recognized as an effective first-line therapeutic (Langtry and Markham, 1999; Boyce and Umland, 2001; Sadovsky et al., 2001), although some cardiovascular side effects are reported (Jackson, 1999; Meinhardt et al., 1999; Zusman et al., 1999; Kloner, 2000). Evaluation of whether this drug is safe for long-term usage must await future data. This inhibitor inactivates a cyclic GMP-degrading enzyme, PDE V, and potentiates and prolongs the pressor response of the corpus cavernosum and the penile erection to activated parasympathetic, nitrergic nerves originating in the pelvic plexus. This action is caused possibly by an increase in the accumulation of cyclic GMP in cavernous smooth muscle that is produced via guanylyl cyclase activated by nerve-derived NO (Burnett et al., 1992; Rajfer et al., 1992).

PDE V is reportedly present in blood vessels (Silver et al., 1998; Hanasato et al., 1999; Kotera et al., 2000) including cerebral arteries (Kruuse et al., 2001). Therefore, vasodilatation mediated by NO from nerves innervating blood vessels and the corpus cavernosum and from the endothelium if impaired by cardiovascular and metabolic dysfunction might be improved by a depression of PDE V-induced cyclic GMP degradation. Unless selective inhibitors that act on different vascular beds in humans are obtained, systemic applications may be limited because of possible side effects on the circulation.

It has been demonstrated that increased rate of cyclic GMP hydrolysis by PDE V may be a major factor contributing to the impairment of NO-mediated cerebral vasodilatation after subarachnoid hemorrhage in rats (Sobey and Quan, 1999). If this is the case in humans, PDE V inhibitors may be used as agents for prophylaxis of cerebral vasospasm. On the basis of immunohistochemical, biochemical, and pharmacological studies with guinea pig basilar arteries, Kruuse et al. (2001) have determined the presence of PDEs IA, IB, and V and have suggested that targeting PDEs with selective inhibitors may be a new way of treating cerebrovascular disease. PDE V inhibitors increase the magnitude of neurogenic relaxation and prolong the response to electrical stimulation and nicotine in canine and monkey cerebral arteries (K. Ayajiki, T. Okamura, and N. Toda, unpublished data).

New approaches to pulmonary hypertension therapy with PDE V inhibitors have been introduced. Orally applied PDE V inhibitors including sildenafil alone or in combination with inhaled iloprost, a stable analog of prostacyclin, were effective in reducing the pulmonary arterial pressure in experimental animals (Cohen et al., 1996; Weimann et al., 2000), healthy volunteers (Zhao et al., 2001), and patients with adult and childhood primary pulmonary hypertension (Abrams et al., 2000; Wilkens et al., 2001). The pulmonary hypotensive effect was observed in doses insufficient to lower systemic arterial pressure. In the isolated perfused lung preparation, sildenafil was more efficient in inhibiting the hypoxia-induced pulmonary hypertension in wild-type mice than that in eNOS-deficient mice, suggesting that the eNOS-NO-cyclic GMP pathway is involved (Zhao et al., 2001). They also postulated that sildenafil has beneficial effects even when eNOS activity is impaired. NO derived from perivascular nerves may also be responsible for the effect. Sildenafil ameliorates the effects of inhaled NO withdrawal (Atz and Wessel, 1999).

In patients with circulatory disturbances of the brain, eye, and uterus that are heavily innervated by nitrergic nerves, PDE-V inhibitors are expected to improve circulation possibly by raising the vasodilator nerve function. Studies using Doppler ultrasonography demonstrated increased uterine artery blood flow by sildenafil in patients undergoing in vitro fertilization (Sher and Fisch, 2000).

B. Ginsenosides

Panax ginseng, one of the most famous Chinese herbs, has been used as an aphrodisiac, hypotensive, cardiotonic, and sedative substance. It was noted that the aqueous ginseng extract, mainly containing ginsenoside Rb_1, augmented the relaxation of isolated monkey cerebral arteries elicited by transmural electrical stimulation or nicotine but did not affect the response to exogenous NO (Toda et al., 2001). Ginsenosides also potentiate the NO-mediated, neurogenic relaxation of isolated rabbit corpus cavernosum (Chen and Lee, 1995b) and have the ability to liberate NO from the endothelium and scavenge oxygen free radicals, including superoxide and hydroxyl radicals (Gillis, 1997). These actions of ginsenosides may account for the aphrodisiac, hypotensive, and other cardiovascular effects of Panax ginseng.

In contrast to the potentiating action of PDE inhibitors, neurogenic responses of the isolated monkey artery are prolonged by ginsenoside only in parallel with the increased magnitude of responses, and the response to exogenous NO is not enhanced, suggesting that the PDE inhibitory action is not involved (Toda et al., 2001). Since ginsenoside additionally increases the neurogenic response that has already been augmented by treatment

with atropine (Toda et al., 1997b), an inhibition of prejunctional muscarinic receptors is not responsible for potentiation by the Chinese herb. Mechanisms of the potentiating action must be elucidated in future studies.

C. Free Radical Scavengers

Edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), a free radical scavenger (Yamamoto et al., 1996), was revealed to be effective in clinically preventing and treating neurological and psychological symptoms in patients after cerebral ischemic insult (Houkin et al., 1998; Otomo et al., 1998). Studies with the cerebral artery occlusion model have shown the effectiveness of this scavenger on ischemic brain damage (Abe et al., 1988; Watanabe et al., 1994; Kawai et al., 1997). The major mechanism for the action of the drug is thought to be scavenging hydroxy radicals generated by ischemia that results in decreased lipid peroxidation of neuronal cell membranes and in preserved endothelial functions for beneficial actions of NO in the brain and circulatory system.

Formation of free radicals, including superoxide anions, is an important mechanism underlying ischemic brain damage (Framm et al., 1978; Kinouchi et al., 1991; Chan, 1992; Siesjo et al., 1992). As already mentioned in this review, experimentally generated superoxide anions do not interfere with the action of NO derived from the endothelium and perivascular nerve in isolated blood vessels. However, after endogenous SOD is inactivated, the generated superoxide depresses the response to neurogenic NO (Okamura et al., 1998c; Tanaka et al., 1999). In the case of increased formation of superoxide anions and decreased activity of SOD, development of cardiovascular dysfunction would be limited by scavenging of free radicals that restore the actions of neurogenic and endothelial NO.

D. α_2-Adrenoceptor Antagonists

Prejunctional α_2 adrenoceptors are involved in the inhibition of transmitter release in many efferent nerves, including nitrergic nerve, innervating peripheral blood vessels (Simonsen et al., 1997). Inhibition of this receptor potentiates the release of neurotransmitter. Augmented nitrergic nerve function by an increase in NO release following α_2 blockers is expected only when the blood vessel is innervated by this nerve predominantly over innervation by adrenergic nerves, since α_2-adrenoceptor blockade also increases the release of norepinephrine. As far as the isolated blood vessels used are concerned, predominance of nitrergic, over adrenergic, innervation is seen only in cerebral (although the functional role of prejunctional α_2 adrenoceptor is not observed in this artery), retinal, ciliary, and uterine arteries and corpus cavernosum.

E. Antimuscarinic Agents

As described in an earlier section, neurogenic acetylcholine acts on muscarinic receptors in nitrergic nerve endings and inhibits the release of NO and the NO-mediated vasodilator response of monkey, porcine, and bovine cerebral arteries (Toda and Ayajiki, 1990; Ayajiki et al., 1993; Toda et al., 1997b; Liu and Lee, 1999; Tanaka et al., 1999). The M_2 receptor subtype is likely involved. Nonselective and M_2-selective muscarinic receptor antagonists significantly augment the response to nitrergic nerve stimulation. If this is the case in human cerebral arteries, M_2 receptor blockers would be used to elicit vasodilatation by increasing the release of NO from the nerve. The same may be expected in the retinal, ciliary, and uterine arteries and the corpus cavernosum, where nitrergic innervation for the control of smooth muscle tone is predominant over adrenergic innervation.

Prejunctional inhibition by acetylcholine of autonomic nerve functions is also seen in peripheral vasculature. The vasoconstrictor response to adrenergic nerve stimulation is attenuated by prejunctional actions of acetylcholine on the M_2 muscarinic subtype in canine saphenous vein (O'Rourke and Vanhoutte, 1987), feline cerebral artery (Alonso et al., 1991), and guinea pig carotid artery (Casado et al., 1994) or M_2 and M_3 subtypes in the bovine cerebral artery (Ferrer et al., 1992). In vasculature in which the vascular size is under predominant control of adrenergic nerves over nitrergic nerves, vasodilatation due to decreased adrenergic nerve function would be induced by neurogenic acetylcholine. If the muscarinic receptor subtypes responsible for the prejunctional inhibition in adrenergic and nitrergic nerves differ in the same mammals, one could expect peripheral vasodilatation by eliminating the inhibitory action of acetylcholine on nitrergic nerves but not on adrenergic nerves.

F. Neuronal Nitric-Oxide Synthase Inhibitors

Development of NOS inhibitors selective for nNOS, eNOS, and iNOS has received much attention. The selective nNOS inhibitors obtained up to 1997 are summarized in a review article by Moore and Handy (1997). Later on, the following inhibitors were synthesized: *N*-(3-(aminomethyl)phenyl)acetamidine (Collins et al., 1998), HMN-1180 [1-(5-isoquinolinyl sulfonyl)-7methylhomopiperazine] (Nishio et al., 1998), N^{ω}-nitroarginine-containing dipeptide amides (Huang et al., 1999, 2001), BN80933 (Chabrier et al., 1999), ARL17477 (O'Neill et al., 2000), 2-thiouracil (Palumbo et al., 2000), *N*-phenacyl imidazoles (Sorrenti et al., 2000) and (4*S*)-*N*-(4-amino-5-[aminoakyl]aminopentyl)-*N*′-nitroguanidines (Hah et al., 2001). An aplysinopsin-type indole alkaloid, a novel nNOS-selective inhibitor, was isolated from the marine sponge *Hyrtios erecta* (Aoki et al., 2001). Ni^{2+} is a competitive

reversible inhibitor of nNOS (Palumbo et al., 2001) and guanabenz is a metabolism-based irreversible inhibitor of nNOS (Noguchi et al., 2000). Developments of new molecules endowed with inhibitory properties against various isoforms of NOS, including nNOS, are reviewed by Salerno et al. (2002).

Neuronal NOS-selective inhibitors may not be expected to have much therapeutic usefulness in the field of cardiovascular diseases, because the actions of NO formed by nNOS in the periphery are mostly beneficial in physiological regulation. However, it was suggested that nNOS-derived NO contributes to the development of a hyperdynamic circulation consisting of low blood pressure, low systemic vascular resistance, and high cardiac output in liver cirrhosis (Xu et al., 2000). From a possible role of NO liberated from the perivascular nerve, brain tissue, or endothelium in triggering a migraine attack, new approaches to the pharmacological treatment of migraine in reference to NOS inhibition are suggested (Olesen and Jansen-Olesen, 2000).

Interest has been directed to finding selective nNOS inhibitors for the protection of nerve cells in the brain and retina from apoptosis and necrosis following ischemic insult. In addition, in the circulatory system, development of selective inhibitors of nNOS would contribute to analyzing the physiological role of nerve-derived NO and thus giving us clues to open new concepts for pharmacological therapy. Tumor growth largely depends on the development and distribution of blood vessels and increased blood supply. Although how nitrergic nerves contribute to a rise in tumor blood flow has not been elucidated, preferential actions of NOS inhibitors on the tumor vascular resistance over that of normal tissue (Tozer et al., 1997) may provide a possibility for new tumor therapies that utilize NOS inhibition.

G. Compounds That Suppress the Action of Endogenous Nitric-Oxide Synthase Inhibitors

Hibbs et al. (1987) have found that L-arginine-dependent macrophage activation is inhibited by guanidino methylated derivatives of L-arginine, such as L-NMMA and N^G,N^G-dimethyl-L-arginine (asymmetric dimethylarginine, ADMA). Along the line of studies on monomethyl arginine in reference to NO synthesis (Palmer et al., 1988b), accumulated ADMA, leading to impaired NO synthesis, is postulated to contribute to vascular and immune dysfunction associated with chronic renal failure (Vallance et al., 1992a). Methylarginines are biosynthesized via protein methylase-I (Paik and Kim, 1993) or protein arginine methyltransferase (Smith et al., 1999). Distribution of free methylarginines is detected in rat tissues and bovine brain (Ueno et al., 1992). The biological significance of endogenous methylarginines is summarized in a review article by Leiper and Vallance (1999). Symmetric dimethylarginine has no inhibitory effect on NOS (Vallance et al., 1992b). ADMA and symmetric dimethylarginine are present in the concentration range of 0.5 to 1 μM in the plasma of healthy volunteers (Leiper and Vallance, 1999).

Plasma concentrations of the endogenous NOS inhibitor increased in patients with renal failure (Vallance et al., 1992a; MacAllister et al., 1996; Anderstam et al., 1997; Kielsein et al., 1999), diabetes mellitus (Miyazaki, 1999), hypertension (Surdacki et al., 1999; Fujiwara et al., 2000), pre-eclampsia (Fickling et al., 1993; Holden et al., 1998), and hypercholesterolemia (Boger et al., 1998). Involvement of this inhibitor in the pathogenesis of these diseases has been suggested, but ideas against this have also been raised (King et al., 1995; Lopez-Jaramillo et al., 1996; Walker et al., 2001). Intravenous injections of ADMA dose-dependently increased systemic blood pressure in association with renal, mesenteric, and hindquarter vasoconstriction in conscious rats (Gardiner et al., 1993). Studies using the cranial window method demonstrated that the topical application of ADMA induced basilar arterial constriction in anesthetized rats and rabbits (Faraci et al., 1995). Mean IC_{50} values of ADMA were estimated as 2.3 and 1.8 μM for nNOS of rat cerebrum and cerebellum in their study. Intra-arterial infusion of ADMA vasoconstricted forearm arteriolar beds of healthy volunteers (Calver et al., 1993). In anesthetized dogs, the intracavernous injection of ADMA (0.25–0.5 mg/kg), although less potent than L-NA, impaired the pressor response to nitrergic nerve stimulation of the corpus cavernosum and the penile erection in anesthetized dogs (Ayajiki et al., 1997a).

A novel protein inhibiting nNOS has been discovered and was designated as protein inhibitor of nNOS (PIN; Jaffrey and Snyder, 1996). Later, this molecule was reported to inhibit all NOS isozymes (Hemmens et al., 1998). Localization of PIN was demonstrated in the rat kidney, and its inhibition of nNOS was suggested to have important implications for the regulation of renal function (Roczniak et al., 2000).

In addition to L-arginine, compounds that suppress actions of ADMA and its synthesizing processes or accelerate its degrading pathways, such as dimethylarginine dimethylaminohydrolase (Ogawa et al., 1987), and alanine:glyoxylate aminotransferase-2 (Ogawa et al., 1990), might be utilized for prophylaxis or therapy in patients with diseases in which increased dimethylarginine levels are causally involved. In addition, if the blockers become available, pathogenic actions of the endogenous inhibitors could be clarified. Information about the pathological implications of PIN, if any, in humans would hasten the development of a pharmacologically effective strategy.

XII. A Possible Reason for Predominant Nitrergic Nerve Function in the Cerebral Artery and Corpus Cavernosum Compared with the Peripheral Vasculature

In isolated cerebral arteries from various mammals including humans, adrenergic neurogenic vasoconstriction is, if any, only slight (Toda and Fujita, 1973; Hardebo et al., 1986; Bevan et al., 1998). This observation is based on the fact that NO-mediated relaxation induced by nerve stimulation was not augmented by treatment with α-adrenoceptor antagonists and adrenergic neuron blocking agents. In addition, reversal of the neurogenic relaxation to a contraction by NOS inhibitors was inconsistent, and the magnitude of an α-adrenoceptor antagonist-sensitive contraction, if induced, was quite small (Toda and Okamura, 1990c, 1992c). Less constrictor action of sympathetic nerve stimulation in arteries of the brain than in those in the periphery was also reported in vivo (Faraci and Heistad, 1990). On the other hand, the myogenic contractions were evident in cerebral arteries (Toda et al., 1978; Nakayama, 1982). Cerebroarterial smooth muscle contracted in response to stretch; this contraction seems to be mediated by an increased influx of Ca^{2+} (Kimura et al., 2000; Obara et al., 2001). It is suggested that herbimycin-sensitive protein tyrosine kinase activity is involved in Ca^{2+} influx through L-type channels (Matsumoto et al., 1997; Kimura et al., 2000). In the perfused cerebral artery, a pressure increment induced vasoconstriction and an elevation of wall tension in association with either increased amounts of Ca^{2+} in the vascular wall or increased Ca^{2+} sensitivity (Gokina et al., 2001).

The greater contribution of the vasodilator nerves to regulate the tone in cerebral vasculature than in peripheral blood vessels has led us to speculate that under physiological conditions in vivo, in which cerebral arteries are constricted, blood flow is controlled mainly by the degree of vasodilator nerve activity. The maintained vascular tone in the brain may be due to transmural pressure, circulating vasoconstrictors, and weak adrenergic nerve activity. In contrast, in the periphery except for the coronary artery, it is well recognized that vascular tone is regulated mainly by adrenergic nerve activity. Vasodilatation appears to be induced mainly by a decrease in adrenergic nerve activity and an increase in nitrergic nerve activity. Figure 7 includes speculative schemes for the control of vessel diameter by NO derived from the nerve and endothelium in cerebral and peripheral arteries. The data pertaining to the cerebral arteries were obtained from studies on anesthetized dogs and monkeys treated with NOS inhibitors, C_6, phentolamine, and atropine, or subjected to denervation of the unilateral pterygopalatine ganglion (Toda et al., 1993d, 2000a,b). In anesthetized phentolamine-treated dogs, the diameter of the basilar artery was depressed 37% by intravenous L-NA, but it was reduced only 12% by the

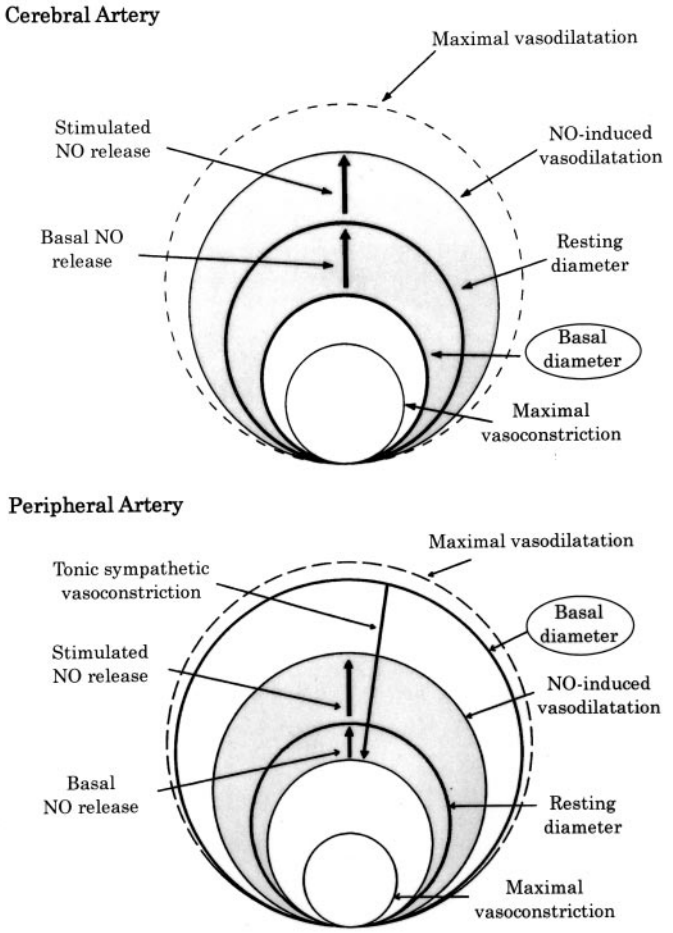

FIG. 7. Schematic presentation of the diameter of cerebral and peripheral arteries as affected by NO (basal and stimulated) released from the nitrergic nerve and endothelium and by sympathetic nerve activation (only for peripheral artery). Basal diameter, the diameter without tonic sympathetic discharge and basal and stimulated release of NO; resting diameter, the diameter under influences of basal release of NO from the nerve and endothelium on the basal diameter; NO-induced vasodilatation, the diameter increased from the resting diameter by stimulated release of NO.

NOS inhibitor after treatment with C_6 (Toda et al., 1993b). About two-thirds of the vasodilatation is associated with NO derived from the nerve that is activated by continuing impulses from the vasomotor center. The remaining one-third may be due to NO from the endothelium.

Damage of the pterygopalatine ganglion and treatment with either ganglionic blocking agents or NOS inhibitors constricted cerebral arteries, suggesting that sustained vasodilator nerve activation reduces the arterial myogenic tone. Cerebroarterial control by inhibitory nerves resembles the control of sphincters in the gastrointestinal and lower urinary tracts, in which the smooth muscle is contracted under resting conditions and relaxed by nerve activation when necessary for discharge of bile, urine, etc.

The isolated corpus cavernosum muscle relaxed in response to NO released from inhibitory nerves; the relaxation was abolished or reversed to a slight contrac-

tion by NOS inhibitors (see *Section VII.*). Relaxation of the NO-mediated cavernous muscle was responsible for a rapid rise of intracavernous pressure and penile erection. Participation of cholinergic and adrenergic neurogenic contractions in inhibiting the erection is at best minimal, since atropine and phentolamine did not augment the response to inhibitory nerve stimulation in vivo (Ayajiki et al., 1997a). The cavernous muscle is thought to interfere with cavernous inflow of blood from the penile artery under resting conditions, and the inflow occurs only when the muscle is relaxed by activation of nitrergic nerves. This phenomenon is analogous to that seen in the gastrointestinal and urinary sphincters. The functional properties of these sphincters are quite similar to those of the "sphincter type" cerebral artery, as described above.

XIII. A Proposal for a New Classification of Efferent Parasympathetic Innervation in Vascular and Nonvascular Smooth Muscle

In parasympathetically innervated blood vessels and corpus cavernosum, a novel neurotransmitter NO mediates inhibitory responses, as already described in this review. Functional predominance of the nitrergic nerve over adrenergic and cholinergic nerves is clearly seen in the cerebral artery and corpus cavernosum in primate as well as nonprimate mammals. This is also the case in nonvascular smooth muscles in particular sphincters, such as pylorus, sphincter of Oddi, ileocolonic junction (Huang et al., 1993; Rand and Li, 1995b; Tanobe et al., 1995; Sanders and Keef, 2000), and bladder neck and urethra (Burnett et al., 1997; Gibson and Lilley, 1997; Andersson and Alm, 2000). In the classical table of autonomic efferent nerves and their functional roles in pharmacology and physiology textbooks, relaxations of vascular and cavernosal smooth muscle and those of sphincters are described as being due to "cholinergic impulses". This term may lead to the misunderstanding, namely that acetylcholine released from cholinergic nerves plays the role. Therefore, we propose a new version of the table: that the parasympathetic section is divided into "cholinergic" and "nitrergic" and that the involved neurotransmitters are clearly indicated as being acetylcholine and NO, respectively. Cerebral vasodilatation, penile erection, and relaxation of sphincters should be shown to be a result of nitrergic nerve activation. Introduction of nitrergic vasodilatation in systemic blood vessels, except for the cerebral vasculature, in the table would better wait until solid information is available concerning physiological roles of the nerve in primates in vitro and in vivo.

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