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Cerebral Blood Flow Regulation by Nitric Oxide: Recent Advances

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Abstract——**Nitric oxide (NO) is undoubtedly quite an important intercellular messenger in cerebral and peripheral hemodynamics. This molecule, formed by constitutive isomers of NO synthase, endothelial nitric-oxide synthase, and neuronal nitric-oxide synthase, plays pivotal roles in the regulation of cerebral blood flow and cell viability and in the protection of nerve cells or fibers against pathogenic factors associated with cerebral ischemia, trauma, and hemorrhage. Cerebral blood flow is increased and cerebral vascular resistance is decreased by NO derived from endothelial cells, autonomic nitrergic nerves, or brain neurons under resting and stimulated conditions. Somatosensory stimulation also evokes cerebral vasodilatation mediated by neurogenic NO. Oxygen and carbon dioxide alter cerebral blood flow and vascular tone mainly via constitutively formed NO. Endothelial dysfunction impairs cerebral hemodynamics by reducing the bioavailability of NO and increasing the production of reactive oxygen species (ROS). The NO-ROS interaction is an important issue in discussing blood flow and cell viability in the brain. Recent studies on brain circulation provide quite useful information concerning the physiological roles of NO produced by constitutive isoforms of nitric-oxide synthase and how NO may promote cerebral pathogenesis under certain conditions, including cerebral ischemia/stroke, cerebral vasospasm after subarachnoid hemorrhage, and brain injury. This information would contribute to better understanding of cerebral hemodynamic regulation and its dysfunction and to development of novel therapeutic measures to treat diseases of the central nervous system.**

I. Introduction

The discovery of nitric oxide (NO[1]), a lipophilic gaseous molecule constitutively generated from endothelial cells and nerve cells or fibers, opened a new era of understanding mechanisms underlying the regulation of cardiovascular functions and their disturbances (Ignarro et al., 1987; Palmer et al., 1987; Furchgott, 1988). Developmental investigations on NO synthesis inhibitors, chemically synthesized (Palmer et al., 1988; Mülsch and Busse, 1990; Toda et al., 1990) or endogenously generated (Vallance et al., 1992), enabled biological researchers to clarify how NO contributes to cardiovascular and central/peripheral nervous system functions. NO formed via endothelial (eNOS) and neuronal NO synthase (nNOS) causes vasodilatation, hypotension, blood flow increase, inhibitions of platelet aggregation and adhesion, and a decrease in smooth muscle proliferation and exerts other beneficial actions as an antioxidant. It also acts as a neurotransmitter or neuromodulator. On the other hand, unregulated production of NO through inducible or immunological NOS (iNOS) during inflammation or NO formed by nNOS in the brain evokes nitrative stress, leading to neurodegeneration and apoptosis.

[1] Abbreviations: NO, nitric oxide; 7-NI, 7-nitroindazol; ACh, acetylcholine, BK, bradykinin; ADMA, asymmetric dimethylarginine; AF-DX 116, 11-([2-[(diethylamino)methyl]-1-piperdinyl]acetyl)-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4]benzodiazepine-6-one; AF-SX 384, *N*-[2-[2-[(dipropylamino)methyl]-1-piperidinyl]ethyl]-5, 6-dihydro-6-oxo-11*H*-pyrido-[2,3-*b*][1,4]benzodiazepine-11-carboxamide; Akt, serine/threonine protein kinase; ANG, angiotensin; ARL 17477, *N*-[4-(2-{[3-chlorophenyl)methyl]amino}ethyl)phenyl]-2-thiophenecarboximidamide dihydrochloride; AT_1, angiotensin type 1 receptor; BH_4, tetrahydrobiopterin; CO, carbon monoxide; COX, cyclooxygenase; DDAH-1, dimethylarginine dimethylaminohydrolase-1; DY-9760e, 3-(2-(4-(3-chloro-2-methylphenyl)1-piperazinyl)ethyl)5,6-dimethoxy-1-(4-imidazolylmethyl)-1*H*-indazol dihydrochloride 3.5 hydrate; EDHF, endothelium-derived hyperpolarizing factor; EET, eicosatrienoic acid; EGCG, (−)-epigallocatechin gallate; eNOS, endothelial NOS; EPO, erythropoietin; ERK, extracellular signal-regulated kinase; ET, endothelin; GR, glucocorticoid receptor; HDT, head-down tail suspension; HO, heme oxygenase; ICI 118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; ICI 182,780, fulvestrant; ICI 192,605, 4(*Z*)-6-(2-*o*-chlorophenyl-4-*o*-hydroxyphenyl-1,3-dioxan-*cis*-5-yl)hexenoic acid; IL, interleukin; iNOS, immunological or inducible NOS; IPC, ischemic preconditioning; L-NA, N^G-nitro-L-arginine; L-NAME, L-NA methylester; L-NMMA, N^G-monomethyl-L-arginine; LPS, lipopolysaccharide; MEK, mitogen-activated protein kinase/ERK kinase; MK-801, 5*H*-dibenzo-[*a*,*d*]cyclohepten-5,10-imine (dizocilpine maleate); NMDA, *N*-methyl-D-aspartate; nNOS, neuronal NOS; NOS, nitric-oxide synthase; NOx, $NO_2 + NO_3$; ODQ, 1*H*-[1,2,4]oxalodiazolol[4,3*a*]quinoxalin-1-one; OVX, ovariectomized; oxyHb, oxyhemoglobin; PARP-1, PARP, poly(ADP-ribose polymerase) 1; PDE-5, phosphodiesterase type 5; PG, prostaglandin; PGI_2, prostacyclin; PI_3, phosphatidylinositol-3; rHuEPO, recombinant human EPO; ROS, reactive oxygen species; SAH, subarachnoid hemorrhage; SHR, spontaneously hypertensive rat(s); SHRSP, stroke-prone SHR; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α; tPA, tissue plasminogen activator; U-II, urotensin-II; VEGF, vascular endothelial growth factor; WKY, Wistar-Kyoto rat(s); Y-27632,*trans*-4-[(1*R*)-1-aminoethyl]-N-4-pyridinylcyclohexanecarboxamide dihydrochloride.

Cerebral blood flow is one of the important factors in the regulation of brain functions. Cerebral blood flow and vascular smooth muscle tone are regulated by NO derived from endothelial cells (Moncada et al., 1991), autonomic nitrergic nerves (Toda and Okamura, 2003), or brain neurons (Bhardwaj et al., 2000). NO plays pivotal roles in regulating cerebrovascular effects of increased or decreased oxygen, elevated carbon dioxide, and carbon monoxide and cerebrovascular autoregulation as well. Information from recent studies on cerebral blood flow regulation by NO is expected to provide new insight about the physiological control of cerebrovascular functions and the pathological mechanisms involved in genesis of central nervous system diseases and to supply clues for developing novel therapeutic strategies for cerebral dysfunctions.

The literature since the discovery of endothelium-derived relaxing factor by Furchgott and Zawadzki (1980) contains numerous reports about the interactions between NO and cardiovascular function and dysfunction in the brain. This review article covers recent advances in these investigations, mainly including those published in this century, on the regulation by NO of cerebral blood flow under physiological and pathological conditions, including cerebral ischemia, stroke and trauma, and vasospasm after subarachnoid hemorrhage (SAH). Possible therapeutic measures using NO, NO release-modulating substances, and NOS inhibitors for these diseases are also summarized.

II. Synthesis and Actions of Nitric Oxide

A. Nitric-Oxide Synthase Isoforms and Nitric Oxide Production

NO is synthesized together with L-citrulline by NOS from the precursor L-arginine in the presence of oxygen and cofactors, including NADPH, tetrahydrobiopterin (BH_4), heme, FAD, FMN, and calmodulin (Alderton et al., 2001). The enzyme has domains for each of the cofactors except for BH_4, which is required for dimerization of the enzyme (Andrew and Mayer, 1999). All of the cofactors are required to produce NO, and BH_4 insufficiency results in uncoupled NOS, which produces superoxide anions instead of NO. It is known that eNOS uncoupling causes endothelial dysfunction, leading to vascular and metabolic disorders such as hypertension, hyperlipidemia, atherosclerosis, insulin resistance, and diabetes mellitus (Channon, 2004; Shinozaki et al., 2004). Recently, eNOS uncoupling in the cerebral artery has been reported to participate in hypoxic-ischemic brain injury (Fabian et al., 2008). The increase in the intracellular Ca^{2+} concentration via transmembrane influx and/or release from intracellular storage sites activates constitutive NOS (eNOS and nNOS), but iNOS does not require Ca^{2+} for activation. eNOS can also be activated through phosphorylation of $Ser^{1177/1179}$ via the phosphatidylinositol-3 (PI_3) kinase-serine/threonine protein kinase (Akt) pathway (Dimmeler

et al., 1999). eNOS binds to caveolin-1 in the caveolae and microdomains of the endothelial plasma membrane and intracellularly migrates in response to increased cytosolic Ca^{2+} in the presence of calmodulin; thereby, the enzyme becomes activated for NO synthesis. On the other hand, nNOS is mostly a soluble enzyme present in the cytoplasm. NO production can be affected by L-arginine availability. L-Arginine is produced from L-citrulline through argininosuccinate synthetase and argininosuccinate lyase inside the cell (Wiesinger, 2001). L-Arginine is also supplied by uptake from the extracellular space through a cation amino acid transporter system (system y+).

Several NOS inhibitors, which have been used to demonstrate the functional roles of endogenous NO, are as follows: N^G-monomethyl-L-arginine (L-NMMA) (Palmer et al., 1988), N^G-nitro-L-arginine (L-NA) (Rees et al., 1990; Toda et al., 1990), L-NA methyl ester (L-NAME) (Rees et al., 1990), and asymmetric dimethylarginine (ADMA) (Vallance et al., 1992) as nonselective inhibitors; 7-nitroindazol (7-NI) (Moore et al., 1993) as a relatively selective inhibitor against nNOS; and aminoguanidine (Griffiths et al., 1993), N^6-iminoethyl-L-lysine (Moore et al., 1994), and W1400 (Mancinelli et al., 2001) as iNOS-selective inhibitors.

B. Nitric Oxide Actions and Signal Transduction

NO derived from endothelial cells causes vasodilatation, a decrease in vascular resistance, lower blood pressure, inhibition of platelet aggregation and adhesion, inhibition of leukocyte adhesion and migration, and reduction of smooth muscle proliferation, thus leading to prevention of atherosclerosis. These NO functions are mediated by cyclic GMP synthesized through soluble guanylyl cyclase, a heme-containing enzyme, which is directly activated by NO. The cyclic GMP-dependent mechanism is involved in vasodilatation caused by various NO donors such as nitroglycerin, sodium nitroprusside (SNP), and sodium nitrite (Moncada et al., 1991).

NO derived from peripheral efferent (nitrergic) nerves innervating blood vessels (Toda and Okamura, 1990b, 2003), gastrointestinal tract, (Bult et al., 1990; Toda and Herman, 2005), penis (Ignarro et al., 1990; Toda et al., 2005), and trachea (Tucker et al., 1990) also causes vasodilatation, blood flow increase, smooth muscle relaxation, and penile erection. Cyclic GMP also mediates these responses. Phosphodiesterase type 5 (PDE-5) degrades cyclic GMP to yield 5′-GMP. PDE-5 inhibitors such as sildenafil potentiate and prolong the actions of NO and cyclic GMP. Recently, a cyclic GMP-independent mechanism such as *S*-glutathiolation or activation of sarco/endoplasmic reticulum Ca^{2+} ATPase (Cohen and Adachi, 2006) and NO donors that activate Ca^{2+}-dependent K^+ channels (Bolotina et al., 1994; Plane et al., 1998) have been reported to participate in NO-induced muscle relaxation.

In the central nervous system, NO derived from nNOS acts as a neuromodulator or neurotransmitter for the regulation of synaptic plasticity, the sleep-wake cycle, and hormone secretion. Glutamate and related amino acids, such as *N*-methyl-D-aspartate (NMDA), stimulate NO formation via increasing intracellular Ca^{2+} concentration. Overproduction of NO synthesized by nNOS has been reported in many clinical disorders, including acute and chronic neurodegenerative diseases (Calabrese et al., 2007).

NO synthesized by iNOS modulates inflammation through multiple pathways and plays an important role in the regulation of immune reactions. Overproduction of NO by iNOS has been implicated in various pathological processes, including tissue injury and cell apoptosis caused by ischemia and inflammation.

Excessive production of NO is clearly neurotoxic, but the mechanism underlying the toxic action is not fully understood. NO is metabolized to peroxynitrite in the presence of superoxide anion (O_2^-), which binds directly to DNA and causes its structure to change, resulting in cell injury. On the other hand, NO is known to inhibit cytochrome *c* oxidase, the terminal complex of the mitochondrial respiratory chain (Erusalimsky and Moncada, 2007). In most cells, including neurons and astrocytes, NO reversibly and irreversibly modulates oxygen consumption, a phenomenon through which NO induces a signal to specific pathways relevant to neuronal survival. NO may modulate the balance between glucose consumption through the glycolytic pathway and the pentose phosphate pathway in neurons (Bolaños et al., 2007). This may relate to the mechanisms of neurodegeneration and enhancement of apoptosis due to oxidative and nitrosative stress.

C. Nitric Oxide Degradation

NO synthesized by NOS or generated from NO donors via nonenzymatic processes is rapidly inactivated by oxidation to nitrite or nitrate. NO is scavenged by superoxide-generating agents such as pyrogallol and hydroquinone, oxyhemoglobin (oxyHb), and carboxy-2-phenyl-4,4,5,5-tetrametyl-imidazoline-okyl 3-oxide (Akaike et al., 1993). Methylene blue, oxyHb, and 1*H*-[1,2,4]oxadiazolo[4,3*a*]-quinoxalin-1-one (ODQ) (Garthwaite et al., 1995) inhibit the activity of soluble guanylyl cyclase. Under certain conditions in which superoxide and NO are sufficiently generated, these molecules form peroxynitrite, which is another free radical species that elicits biological actions.

III. Physiological Control of Cerebral Blood Flow

A. Cerebral Blood Flow Regulation by Endothelium-Derived Nitric Oxide

There are number of reports implicating the involvement of basal NO release in cerebral blood flow (Faraci, 1990), vascular tone, vascular resistance, and vascular growth (Baumbach et al., 2004) under resting conditions in various mammals including mice, rats, dogs, pigs, and goats (reviewed by Toda and Okamura, 2003). This release has been elucidated by determining the decrease in

blood flow and vasoconstriction induced by NOS inhibitors, NO scavengers, guanylyl cyclase inhibitors (Sobey and Faraci, 1997), and endothelial disruption. On the other hand, stimulated release of NO from the endothelium has been demonstrated in cerebral vasculatures in response to acetylcholine (ACh), bradykinin (BK), or the Ca^{2+} ionophore A23187.

1. Basal Release of Nitric Oxide. Rats injected intracisternally with replication-defective adenovirus containing the bovine eNOS gene developed transient increases in cerebral blood flow (Lüders et al., 2000). There is evidence suggesting that adenosine-induced vasodilatation via activation of A_{2B}-adenosine receptors in the rat pial artery is coupled to the production of NO (Shin et al., 2000). After administration of L-NAME to near-term fetal sheep, cortical blood flow decreased, arterial blood pressure increased, and cerebrovascular resistance increased (Hunter et al., 2003). In lambs, L-NA infusion produced increases in cerebral vascular resistance and decreases in cerebral blood flow during sleep, with the greatest changes occurring in active sleep, which was characterized by widespread neural activation and elevated cerebral blood flow; characteristic differences in cerebral vascular resistance and blood flow among active sleep, quiet sleep, and quiet wakefulness disappeared after treatment with L-NA (Zoccoli et al., 2001). Acute NOS blockade by intravenous L-NAME injection induced a dramatic decrease in hypothalamic blood flow in rats, whereas chronic oral administration of L-NAME did not alter the blood flow; reversal of chronic NOS blockade by L-arginine infusion evoked hypothalamic hyperemia, suggesting the appearance of a compensatory vasodilator mechanism (Hortobágyi et al., 2007). These authors obtained data indicating that the compensatory mechanism is independent of vasodilator prostanoids but reduced release of vasoconstrictor prostanoids may contribute to the normalization of hypothalamic blood flow after chronic loss of NO. Chronic treatment for 3 months with L-NAME in the drinking water increased mean cerebral arteriolar pressure and pulse pressure in Wistar-Kyoto rats (WKY) to levels lower than those in stroke-prone spontaneously hypertensive rats (SHRSP). The cross-sectional area of the vessel wall was greater in L-NAME-treated WKY and SHRSP than in untreated WKY, and the external diameter was less in L-NAME-treated WKY than in untreated WKY but greater than in SHRSP (Chillon and Baumbach, 2004). Cerebral arterioles seem to undergo hypertrophy and remodeling in WKY with L-NAME-induced hypertension. Jesmin et al. (2004a) noted that there was a marked reduction in the profile of cerebral blood flow and angiogenic factors of SHRSP, compared with that in age-matched WKY and SHR, and reduction in levels of endothelial growth factor (VEGF) and fibroblast growth factor occurred earlier in development before the juvenile period and probably preceded reduction in regional cerebral blood flow in SHRSP. They also obtained evidence suggesting that the decrease in NO, possibly responsible for reduced blood flow, may largely be due to a decrease in eNOS expression in juvenile male SHRSP. Vascular expression of dimethylarginine dimethylaminohydrolase-1 (DDAH-1), an ADMA-hydrolyzing enzyme, was increased and plasma levels of ADMA were reduced in DDAH-1 transgenic mice. Contraction of the aorta to L-NAME was increased, and relaxation of the carotid artery to ACh was enhanced in the transgenic mice; in vivo, ADMA reduced responses of cerebral arterioles to ACh in nontransgenic mice, and this inhibitory effect was absent in the transgenic mice (Dayoub et al., 2008). Overexpression of DDAH-1 seems to increase basal levels of vascular NO and protects against ADMA-induced endothelial dysfunction in the cerebral circulation.

In addition to subprimate mammals, Japanese monkeys also responded to NOS inhibitors with constriction of the anterior and middle cerebral arteries, and the effect was reversed by L-arginine (Toda et al., 2000a). Joshi et al. (2003) noted that in healthy baboons during isoflurane anesthesia, intracarotid SNP did not increase cerebral blood flow, although it decreased proximal arterial tone, as demonstrated by the increase in cyclic GMP content measured in these vessels in vitro.

Chronic head-down tail (HDT) suspension in rats to induce headward fluid shifts and elevations in arterial pressure was suggested to result in an attenuated basal release of NO or a diminished sensitivity of cerebral artery smooth muscle cells to NO (Geary et al., 1998). Basal tone and vasoconstrictor responses to increases in transmural pressure, shear stress, and K^+ were greater in middle cerebral arteries isolated from HDT rats compared with controls. L-NAME and endothelium denudation abolished these differences between HDT and control rats, and HDT was associated with lower levels of middle cerebral artery eNOS protein; cerebral blood flow in select regions was lower and cerebral vascular resistance higher during standing and head-up tilt in HDT rats, indicating that chronic cephalic fluid shifts enhanced basal tone and vasoconstriction through a down-regulation of eNOS signaling mechanism (Wilkerson et al., 2005). Alterations in cerebral autoregulation associated with microgravity or prolonged bed rest may be the result of diminished levels of cerebral artery endothelial NO.

There is evidence suggesting that cyclic AMP has opposing effects on NO-stimulated cyclic GMP increases (Xu et al., 2004). Results from studies on pial arteriolar diameter changes in anesthetized rats that received knockdown of the cyclic GMP efflux protein multidrug resistance protein 5 or were treated with inhibitors of phosphodiesterase-5 (PDE-5) led the authors to conclude that the effect of cyclic AMP to reduce cyclic GMP efflux seems to predominate over cyclic AMP stimulation of cyclic GMP hydrolysis.

In summary, on the basis of studies using NOS inhibitors or those on measurement of plasma ADMA and DDAH-1 levels, there is evidence suggesting that basal release of NO produced by eNOS contributes to in-

creases in cerebral blood flow in various mammals including monkeys and also to prevention of cerebrovascular hypertrophy and remodeling.

2. Stimulated Release of Endothelium-Derived Nitric Oxide. Cerebral vasodilatation can occur by stimulated release of endothelium-derived relaxing factor (NO, EDHF, and vasodilator prostaglandins). In anesthetized rats, L-NA almost abolished cerebral vasodilator responses to ACh and BK. 5-*N,N*-Hexamethyleneamiloride, an inhibitor of the Na^+/H^+ exchanger, did not affect the baseline diameter of the basilar artery but inhibited the vasodilator response to ACh and BK, without affecting vasodilatation produced by SNP; monomethylamine hydrochloride, which causes intracellular alkalinization, enhanced ACh-induced vasodilatation in the presence of a Na^+/H^+ exchanger inhibitor (Kitazono et al., 2001). Intracellular alkalinization produced by activation of the Na^+/H^+ exchanger may enhance NO production in the basilar arterial endothelium. ACh lost the ability to dilate cerebral arteries and arterioles in mice lacking the muscarinic ACh receptor M_5 subtype (Yamada et al., 2001). Light/dye endothelial injury inhibited cerebral vasodilator responses to hypercapnia and BK in anesthetized newborn and juvenile pigs. Juvenile, but not newborn, hypercapnic and BK vascular responses were sensitive to soluble guanylyl cyclase inhibition, and indomethacin inhibited BK responses in newborns, whereas juvenile responses were markedly inhibited by L-NAME and mildly decreased by indomethacin (Willis and Leffler, 2001). It seems that newborn cerebrovascular responses are largely NO-independent, but NO becomes more important with maturation. Topical application of the inhibitor of copper/zinc superoxide dismutase (SOD) attenuated cerebral vasodilator responses to ACh, BK, and arachidonate but not to SNP in anesthetized rabbits, and these inhibitory effects were reversed by a superoxide scavenger (Didion et al., 2001). Dilatation of cerebral arterioles to ACh was reduced in copper/zinc SOD knockout mice compared with wild-type mice (Didion et al., 2002). These results suggest that endogenous SOD limits superoxide levels in the cerebral microcirculation and that NOS- and cyclooxygenase (COX)-mediated responses are dependent on normal activity of SOD. On the basis of data indicating that the responses of cerebrocortical microflow to L-NAME, indomethacin, and ACh were abolished in rats subjected to chronic vasopressin-induced hyponatremia, Kozniewska and Radomska (2001) suggested that attenuation of cerebral blood flow during chronic hyponatremia is, at least in part, due to the withdrawal of basal vasodilator tone produced by NO and PGI_2 in cerebral circulation under physiological conditions. Pial arteriolar dilatation in response to hypercapnia or BK was inhibited by L-NAME only in indomethacin-treated piglets, and NOS activity, but not eNOS expression, increased after chronic indomethacin treatment, suggesting that chronic inhibition of COX can increase the contribution of NO to cerebrovascular circulatory control (Zhang and Leffler, 2002).

In awake goats, injection of vasopressin into the cerebral circulation increased the resting cerebrovascular resistance, and this effect was reduced by an antagonist of vasopressin V_1 receptors and was augmented by L-NAME but not by indomethacin (Fernández et al., 2001). The vasopressin-induced vasoconstriction may be mediated by V_1 receptors and also modulated by NO. From studies on isolated canine ciliary arteries, the contractions and NO release from the endothelium induced by vasopressin are both suggested to be mediated via vasopressin V_1 receptors (Okamura et al., 1997). There are data indicating that NO is involved in acetazolamide-induced cerebral blood flow stimulation possibly by acting as a modulator rather than as a mediator in rats (Tuettenberg et al., 2001). Pial arteriolar dilatation in response to ACh and ADP was reduced by L-NAME but not by an nNOS-selective inhibitor, and suffusion of the heat shock protein 90 blocker 17-(allylamino)-17-demethoxygeldanamycin, the PI_3 kinase inhibitor wortmannin, or the tyrosine kinase blocker tryphostin 47 was accompanied by reductions in ACh-induced dilatation but no changes in the responses to ADP (Xu et al., 2002). Muscarinic and purinergic receptor-mediated eNOS activation in cerebral arterioles was suggested to involve distinctly different signal transduction pathways.

Intravenous angiotensin (ANG) II attenuated the cerebral blood flow increase induced by mechanical stimulation of vibrissae, and this effect was blocked by the AT_1 receptor antagonist losartan or by SOD and was not observed in mice lacking the gp91 phox subunit of NAD(P)H oxidase; AT_1 and gp91 phox immunoreactivities were present in the endothelium and adventitia of neocortical arterioles (Kazama et al., 2004). ANG II seems to impair functional hyperemia, possibly mediated by neurogenic NO, by activating AT_1 receptors and inducing reactive oxygen species (ROS) production via a gp91 phox-containing NAD(P)H oxidase. ANG II attenuated the cerebral blood flow increase in mice produced by topical application of ACh and by whisker stimulation, and it also increased the nitration marker 3-nitrotyrosine in cerebral blood vessels, an effect dependent on NO and NOX-2-derived ROS (Girouard et al., 2007). Peroxynitrite, formed from NO and superoxide, seems to contribute to the deleterious cerebrovascular effects of ANG II.

Local application of ADP in anesthetized wild-type mice produced vasodilatation that was not altered by indomethacin but was partially reduced by L-NA or ODQ. In eNOS-deficient mice, responses to ADP were preserved, and in the absence of L-NA, responses to ADP were markedly reduced by charybdotoxin plus apamin in both wild-type and eNOS-deficient mice (Faraci et al., 2004). A significant portion of the response to ADP in cerebral microvessels may be mediated by an EDHF but not by an eNOS-dependent mechanism. In intact and ovariectomized (OVX), 17β-estradiol-treated nascent fe-

male rats, both chronic and acute NOS inhibition by L-NA, combined with indomethacin, depressed ADP-induced pial arteriolar dilatation, and subsequent application of the gap junction inhibitory peptide Gap 27 had no further effect; however, ADP reactivity was retained in OVX rats after combined treatment with L-NA and indomethacin, but it was attenuated by Gap 27 (Xu et al., 2003). The increased EDHF-like function in chronic estrogen-depleted rats is not due to eNOS deficiency but possibly is attributable to a direct effect of estrogen to modulate EDHF-mediated cerebral vasodilatation. The importance of EDHF relative to NO has also been reported in association with nitroglycerin (Watanabe et al., 2008) and anesthetics (Bryan et al., 2005).

Dilatation of the rat basilar artery in vivo induced by raising cerebrospinal fluid K^+ concentrations (3–30 mM) was inhibited by the inwardly rectifying K^+ channel inhibitor $BaCl_2$, but neither ouabain nor L-NA had any effect on K^+-induced vasodilatation; the administration of K^+ hyperpolarized smooth muscle in isolated segments of basilar artery, with the effect being abolished by $BaCl_2$ (Chrissobolis et al., 2000). The induced basilar artery dilatation in vivo seems to partly involve hyperpolarization mediated by inwardly rectifying K^+ channel activity and possibly another mechanism that does not involve hyperpolarization, activation of Na^+,K^+-ATPase or NO. On the other hand, K^+ (0.5–10 mM)-induced relaxations in isolated canine basilar, middle cerebral, and posterior cerebral arteries were abolished or reversed to contractions by ouabain or substitution of LiCl for NaCl in the bathing media, enhanced by a reduction of extracellular K^+ concentrations, and decreased when the temperature of the bathing media was lowered (Toda, 1974). Similar findings were also obtained in human, feline, and rabbit cerebral arteries (Toda, 1976). Stimulation of the electrogenic Na^+ pump seems to be involved in the K^+-induced cerebroarterial dilatation in the animal species used. Whether the different mechanisms involved are due to differences in animal species (rat versus dog, cat, rabbit, and human) or experimental methods (in vivo versus in vitro) remains to be determined.

3. Role of Superoxide Anions. In anesthetized mice equipped with a cranial window, superfusion with NAD(P)H increased cerebral blood flow, with the effect being attenuated by the free radical scavenger manganese(III) tetrakis(4-benzoic acid)porphyrin chloride, a peptide inhibitor of NAD(P)H oxidase (gp91ds-tat), or by L-NA, the stimulating effect of NAD(P)H was inhibited in gp91-null mice, and NAD(P)H increased the production of ROS, an effect not observed in gp91-null mice (Park et al., 2004). The authors suggested that the mechanisms of cerebral blood flow increases produced by NAD(P)H include NAD(P)H oxidase-dependent and -independent factors. On the other hand, NADH produced Tiron (a scavenger of superoxide)-sensitive dilatation of rabbit cerebral arterioles in vivo. NADH in low concentrations produced relaxations, whereas higher concentrations produced contractions of the isolated basilar artery, NAD(P)H produced only contractions, and NADH- and NAD(P)H-induced increases in superoxide levels were reduced in the presence of SOD or a NAD(P)H oxidase inhibitor (Didion and Faraci, 2002). NADH- or NADPH-induced changes in cerebral vascular tone seem to be mediated by superoxide that is probably produced via NAD(P)H oxidase but not xanthine oxidase or NOS. Faraci (2006) obtained data suggesting that low concentrations of ROS may function as signaling molecules involved in normal regulation of cerebral vascular tone. In 12-month-old mice, the cerebral blood flow increase evoked by whisker stimulation or by ACh and BK was attenuated in association with increased ROS production in neurons and cerebral blood vessels; the cerebrovascular impairment was reversed by a ROS scavenger or by gp91 ds/tat, and it was not observed in mice lacking the NOX-2 subunit of NAD(P)H oxidase (Park et al., 2007). NOX-2 may be a critical source of the neurovascular oxidative stress mediating the deleterious cerebrovascular effects associated with increasing age. In the mice with dominant-negative mutation of peroxisome proliferator-activated receptor γ, ACh-induced vasodilation was impaired and superoxide levels were increased in cerebral arterioles, suggesting that peroxisome proliferator-activated receptor γ plays a role in protecting blood vessels by suppression of oxidative stress (Beyer et al., 2008). In short, superoxide anions mainly produced via NAD(P)H oxidase interact with NO, involving decreases in cerebral blood flow, endothelial dysfunction, and impairment of cell viability.

4. Role of Estrogen and Other Hormones. In estrogen-depleted rats, decreased eNOS function in pial arterioles in vivo was not reversed by the up-regulation of eNOS or the down-regulation of caveolin-1 but normalized only when eNOS up-regulation and caveolin-1 down-regulation were combined (Xu et al., 2001). The long-term deprivation of estrogen by ovariectomy in rats decreased serum levels of estradiol and plasma concentrations of NO metabolites, and the thrombotic tendency was increased and vessel diameter and blood flow in pial arterioles were reduced after ovariectomy, suggesting that estrogen may mediate beneficial effects on the cerebral microcirculation and modulate cerebral thrombotic mechanisms via increased NO bioavailability in female rats (Ono et al., 2002). Stirone et al. (2003) noted that in vivo estrogen treatment resulted in a 100% increase in eNOS mRNA copy number and increased eNOS protein levels by 47% in mouse cerebral blood vessels. Estrogen seems to modulate eNOS at the transcriptional level in blood vessels in vivo. The Rho-kinase inhibitor Y-27632 was more potent as a cerebral vasodilator in male rats than in female rats. In OVX rats, vasodilator responses to Y-27632 resembled responses in males, and treatment of OVX rats with 17β-estradiol normalized the effect of Y-27632 so that they were equivalent to the responses in intact female controls; L-NAME

caused greater constriction of the basilar artery in females than males (Chrissobolis et al., 2004). It seems that vascular Rho-kinase function is suppressed by estrogen in female rats, and the higher NO activity in females is estrogen-independent. In cerebral blood vessels from control mice, chronic treatment with estrogen increased protein levels of eNOS but had no effect on COX-1 protein and PGI_2 production, in L-NAME-treated mice, cerebrovascular COX-1 levels and PGI_2 production, as well as eNOS protein, were greater in estrogen-treated animals, and in vessels from eNOS-knockout mice, estrogen also increased levels of COX-1 protein, but no effect on PGI_2 production was detected (Li et al., 2004). It seems that estrogen increases eNOS protein in cerebral microvessels and decreases cerebrovascular Rho-kinase function, thus improving cerebral microcirculation.

Levels of vascular VEGF, VEGF receptors, eNOS, phosphorylated Akt, estrogen receptor-α, aromatase, and cerebral capillary density in juvenile male SHRSP, a model for attention-deficit/hyperactivity disorder, were down-regulated, whereas androgen receptor levels were up-regulated, compared with age-matched controls; castration, estrogen, and an androgen receptor antagonist (flutamide) counteracted these effects (Jesmin et al., 2004b). The authors postulated that changes in androgen metabolism in the frontal cortex of male SHRSP may lower levels and/or activity of VEGF and its signaling cascade and, subsequently, reduce regional cerebral blood flow. These findings could help explain the pathogenesis of reduced regional cerebral blood flow and male preponderance in attention deficit/hyperactivity disorder.

Tripeptide thyrotropin-releasing hormone induced an increase in cerebral blood flow in rats, and L-NAME, but not 7-NI and D-NAME, reduced this effect, indicating that the cerebral vasodilatation induced by this hormone may be mediated by endothelial NO (Koskinen and Koch, 2003).

5. Studies on Humans. Basal NO release seems to be important in controlling human cerebral blood flow (White et al., 1998; Joshi et al., 2000). On intravenous infusion of L-arginine, cerebral blood flow velocity increased and mean blood pressure decreased in healthy volunteers; however, the hypotensive effect did not differ between older (average age 70.2 years) and younger (28.8 years) groups. The cerebral circulation in the older group showed a blunted, smaller, and more easily saturated vasomotor response compared with that in the younger group (Okamoto et al., 2001). NO may be involved in the increased cerebral blood flow velocity. Basal cerebral blood flow was lower in old (78 years) compared with young subjects (25 years). Intravenous infusion of L-NMMA increased mean arterial pressure in both groups, decreased cerebral blood flow in the old subjects, and did not influence cerebral circulation in the young subjects (Kamper et al., 2004). In elderly individuals, cerebral blood flow seems to be impaired and dependent on the intactness of the NO pathway. In healthy volunteers, intravenous L-NMMA decreased regional cerebral blood flow, but did not affect middle cerebral artery blood velocity; L-NMMA did not affect acetazolamide-induced increases in middle cerebral artery blood velocity nor regional cerebral blood flow, suggesting that the basal tone of human cerebral arterioles but not that of the conduit arteries is NO-dependent (Lassen et al., 2005). Katona et al. (2006) provided evidence indicating that cerebral blood flow velocities but not cerebral vascular resistance values may be associated with serum NO and endothelin (ET)-1 concentrations in healthy and hypertensive adolescents.

The use of NO synthase inhibitors in humans is limited because only relatively low concentrations of L-NMMA are permitted in human experimental use. Therefore, the role of NO may be underestimated.

Intracarotid administration of SNP in doses sufficient to decrease mean arterial pressure in sedated humans did not augment cerebral blood flow (Joshi et al., 2002). Intravenous nitroglycerin at therapeutic doses in awake humans did not alter global or regional cerebral blood flow (White et al., 2000a). Intravenous infusion of SNP decreased mean blood pressure and regional cerebral vascular resistance, resulting in unaffected cerebral blood flow in healthy subjects. SNP infusion blunted the vasoconstrictor responses to hypocapnia and augmented the vasodilator response to hypercapnia, indicating that exogenously administered NO selectively affects the CO_2-dependent, chemoregulatory mechanism (Lavi et al., 2003). Therefore, the CO_2-NO axis may be a cardinal pathway for cerebral blood flow regulation in humans.

In short, basal and stimulated release of NO from the endothelium plays important roles in the cerebral blood flow increase in humans. Elderly individuals seem to have impaired NO availability and also increased production of counteracting molecules, ROS.

B. Autoregulation

In vital organs, particularly the brain, blood flow is constantly maintained when blood pressure changes rapidly within a limited range. The mechanism of this phenomenon, termed autoregulation, has been intensively investigated; however, the involvement of NO in this mechanism is still controversial.

1. Studies on Experimental Animals. There is evidence supporting the idea that NO is involved in the physiological autoregulation of cerebral blood flow in rats (Tanaka et al., 1993; Preckel et al., 1996; Jones et al., 1999; Sugimoto et al., 2000), cats (Kobari et al., 1994), and newborn pigs (Hardy et al., 1999). NO synthesis exerted an important influence on the pressure-flow relationships of the internal and external carotid artery circulations, as L-NMMA increased input perfusion pressure at any given flow rate; however, in the presence of NO synthesis, hydraulic conductance increased rapidly with flow in the internal carotid artery, thereby stabilizing perfusion pressures over a wide range of flow rates, whereas this phe-

nomenon was not evident in the external carotid artery territory (Ujiie et al., 2002). A possible explanation for the apparently greater functional effects of NO in the internal carotid artery than in the external carotid artery may be that there are differences in the NO availability between the two circulations. Jones et al. (2003) found that NOS inhibition depressed the autoregulatory pattern in rats, decreasing the seemingly paradoxical increase in cerebral blood flow as blood pressure decreased. NO probably increases cerebral blood flow near the lower limit and augments the hypotensive portion of the autoregulatory curve. Newborn lambs showed cerebral autoregulation before severe hypoxia-ischemia but lacked the autoregulatory ability of the cerebral vascular bed after hypoxia-ischemia; autoregulation was restored in L-NA-treated lambs, suggesting a role for NO-induced vasodilatation in the impairment of cerebral blood flow autoregulation after birth asphyxia (Dorrepaal et al., 2001).

In contrast, no effects of NOS inhibitors on autoregulation have been reported in anesthetized (Wang et al., 1992; Buchanan and Phillis, 1993) and conscious rats (Kelly et al., 1994; Takahashi et al., 1995), dogs (Saito et al., 1994), and cynomolgus monkeys (Thompson et al., 1996). Low concentrations of superoxide anions generated by subdural perfusion of xanthine/xanthine oxidase and catalase decreased basal cerebral blood flow in rats, whereas higher concentrations of superoxide increased basal blood flow and impaired autoregulation; neither inhibition of NO synthesis nor the addition of deferoxamine had any effect at higher concentrations of superoxide; superoxide increased the activity of Ca^{2+} activated K^+ channels in cerebral vascular smooth muscle cells (Zagorac et al., 2005). Superoxide anions seem to increase basal cerebral blood flow and impair autoregulation, probably through the activation of Ca^{2+}-activated K^+ channels but not NO production.

Sevoflurane increased brain tissue NO_2 and impaired cerebral blood flow autoregulation in rats, and administration of L-NAME restored the autoregulation, suggesting that this volatile anesthetic impairs cerebrovascular autoregulation by mechanisms secondary to increases in perivascular NO availability (Werner et al., 2005).

2. Studies on Humans. To calculate the autoregulation index in healthy subjects, the rate of rise of middle cerebral artery blood flow velocity, compared with that of arterial blood pressure, was determined after a stepwise fall in arterial blood pressure (White et al., 2000b). The authors noted that the mean change in autoregulatory index after norepinephrine at a similar pressor dose was greater than the change after the bolus injection of L-NMMA, and they concluded that NO seems to at least partly mediate the dynamic phase of cerebral autoregulation, and reduced NO release may play a role in the impaired cerebral autoregulation seen in patients with cerebral ischemia.

On the other hand, in healthy volunteers, intravenous L-NMMA increased mean blood pressure but did not change cerebral blood flow velocity, resulting in an increased cerebrovascular resistance index. Similar changes were observed after phenylephrine infusion, and during baseline tilt, steady-state blood pressure increased and cerebral blood flow velocity decreased to a similar extent in L-NMMA-treated subjects and untreated subjects (Zhang et al., 2004). Inhibition of tonic production of NO does not seem to alter dynamic cerebral autoregulation in humans. Pressure-dependent autoregulation was preserved in patients with endothelial dysfunction (Lavi et al., 2006).

Whether or not endogenous NO participates in the autoregulation of cerebral blood flow in a variety of experimental animals and healthy subjects is still controversial. The basis of these discrepancies in reported findings has yet to be elucidated.

C. Influences of Carbon Dioxide, Oxygen, and Carbon Monoxide

1. Hypercapnia

a. Studies on Experimental Animals. The increase in cerebral blood flow by hypercapnia has been reported to be either dependent on or independent of endogenous NO (reviewed by Toda and Okamura, 2003). The hypercapnia-induced cerebral blood flow increase was reduced by either 7-NI or indomethacin in rats; the attenuation by indomethacin was diminished by 7-NI, and 7-NI had less effect in the presence of indomethacin (Heinert et al., 1999). It seems that the pathways involved in the hypercapnic responses mediated by NO formed by nNOS are distinct from those mediated by COX products; however both may interact synergically. Light/dye endothelial injury inhibited hypercapnic cerebrovascular dilatation in anesthetized juvenile pigs, which were sensitive to L-NAME and soluble guanylyl cyclase inhibition, indicating that endothelial NO may participate significantly in the hypercapnic vasodilatation (Willis and Leffler, 2001). L-NAME inhibited pial arteriolar dilatation in response to hypercapnia in piglets chronically treated with indomethacin but not in controls, topical SNP or iloprost, a stable analog of PGI_2, restored hypercapnia-induced dilatation, and pial arterioles of control piglets and those chronically treated with L-NAME constricted in response to ACh, whereas those of indomethacin-treated piglets dilated in response to ACh; this response was inhibited by L-NAME (Zhang and Leffler, 2002). Chronic inhibition of COX may increase the contribution of NO to cerebrovascular circulatory control. L-NAME completely and indomethacin markedly inhibited the hypercapnia-induced increase in corticocerebral blood flow in conscious rabbits (Csete et al., 2001). Ances et al. (2001) obtained data suggesting that estrogen may modulate the up-regulation of the cerebral blood flow response observed after transient hypercapnia in female rats. There are findings supporting the idea that estrogen increases NO availability in cerebral vasculatures (see Section III.A.4). On the other hand, Xu et al. (2003)

reported that in the presence of L-NAME, normal CO_2 reactivity was observed in female rats, whereas a 50% reduction in CO_2 reactivity was seen in males.

Ventilation of pigs with high CO_2 caused an increase in cerebral blood flow at 30 min, which declined and gradually rose at 6 to 8 h; the latter increase was associated with PGE_2 elevation, nitrite formation, eNOS mRNA expression, and in situ NOS reactivity. Treatment of pigs with the COX inhibitor diclofenac or L-NA prevented the secondary cerebral blood flow increase during hypercapnic acidosis. In addition, acidosis produced changes similar to those seen with hypercapnia, which were prevented by diclofenac, by a Ca^{2+} channel inhibitor, and by the ATP-sensitive K^+-channel blocker glybenclamide (Najarian et al., 2000). In prolonged hypercapnic acidosis, the secondary increase in cerebral blood flow is closely associated with induction of eNOS expression; this seems to be mediated by PGE_2 generated by an ATP-sensitive K^+ and Ca^{2+} channel-dependent process. In superfused brain slices from rat cerebral cortex, ATP-sensitive K^+ channels were suggested to play a major role in cerebral vasodilator responses to decreased pH accompanied by hypercapnia, but not hypercapnia itself (Nakahata et al., 2008). The CO_2-evoked increase in cerebral arteriolar diameter in rats was depressed by selective adenosine A_{2A} receptor antagonists and also by nonselective NOS inhibitors (Phillis et al., 2004). Pial arterial vasodilatation to hypercapnia was reduced by intravenous infusion of L-glutamine in rats, and coinfusion of L-arginine with glutamine maintained the hypercapnic vasodilatation; infusion of ammonium acetate at a rate to produce increases in cortical tissue glutamine concentrations resulted in no hypercapnic vasodilatation, and coinfusion of L-arginine reversed the effect (Okada et al., 2000). Glutamine probably acts by limiting L-arginine availability. Puscas et al. (2000) provided evidence suggesting that inhibitions by NO and PG of carbonic anhydrase I activity is involved in cerebral vasodilatation produced by hypercapnia.

b. Studies on Humans. In healthy young subjects, hypercapnia increased mean flow velocity in the middle cerebral artery, and the response was blunted by L-NMMA infusion, supporting the concept that NO has a role in hypercapnia-induced vasodilatation in humans (Schmetterer et al., 1997). In patients with impaired vasomotor reactivity to CO_2, L-arginine infusion increased CO_2 vasoreactivity (Zimmermann and Haberl, 2003). In diabetic or hypertensive patients with endothelial dysfunction, cerebral CO_2 vasoreactivity was impaired, and SNP offset this disparity, indicating that NO may be involved in CO_2-dependent cerebral blood flow regulation (Lavi et al., 2006). On the other hand, Meadows et al. (2005) noted the lack of correlation between total nitrite + nitrate levels and hypercapnic cerebral vascular reactivity in healthy male individuals. According to Ide et al. (2007), hypercapnia-induced increases in mean arterial pressure, heart rate, and middle cerebral artery blood velocity were similar with and without L-NMMA in young adult volunteers, suggesting that NO is not required for the cerebrovascular responses to hypercapnia

In summary, the hypercapnia-induced cerebral blood flow increase in experimental animals seems to be associated with NO formed by nNOS, eNOS, or both and with eNOS mRNA expression. Studies in volunteers provide evidence for and against the role of NO. Again, these studies are limited by the low doses of L-NMMA used in human studies.

2. Hyperbaric Oxygen. Hyperbaric oxygen has been advocated for the treatment of various vascular dysfunctions, including ischemic stroke, air embolism, and carbon monoxide (CO) poisoning.

a. Studies on Experimental Animals. In rats exposed to hyperbaric oxygen at 5 atm, regional cerebral blood flow decreased, brain NOx levels decreased, and estimated hydroxyl radical production increased; the decrease in cerebral blood flow in response to hyperbaric oxygen was abolished by manganese SOD administration into the circulation, suggesting that impaired cerebral blood flow with hyperbaric oxygen is associated with a decrease in effective NO concentrations and an increase in ROS production in the brain (Demchenko et al., 2000). Decreases in regional cerebral blood flow in response to hyperoxia were observed in wild-type and SOD3-transgenic mice, whereas SOD3-deficient mice did not show cerebral vasoconstriction. Cerebral blood flow was reduced in these three groups, but SOD3-transgenic mice showed larger decreases in cerebral blood flow, implying that extracellular SOD promotes NO vasodilatation by scavenging superoxide anions, whereas hyperoxia opposes NO and promotes cerebral vasoconstriction by enhancing endogenous superoxide generation and decreasing the basal vasodilator effects of NO (Demchenko et al., 2002). The decrease in the diameter of perfused middle cerebral artery branches induced by exposure to L-NAME was less in the arteries from newborn lambs exposed to extracorporeal membrane oxygenation compared with those in control animals, suggesting that oxygenation leads to a decrease in basal production of NO, possibly associated with an impairment of cerebral arterial endothelial function (Ingyinn et al., 2000). Mice lacking eNOS and nNOS genes showed decreased cerebrovascular reactivity to L-NAME and ACh. In response to hyperbaric oxygen, wild-type and nNOS-knockout mice showed decreases in regional cerebral blood flow over 30 min, but eNOS-knockout mice did not, and after 60 min of exposure to hyperoxia, cerebral blood flow increased more in wild type mice than in eNOS-knockout or nNOS-knockout mice (Atochin et al., 2003b). Modulation of eNOS-derived NO by hyperbaric oxygen may be responsible for the early vasoconstrictor responses, whereas late vasodilatation induced by hyperoxia probably depends on both eNOS and nNOS. In rat brains exposed to hyperbaric oxygen,

ADMA and L-arginine levels were higher and the NOx level was lower than control levels; significant correlations between ADMA and L-arginine and between ADMA and NOx were detected (Akgül et al., 2007). ADMA-induced NOS inhibition may be responsible for the early-phase hyperoxic vasoconstriction.

Before convulsive activity evoked by hyperbaric oxygen appeared on the electroencephalogram in rats, cerebral blood flow increased; pretreatment with L-NAME or 7-NI prevented the development of hyperoxic hyperemia and paroxysmal spikes on the electroencephalogram (Moskvin et al., 2003). Hyperbaric oxygen is suggested to induce changes in cerebral blood flow, which modulates its neurotoxic action via NO synthesized possibly by nNOS.

b. Studies on Humans. In young healthy subjects, L-NMMA did not affect the hyperoxia-induced decrease of mean flow velocity in the middle cerebral artery (Schmetterer et al., 1997).

3. Hypoxia

a. Studies on Experimental Animals. In 0.75 gestation sheep, L-NAME blunted the increase in cerebral blood flow after hypoxia, compared with that seen during normoxia, suggesting that NO plays a role in fetal cerebrovascular control during normoxia and hypoxia (Coumans et al., 2003). In near-term fetal sheep, increases in cortical blood flow and cortical release of cyclic GMP evoked by hypoxia were attenuated after L-NAME administration (Hunter et al., 2003). The increase in the expression of nNOS in the cortex of newborn rats seen during the early postnatal days was higher in the hypoxic group than that in the control group; immunoreactivity for iNOS was also higher in the cortex of hypoxic rats (Fernández et al., 2003). The authors suggested that overproduction of NO in the brain of hypoxic animals may constitute an effort to reestablish normal blood flow. In the llama fetus during normoxemia, L-NAME produced an increase in mean blood pressure, an increase in cerebral vascular resistance, and no change in cerebral blood flow. During hypoxemia, vascular resistance was decreased compared with that during normoxemia, and it was increased after treatment with L-NAME, suggesting that NO has an important role in maintaining normal vasodilator tone during normoxemia and hypoxemia in cerebral vascular beds and that NOS inhibition unmasks other vasodilator substances that seem to play a role in the control of cerebral hemodynamic responses to acute hypoxemia (Sanhueza et al., 2005).

In contrast, there was evidence suggesting no contribution of NO to the vascular response to hypoxia. Administration of L-NAME increased blood pressure and decreased microsphere-determined forebrain cerebral blood flow during normoxia in fetal sheep, and increases in cerebral blood flow by hypoxia in control and L-NAME-treated groups were similar at 0.6 and 0.9 gestations, suggesting that NO does not play an important role in hypoxic vasodilatation in brain at either 0.6 or 0.9 gestation (Harris et al., 2001). There was no change in the activity or subcellular distribution of NOS activity in brain tissues from llama fetuses after a 24-h exposure to hypoxia (Galleguillos et al., 2001).

In isolated guinea pig basilar arteries, hypoxia enhanced the vasodilator response to the NO donor *S*-nitroso-*N*-acetylpenicillamine, and in the presence of L-NA, hypoxia no longer enhanced this response, suggesting that this response enhancement may be explained by hypoxia-induced inhibition of basal NO synthesis (Movahed et al., 2003). The hypoxia-induced increase in transport of [^{14}C]sucrose across primary bovine brain microvessel endothelial cell monolayers compared with normoxia was attenuated by either posthypoxic reoxygenation or inhibition of NOS; total NO and expression of iNOS were increased in the endothelial cells after hypoxic exposure (Mark et al., 2004). Hypoxia-induced blood-brain barrier breakdown may be diminished by NOS inhibition and decreased the concentration of NO metabolites and/or reoxygenation. Exposure to hypoxia of brain capillary endothelial cells resulted in increased tight junction permeability, with a decrease in transendothelial electrical resistance, and induced the expression of both occludin and glucose transporter 1 mRNA in endothelial cells. The decrease in the electrical resistance due to hypoxia was inhibited by anti-interleukin-1 antibody and NOS inhibitor (Yamagata et al., 2004). Based on these results, the authors concluded that the expression of occludin and glucose transporter 1 mRNA may be sensitive to exposure to hypoxia and that the changes in permeability in endothelial cells are associated with interleukin-1β and NO.

b. Studies on Humans. In young volunteers, administration of L-NMMA during normoxia did not affect cerebral blood flow, blood pressure, or heart rate; hypoxia increased cerebral blood flow, and after L-NMMA, the augmented cerebral blood flow returned to baseline, indicating that hypoxia-induced cerebral vasodilatation in humans seems to be mediated by NO (Van Mil et al., 2002). On the other hand, Ide et al. (2007) found that hypoxia-induced increases in mean arterial pressure, heart rate, and middle cerebral artery blood velocity in humans were similar with and without administration of L-NMMA. In this study, administration of L-NMMA elevated blood pressure and decreased heart rate under normoxia. Therefore, systemic effects of L-NMMA on the baseline hemodynamics under normoxia may affect the responsiveness of the cerebral artery to L-NMMA. In the human endothelial cell line, RhoA protein levels and Rho-kinase expression were increased, and eNOS expression was decreased after a 5-h exposure to hypoxia; the hypoxia-induced decrease in eNOS expression was inhibited when the endogenous Rho-kinase activity was inhibited and enhanced by expression of the constitutively active form of Rho-kinase (Jin et al., 2006b). At-

tempts to down-regulate RhoA and Rho-kinase by multiple drugs, such as statins and Rho-kinase inhibitors, may provide endothelial and cardiovascular benefits through up-regulation of eNOS. In summary, endogenous NO seems to play important roles in increasing cerebral blood flow in the fetus and in newborn animals. This may also be the case in young subjects.

4. Carbon Monoxide. CO, an endogenously derived gas formed from the breakdown of heme by the enzyme heme oxygenase (HO), exerts a beneficial effect in maintaining cardiovascular homeostasis in relation to NO availability. The vasodilatory (nonhypoxic) effects of CO on cerebral blood flow in adult rats were mediated by NO; aging rats exposed to CO and to air under the same conditions did not show significant changes in cerebral blood flow, indicating that the brain vasodilatory response to CO does not seem to be active in the aging rat (Mendelman et al., 2000). Intraperitoneal administration of the HO inhibitor zinc deuteroporphyrin 2,4-bis glycol had no effect on the resting cerebral blood flow in rats but increased hypothalamic NOS activity without changing cerebrospinal fluid cyclic GMP concentration. After treatment with NOS inhibitors, the diminished cerebral blood flow was further reduced by the HO inhibitor (Horváth et al., 2003). Endogenous CO may contribute to the cerebral vasodilatation in pathophysiological states associated with diminished NO synthesis. In anesthetized pigs, pial arterioles dilated in response to SNP, CO, or the CO-releasing molecule Mn_2CO_{10}, and this molecule did not cause an increase in the cerebrospinal fluid cyclic GMP concentration. A cyclic GMP clamp with a threshold dilator level of 8-bromo-cyclic GMP and ODQ restored the dilatation to Mn_2CO_{10} that had been blocked by ODQ alone. Inhibition of the pial arteriolar dilatation to glutamate by L-NA was similar to that by HO inhibitors (Koneru and Leffler, 2004). These authors suggested that the permissive role of NO in CO- and glutamate-induced vasodilatation involves maintaining the cyclic GMP levels necessary to allow CO to cause dilatation independently of increasing cyclic GMP. Leffler et al. (2005a) obtained data indicating that activation of cyclic GMP-dependent protein kinase seems to be the predominant mechanism of the permissive actions of NO and PGI_2 for CO-induced pial arteriolar dilatation in piglets. In isolated cerebral microvessels from piglets, L-NA or ODQ blocked glutamate stimulation of HO-2 activity and CO production (Leffler et al., 2005b). Glutamate may activate NOS, producing NO that leads to CO synthesis via a cyclic GMP-dependent elevation of HO-2 catalytic activity. In newborn and 7-week-old pigs, L-NA inhibited the cerebrovascular dilatation to CO; in contrast, the CO-induced dilatation in juvenile piglets or rats was not reduced by NOS inhibition (Holt et al., 2007).

In rats, blockade of the HO activity by zinc protoporphyrin IX dilated pial arterioles, and the dilator effect was dependent on the local NOS activities and was abolished by CO supplementation. Upon CO synthesis inhibition, regional NO formation was augmented in subdural mesothelial cells, and this effect was attenuated by the application of CO (Ishikawa et al., 2005). CO derived from HO-2 seems to serve as a tonic vasoregulator antagonizing NO-mediated vasodilatation in the rat cerebral microcirculation. There is evidence suggesting that CO is not a dilator of rat, mouse (Andresen et al., 2006), and rabbit cerebral arteries (Brian et al., 1994). More data are required to resolve this discrepancy.

NO concentrations and cerebral blood flow were increased by exposure to CO poisoning associated with activation of nNOS that was inhibited by MK-801, and elevations of NO were inhibited by infusion of SOD. When injected with MK-801 or 7-NI, rats did not exhibit CO-mediated nitrotyrosine formation; elevation of nitrotyrosine was seen in CO-poisoned wild-type mice but not in nNOS-knockout mice (Thom et al., 2004). It seems that CO exposure initiates perivascular processes including oxidative stress that triggers activation of NMDA nNOS.

In short, cerebral vasodilator effects of CO are mediated by NO or by NO-independent mechanisms; different mechanisms may be partly dependent on the animal species used. Cyclic GMP may be involved in the vascular response. CO poisoning probably triggers activation of the NMDA neuronal NOS pathway.

D. In Vitro Cerebral Arterial Tone as Affected by Endothelial Nitric Oxide

1. Basal Release of Nitric Oxide. There are literature reports indicating that NOS inhibitors produced cerebral vasoconstriction in isolated cerebral arteries and arterioles from various animal species (Toda and Okamura, 1992; Rosenblum, 1997; Yamakawa et al., 1997), including Japanese monkeys (Toda et al., 1993a). L-NA elicited a gradual constriction in isolated, perfused bovine and human intracortical penetrating arterioles, indicating the presence of constitutive NO release (Elhusseiny and Hamel, 2000). In isolated rat middle cerebral arteries, blocking the basal release of NO by L-NA or guanylyl cyclase inhibition by ODQ induced vasomotion, which was enhanced and became irregular after UTP administration; the thromboxane receptor antagonist ICI 192,605 attenuated the vasomotion induced by L-NA and UTP, suggesting that the lack of NO in cerebral vessels provokes vulnerability to chaotic vasomotion that is mediated by activation of thromboxane receptors (Lacza et al., 2001). In cannulated and pressurized endothelium-intact cerebral arteries, indomethacin produced no effect on vascular tone in either neonatal or adult mice, but subsequent addition of L-NAME constricted both neonatal and adult arteries at various pressures. In the presence of indomethacin and L-NAME, intrinsic tone was greater in neonatal arteries than in adult arteries, but when the endothelium was removed, tone was similar in neonatal and adult arteries at all

pressures (Geary et al., 2003). The contribution of endothelial vasoactive factors (NO and PGI_2) to intrinsic vascular tone seems to be highly age-dependent. Geary and Buchholz (2003) noted that aging alters rat middle cerebral artery tone and $[Ca^{2+}]_i$ responses through endothelium-derived NOS-sensitive and -insensitive mechanisms. Treatment with L-NAME and indomethacin revealed that both endothelium-derived NO and vasodilator prostanoids were responsible for the reduced vascular tone in arteries from near-term fetal sheep (Geary et al., 2004). The authors suggested that development is associated with alterations in cerebral vascular smooth muscle, endothelium, NOS, and COX responses to intravascular pressure. In pressurized cerebral arteries, L-NA-induced contraction was significantly greater in the postpartum rats compared with nonpregnant or late-pregnant rats, suggesting that basal release of NO increases after delivery (Cipolla et al., 2004).

In cerebral blood vessels from control mice, estrogen increased protein levels of eNOS but had no effect on COX-1 protein and PGI_2 production; after treatment with L-NAME, cerebrovascular COX-1 levels and PGI_2 production, as well as eNOS protein, were greater in estrogen-treated mice. In vessels from eNOS-knockout mice, estrogen increased levels of COX-1 protein, but no effect on PGI_2 production was detected (Li et al., 2004). Cerebral blood vessels of control mice do not seem to exhibit effects of estrogen on the PGI_2 pathway; however, when NO production is dysfunctional, the impact of estrogen on a COX-sensitive vasodilator is probably revealed. Stirone et al. (2005) found that treatment with 17β-estradiol increased NO production in cerebral arteries isolated from OVX rats, and this effect was decreased by membrane cholesterol depletion, the estrogen receptor antagonist ICI 182,780, and inhibitors of PI_3 kinase. 17β-Estradiol increased phosphorylation of both eNOS and Akt. In addition, long-term estrogen exposure increased levels of cerebrovascular phosphorylated Akt and phosphorylated eNOS as well as basal NO production. The authors suggested that estrogen signaling via nontranscriptional kinase mechanisms has long-term consequences for vascular function. Treatment of bovine microvascular and human umbilical endothelial cells with 17β-estradiol increased phosphorylation of Akt followed by phosphorylation of eNOS, and ICI 182,780 inhibited eNOS phosphorylation. 17β-Estradiol dilated cerebral microvascular vessels, and this effect was blocked by wortmannin (Florian et al., 2004).

Martens and Kojda (2001) provided evidence suggesting that porcine cerebral conductance arteries seem to have a reduced expression and/or activity of a cellular enzymatic electron transport systems, such as the cytochrome P450 enzymes, which are necessary to bioconvert organic nitrates to NO.

2. Stimulated Release of Nitric Oxide. Chemical and physical stimuli release vasodilator factors from the cerebral vascular endothelium. In an isolated, perfused guinea pig brain preparation, ACh exerted two simultaneous and opposite effects, characterized by a slow direct constriction concealed in physiological conditions by a fast vasodilatation mediated through the release of NO from endothelial cells (Librizzi et al., 2000). ACh dilated isolated and pressurized bovine and human intracortical penetrating arterioles, and the effect was blocked by L-NA; the rank order of potency of muscarinic receptor antagonists was 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide $\gg$ pirenzepine $>$ AF-DX 384, suggesting the involvement of the M_5 muscarinic receptor subtype in NO-mediated dilatation in bovine and human cerebral arterioles (Elhusseiny and Hamel, 2000). NO-mediated, ACh-induced vasodilatation in isolated perfused basilar arteries was not altered in heterozygous manganese SOD-deficient mice compared with wild-type controls, but vasoconstriction induced by arginine vasopressin was increased in manganese SOD-deficient mice (Faraci et al., 2006). On the other hand, vasodilator responses to ACh were reduced in pial arterioles in manganese SOD-deficient mice in vivo. In the cerebral microcirculation, there may be superoxide-mediated impairment of the response to ACh.

Acidosis induced dilatation of isolated rat cerebral arterioles, and this effect was inhibited by KCl, glybenclamide, or $BaCl_2$ and also by L-NMMA or L-NA, suggesting that endothelial NO and smooth muscle ATP-sensitive K^+ channels contribute to acidosis-induced cerebroarterial dilatation (Horiuchi et al., 2002). In human brain capillary endothelial cells, ET-1-stimulated Ca^{2+} mobilization was inhibited by NOR-1, an NO donor, and this inhibition was prevented by ODQ or Rp-8-CPT-cyclic guanine monophosphate synthetase, an inhibitor of protein kinase G; NOR-1 reduced Ca^{2+} mobilization induced by the G protein activator mastoparan, inositol trisphosphate, or the Ca^{2+}-ATPase inhibitor thapsigargin (Chen et al., 2003). The functional interrelationship between ET-1 and NO seems to play a role in regulating human cerebral capillary tone and microcirculation.

In isolated rat middle cerebral arteries, the addition of K^+ (20–60 mM) caused dilatation. Although the vasodilator effect of 20 mM K^+ was unaffected by L-NA, NOS inhibition resulted in vasoconstriction at 40 mM K^+ or higher, suggesting a modulator role of NO in the actions of K^+ at 40 mM or higher (Schuh-Hofer et al., 2001). Similar results were also obtained in rat cerebral arteries exposed to high K^+/osmolarity (Golding et al., 2001). Toda (1974, 1976) suggested that relaxations of canine and human cerebral arteries in response to K^+ (0.5–10 mM) are mediated by activation of the electrogenic Na^+ pump. Yamazaki and Kitamura (2001) provided evidence from a patch-clamp study on pieces of arterioles dissected from the rat cerebral pial membrane that BK seems to cause NO to move from the endothelium to smooth muscle, where it inhibits an ET-1-evoked oscil-

latory, Ca^{2+}-activated Cl^- current via the NO-cyclic GMP pathway.

Not only endothelium-derived NO but also vasodilator prostanoids and EDHF are involved in relaxation of cerebral arteries and arterioles in vitro under basal and stimulated conditions. An increase in NO production in endothelial cells through the Akt/protein kinase B-dependent pathway is seen in cerebral microvessels in response to 17β-estradiol.

E. Involvement of Nerve-Derived Nitric Oxide in the Regulation of Cerebral Blood Flow and Vascular Tone

NO liberated from nitrergic nerves under resting conditions contributes to the control of cerebral blood flow, thus playing important roles in the maintenance of physiological brain functions. Activation of parasympathetic nuclei results in increased NO release from postganglionic nitrergic nerves, leading to cerebral vasodilatation and blood flow increase (Toda and Okamura, 2003).

1. In Vivo Studies

a. Basal Release of Nitric Oxide. Intraperitoneal injections of 7-NI, an in vivo inhibitor of the neuronal isoform of NOS, lowered baseline cerebral blood flow in unanesthetized rats (Montécot et al., 1997; Gotoh et al., 2001), cerebral capillary flow in anesthetized rats (Hudetz et al., 1998), and global cerebral blood flow in cats (Hayashi et al., 2002). In anesthetized dogs, unilateral denervation of vasodilator nerves from the pterygopalatine ganglion to the artery constricted middle cerebral and posterior communicating arteries. L-NA produced no or only a slight vasoconstriction in the denervated side, whereas clear vasoconstriction was observed with this inhibitor in the innervated side (Fig. 1) (Toda et al., 2000a). The parasympathetic nitrergic nerves innervating the cerebral vasculature are hypothesized to tonically dilate arteries and arterioles (Okamura et al., 2002; Toda and Okamura, 2003). Acute administration of 7-NI reduced cerebral blood flow in rats to the same extent in both chronic saline- and L-NAME-treated groups, suggesting that residual NOS activity in brain is sufficient to provide tonic, NO-dependent cerebrovascular dilator tone (Kelly et al., 2000). Erythrocyte velocity in rat cerebral capillaries increased, as arterial hematocrit was reduced; pretreatment with 7-NI abolished the increase in velocity, leading to the conclusion that NO from a neural source may contribute to the increase in capillary erythrocyte flow during hemodilution (Hudetz et al., 2000). There was evidence suggesting that NO released from parasympathetic fibers contributes to cerebral vasodilatation during acute hypertension in anesthetized rats (Talman and Nitschke Dragon., 2007). On the other hand, Stefanovic et al. (2007) found that 7-NI had no effect on cerebral blood flow in anesthetized rats.

b. Stimulated Release of Nitric Oxide.

i. Efferent nitrergic nerve stimulation. Electrical stimulation of parasympathetic nerves around the sphenopalatine ganglion increased cortical blood flow in anesthetized rats, which was reduced by treatment with L-NAME (Morita-Tsuzuki et al., 1993). Stimulation of the pterygopalatine ganglion or geniculate ganglion in anesthetized dogs and Japanese monkeys induced vasodilatation of ipsilateral anterior and middle cerebral arteries, and intravenous L-NA abolished and L-arginine restored the response (Toda et al., 2000a,b). Histochemical examination using an axonal transport method has demonstrated that NOS-containing neurons in the sphenopalatine ganglion innervate the middle cerebral artery of rats (Minami et al., 1994). It is hypothesized that NO liberated as a neurotransmitter from nitrergic nerves originating from the superior salivatory nucleus through the geniculate ganglion, greater petrosal nerve, and pterygopalatine ganglion mediates dilatation of cerebral arteries (Toda and Okamura, 2003). According to Faraci and Heistad (1990), large cerebral arteries are important determinants of local microvascular pressures and also contribute to total cerebrovascular resistance.

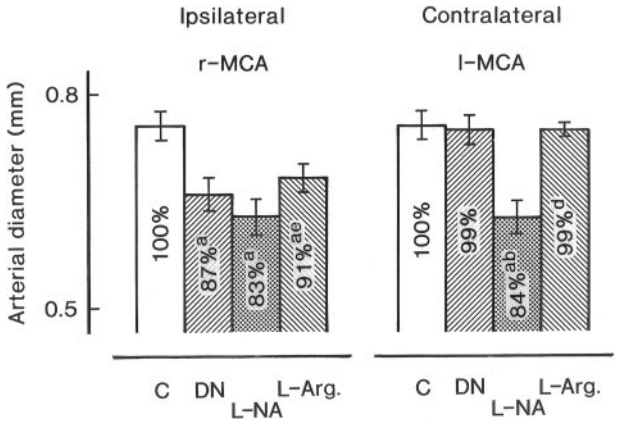

FIG. 1. Effects of L-NA and L-arginine (L-Arg.) on the diameter of denervated (DN, left panels) and innervated (right panels) middle cerebral arteries (MCAs) in anesthetized dogs. MCAs subjected to denervation of the pterygopalatine ganglion (ipsilateral side) showed vasoconstriction by 13% (significantly different from control, C), and treatment with L-NA did not produce additional constriction. On the other hand, contralateral arteries without denervation constricted in response to L-NA by 16% (significantly different from control and ipsilateral DN) and the arterial diameter was restored by injections of L-arginine (15%, significantly different from L-NA). DN in the contralateral, left MCA denotes the diameter measured when the other side (right MCA) was denervated. [Reproduced from Toda N, Ayajiki K, Tanaka T, and Okamura T (2000) Preganglionic and postganglionic neurons responsible for cerebral vasodilation mediated by nitric oxide in anesthetized dogs. *J Cereb Blood Flow Metab* **20:**700–708. Copyright © 2000 Nature Publishing Group. Used with permission.]

ii. Somatosensory stimulation. During vibrissal stimulation, regional blood flow equally increased in cortical barrel fields in wild-type and eNOS-knockout mice, and L-NA inhibited the response in both groups, suggesting that coupling of blood flow and metabolism seems to be nNOS- but not eNOS-dependent (Ayata et al., 1996). Conversely, Ma et al. (1996) noted that vibrissal stimulation increased regional cerebral blood flow in nNOS-knockout mice to an extent similar to that in wild-type mice, and the blood flow response was partially inhibited by L-NA in

wild-type mice but was not affected in nNOS-knockout mice. The authors suggested that in mice deficient in nNOS expression, endothelial NO production does not mediate the blood flow coupling to neuronal activity, and NO-independent mechanisms couple regional cerebral blood flow and metabolism during whisker stimulation. However, the following data support the participation of neurogenic NO in the stimulation-induced increase in cerebral blood flow. Whisker stimulation for 10 min led to a continuous and persistent cortical blood flow increase in anesthetized rats; after nNOS inhibition, the blood flow response was depressed (Bonvento et al., 2000). Regulation of the cerebral blood flow in response to increased neuronal activity seems to be a dynamic and tonic process, in which nNOS plays an essential role. Increases in cerebral blood flow during vibrissal stimulation in unanesthetized rats were reduced by L-NAME in the ventroposteromedial thalamic nucleus and reduced by 7-NI in both the ventroposteromedial thalamic nucleus and barrel cortex (Gotoh et al., 2001). In wild-type mice, upper lip stimulation increased blood flow in Crus II, a region of the cerebellar cortex that receives trigeminal sensory afferents, and the blood flow response was reduced in cyclin D2-null mice that had a profound reduction in the number of stellate neurons in the cerebellar monolayer; 7-NI attenuated the vascular response to Crus II activation in wild-type mice but not in mice lacking cyclin D2 (Yang et al., 2000). Stellate neurons are probably the major source of NO mediating the vascular response. Increased blood flow in the Crus II in response to upper lip stimulation seen in wild-type mice was attenuated in nNOS-null mice; the increase in cerebellar blood flow by superfusion of Crus II with glutamate or by systemic administration of harmaline was also attenuated in nNOS-knockout mice, implying that nNOS-derived NO is a critical link between glutamatergic synaptic activity and blood flow in the activated cerebellum (Yang et al., 2003).

Ayajiki et al. (2005) provided evidence indicating that NO released from parasympathetic nerves and neuropeptide(s) released antidromically from sensory nerves seem to be responsible for the increase in cerebral blood flow in the rat. Electrical somatosensory stimulation in the unilateral forepaw elicited an increase in cerebral blood flow in the cat contralateral somatosensory cortex and the ipsilateral cerebellum, and 7-NI decreased cerebral blood flow both during rest and activation, suggesting that neuronal NO has an important role as a mediator of regional neurovascular coupling (Hayashi et al., 2002). 7-NI also attenuated cerebral blood flow responses to electrical stimulation of the forepaw and decreased the amplitude of somatosensory-evoked potentials (Stefanovic et al., 2007). Electrical forepaw stimulation resulted in an immediate increase in NO in the rat somatosensory cortex followed by cortical blood flow increase (Buerk et al., 2003). Some evidence suggested that an epoxygenase, presumably localized in astrocytes, and NOS are both required for generating the cortical hyperemic response in rats evoked by electrical forepaw stimulation (Peng et al., 2004). It was suggested that the increase in cerebral blood flow in the hippocampus during walking in rats is attributable to activation of nicotinic receptors by ACh released from vasodilator nerves projecting to the hippocampus, resulting in the production of NO (Nakajima et al., 2003).

iii. Hypercapnia and hyperoxia. Administration of 7-NI reduced the vasodilator and cerebral blood flow responses to hypercapnia in anesthetized rats, suggesting that NO synthesized by nNOS participates in hypercapnic hyperemia (Wang et al., 1995; Okamoto et al., 1997). From studies using mice lacking nNOS or eNOS and wild-type mice, Atochin et al. (2003b) obtained evidence suggesting that cerebral vasodilatation induced by 60-min exposure to hyperbaric oxygen depends on both nNOS and eNOS. The cerebral blood flow response as well as blood oxygenation level-dependent signal intensity changes after electrical stimulation were abolished after application of 7-NI in rats (Burke and Bührle, 2006).

iv. Response to N-methyl-D-aspartate. NMDA (Faraci and Breese, 1993) and kainic acid (Faraci et al., 1994) increase cerebral blood flow via neuronally derived NO. Microdialysis perfusion of NMDA doubled blood flow in rat striatum and increased NO production; perfusion of L-NA inhibited the blood flow response to NMDA, and an epoxygenase substrate inhibitor blocked the increase in blood flow without decreasing NO recovery (Bhardwaj et al., 2000). These authors concluded that both the P450 epoxygenase and NOS pathways seem to be involved in the local cerebral blood flow response to NMDA receptor activation. A direct cortical application of NMDA increased regional blood flow and oxygen consumption in rats, and 7-NI attenuated the effects of NMDA on cerebral blood flow and decreased the oxygen consumption during NMDA receptor stimulation (Chi et al., 2003). NMDA induced both NO flux and cerebellum vasodilatation that were abolished by treatment of rats with an NOS inhibitor and tetrodotoxin, suggesting that NO-producing interneurons mediate this vasomotor response (Rancillac et al., 2006). These authors also demonstrated that electrical stimulation of single nitrergic stellate cells was sufficient to release NO and dilate intraparenchymal and upstream pial microvessels. Harris et al. (2008) obtained findings suggesting that NO-dependent cerebral vasodilatation in response to NMDA receptor activation was present as early as 0.65 gestation in fetal sheep and increased further during the last trimester.

c. Involvement of Neurogenic Nitric Oxide in Diseases. There are recorded studies indicating the participation of neurogenic NO in some diseases. The selective nNOS inhibitor ARL 17477 and the nonselective inhibitor L-NA reduced intraischemic cortical cerebral blood flow to a similar extent in rats subjected to forebrain ischemia, suggesting that the nNOS contribution to intraischemic

vasodilatation in vulnerable brain regions is greater than that of eNOS (Santizo et al., 2000). Combined treatment with 7-NI and propranolol but not treatment with 7-NI or propranolol alone was accompanied by a reduction in the hypoglycemia-induced pial arterial dilatation; in the presence of tetrodotoxin, the arterial response was lost in rats (Santizo et al., 2001). β-Adrenoceptors and nNOS-derived NO seem to interact in contributing to hypoglycemia-induced cerebral vasodilatation, and the vasodilating signal may be transmitted via a neuronal pathway. In anesthetized rats, without the nNOS inhibitor propyl-L-arginine, the pial arterial diameter abruptly increased when mean arterial pressure rose, whereas with nNOS inhibition the diameter increased to a much lesser extent even when arterial pressure was similarly raised; the nNOS inhibitor attenuated pial arterial dilatation induced by NMDA but not that induced by ACh delivered into a cranial window (Talman and Nitschke Dragon, 2007). NO released from parasympathetic fibers seems to contribute to cerebral vasodilatation during acute hypertension. Topical application of NMDA or kinate produced pial arteriolar dilatation in alcohol-fed (8–12 weeks) rats to a lesser extent than nonalcohol-fed rats; treatment with L-NAME inhibited dilatation of pial arterioles in response to NMDA, implying that chronic alcohol consumption impairs nNOS-dependent pial arteriolar dilatation (Sun et al., 2002). Impaired reactivity of cerebral blood vessels to neuronal activation may contribute to the pathogenesis of cerebrovascular disorders observed during chronic alcohol consumption. At clinically relevant concentrations, halothane dilated rat intracerebral arterioles (Staunton et al., 2000). This dilatation in hippocampal microvessels was mediated by neurogenic NO but not NO derived from the endothelium.

d. Interactions between Nitric Oxide from Nitrergic Nerves and Other Neurotransmitters. The nicotine-induced increase in the rat cortical blood flow, mediated by nerve-derived NO, was attenuated by propranolol and the β_2-adrenoceptor antagonist ICI 118,551, but not by the β_1-adrenoceptor antagonist metoprolol (Uchida et al., 2002). According to Toda and Okamura (2003), 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 from various mammals.

Increases in rat meningeal blood flow caused by NO has been suggested to depend on the release and vasodilatory action of calcitonin gene-related peptide from dural afferents (Strecker et al., 2002a,b). The interaction of NO and calcitonin gene-related peptide may be relevant for the development of vascular headaches.

e. Histological Demonstration of Nitrergic Innervation in Cerebral Vasculature. Numerous histochemical and immunohistochemical studies have demonstrated the innervation of nitrergic nerves in cerebral arteries and arterioles from a variety of mammals (Toda and Okamura, 2003), including humans (Nozaki et al., 1993; Gorelova et al., 1996) and monkeys (Yoshida et al., 1994; Toda et al., 1997). It was demonstrated on the electron microscopic level that most perivascular NOS neuronal elements corresponded to nerve terminals and a majority of these were located in the immediate vicinity of the blood vessels; NOS terminals abutting on cortical NOS neurons were primarily nonjunctional (Tong and Hamel, 2000). The authors raised the possibility that nitrergic basal forebrain neurons are involved in the blood flow response observed after stimulation of the basal forebrain. Using the fluorescent imaging method, Brown et al. (2000) provided evidence suggesting that tonic activity in perivascular nitrergic nerve fibers lying in close proximity to intraparenchymal microvessels may be a source of dilator tone within the parenchyma. Double-labeling studies revealed that cytochrome P450 2C11 epoxygenase and soluble epoxide hydrolase immunoreactivity predominantly colocalized with nNOS immunoreactivity within perivascular nerves (Iliff et al., 2007). The presence of enzymes involved in production and inactivation of eicosatrienoic acids within extrinsic parasympathetic and sensory vasodilator fibers suggests a novel role for eicosatrienoic acids in the neurogenic control of cerebral arteries.

From studies on Wistar rats of various postnatal days, Morys et al. (2002) found that NOS and neuropeptide Y immunoreactivities in rat hippocampus were weak in the newborn rat. Then the levels increased until the 7th day of postnatal life, and thereafter until day 14 they were maintained at the similar levels; until the 10th day of life, the immunoreactive cells were immature.

f. Summary of the Possible Role of Neuronal Nitric-Oxide Synthase-Derived Nitric Oxide. The hypothesis that NO formed through nNOS contributes to the increase in cerebral circulation is supported by the following observations. 1) Cerebral vasodilatation or increased cerebral blood flow is evoked by efferent nerve stimulation by electrical pulses or ganglionic stimulants such as nicotine. The responses are susceptible to NOS inhibitors and soluble guanylyl cyclase inhibitors, and light and/or electron microscopy reveals that nNOS-immunoreactive nerve fibers are located in close proximity to vascular smooth muscle cells. 2) Cerebral arteriolar dilatation or a blood flow increase induced by somatosensory stimulation is blocked by selective nNOS inhibitors, such as 7-NI, concomitant with a histochemical determination of nNOS-positive nerve fibers in the immediate vicinity of intracerebral arterioles. 3) Cerebral arteriolar dilatation or the blood flow increase induced by NMDA receptor agonists is inhibited by 7-NI, and increased NO levels are also observed in brain tissues. 4) Cerebral vasodilator responses to stimulation of efferent or afferent nerves are reduced in nNOS-, but not in eNOS- and iNOS-deficient mice. Figure 2 summarizes the origins of NO formed by nNOS and eNOS that acts

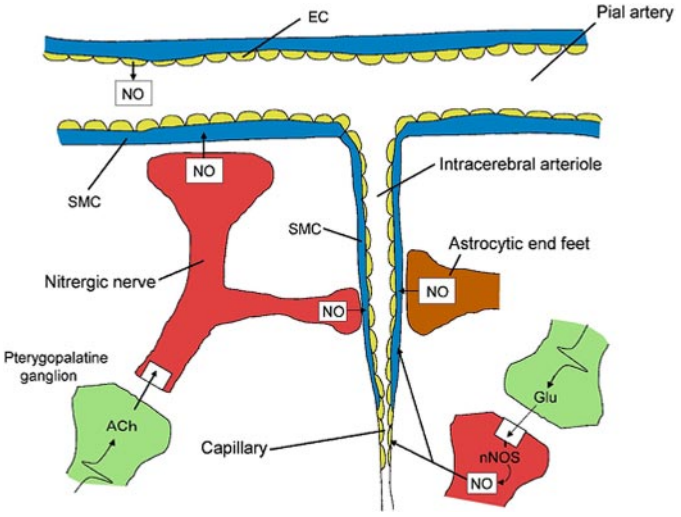

FIG. 2. Possible sites of NO release for the control of cerebral blood flow. Endothelial cells (EC) of pial arteries, intracerebral arterioles, and capillaries liberate NO formed through eNOS and superoxide anions synthesized via NADPH oxidase; PGI_2 and EDHF may also be released from EC. The major sites of releasing NO derived from nNOS are parasympathetic postganglionic (nitrergic) nerves innervating cerebral arteries and arterioles and nNOS-containing neurons innervated by glutamatergic nerves. Astrocytes are hypothesized to be a site of NO generation or act as intermediaries in neurovascular signaling. SMC, vascular smooth muscle cells; Glu, glutamate

on cerebral arterial and arteriolar smooth muscle cells and capillaries.

The cerebrovascular responses would be mediated mainly through NO liberated as a neurotransmitter from nitrergic efferent nerves, even though stimuli are applied on afferent and efferent neurons. Vasodilator responses impaired by in vivo treatment with 7-NI can possibly be elicited by NO formed via nNOS; however, this nNOS is not necessarily the enzyme located in nitrergic nerve fibers. In particular, NMDA-induced, NO-mediated cerebral vasodilatation may be associated with NO formed in nNOS-containing neurons adjacent to parenchymal arterioles and capillaries because released NO is diffusible to neighboring tissues, including vasculatures (Bach-y-Rita, 1993). However, concentrations of this unstable molecule are expected to exponentially decay as it transverses the longer distance to vascular smooth muscle cells compared with the much shorter space of the synaptic cleft; thus, NO efficiency may be less when it is liberated from NO-containing neurons in the brain than from nitrergic nerves. The signaling pathway involved in the cerebral blood flow response to NMDA receptor activation may be more complex than simple NO diffusion from neurons to vascular smooth muscle (Bhardwaj et al., 2000). Recent studies have raised possibilities that astrocytes are one of the sites of NO generation or act as intermediaries in neurovascular signaling.

2. In Vitro Studies

a. Basal Release of Nitric Oxide. 7-NI failed to alter the resting tension in isolated rat middle cerebral arteries (Benyó et al., 1999). The authors suggested that cerebrovascular nNOS is not involved in the resting NO production and that nNOS-derived NO, possibly from neurons and/or glial cells, seems to contribute to the maintenance of the resting cerebral blood flow in vivo.

b. Stimulated Release of Nitric Oxide. Isolated cerebral arteries from various mammals, including the human, dog, monkey, cat, pig, cow, sheep, guinea pig, and rat, showed relaxation in response to nicotine or other nicotinic receptor agonists, and this was abolished by treatment with NOS inhibitors or ganglionic blocking agents. Electrical field stimulation also produced relaxations of these cerebral arteries that were sensitive to NOS inhibitors and tetrodotoxin. The data are summarized in our previous review article (Toda and Okamura, 2003). These findings, together with the histological demonstration of NOS-containing neurons in the immediate vicinity of cerebral arterial smooth muscle, led us to hypothesize that NO liberated from vasodilator nerves plays a pivotal role as a neurotransmitter in eliciting cerebral vasodilatation (Toda and Okamura, 1990a,b; Toda et al., 1990). On the other hand, Elhusseiny and Hamel (2000) did not find any relaxation in bovine intracortical arterioles in response to nicotine.

c. Interactions between Nitrergic Nerve and Other Autonomic Nerves. Histochemical studies with antibodies against tyrosine hydroxylase and cholinesterase/choline acetyltransferase 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 nitrergic neurons and adrenergic or cholinergic neurons.

In isolated sheep middle cerebral arteries, electrical stimulation-evoked norepinephrine release decreased in the presence of L-NAME; the effect of this inhibitor was reversed by the addition of an NO donor, suggesting that NO released from nitrergic nerves augments stimulation-evoked norepinephrine release from adrenergic nerves (Mbaku et al., 2000). In the rat cerebral cortex, NMDA-induced norepinephrine release was inhibited by an NOS inhibitor (Montague et al., 1994). The direct application of NO enhanced K^+-induced norepinephrine release from the rat cerebral cortex and hippocampus (Martire et al., 1998). The opposite results were obtained with the rat hypothalamus, in which SNP inhibited whereas an NOS inhibitor enhanced K^+-induced norepinephrine release (Seilicovich et al., 1995).

Relaxations caused by electrical field stimulation of isolated monkey cerebral arteries, sensitive to NOS inhibitors, were inhibited by ACh; the attenuation was reversed by either atropine or the muscarinic M_2-receptor subtype antagonist AF-DX 116, but not by M_1, M_3, and M_4 receptor antagonists; in the absence of exogenous ACh, atropine potentiated and physostigmine inhibited the response to nitrergic nerve stimulation (Toda et al., 1997). The effects of nitrergic nerve fiber activation on the monkey cerebral artery seem to be inhibited

by nerve-released ACh acting on prejunctional muscarinic receptors, possibly of the M_2 subtype. Voltage-dependent, ω-conotoxin-sensitive Ca^{2+} currents in the cultured rat sphenopalatine ganglion were inhibited by ACh and a muscarinic M_2 receptor agonist; the inhibitory effects were reversed by atropine and a M_2 receptor antagonist but were not affected by M_1, M_3, and M_4 receptor antagonists (Liu et al., 2002b). The sphenopalatine/pterygopalatine ganglia are major sources of nNOS and vasoactive intestinal peptide and send postganglionic nitrergic and peptidergic neurons to middle cerebral arteries (Edvinsson et al., 2001).

F. Astrocyte-Derived Nitric Oxide and Other Vasodilators

Histochemical study demonstrated the subcellular distribution of NADPH-diaphorase (NOS) in the rat striatal astroglia; electron microscopic examination revealed disposition of the NADPH-diaphorase reaction product predominantly in mitochondria of astrocytic "end feet" (Calka and Wolf, 2003). The authors have suggested that astroglial NADPH diaphorase/NOS may be involved in adaptation of the local blood flow in the striatal microenvironment. The gap junction protein connexin-43 and purinergic P_{2Y2} and P_{2Y4} receptors were expressed in end feet abutting capillaries and larger cerebral vessels; ATP mobilized cytosolic Ca^{2+} in astrocytic end feet, whereas electrical stimulation triggered Ca^{2+} waves propagating along the vessel wall (Simard et al., 2003). It was speculated that Ca^{2+} signaling may play a role in astrocytic function related to blood flow regulation and that astrocytes are in a position for sensing neuronal activity and communicating with blood vessels in the brain parenchyma. The dilatation of arterioles triggered by neural activity in rat cortical slices was dependent on glutamate-mediated $[Ca^{2+}]_i$ oscillations in astrocytes, and inhibition of these Ca^{2+} responses resulted in the impairment of stimulated vasodilatation; a COX product was involved in this astrocyte-mediated control of arterioles, suggesting that neuron-to-astrocyte signaling is a key mechanism in functional hyperemia (Zonta et al., 2003). Filosa et al. (2004) obtained data suggesting that astrocytic Ca^{2+} changes signal the cerebral microvasculature. Koehler et al. (2006) suggested that astrocytes act as intermediaries in neurovascular signaling and that neurally derived NO, COX-2 metabolites, epoxyeicosatrienoic acids, and adenosine couple increased cerebral blood flow to increased neuronal activity. The production of PGE_2 or epoxyeicosatrienoic acid, the level of NO, and the activation of Ca^{2+}-activated K^+ channels in astrocytes seem to participate in cerebral vasodilatation (Gordon et al., 2007). More work is needed to clarify the contributions of astrocytes to vascular dynamics.

IV. Cerebral Blood Flow Regulation by Nitric Oxide under Pathological Conditions

A. Cerebral Ischemia and Stroke

1. Ischemia and Endothelial Nitric-Oxide Synthase-Derived Nitric Oxide. In rats that underwent reversible occlusion of the right middle cerebral artery, eNOS protein occurred in all blood vessels and microvessels in the ischemic striatum after 24 to 168 h of reperfusion, and eNOS expression was also demonstrated; levels of brain NOS did not increase after ischemia (Veltkamp et al., 2002). Ischemia associated with occlusion of the bilateral common carotid arteries induced increases in NO end-products (NOx) during ischemia and prominently after reperfusion, and such increases were abolished by L-NAME but only slightly reduced by 7-NI; the increase in the quantity of NO end-products became prominent and was persistent after reperfusion in rats in which the cerebral blood flow during ischemia was in the range between 22.7 and 60 ml/100 g/min, whereas in animals in which the cerebral blood flow during ischemia fell below 22.7 ml/100 g/min, the end-products decreased during ischemia increased transiently after reperfusion. (Uetsuka et al., 2002). The increase in NO seems to be derived from eNOS, and NO synthesis during and after ischemia may be related to cerebral blood flow. In anesthetized goats subjected to a 60-min occlusion of the left middle cerebral artery followed by a 60-min reperfusion, ipsilateral middle cerebral artery resistance was decreased, and the cerebral vasodilatation to ADP but not to SNP was reduced; the relaxation to ADP but not to SNP was less in isolated left (ischemic) middle cerebral arteries than in right (control) ones (Sánchez et al., 2006). These authors obtained further evidence suggesting that ischemia-reperfusion produces cerebrovascular endothelial dysfunction, which may be associated with decreased NO availability, decreased release of EDHF (Chen et al., 1988), and increased production of vasoconstrictor prostaglandins and that these alterations may be related with increased production of superoxide anions. In wild-type mouse brains subjected to middle cerebral artery occlusion, endothelial caveolin-1 and caveolin-2 and eNOS protein levels were increased; conversely, the protein levels of eNOS remained unchanged and the cerebral volume of infarction was markedly increased in caveolin-1-knockout, ischemic mouse brains, suggesting that caveolin-1-deficient, ischemic brains show impaired angiogenesis and increased apoptotic cell death (Jasmin et al., 2007). Adiponectin-deficient mice exhibited enlarged brain infarction and increased neurological deficits after ischemia-reperfusion and also decreased cerebral blood flow during ischemia compared with wild-type mice; phosphorylation of eNOS in ischemic brain tissues and the production of NO metabolites in plasma were attenuated in adiponectin-deficient mice. Conversely, adenovirus-mediated supplementation of adiponectin reduced cere-

bral infarct size in wild-type and adiponectin-knockout mice but did not affect brain infarction in mice deficient in eNOS (Nishimura et al., 2008). Adiponectin seems to exert a cerebroprotective action through an eNOS-dependent mechanism.

In three groups of rats subjected to chronic brain hypoperfusion and treated with 7-NI, aminoguanidine, or iminoethyl-L-ornithine (relatively selective inhibitors of nNOS, iNOS, and eNOS, respectively), only rats administered the eNOS inhibitor showed a worsened water maze performance, suggesting that vascular NO derived from eNOS may play a critical role in spatial memory function during brain hypoperfusion by keeping cerebral blood flow optimal (de la Torre and Aliev, 2005). NOx levels in plasma, ischemic brain tissue, and cerebrospinal fluid increased after transient middle cerebral artery occlusion in rats. ADMA levels were unchanged in plasma, but decreased in cerebrospinal fluid after the occlusion; the ADMA/NOx ratio in cerebrospinal fluid decreased after ischemia (Xu et al., 2007). The combined increase in NOx and decrease in ADMA suggests occurrence of marked activation of NOS. There is evidence indicating that RhoA/Rho-kinase activity is increased in cerebral vascular diseases, such as ischemic brain injury, cerebral vasospasm, hypertension, and diabetes, not only in vascular muscle but also in the endothelium that results in decreases in NO production and increases in cerebral vascular tone (Didion et al., 2005; Chrissobolis and Sobey, 2006). Sercombe et al. (2001) provided evidence indicating that in the SHR with focal cerebral ischemia, cerebral infarct expansion may be limited by chronic treatment with L-NAME through a progressive reduction in cerebral artery response to vasoconstrictor neurotransmitters, concomitant with augmented responses to the guanylyl cyclase-independent vasodilator papaverine and some recovery of NO-mediated dilatation to ACh.

Intrauterine ischemia-hypoxia insult applied by uterine artery occlusion on day 21 of pregnancy caused increases in both activities and mRNA levels of eNOS, whereas there were no changes in nNOS mRNA levels, suggesting that eNOS may contribute to the maintenance of cerebral blood flow against ischemia or hypoxia in fetal rats (Nakata et al., 2002). Brachiocephalic occlusion increased nNOS (hippocampus and brainstem), iNOS (hypothalamus), and eNOS (hippocampus and cortex) at the protein level in fetal sheep brain, estradiol increased nNOS (brainstem and cortex), iNOS (hippocampus and hypothalamus), and eNOS (brainstem and cerebellum), and the combination of occlusion and estradiol produced smaller changes in NOS isomers than those produced by either stimulus (Wood and Giroux, 2006).

Voluntary training on running wheels or exercise on treadmill apparatus reduced cerebral infarct size and functional deficits, improved endothelium-dependent vasodilatation, and augmented cerebral blood flow in wild-type mice, whereas the neuroprotective effects were absent in eNOS-deficient mice, indicating that the enhanced eNOS activity by physical training seems to be the mechanism by which this modality protects against cerebral injury (Endres et al., 2003). Continuous voluntary running on wheels conferred long-term up-regulation of eNOS in the vasculature and of endothelial progenitor cells in the spleen and bone marrow: 4 weeks after the ischemic insult, trained animals showed higher numbers of newly generated vascular cells, increased density of perfused microvessels, and sustained augmentation of cerebral blood flow within the ischemic striatum; the protective effects of physical training were abolished in animals treated with NOS inhibitors or in those lacking eNOS expression (Gertz et al., 2006).

On the other hand, there is evidence for neuroprotection by NOS inhibition against ischemic injury. The novel calmodulin-dependent NOS inhibitor DY-9760e, which inhibits eNOS activity and, in turn, protein tyrosine nitration, inhibited blood-brain barrier disruption induced by microsphere embolism and also inhibited cleavage of poly(ADP-ribose) polymerase 1 (PARP-1) as a marker of the apoptotic pathway in vascular endothelial cells in rats (Han et al., 2006). Microsphere embolism-induced eNOS expression in vascular endothelial cells probably mediates blood-brain barrier disruption and, in turn, brain edema.

Urotensin-II (U-II) is a vasoactive factor with pleiotropic effects that is identified in mammalian species including humans (Bousette and Giaid, 2006). U-II and its receptor are expressed as mRNA and protein in rat neuromicrovascular endothelial cells, and U-II exerts a pro-angiogenic effect (Spinazzi et al., 2006). Administration of U-II into the lateral cerebral ventricle increased cerebral blood flow in anesthetized rats, and this effect was reduced by NOS inhibition. U-II administration after the induction of cerebral ischemia failed to alter residual cerebral blood flow in the affected hemisphere, but after reperfusion, U-II-treated rats displayed a hyperperfusion; treatment with U-II increased the volume of infarction, suggesting an exacerbation by U-II of brain damage associated with an ischemic insult (Chuquet et al., 2008).

In summary, ischemia with reversible occlusion of the cerebral artery causes increments in endothelial caveolin and eNOS protein levels, plasma NOx concentrations, and cerebral blood flow that seem to play a role in minimizing impaired memory function. Physical training protects against cerebral injury after ischemic insult due possibly to enhanced eNOS activity and NO formation. The increased RhoA/Rho-kinase activity in ischemic brain injury decreases NO production.

2. Ischemia and Neuronal Nitric-Oxide Synthase. Female mice lacking the nNOS gene exhibited exacerbated histological injury after middle cerebral artery occlusion relative to that in wild-type females, unlike the protection observed in male nNOS-knockout mice, and treatment with 7-NI increased infarction in female wild-type mice,

but protected male mice. Unlike male PARP-1 knockout mice, female PARP-1 knockout littermates sustained grossly increased ischemic damage relative to that in sex-matched wild-type mice, and loss of PARP-1 resulted in reversal of the neuroprotective activity by the female sex steroid (McCullough et al., 2005). Cell death pathways involving NO and PARP ischemic neurotoxicity may be operant solely in male brain, and the integrity of nNOS/PARP-1 signaling is paradoxically protective in the female. Although deletion of HO-2 resulted in greater stroke damage (Doré et al., 2000), the pharmacological inhibition of nNOS or its gene deletion conferred neuroprotection in mice with transient cerebral ischemia (Eliasson et al., 1999; Wei et al., 1999). In double knockout (HO-2 and nNOS) mice lacking both genes, no difference was observed in the volume of infarction, neurological signs, and decrease in relative cerebral blood flow during ischemia associated with middle cerebral artery occlusion compared with wild-type mice, suggesting that the deleterious action of nNOS would counteract the role of HO-2 in neuroprotection (Namiranian et al., 2005).

3. Ischemia and Inducible Nitric-Oxide Synthase. Nitrotyrosine, a marker of peroxynitrite, was detected after a 15-h reperfusion only in wild-type mice, in which the left middle cerebral artery was occluded for 2 h, but not in iNOS-knockout or sham-operated mice; nitrotyrosine was seen predominantly in the vascular wall in the peri-infarct region of the cerebral cortex in wild-type mice (Hirabayashi et al., 2000). It seems that iNOS is responsible for nitrotyrosine formation in the later phase of reperfusion and that vascular endothelium is the major site of the reaction. The expression of iNOS was detected at 22 to 70 h after reperfusion in the vascular wall and cortex of rats subjected to transient middle cerebral artery occlusion, and nitrotyrosine in the vascular wall and cortex and NOx levels in the circulation increased 4 to 46 h after reperfusion, suggesting that marked increases in the NOx level might reflect the expression of iNOS (Suzuki et al., 2002). Hyperglycemia induced before ischemia in rats resulted in earlier infarction, which correlated with increased immunoreactivity for iNOS, manganese SOD, and nitrotyrosine (Ste-Marie et al., 2001). Based on the observed time courses of the occurrence of ischemic infarction, oxidative stress, iNOS expression, and neutrophil infiltration, Lerouet et al. (2002) concluded that the delayed iNOS activity and neutrophil infiltration may not contribute to ischemic brain damage in rats. Prüss et al. (2008) noted that with the use of three different strains of iNOS-deficient mice and wild-type controls, infarct size was independent of iNOS deletion and thus suggested that iNOS is not a universal mediator of brain damage after cerebral ischemia.

4. Ischemic Preconditioning. Huang (2004) summarized the effects of NO after cerebral ischemia, the signaling pathways through which NO acts, and its potential roles in cerebral ischemic preconditioning (IPC).

Wild-type mice subjected to IPC showed decreases in the neurological deficit and infarct size induced by ischemic insult, whereas neither eNOS nor nNOS knockout mice showed protection from IPC; blood flow decreases in core ischemic areas were the same in all groups, suggesting that NO formed by eNOS or nNOS may play a role in the molecular mechanisms of protection (Atochin et al., 2003a). The early and late phases of local IPC and the late phase of remote IPC were found to have endothelium-protecting actions evidenced as improvements in the recovery of cerebral blood flow in the post-ischemic period in preconditioned rats, with lower levels of endothelial desquamation and cerebral edema (Vlasov et al., 2005). Preconditioning with lipopolysaccharide (LPS), as a preconditioning stimulus, prevented ischemic cell death by reducing lesion size, and reduction of local cerebral blood flow in the peri-infarct area was inhibited in the LPS group, which was correlated with eNOS expression (Furuya et al., 2005). Preservation of local cerebral blood flow, which is relevant to sustained up-regulation of eNOS, may be one factor exerting an inhibitory effect against infarct evolution in the LPS-induced tolerant state.

On the other hand, infarct volume in the brain was reduced 24 h after IPC, treatment with aminoguanidine abolished the IPC-induced protection, and IPC failed to induce ischemic tolerance in iNOS-null mice. IPC increased the resistance to Ca^{2+}-mediated depolarization in isolated brain mitochondria in wild-type mice, whereas in iNOS-null mice, IPC failed to induce such resistance (Cho et al., 2005). NO derived from iNOS may be beneficial by promoting ischemic tolerance through signaling, resulting in mitochondrial protection. Administration of LPS as a preconditioning stimulus, 24 h before ischemic insult reduced the infarct volume and improved ischemic cerebral blood flow, and these effects were not observed in mice lacking iNOS or the NOX-2 subunit of the superoxide-producing enzyme NAD(P)H oxidase, suggesting a mediation by peroxynitrite formed from iNOS-derived NO and NOX-2-derived superoxide in neuroprotection and vasoprotection against ischemic insult (Kunz et al., 2007).

In short, neurological deficit and infarct size induced by ischemic insult are decreased by ischemic preconditioning through increased NO production by either cNOS or iNOS.

5. Studies on Patients with Stroke. In patients with ischemic stroke, differences in the mean middle cerebral arterial velocity between rest (prestimulation) and L-arginine stimulation were lower in the ischemic hemispheres compared with the healthy ones, but did not differ between healthy hemispheres in healthy and ischemic groups, suggesting that cerebrovascular reactivity to L-arginine is impaired in patients with recent stroke (Zvan et al., 2002). The mean middle cerebral arterial

velocity increase during L-arginine infusion was impaired in asymptomatic patients with cardiovascular risk factors, compared with the healthy control subjects; however, patients with lacunar infarctions did not show any additional impairment of cerebral endothelial function (Pretnar-Oblak et al., 2006b). A negative correlation was established between the initial levels of blood NO and the erythrocyte aggregability index, as well as between the blood NO_2 and NO initial levels and the ischemic lesion size (Beridze et al., 2004). Endothelium-derived NO seems to have a positive impact on restoration of cerebral blood flow in the initial stage of acute brain ischemia.

Patients with a history of stroke or transient ischemic attack had higher cerebral blood flow velocity responses to L-arginine than patients with cardiovascular risk factors but no previous cerebrovascular event; there was a significant association of enhanced L-arginine reactivity with previous stroke/transient ischemic attack and elevated fibrinogen levels but not with age, intima-media thickness, hypertension, cholesterol, or other risk factors (Zimmermann et al., 2004). The authors concluded that enhanced L-arginine reactivity is a potential marker for cerebral endothelial dysfunction. Taffi et al. (2008) noted that plasma levels of peroxynitrite were higher in patients with ischemic stroke, whereas plasma NO levels were higher in control subjects; peroxynitrite levels were higher among patients with nonlacunar stroke, whereas NO levels were higher among lacunar stroke patients. Changes in NO metabolism were suggested to be markers of brain injury in patients with ischemic stroke.

6. Therapeutic Measures for Cerebral Ischemic Injury

a. L-Arginine and Nitric Oxide Donors. L-Arginine was effective in reducing the areas of necrosis in rats with ischemic stroke (Hong and Hwang, 2000). In the rabbit cerebral ischemia-reperfusion model, intracarotid administration of the NO donor proline NO decreased free radical levels, and in the rat ischemia-reperfusion model, proline NO reduced the brain infarction volume. In both experimental groups, proline NO did not affect regional cerebral blood flow (Pluta et al., 2001). These beneficial effects of the NO donor may be associated with ROS scavenging by NO. Administration of the NO donor 3-morpholinosydnonimine (SIN-1) reduced infarction volume in normoglycemic rats and, to a lesser extent, in hyperglycemic rats (Coert et al., 2002). The same authors (Coert et al., 2003) noted that 7-NI also reduced infarction volume that was dependent on serum glucose levels, as did SIN-1. The authors hypothesized that the NO donor and nNOS inhibitor have varying efficiencies, depending on the intracellular pH under cerebral ischemia and different serum glucose levels. However, the dynamic changes of NO in decreasing cellular pH may be too speculative, and a more valid rationale for the discrepant findings would be required. It may be plausible that the beneficial effect of SIN-1 is due to improvement of cerebral blood flow, and the neuroprotective effect of 7-NI is associated with decreased production of neurogenic NO that participates in ischemic brain injury. The ischemia-induced increase in NO production may enhance intracellular synthesis of more neurotoxic radicals such as peroxynitrite.

Sodium nitrite reduced infarction volume and enhanced local cerebral blood flow and functional recovery in ischemic rats, and the effects were abolished by carboxy-2-phenyl-4,4,5,5-tetrametyl-imidazoline-okyl 3-oxide (Jung et al., 2006). An NO donor reduced neuropathologic injury resulting from hypoxia-ischemia in the postnatal day 7 rat. NO levels decreased in both ischemic and contralateral hemispheres during hypoxia-ischemia, and this response was prevented by treatment with an NO donor. After prolonged hypoxia-ischemia, cerebral blood flow remained decreased in the ischemic hemisphere, and this effect was prevented by an NO donor (Wainwright et al., 2007). The neuroprotective effects of the NO donor may be due to an increase in the rate of cerebral blood flow recovery. On the basis of the literature reported so far, Willmot et al. (2005b) proposed that NO donors and L-arginine reduce stroke lesion in permanent and transient models of ischemia and that this may be mediated, in part, by increased cerebral perfusion in permanent models of ischemia. Administration of *S*-nitrosoglutathione after the onset of focal cerebral ischemia in rats reduced infarct volume, improved cerebral blood flow, and reduced the expression of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and iNOS; the number of apoptotic cells and the activity of caspase-3 were also decreased, suggesting that this compound protects the brain against ischemia-reperfusion injury by modulating the NO system, resulting in a reduction in inflammation and neuronal cell death (Khan et al., 2005)

b. Nitric Oxide Modulators. Administration of L-kinurenine (precursor of kinurenic acid, known as an endogenous antagonist of the excitatory amino acid receptors) increased corticocerebral blood flow in conscious rabbits, and this effect was pronounced in rabbits with carotid occlusion; atropine or L-NAME prevented L-kinurenine-induced increase in the normal and the ischemic cerebral blood flow, suggesting that the effect of L-kinurenine was mediated through activation of the cholinergic and nitrergic pathways (Sas et al., 2003). Several modalities, including HMG-CoA reductase inhibitors (statins), steroid hormones, nutrients, and physical activity, that up-regulate eNOS expression and/or activity have been identified to lead to enhanced cerebral blood flow and protection from ischemic stroke (Endres et al., 2004). Bolus infusion of L-3,5,3′-triiodothyronine to mice undergoing transient focal cerebral ischemia increased Akt activity in the brain, reduced cerebral infarct volume, and improved neurological deficit scores; these effects were attenuated or absent in eNOS-knockout and thyroid hormone receptor ($TR_{\alpha 1, \beta}$)-knockout mice and were abolished in wild-type mice

treated with a triiodothyronine antagonist, indicating that the activation of Akt and eNOS seems to contribute to the vasodilatory and neuroprotective effects of thyroid hormone (Hiroi et al., 2006). Rho-kinase inhibitors reduced the area of ischemic cortex in wild-type mice but not in eNOS-knockout mice (Shin et al., 2007). The effect of Rho-kinase inhibitors on cerebral blood flow in the ischemic cortex is probably endothelium-dependent, and Rho-kinase may negatively regulate eNOS activity in ischemic brain.

c. Nitric-Oxide Synthase Inhibitors. In contrast to the neuroprotective effects of NO donors, there are reports in the literature suggesting that NO synthesis inhibition is beneficial in reducing functional and histological impairments induced by ischemic insult. Pretreatment of newborn pigs with L-NAME induced severe hypotension and reduced cerebral microcirculation during hypoxia, and NO synthesis inhibition during reoxygenation blunted the increase in NO concentration without the undesirable side effect of reducing cerebral blood flow, suggesting a beneficial effect of L-NAME in the reoxygenation phase (Kutzsche et al., 2002). Selective nNOS and iNOS inhibitors may be candidate treatments for acute ischemic stroke (Willmot et al., 2005a). Aminoguanidine attenuated heatstroke-induced hyperthermia, arterial hypotension, intracranial hypertension, cerebral ischemia, and neuronal damage and inhibited the increase in iNOS-dependent NO formation in the brain. iNOS inhibition also attenuated the increase in hypothalamic ischemia and damage markers associated with heatstroke (Chang et al., 2004).

d. Drugs Possessing Anti-Inflammatory Effects. N-Acetylcysteine, a precursor of glutathione and a potent antioxidant, administered after ischemic events in experimental stroke in rats decreased the infarct area and infarct volume, improved neurological scores and glutathione levels, and reduced the expression of proinflammatory cytokines and apoptotic cell death in ischemic brain, suggesting that administration of *N*-acetylcysteine even after ischemia onset may protect the brain from free radical injury, apoptosis, and inflammation (Khan et al., 2004). Administration of the peroxisome proliferator-activated receptor-γ activators troglitazone or pioglitazone before and at the time of cerebral infarction after middle cerebral occlusion in rats reduced infarction volume, improved neurological function, and reduced inflammation, as evidenced by decreased protein and mRNA for interleukin-1β, COX-2, and iNOS (Sundararajan et al., 2005). The beneficial effects of these drugs are probably due to reduced expression of inflammatory mediators, which exacerbate ischemic injury after stroke. In anesthetized rats, the heatstroke-induced arterial hypotension, cerebral ischemia, and hypoxia and increased levels of iNOS-dependent NO in the striatum were reduced by pretreatment with human umbilical cord blood cells but not with human peripheral mononuclear cells (Chen et al., 2006). Khan et al. (2007) provided evidence suggesting that caffeic acid phenethyl ester is a promising drug candidate for ischemic stroke treatment owing to its inhibition of oxidative stress and inflammation.

e. Miscellaneous. Reperfusion after forebrain ischemia increased the phosphorylation level of extracellular signal-regulated kinase (ERK) 2 in the gerbil hippocampus, and administration of a specific inhibitor of MEK (mitogen-activated protein kinase/ERK kinase) reduced infarct volume after focal ischemia in mice, indicating that MEK inhibition may protect the brain against reperfusion injury (Namura et al., 2001). In addition, the MEK inhibitor protected mouse primary cultured cortical neurons against oxygen deprivation as well as NO toxicity. There has been evidence for an involvement of the MEK/ERK pathway in oxidative injury and NO-induced apoptosis (Bhat and Zhang, 1999; Ishikawa et al., 2000; Satoh et al., 2000). Intravenous hexasulfobutylated C60, a free radical scavenger, reduced the total volume of infarction and increased the NO content in the plasma of rats subjected to focal cerebral ischemia by clipping the common carotid artery, suggesting that the beneficial effect may be related to its antioxidant property and to the up-regulation of NO production (Huang et al., 2001). The Rho-kinase inhibitor hydroxyfasudil increased eNOS mRNA and protein expression in human vascular endothelial cells that correlated with an increase in eNOS activity and NO production. Fasudil increased cerebral blood flow to both ischemic and nonischemic brain areas and reduced cerebral infarct size in mice; however, the neuroprotective effects were absent in eNOS knockout mice (Rikitake et al., 2005). Collagen-stimulated expression of the platelet activation marker P-selectin was increased in OVX rats after reperfusion in rats subjected to forebrain ischemia, and this effect was reversed by estrogen treatment. No differences were detected in platelet eNOS expression between intact, OVX, and OVX plus estrogen-treated rats or in cerebral blood flow between OVX and OVX plus estrogen-treated rats (Littleton-Kearney et al., 2005).

B. Cerebral Vasospasm after Subarachnoid Hemorrhage

1. Studies on Experimental Animals. In anesthetized dogs treated with phentolamine, intracisternal applications of oxyHb and L-NA reduced the basilar artery diameter by about 50% (Toda et al., 1991) and 35% (Toda et al., 1993b), respectively. In Japanese monkeys, the basilar artery diameter was decreased by 28 and 49% after cisternal injections of L-NA (Okamura et al., 1995) and oxyHb (Toda et al., 1991), respectively. OxyHb, possibly produced from blood clots via erythrocyte lysis in animals subjected to SAH, is a strong scavenger of NO (Martin et al., 1985). Cerebral vasospasm after SAH would therefore be associated with impaired bioavailability of NO derived from the nitrergic nerve and endothelium and also with non-NO factors such as vasocon-

strictor COX products, ROS, ET-1, and RhoA/Rho-kinase (Toda and Okamura, 2003).

From studies on rats receiving intravenous L-NAME before or 15, 30, or 60 min after SAH, Schwartz et al. (2000) obtained data suggesting that NO-mediated cerebral vasodilatation is limited during the first 30 min of SAH and is restored by 60 min after SAH. Cortical arterioles isolated from SAH rats demonstrated attenuated dilatation to the endothelium-dependent vasodilator adenosine; eNOS protein concentration was decreased, but eNOS mRNA was increased after SAH, indicating that acute microvascular endothelial dysfunction may occur after SAH and contribute to microvascular vasospasm (Park et al., 2001). During the acute stage after SAH, the lower limit of cerebral blood flow autoregulation shifted to the higher arterial pressure in association with suppressed vasodilatation in response to acute hypotension, which was accompanied by increased expression of eNOS mRNA and increased production of superoxide anions in rat cerebral vessels. In addition, the increased superoxide production was further enhanced under pretreatment with L-NAME, and L-arginine administration restored hemodynamic alterations and reduced superoxide levels; rats that received polyethylene glycol superoxide dismutase and catalase showed recovery of impaired autoregulatory vasodilatation (Cho et al., 2003). Endogenous NO seems to play an important role in the preservation of cerebral blood flow autoregulation during the acute stage after SAH via its capability to scavenge superoxide anions. Cerebrospinal fluid levels of ADMA correlated directly with the degree of delayed vasospasm after SAH in cynomolgus monkeys, and levels of NO_2 and NO_3 as well as those of L-citrulline in cerebrospinal fluids were decreased in animals with vasospasm, suggesting that endogenous inhibition of NOS by ADMA may be involved in the development of delayed cerebral vasospasm (Jung et al., 2004).

Despite the narrowing of arterial diameter and reduced expression of eNOS, expressions of phosphorylated Akt and phosphorylated eNOS were increased in spastic basilar arteries of rabbits subjected to SAH; gene transfer of human erythropoietin reversed the cerebral vasospasm, augmented endothelium-dependent relaxations to ACh, and further increased the expression of phosphorylated Akt and eNOS (Santhanam et al., 2005). Phosphorylation of Akt/eNOS may contribute to the protective effect of erythropoietin against cerebral vasospasm induced by SAH (Santhanam and Katusic, 2006).

Takeuchi et al. (2006) provided evidence that the fall in cerebral blood flow after SAH was largely due to the release of vasoactive factors by clotting blood rather than the scavenging of NO by hemoglobin in rats and that 20-hydroxyeicosatetraenoic acid contributed to the vasoconstrictor response of cerebral vessels to both hemoglobin and blood. Immunohistochemical expression of eNOS was comparable in control rats and those sacrificed on day 3 and day 5 after SAH; the soluble guanylyl cyclase α- and β-subunits were diminished on day 3, but recovered by day 5; the relaxation of basilar arteries to ACh and 8-bromo-cyclic GMP was virtually identical in controls and during cerebral vasospasm (Vatter et al., 2007b). Relaxations dependent on the endothelium, NO, and cyclic GMP seem to persist during cerebral vasospasm in rats. There was evidence for a role of oxyHb in impaired autoregulation (i.e., enhanced myogenic tone) in small cerebral arteries during SAH in rabbits (Ishiguro et al., 2002).

There are findings suggesting that endothelial and nonendothelial NO counteract the contractile response of cerebral arteries to ET-1. Furthermore, although SAH does not modify the effect of nonendothelial NO, the absence of endothelial NO after SAH may contribute to the hyperreactivity of cerebral arteries to ET-1 and, thereby, to the development of cerebral vasospasm (Alabadí et al., 1997). Regulatory features of NO and ET-1 and the possibility of their relationship to SAH are reviewed in a brief report (Thomas, 1997). In basilar artery rings obtained from rats with SAH, relaxation to the selective ET_B receptor agonist sarafotoxin 6c was reduced compared with that in artery rings from sham-operated rats, whereas endothelium-dependent ACh-induced relaxation was not altered. Immunoreactivity for the ET_B receptor was observed exclusively in the endothelium, suggesting that the loss of the ET_B receptor-mediated relaxation after SAH is independent of endothelial NO (Konczalla et al., 2006; Vatter et al., 2007a).

2. Therapeutic Measures in Animals. In anesthetized rats, the NO donor *S*-nitrosoglutathione reversed acute vasoconstriction and prevented ischemic brain injury after SAH (Sehba et al., 1999). The authors implied that acute vasoconstriction contributes to SAH-induced ischemic brain injury and this is mediated by decreased availability of NO. In a rabbit SAH model, Göksel et al. (2001) provided results supporting the contribution of the "NO shortage" concept in the pathogenesis of cerebral vasospasm. Overconsumption of L-arginine during the post-SAH period may cause this shortage; therefore, L-arginine supplementation may be useful for the prophylaxis and treatment of cerebral vasospasms. SAH induced an immediate and persistent decrease in regional cerebral blood flow in rats, decreased serum NO_2/NO_3 levels, increased plasma ET-1 levels, and damage of neurons in the hippocampus CA1 region. Treatment with intraperitoneal L-arginine alleviated these pathological alterations (Sun et al., 2003b). L-Arginine seems to increase cerebral blood flow by enhancing NO levels and decreasing ET-1 levels in blood, resulting in a protective effect on cerebral ischemic injury after SAH. Cerebral blood flow measurements in rabbits revealed resolution of vasospasm after SAH with short-term intracisternal and intracarotid L-arginine infusion (Özüm et al., 2007). On the other hand, in cynomolgus monkeys, brief intracarotid and continuous intravenous infusion of L-arginine did not influence the incidence or degree of

cerebral vasospasm after SAH (Pluta et al., 2000). Whether the discrepancy in effectiveness of L-arginine is due to differences in animal species, route of drug administration, or dosage has not been determined. If an increase in cerebrospinal fluid ADMA levels is one of pathogenic factors provoking vasospasm after SAH, then increased supply of the NOS substrate, L-arginine, would be expected to minimize the action of this endogenous NOS inhibitor.

The endogenous NOS inhibitor ADMA in cerebrospinal fluids was increased by the presence of bilirubin-oxidized fragments, and disappearance of ADMA-hydrolyzing enzyme immunoreactivity in cerebral arteries in spasm decreased ADMA elimination; cerebrospinal fluid ADMA levels were associated with the degree and times of vasospasm (Pluta, 2005, 2006). Therefore, stimulation of ADMA-hydrolyzing enzymes and/or exogenous delivery of NO were suggested as therapeutic modalities to prevent and treat SAH.

In copper/zinc SOD-1 transgenic rats, copper/zinc SOD-1 activity increased in association with the increase in copper/zinc SOD-1 mRNA and protein expression in cerebral vasculature of both sham-operated and SAH animals. The SAH-induced increase in superoxide anions was suppressed with increased NO production, and blunted cerebral vasodilatation and impaired autoregulation after SAH were restored, providing a rational basis for application of copper/zinc SOD gene therapy (Shin et al., 2003).

These findings, together with those seen in SAH animals treated with PDE-5 inhibitors (refer to section V.D), lead to the hypothesis that NO has a potential to prevent cerebral vasospasm and ischemic brain injury after SAH through mechanisms including cerebral blood flow increase, platelet aggregation inhibition, superoxide scavenging, antagonism against the production of ET-1 (Ahlborg and Lundberg, 1997), and inhibition of the Rho-kinase activity (Chitaley and Webb, 2002) or inhibition of the Rho-kinase mediated vasoconstriction (Mills et al., 2001).

3. Studies on Human Materials and Patients. In tissues from SAH patients, decreased extracellular concentrations of NOx in cerebral white matter of the vascular territory have been demonstrated. Furthermore, overall NOx correlated intraindividually with energy- or damage-related compounds, glucose, lactate, and glutamate (Sakowitz et al., 2001, 2002). These alterations may be related to reduced NO availability because of the hypothetical NO scavenging by products of hemolysis. Woszczyk et al. (2003) noted that patients with cerebral vasospasm after SAH showed higher levels of NOx in cerebrospinal fluids than patients with a uncomplicated follow-up between day 2 and 8; the increase in NOx correlated with the flow velocities in transcranial Doppler measurement. Raabe et al. (2002) found that in 6 of 13 patients with severe, medically refractory vasospasm, intracerebroventricular SNP improved cerebral oxygenation and blood flow and suggested that NO may be more effective when initiated early and administered continuously. Even though transdermal nitroglycerin reduced blood pressure, lowered intracarotid transcranial Doppler velocities, and increased the Lindegaad ratio, higher cerebral blood flow was measured in the nitroglycerin group (Reinert et al., 2004). The authors suggested that SAH therapy with nitroglycerin might be an effective treatment with minimal risk of increasing the incidence of delayed ischemic neurological deficits; however, the exact timing of onset, duration, and reduction of nitroglycerin administration with respect to the appearance of vasospasm may have a strong impact on the success of such a therapy. According to Keyrouz and Diringer (2007), NO donors, HMG-CoA reductase, ET-1 antagonists, and magnesium sulfate are the most promising for SAH therapy. Starke et al. (2008) provided evidence that patients with the T allele of the eNOS gene are more likely to have severe cerebral vasospasm after aneurysmal SAH.

Impaired bioavailability of NO and vasoconstrictor factors such as prostanoids, ROS, ET-1, ADMA, and RhoA/Rho-kinase are involved in the genesis of cerebral vasospasm after SAH. Reduced ability of NO to interfere with the synthesis of ET-1 and RhoA/Rho-kinase and the actions of ROS and ADMA may contribute to promotion of the vasospastic response.

C. Traumatic Head Injury

1. Role of Constitutive Nitric-Oxide Synthase-Derived Nitric Oxide. In a closed-head injury model, female rats showed a less dramatic reduction in and better recovery of cerebral blood flow than males; postinjury cerebral blood flow was higher in female and male rats given 17β-estradiol injections that did not alter mean blood pressure (Roof and Hall, 2000). The authors hypothesized, based on known effects of estrogen, that the beneficial microvascular effects of estrogen are most likely a combination of eNOS induction and an antioxidant effect. Sustained severe head injury resulted in a uniform decline in the tissue concentrations of NO metabolites in rat cortex, hippocampus, cerebellum, and brainstem that occurred in the first 5 min after impact trauma, and there seemed to be a relationship between degree of decline in NO levels and amount of trauma (Tuzgen et al., 2003). This decline may be linked to and may even cause the global decrease in cerebral blood flow that occurs in the initial stages of traumatic brain injury. In the contused brain tissue at the impact site after injury, the eNOS-deficient mice had a greater reduction in cerebral blood flow than in the wild-type mice. L-Arginine administration increased blood flow postinjury and reduced contusion volume only in the wild-type mice, suggesting an important role for NO produced by eNOS in the preservation of cerebral blood flow in contused brain (Hlatky et al., 2003b). In anesthetized newborn pigs, topical application of urokinase or tissue plas-

minogen activator (tPA) elicited pial artery dilatation associated with increased cyclic GMP in cerebrospinal fluid that was blunted by L-NA; the artery dilatation was blocked after fluid percussion brain injury (Armstead et al., 2004). Altered NO function seems to contribute to the impairment of urokinase- and tPA-induced cerebrovasodilatation after brain injury. In patients with severe traumatic injury, microdialysate NOx values were the highest within the first 24 h after trauma and gradually declined over the 5 postinjury days. There was a significant relationship between regional cerebral blood flow and dialysate NOx levels, and the NOx levels in patients with critical reduction of regional cerebral blood flow were lower than those in patients with normal cerebral blood flow (Hlatky et al., 2003a). NO may play some role in the abnormalities of cerebral blood flow that occur after traumatic injury.

Western blot analysis and immunohistochemistry revealed that phosphorylated eNOS was expressed in the endothelial cells of microvessels within the gray matter during the subacute stage after spinal cord injury in mice, suggesting that eNOS activation and an increase in spinal cord blood flow may be involved in protecting and repairing the responses (Osuka et al., 2008).

In summary, there is a relationship between cerebral blood flow/NO levels and the amount of trauma in experimental animals and patients with head injury. NO produced by eNOS seems to play an important role in the preservation of cerebral blood flow in traumatic brain injuries.

2. Role of Inducible Nitric-Oxide Synthase-Derived Nitric Oxide. After head trauma in rats, cortical perfusion was reduced. A reduction in the lumen of microvessels and distortion of their shape, together with ET-1 expression and up-regulation of iNOS mRNA synthesis in the endothelial cells, were observed after the 4th h post-trauma; only ET-1 expression was further increased at 24 h after injury (Petrov and Rafols, 2001). A reciprocal interaction in the synthesis of these two molecules may underlie the control of microvascular autoregulation after traumatic brain injury. Steiner et al. (2004) provided evidence suggesting that NO, generated primarily by iNOS, suppresses ET-1 production and that a decrease in NO results in up-regulation of ET-1 via transcriptional and translational mechanisms. Laser Doppler flow velocity decreased in rats subjected to moderate or severe traumatic brain injury; brain tissue NO levels decreased after moderate injury, and IL-6 and TNF-α mRNA expression increased after both moderate and severe injury (Ahn et al., 2004). There were reductions in cerebral blood flow in the hemisphere and cortex at 24 h after controlled cortical impact. Recovery of cerebral blood flow at 72 h after injury was reduced in iNOS knockout mice versus wild-type mice, suggesting a contribution of iNOS to delayed post-traumatic hyperemia (Foley et al., 2008). In short, there is a reciprocal interaction between iNOS expression and ET-1 up-regulation that may underlie the control of cerebral blood flow after traumatic brain injury.

3. Therapeutic Measures. The cerebral vasodilatation to CO_2 was attenuated in rats with cortical spreading depression elicited by a controlled cortical impact, and topical superfusion with the NO donor *S*-nitroso-*N*-acetylpenicillamine restored the hypercapnic vasodilatation, supporting the hypothesis that NO production is reduced after traumatic brain injury and that the NO donor has a potential beneficial role in the clinical management of head injury (Zhang et al., 2002). In anesthetized mice and rats that were subjected to cortical impact injury, cerebral blood flow decreased at the impact site, and L-arginine administration restored the blood flow (Liu et al., 2002a). L-Arginine administration resulted in an increase in tissue NO concentrations and an improvement in cerebral blood flow at the site of traumatic brain injury in rats, and similar beneficial effects were elicited by administration of SOD in combination with catalase (Cherian and Robertson, 2003). Free radical production after trauma may contribute to the reduction in cerebral blood flow by inactivating NO. Administration of L-arginine increased regional cerebral blood flow in the injured brain tissue in rats, but brain tissue partial pressure of oxygen was not increased (Mendez et al., 2004). L-Arginine and BH_4 administration both resulted in preservation of tissue NO concentrations and an improvement in cerebral blood flow (Cherian et al., 2004a). In mice, cortical blood flow in the traumatized brain was increased by L-arginine at 3 h, but not at 24 h, after trauma, and brain water content was lower in the L-arginine-treated group than in the vehicle group only at 3 h after trauma (Lundblad and Bentzer, 2007). L-Arginine seems to improve cortical blood flow and reduce brain edema formation in the early phase after a brain trauma, whereas no circulatory effects are seen after prolonged treatment. On the other hand, Prough et al. (2006) noted that the improvement in cerebral blood flow, intracranial pressure, and mean arterial pressure produced by hypertonic saline alone after traumatic brain injury was not enhanced by the addition of L-arginine.

Treatment with L-NAME alone or combined with the nitrone radical scavenger 2-sulfo-phenyl-*N*-*tert*-butyl nitrone reduced eNOS and nNOS activity but did not affect iNOS activity or iNOS immunoreactivity; these treatments reduced neuronal degeneration and nitrotyrosine immunoreactivity at 24 h and increased neuronal survival at 6 days after secondary traumatic brain injury (Gahm et al., 2005). Post-traumatic treatment with these compounds does not seem to inhibit early beneficial NO-related effects but may limit peroxynitrite formation, promoting neuronal survival. In short, increased production of NO by eNOS or iNOS seems to play a role in the preservation of cerebral blood flow under traumatic brain injury.

V. Pharmacological Implications of Nitric Oxide and Nitric Oxide-Related Agents for Miscellaneous Brain Diseases

A. L-Arginine

L-Arginine or nitroglycerin reversed the attenuation of cerebral vasodilatation to hypercapnia seen under ketamine anesthesia in rabbits (Nagase et al., 2002). In patients with cardiovascular risk factors and impaired vasomotor reactivity, L-arginine infusion improved impaired reactivity to CO_2 of cerebral blood vessels (Zimmermann and Haberl, 2003). L-Arginine administration during the acute phase improved symptoms in patients with mitochondrial myopathy, encephalopathy, and stroke-like episodes (Kubota et al., 2004; Koga et al., 2005, 2006).

B. Nitric Oxide Inhalation

In fetal sheep, acute pulmonary vasodilatation caused by NO inhalation did not change left ventricular output, cerebral blood flow, or cerebral oxygen consumption, despite an increased systemic-to-pulmonary shunt across the ductus arteriosus (Rosenberg et al., 1995). In anesthetized pigs, inhaled NO increased cerebral blood volume and cerebral transit time, whereas cerebral blood flow remained unchanged, suggesting a vasodilator action of inhaled NO in the cerebral vasculature, which may occur preferentially in the venous component (Kuebler et al., 2003). From studies evaluating the impact of inhaled NO on brain growth and cerebral injury in a premature baboon model, Rees et al. (2007) provided evidence indicating that there was neither a protective nor a major injurious effect of NO therapy on the developing brain.

There was evidence that inhaled NO therapy, compared with 100% oxygen therapy, was associated with a decreased risk of cerebral palsy in preterm infants with persistent pulmonary hypertension of the newborn (Tanaka et al., 2007b). After the onset of inhaled NO, the velocity time integral of flow in the middle cerebral artery was acutely decreased in newborns with pulmonary hypertension, who experienced an acute increase in oxygenation (Day, 2001). In a child with traumatic brain injury, peripheral vascular resistance was decreased during the inhaled NO trial, but middle cerebral artery blood flow, jugular bulb oxygen saturations, and intracranial pressure were not changed (Vavilala et al., 2001).

C. Nitric Oxide Donors

In patients with recent ischemic or hemorrhagic stroke, transdermal nitroglycerin lowered blood pressure but did not affect platelet aggregation or expression of adhesion molecules (Bath et al., 2001). In addition, there was no change in cerebral blood flow, cerebral perfusion pressure, or cerebral steal in these patients treated with the NO donor (Willmot et al., 2006). In hypertensive pregnant patients, estimated cerebral perfusion pressure was unaltered by sublingual isosorbide dinitrate, despite decreases in maternal blood pressure and blood flow velocities in the middle cerebral artery (Nevo et al., 2003).

D. Phosphodiesterase-5 Inhibitors

PDE-5 inhibitors are therapeutic measures for penile erectile dysfunction and pulmonary hypertension (Goldstein et al., 1998; Sastry et al., 2004), the effects being associated with a reduced degradation of intracellular cyclic GMP formed via NO liberated from cavernosal nitrergic nerves and endothelial cells (Toda et al., 2005).

1. Studies on Experimental Animals. In SAH rats, vasodilator responses to ACh, SNP, and the PDE-5 inhibitor zaprinast were impaired; in contrast, vasodilatation induced by adenosine and 8-Br-cyclic GMP were similar in control and SAH rats, suggesting that an increased rate of cyclic GMP hydrolysis by PDE-5 may be a major factor contributing to the impairment of NO-mediated cerebral vasodilatation after SAH (Sobey and Quan, 1999). In the vasospastic artery in a canine SAH model, oral sildenafil produced vasodilatation and increased the activity and expression of PDE-5 (Inoha et al., 2002). The authors suggested that PDE-5 inhibitors may be useful therapeutic agents for the prevention of vasospasm after SAH. Sildenafil dilated the basilar arteries in both the sham surgery and SAH groups of rabbits; numbers of the apoptotic endothelial cells per cross-section after SAH in the control and sildenafil-treated groups were similar (Atalay et al., 2006). Zaprinast increased regional cerebral blood flow in the ischemic brain of rats, despite the decreased mean blood pressure, whereas it did not affect cerebral blood flow in the contralateral side. The volume of cerebral infarction was decreased by zaprinast administration (Gao et al., 2005).

In isolated rabbit basilar arteries, sildenafil induced vasodilatation, prevented vasoconstriction, and potentiated the effect of other NO-dependent vasodilators, possibly by enhancing the NO/cyclic GMP signaling pathway (Salom et al., 2006). On the other hand, Kruuse et al. (2005) found that sildenafil in vitro was a poor dilator of guinea pig cerebral arteries unless an NO donor was coadministered.

2. Studies on Patients or Healthy Subjects. In patients suffering from severe pulmonary hypertension, oral sildenafil provoked a reduction of pulmonary arterial pressure and vascular resistance, accompanied by minor changes in systemic vascular resistance; in contrast, cerebral vascular reactivity was improved by the PDE inhibitor, indicative of an improvement in neurovascular coupling in these patients (Rosengarten et al., 2006). In a double-blind, placebo-controlled study in patients with erectile dysfunction, a significant improvement in cerebrovascular reactivity, measured by means

of the breath holding index, was observed by oral administration of 50 mg of sildenafil (Diomedi et al., 2005).

E. 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Inhibitors (Statins)

1. Studies on Experimental Animals. The HMG-CoA reductase inhibitor simvastatin increased cerebral blood flow to ischemic regions of the brain, and mice treated with simvastatin showed smaller cerebral infarctions after middle cerebral artery occlusion. No neuroprotection was observed in eNOS-knockout mice (Laufs et al., 2000a). After eNOS up-regulation by chronic simvastatin treatment, L-arginine amplified and sustained the L-arginine-induced hyperemia; pretreatment with simvastatin enhanced the blood flow within ischemic brain tissue after middle cerebral artery occlusion (Yamada et al., 2000). The HMG-CoA inhibitor atorvastatin up-regulated eNOS in thrombocytes, decreased platelet activation in vivo, and protected from cerebral ischemia in normocholesterolemic mice (Laufs et al., 2000b). Similar results were also obtained with mevastatin (Amin-Hanjani et al., 2001) and rosuvastatin (Laufs et al., 2002). The stroke-protective effects of statins seem to be mediated by eNOS up-regulation. Statins increased eNOS and tPA mRNA levels; in eNOS knockout mice, atorvastatin reduced the volume of ischemic tissue after arterial occlusion by blood clot emboli, whereas, in contrast, statins did not have protective effects in tPA knockout mice after embolic focal ischemia (Asahi et al., 2005). Statins may protect against stroke by multiple mechanisms involving both eNOS and tPA. Zhang et al. (2005) also obtained evidence that combined treatment with atorvastatin and recombinant human tPA exerted a neuroprotective effect in mice when administered after stroke. The survival ratio at 19 weeks of age in SHRSP was higher in the atorvastatin-treated group than in the vehicle group, and statin administration reduced the ADMA levels without affecting the levels of plasma lipids, suggesting that the efficacy of the statin for stroke protection may be involved in the improvement of endothelial function via NO production and reduction of ADMA (Tanaka et al., 2007a).

Although neither simvastatin nor dipyridamole alone at subtherapeutic doses changed cerebral blood flow and aortic cyclic GMP levels in mice, the combination of subtherapeutic doses of these drugs increased cerebral blood flow, conferred stroke protection, and improved neurologic motor deficits, all of which were absent in eNOS-deficient mice, suggesting that the combined therapy has greater benefit in stroke protection than statin alone (Kim et al., 2008).

2. Studies on Patients. Statin administration results in greater NO availability, improved endothelial function, enhanced cerebral blood flow, decreased platelet aggregation, and antioxidant activity (Reiss and Wirkowski, 2007). Recent evidence suggests that sudden withdrawal of statin treatment leads to a rebound effect with down-regulation of NO production, resulting in impaired vascular function.

In patients with subcortical small vessel disease, the cerebral blood flow velocity increase by bolus injection of acetazolamide was greater after pravastatin treatment; there was a negative correlation between the statin-induced enhancement of vasomotor reactivity and the pretreatment blood flow velocity increase (Sterzer et al., 2001). This pilot study provided evidence for an improvement of cerebral vasomotor reactivity by statins in these patients. In patients with lacunar infarctions, L-arginine reactivity was decreased compared with that of healthy control subjects, and systemic endothelial function, evaluated by flow-mediated vasodilatation, was also impaired; atorvastatin treatment reversed the impairments seen in these patients (Pretnar-Oblak et al., 2006a).

F. Erythropoietin

EPO, a hypoxia-inducible hormone, is essential for normal erythropoiesis in bone marrow. EPO receptors are widely distributed in the cardiovascular system (Smith et al., 2003). Tsukahara et al. (1997) found that chronic administration of recombinant human erythropoietin (rHuEPO) increased the steady-state release of NO formed by eNOS in rats. Thoracic aorta segments from rHuEPO-treated rats revealed an increase in NO-dependent relaxation elicited by ACh, and NOS expression in the rHuEPO aorta and plasma NO levels were increased (Kanagy et al., 2003). Injections of EPO into the hippocampal tissue in rats increased NOx levels that were eliminated in the presence of anti-EPO antibody; the increases in NOx levels were blunted by nicardipine but not by MK-801, suggesting that EPO increases NO production by activating voltage-gated Ca^{2+} channels but not through NMDA receptors (Yamamoto et al., 2004). In cross-country skiers, EPO levels significantly increased after intensive training at a moderate altitude of 3100 m. Nitrite/nitrate baseline values were higher in trained subjects compared with untrained subjects, and adaptation to moderate altitude increased nitrite/nitrate levels in untrained subjects (Schena et al., 2002).

Administration of rHuEPO reversed the vasoconstriction of the basilar artery and reduced total damaged neurons in rabbits with experimental SAH, suggesting that EPO is effective in attenuating ischemic brain injury after SAH (Grasso, 2001), thereby reducing the post-SAH morbidity (Grasso et al., 2002). In cerebral arteries made spastic by SAH in rabbits, expression of eNOS was reduced, and expressions of phosphorylated Akt and phosphorylated eNOS were increased. Gene transfer of rHuEPO reversed the vasospasm, augmented NO-mediated relaxation to ACh, and further increased the expressions of phosphorylated Akt and eNOS (Santhanam et al., 2005). The vascular protective effect of rHuEPO against cerebral vasospasm may be mediated by phosphorylation of Akt/eNOS. d'Uscio et al.

(2007) provided evidence that the absence of eNOS transformed EPO from a vasoprotective agent to one that promoted hypertension and adverse and aberrant remodeling of the injured vasculature. In rats that underwent controlled cortical impact injury and were treated with EPO postinjury, the contusion volume was reduced and the neuron density in the CA1 and CA3 regions of the hippocampus was increased (Cherian et al., 2007). The neuroprotective action of EPO against traumatic brain injury may be due to increased cerebral blood flow in response to increased NO production. On the other hand, Calapai et al. (2000) found that treatment with rHuEPO after brain ischemic injury in the gerbil reduced hippocampal CA1 neuronal loss and decreased nitrite/nitrate levels in the hippocampus that had been increased by ischemia, suggesting that the neuroprotective effect of EPO could be due to inhibition of NO overproduction instead.

G. Other Therapeutic Agents

Enalapril prevented diabetes-induced impairment of NOS-dependent pial arteriolar dilatation; eNOS protein was higher in diabetic rats than in nondiabetic rats, and enalapril did not produce a further increase in eNOS, suggesting that the protective role of angiotensin-converting enzyme inhibition may be independent of an alteration in eNOS protein in cerebral microvessels (Trauernicht et al., 2003). The middle cerebral artery of SHR exhibited decreased lumen diameter and increased media thickness, in association with decreased eNOS and increased iNOS protein and mRNA, and these morphological and enzymatic alterations were reversed by long-term treatment with the AT_1 receptor antagonist candesartan (Yamakawa et al., 2003). Their findings are supported by the fact that chronic administration of angiotensin II produced cerebral artery contraction mediated by AT_1 receptors, Rho-kinase, and superoxide (Faraci et al., 2006). In hypertensive patients, candesartan decreased carotid intima-media thickness, possibly by enhancing NO production and decreasing oxidative stress (Ono et al., 2008). High-dose corticosteroids increased eNOS activity, augmented regional cerebral blood flow, and reduced cerebral infarct size, and these effects were abolished by glucocorticoid receptor (GR) blockade and by PI_3 kinase inhibition and were absent in eNOS knockout mice. GR and a mutant GR, which cannot dimerize and bind to DNA, activated PI_3 kinase and Akt in response to corticosteroids (Limbourg et al., 2002). Non-nuclear GR seems to activate eNOS through the PI_3 kinase/Akt pathway that mediates the neuroprotective effect of corticosteroids through augmentation of cerebral blood flow.

In double-blind, placebo-controlled studies on patients with Fabry disease, which is a lysosomal deficiency of α-galactosidase A, Moore et al. (2001) noted that the resting regional cerebral blood flow in the patients treated with α-galactosidase A was reduced, with a decrease in nitrotyrosine staining in dermal blood vessels. A chronic alteration of the NO pathway in Fabry disease with critical protein nitration may be reversible with enzyme replacement therapy.

1. Dietary Factors and Chinese Herbs. Intraperitoneal resveratrol, a polyphenolic antioxidant, either during or after common carotid artery occlusion, decreased delayed neuronal cell death as well as glial cell activation in gerbils, suggesting that resveratrol seems to cross the blood-brain barrier and exerts protective effects against ischemic injury (Wang et al., 2002). During cerebral ischemia in rats, the hydroxy radical level in the hippocampus was elevated. Intravenous administration of resveratrol increased the NO level, decreased the hydroxy radical level, and attenuated the reduction of cerebral blood flow and neuronal cell death (Lu et al., 2006). The neuroprotective effect of this phytoestrogen against cerebral ischemia may be attributed to free radical scavenging and cerebral blood flow elevation due to NO release. Evidence for the important role of NO formed by eNOS in the neuroprotective effect of resveratrol was also obtained in rats subjected to focal cerebral ischemia (Tsai et al., 2007).

In intact rat hippocampus, ischemia increased NOx concentrations, whereas (−)-epigallocatechin gallate (EGCG) inhibited the NO increase without affecting hippocampal blood flow; treatment with SNP reduced the viability of cultured rat hippocampal neurons, whereas EGCG reversed the effect of SNP, suggesting that EGCG may protect against ischemic neuronal damage by acting as an antioxidant (Nagai et al., 2002). Administration of the flavonoid baicalin before the start of heat exposure reduced the hyperthermia, intracranial hypertension, increased levels of NO_2 and glutamate and the lactate/pyruvate ratio in the hypothalamus that occurred during heatstroke; baicalin also suppressed the heatstroke-induced increase in IL-1β and TNF-α levels (Chang et al., 2007). Baicalin may protect against cerebrovascular dysfunction and brain inflammation in heatstroke. Flavonol-rich cocoa induces consistent vasodilatation in healthy subjects by improving endothelial function in an NO-dependent manner (Toda, 2007). On the basis of studies, although still preliminary, on healthy older subjects, Fisher et al. (2006) raised the prospect that increasing cerebral perfusion with cocoa flavanols is promising as a preventive measure against decreased cerebral perfusion with dementia.

In rats fed a diet containing homocysteine that led to hyperhomocysteinemia, cerebral eNOS and glucose transporter-1 levels were reduced and the level of vascular cell adhesion molecule-1 expression was increased. Dietary folic acid supplementation decreased the plasma homocysteine levels and reversed the effects of homocysteine on the endothelial function, glucose transporter protein, and cell adhesion molecule (Lee et al., 2004). In SHRSP, L-arginine, antioxidants, and voluntary exercise reduced blood pressure and thrombotic tendency in

cerebral microvessels; A number of dietary factors (vitamin E, sesamin, and *Ginkgo biloba* extract), possessing antioxidant activity, showed preventive effects on hypertension, cerebral blood flow dysfunction, thrombus formation, and neuronal cell death (Noguchi et al., 2004).

G. biloba extract effectively antagonized SAH-induced changes in rats, including a reduction in the regional cerebral blood flow, a decrease in serum NO levels, and an increase in brain tissue NO, leading to relief from cerebral ischemic damage due to SAH (Sun et al., 2000). These authors (Sun et al., 2003a) also obtained evidence that *G. biloba* extract improved microcirculation in SAH rats by antagonizing the overproduction of ET-1. Jin et al. (2006a) noted that an extract of *G. biloba* promoted the development of NOS- and acetylcholinesterase-positive neurons in the rat embryonic basal forebrain. Shengmai San, a Chinese herbal medicine, and aminoguanidine attenuated heat stress-induced arterial hypertension, cerebral ischemia, and increased levels of brain iNOS-dependent NO formation and serum cytokine formation, suggesting that the neuroprotective effects may be associated with an inhibition of NO overproduction and excessive accumulation of cytokines (Wang et al., 2005).

VI. Summary and Conclusion

This review article summarizes information concerning recent advances in research on cerebral blood flow regulation in reference to NO generated through activation of eNOS, nNOS, and iNOS under physiological and pathophysiological conditions. NO derived from eNOS activation under basal conditions and in response to chemical (neurotransmitters, hormones, and autacoids as well as oxygen and carbon dioxide) and physical stimuli (blood flow and shear stress) contributes to cerebral arterial and arteriolar dilatation, increases in cerebral blood flow, and decreases in cerebral vascular resistance. These NO effects are mediated by soluble guanylyl cyclase activation that leads to increased production of cyclic GMP in vascular smooth muscle cells. NO is also generated for vasodilatation and the blood flow increase from parasympathetic postganglionic (nitrergic) nerve fibers innervating pial arteries and intracerebral arterioles and from neurons in the vicinity of arterioles and capillaries, in which nNOS is activated by elevated $[Ca^{2+}]_i$ through stimulation of NMDA receptors by the neurotransmitter glutamate in the brain. Astrocytes adjacent to parenchymal arterioles and capillaries may be one of the sites of NO generation or act as intermediaries in neurovascular signaling.

Impaired synthesis and actions of NO derived from constitutive NOS are widely regarded as some of the serious pathogenic factors in various brain diseases, such as ischemic stroke and traumatic head injury that elicit cerebral dysfunction and neuronal cell death and cerebral vasospasm after SAH. Substrates of NO synthesis, NO donors, phosphodiesterase-5 inhibitors, and HMG-CoA reductase inhibitors are beneficial, because of increasing NO bioavailability, in the treatment of patients suffering from the above-mentioned diseases having impaired cerebral blood flow. On the other hand, long-lasting overproduction of NO by iNOS and nNOS, in conjunction with the generation of superoxide anions, participates in neurodegeneration. Inhibitors of NOS (particularly iNOS), together with antioxidants, are effective in reversing their deteriorating actions.

Information in this article was obtained mainly from experimental animals. Findings in healthy subjects and patients with cerebral circulatory dysfunction are still limited and conflicting. Determination of the neural networks via which NO regulates cerebral blood flow and the elucidation of the functional roles of astrocytes in nitrergic mechanisms remain important but yet to be achieved goals. Further efforts devoted to advance our understanding of the physiological and pathophysiological actions of NO and its counteractive molecules, ROS, on cerebral blood flow regulation will contribute to development of novel ways of preventing and treating cerebrovascular dysfunction.

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