

New developments in the understanding of cerebral vasoregulation and vasospasm: the endothelin-nitric oxide network

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SUMMARY Endothelins, which are powerful vasoconstrictors, and nitric oxide, which is a powerful vasodilator, together form a balanced system that regulates blood flow in the brain and in other organs. Ongoing research may yield new drugs that act on this system to prevent or reverse cerebral vasospasm in subarachnoid hemorrhage and other conditions.

KEY POINTS Many compounds are involved in cerebral vasoregulation under physiologic and pathologic conditions; of these, endothelins and nitric oxide have attracted considerable attention over the last several years. ■ Endothelins and nitric oxide differ in chemical structure and pharmacological properties: endothelins are potent vasoconstrictor peptides consisting of 21 amino acids; nitric oxide is a free radical with a half-life of only a few seconds and exerts powerful vasodilatory effects. ■ Both are produced by a number of cell types in the brain and interact at various levels to profoundly influence cerebral vessel function.

INDEX TERMS: ENDOTHELINS; NITRIC OXIDE; CEREBROVASCULAR CIRCULATION; VASOCONSTRICTION; VASODILATION; CEREBRAL ISCHEMIA, TRANSIENT
CLEVE CLIN J MED 1995; 62:105-116

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This paper is dedicated to K. Felgenhauer, MD, Professor of Neurology, Director of the Department of Neurology, University of Göttingen, on the occasion of his 60th birthday.

THIS ARTICLE distills new developments that may be of major clinical relevance in understanding cerebral vasoregulation and conditions of vasospasm, briefly summarizes the biology of endothelins (ETs) and nitric oxide (NO), and sketches some of the interactions of a hypothetical ET-NO network in brain vasculature, derived from our own work and that of others. Further, we discuss the pathophysiologic aspects of this network that offer encouraging approaches to future therapy.

Many compounds and mechanisms identified in the last several decades can influence cerebral blood flow under physiologic and pathologic conditions. Most of them have been addressed extensively in a number of excellent reviews.¹⁻¹⁰ Newly discovered factors that have attracted considerable attention in recent years are the ETs and NO, which apparently are components of a well-balanced regulatory arrangement that maintains vascular tone while retaining a high degree of plasticity. In the brain, this counterregulatory system reaches out far beyond blood

vessels to involve neurons and glial cells, thereby potentially reflecting exciting aspects of a functional entity.

ENDOTHELINS: A FAMILY OF VASOCONSTRICTORS

ETs, a recently described family of peptides, are among the most potent vasoconstrictors known and possess an extremely long duration of action. At least three ETs have been identified, each consisting of 21 amino acids. ET-2 and ET-3 differ from ET-1 by two and six amino acids, respectively, and also have somewhat different pharmacologic properties.¹¹⁻¹³ The genes encoding these peptides are located on different chromosomes, in humans on chromosomes 6, 1, and 20.¹⁴⁻¹⁸

Originally isolated from aortic endothelial cells,¹¹ ETs are produced by a number of other cell types as well, including cerebral endothelial cells,^{19,20} heterogeneous populations of neurons,²¹⁻²⁵ glial cells,²⁶⁻²⁸ and certain immune cells.^{29,30} Recently, even vascular smooth muscle cells were shown to produce ETs upon induction with growth factors.³¹ Because of this confusing variety of ET sources, observations of a cell-type-specific regulation of ET-gene expression take on greater importance.³²⁻³⁴

ETs derive from prepropeptides approximately 200 amino acids long; specific proteolytic enzymes produce the biologically active ET.^{11,35,36} The activity of these "endothelin-converting enzymes" (ECEs) seems to differ among organs and tissues,³⁷⁻³⁹ and each ECE mostly cleaves a specific substrate to produce a specific ET.⁴⁰ In addition, the distribution of messenger RNA for ET-1, ET-2, and ET-3 is different in different organs.⁴¹ All this may reflect the different physiologic tasks of these peptides. ECEs not only represent important regulatory elements in ET biology but also possess properties attractive for future therapeutic intervention.

ETs act via specific binding sites—G-protein-coupled receptors—distributed throughout the body, not exclusively associated with vascular structures.⁴² In the brain, these binding sites have been identified mainly in the cerebellum, basal ganglia, hippocampus, brain stem, and choroid plexus.^{25,42-45} Two distinct subtypes of ET receptors have so far been cloned from a number of species. The ET_A subtype preferentially accepts ET-1 and ET-2 as ligands and appears to be the one predominantly responsible for mediating vasoconstriction. In con-

trast, the ET_B subtype is considered nonselective, binding ET-1, ET-2, and ET-3 with comparable affinity. Activation of the ET_B receptor in endothelial cells by low doses of ETs tends to antagonize ET_A-induced effects and leads to the release of potent vasodilators, mainly NO and prostacyclin.⁴⁶⁻⁵¹ Thus, the ratio of ET_A to ET_B activation appears to be critical for the net effect of ETs on vascular tone. This ratio can change: any ET-receptor subtype can undergo up- or down-regulation, in turn altering tissue responsiveness to ETs.^{52,53}

Signal transduction pathways involved in ET-receptor stimulation include phospholipases C, A₂, and D, protein kinase C, tyrosin kinase, receptor-gated or voltage-dependent calcium channels, and sodium-hydrogen antiporters.⁵⁴⁻⁵⁹

ETs not only are potent vasoconstrictors, but also act on other smooth muscle cells such as those in the bronchial tree.⁶⁰ In addition, they display remarkable mitogenic or comitogenic activity in a number of cell types and are able to influence cell differentiation.⁶¹⁻⁶⁶ The characteristics and mode of action of ETs may justify their classification as hormones, neuropeptides, or cytokines.

Subtype-selective ET antagonists, monoclonal antibodies against ETs, and ECE inhibitors have been of tremendous help in the search for the physiologic and pathophysiologic role of ETs.^{38,67-69} Two peptides, BQ123 (an ET_A antagonist)⁶⁸ and IRL1038 (an ET_B antagonist)⁶⁹ have become available for experimental use, but their pharmacokinetic disadvantages make them unsuitable for clinical application. Recently, RO46-2005, a promising, though non-ET-subtype-selective, nonpeptide antagonist, has been described. A structurally modified pyrimidinyl sulfonamide related to oral antidiabetic agents but devoid of hypoglycemic activity, RO46-2005 can be given by mouth, penetrates the blood-brain barrier, and has a half-life of approximately 8 hours.⁷⁰ This or similar compounds may be introduced clinically in the near future.

ETs are potent vasoactive mediators in the brain

ETs can provoke extremely potent and long-lasting vasoconstriction, both in vitro and in vivo, in cerebral blood vessels of all sizes and types, including the microcirculation.⁷¹⁻⁷⁸ Intracisternal application of as little as 10 pMol of ET-1 in dogs induces a pronounced spastic constriction of the vertebrobasilar arteries, which lasts for more than a day.^{71,73} ET-1

can therefore be regarded as a potential mediator of chronic functional narrowing of cerebral vessels. Vasodilatory effects of ETs, apparently concentration-dependent, have also been described for the cerebral circulation and are most likely indirect, ie, they involve other mediators.^{48,50,51,78-81}

Under physiologic conditions, ETs do not penetrate the blood-brain barrier or influence its permeability.^{42,82} ETs thus either require a damaged endothelial cell layer in order to exert their effect in cerebral vessels via the lumen, or they must act from the adventitial side.^{83,84} These observations originally prompted our search for a source of ETs on the outside of cerebral vessels.

In fact, astrocytes (glial cells that profoundly influence the function of both neurons and cerebral endothelial cells) were found to produce ET-1 and ET-3 and, in addition, to express high-affinity binding sites for these peptides.²⁶⁻²⁸ Further, ET-1 release by these cells is subject to selective autostimulation: stimulation of astrocytic ET receptors potentiates further ET-1 release while leaving ET-3 unaffected.²⁸ A similar autostimulation of ETs has been shown in endothelial cells^{85,86} and, upon induction, in vascular smooth muscle cells.³¹ The amount of ET-1 produced by astrocytes in response to autostimulation with ET-1 greatly exceeds that achieved with other stimulants such as norepinephrine or thrombin.^{28,53} Such local autostimulatory amplification within a cerebral microenvironment may be of major pathophysiological significance in a number of conditions ranging from subarachnoid hemorrhage to cerebral infection.

NITRIC OXIDE: A VASODILATORY COUNTERBALANCE

Interest in NO began when Furchgott and Zawadzki⁸⁷ discovered that endothelial cells play an obligatory role in mediating vasorelaxation by releasing a chemical compound in response to different stimulants. This compound, initially termed "endothelium-derived relaxing factor" (EDRF), induces relaxation by activating soluble guanylate cyclase in smooth muscle and by increasing the intracellular concentration of cyclic guanosine monophosphate.⁸⁸ It was subsequently identified as NO, a free radical with high lipid solubility and an extremely short half-life of only a few seconds in biological fluids.^{89,90} However, there is still a debate as to whether a nitroso-thiol compound such as

S-nitroso-cystein eventually accounts for the effect of EDRF.^{91,92}

NO is derived from the guanidino group of its precursor, the amino acid L-arginine. This reaction, which is catalyzed by the enzyme NO synthase (NOS), yields citrulline as a by-product, which may be recycled in the cells via an intermediate compound, argininosuccinate, in a partial urea cycle.^{93,94} Several isoforms of NOS have been described,^{95,96} which are expressed either constitutively (cNOS, eg, in endothelial cells) or upon induction (iNOS). Interestingly, iNOS expression may be triggered in smooth muscle cells of peripheral as well as cerebral arteries by incubation with endotoxin or cytokines.⁹⁷⁻¹⁰¹ Once activated, iNOS results in high amounts of NO, which can contribute, for example, to the pathogenesis of endotoxic shock.¹⁰⁰

Endothelial cells are not the sole source of NO in the brain, since glial cells and neurons also express cNOS. In neurons, NO release is potently stimulated by the excitatory amino acid glutamate via activation of postsynaptic N-methyl-D-aspartate (NMDA) receptors.¹⁰² NMDA-induced neuronal NO release is most pronounced in the cerebellum,^{93,102,103} but also occurs in other brain regions, including the forebrain.^{104,105} In the rat cortex, approximately 1% to 2% of the neurons are stained by NOS antibodies or are positive for nicotinamide-adenine-dinucleotide phosphate- (NADPH-) diaphorase, an enzyme that appears to be highly similar or even identical to neuronal NOS.¹⁰⁵⁻¹⁰⁷

NOS and NADPH-diaphorase have also been identified in nerve fibers surrounding cerebral arteries in rats,^{103,108-111} dogs,¹¹² cats,¹¹³ and humans.¹¹⁰ This "nitroxidergic" innervation, which appears to originate mainly from parasympathetic ganglia, has been hypothesized to be involved in the pathogenesis of cerebral vasospasm and migraine attacks,¹¹⁴ via an imbalance (lack or excess) of NO release.

NOS inhibitors can effectively block the relaxation induced by transmural nerve stimulation in isolated basilar or middle cerebral arteries devoid of a functional endothelium,¹¹²⁻¹¹⁹ indicating that NO from nitroxidergic nerves may mediate the nonadrenergic, noncholinergic relaxation of cerebral blood vessels. Similarly, NO release may be the underlying cause of nonadrenergic, noncholinergic relaxation of smooth muscle cells in the gastrointestinal tract¹²⁰ and the genitourinary system, thereby playing a pivotal role in the control of penile erection.¹²¹

Vascular NO and cerebrovascular tone

The function of NO can be studied by blocking NOS activity with analogues of arginine such as N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA), or its methyl ester (L-NAME).¹²² In large cerebral arteries isolated from different species, NOS inhibitors induce contraction, indicating that basal release of NO contributes to the maintenance of resting tone.^{123–128} Furthermore, NO mediates relaxation in response to a number of vasoactive compounds such as acetylcholine.^{123,125–127,129} However, additional factors important to relaxation may also be released from the endothelium. In rabbit basilar arteries, complete inhibition of muscarinic and histaminergic relaxation can only be achieved by simultaneous application of an NOS inhibitor and indomethacin to block the release of relaxant prostanoids (probably prostacyclin).^{127,130}

Similarly, *in vivo*, superfusion with NOS inhibitors decreased the resting diameter of basilar arteries of rats and inhibited acetylcholine-induced dilation.^{131–133} NO, therefore, participates in regulating the resting diameter and mediates muscarinic dilation of the basilar artery. However, in small pial arteries of rats, topical application of an NOS inhibitor produced no significant vasomotor effect,^{134–138} and comparable results have been obtained in most studies in other species,^{139–142} although somewhat different observations have been reported sporadically.^{143–146}

Thus, basal release of NO does not appear to be a general prerequisite for the adjustment of resting tone in small pial arteries. Upon topical application of acetylcholine, however, these small arteries dilate in a concentration-dependent manner. This dilation is blocked by simultaneous application of an NOS inhibitor,^{133,135,141,143,147} indicating that the apparent noninvolvement of NO in the regulation of resting tension is not due to a lack of NOS activity in the vessel wall.

Topical application of low concentrations of 5-hydroxytryptamine (5-HT) also results in an L-NNA-sensitive dilation of small pial arteries in rats,¹³⁶ while the dilating effect of bradykinin is not modified in the presence of L-NNA.^{135,144} Taken together, there are pronounced regional and mediator-dependent differences in the function of EDRFs, reflecting this system's high plasticity in the regulation of cerebrovascular resistance.

NO may link cerebral blood flow to neuronal activity

In most published studies, NOS inhibitors given systemically decreased the resting cerebral blood flow,^{129,148–157} indicating an increase in total cerebrovascular resistance. This may partly result from constriction of large arteries such as the basilar artery.^{131–133} It may also partly result from constriction of intraparenchymal arteries and arterioles, since superfusion of the parietal cortex with NOS inhibitors decreases regional cerebral blood flow,^{155,156,158} although this does not affect the resting diameter of pial arteries appreciably, as discussed above.

Endothelial cells may supply the tonically released NO that influences intraparenchymal resistance vessels, but the presence of cNOS in neurons and glial cells^{95,159} suggests that these cells also make substantial contributions. These observations led to the hypothesis, based on computer simulation,¹⁶⁰ that NO released from the parenchyma could couple regional cerebral blood flow to local neuronal activity.

We have recently tested this hypothesis using the spreading cortical depression described by Leao¹⁶¹ as a model of cortical activation. The spreading depression is characterized by a transient phase of neuronal hyperactivity caused by a massive release of the excitatory transmitter glutamate,^{162–165} followed by a more sustained period of hypoactivity travelling over the cortex in a wavelike manner. The wavelike spread of increased neuronal firing is accompanied by transient dilation of pial arteries^{166,167} and regional hyperperfusion.^{152,168,169} These effects appear to be mostly indirectly induced, since neither glutamate nor NMDA exerts any direct vasomotor effects in isolated cerebral arteries.^{145,170,171} Both pial arterial dilation and hyperperfusion during a wave of spreading depression can be reduced considerably by local or systemic application of an NOS inhibitor.^{142,152,157} This may point to a role of NO in coupling neuronal activation and arterial dilation under this condition.

Further studies using different methods of cortical activation support the hypothesis that NO links neuronal activity and perfusion. The increase in regional cerebral blood flow induced by electrical stimulation of the tibial nerve can be abolished by intraparenchymal application of an NOS inhibitor.¹⁷² Similarly, cortical hyperperfusion during whisker stimulation can be reduced by systemic application or cortical superfusion with an NOS inhibitor in anesthetized rats.¹⁵⁸ However, in awake rats a

similar degree of hyperperfusion (expressed in percent of resting cerebral blood flow) during whisker stimulation was found in the absence and presence of an NOS inhibitor.^{173,174} Whether this lack of effect of NOS inhibitors on metabolic coupling is due to incomplete inhibition of NOS (as suggested by Irikura and coworkers¹⁷⁵) remains to be established.

Although the exact role of NO in controlling cerebrovascular resistance is still a matter of speculation,^{122,176} it may provide at least part of the link matching metabolic demand with supply.

THE ET-NO NETWORK

The ET-NO network, as deduced from the literature as well as from our own work, is presented in the *Figure*. This network provides a basis for understanding the actions and interactions of ETs and NO in cerebral vasoregulation.

There are a number of potential sources of ET-1 in the cerebral microenvironment. As mentioned above, endothelial cells,^{19,20,177,178} astrocytes,²⁶⁻²⁸ and neurons²¹⁻²⁵ all can produce ET-1 and release it upon stimulation with various factors. ET-stimulating factors, such as norepinephrine, thrombin, interleukin-1, endotoxin, and transforming growth factor-beta, are not equally efficient among cell types and also result in a different temporal ET-response pattern.^{11,28,31} In addition to being produced in cells that reside in the brain, ET can be produced by macrophages that invade it under pathological conditions, eg, meningitis, ischemia, subarachnoid hemorrhage, or human immunodeficiency virus (HIV) encephalopathy.^{29,179-182}

Once ET-1 is present in a certain cerebral microenvironment, it binds not only to vascular smooth muscle cells (causing vasoconstriction), but also to ET_B receptors and, most likely, to ET_A receptors located on endothelial cells^{85,183,184} and astrocytes.^{53,185} Activation of these receptors initiates the

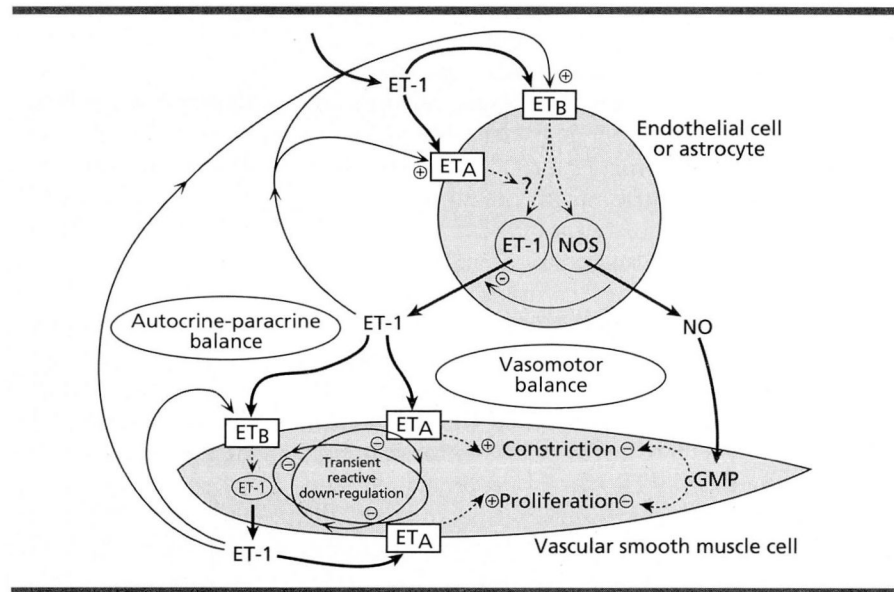


FIGURE. The endothelin (ET)-nitric oxide (NO) network: The players are known but the rules of the game are still obscure. ET-1, ET-2, and ET-3 are different peptides; ET_A and ET_B are receptors. NOS, nitric oxide synthase; cGMP, cyclic guanosine monophosphate.

autostimulatory amplification of ET-1 on one hand^{28,85,86} and, on the other hand, leads to stimulation of NOS and subsequent synthesis of NO.^{78,186-188} As illustrated in the *Figure*, NO is capable of inhibiting ET-1 release, thereby serving as a natural control factor of ET autostimulation.¹⁸⁹

Both NO and ET-1 act on vascular smooth muscle cells, the former inducing vasodilation, the latter provoking vasoconstriction. With respect to smooth muscle proliferation, they also exhibit opposite effects, ET being a stimulator, NO an inhibitor.^{61,190} ET-1-induced vasoconstriction appears to be mediated mainly via ET_A-receptors. There is, however, a high probability that vascular smooth muscle cells, perhaps with regional differences, additionally express an ET_B-type receptor that is predominantly responsible for autoinduction of ET-gene expression in these cells.³¹ Whether ET_B stimulation can lead to iNOS activation, thereby initiating smooth muscle NO production, is still unknown and has therefore not been integrated into the *Figure*. Nevertheless, vascular smooth muscle ET-1 can contribute to the "autocrine-paracrine balance" within its microenvironment.

An additional level of control over ET action apparently consists of a transient reactive down-

regulation of ET-receptor expression. This homologous down-regulation may selectively affect one receptor subtype and leave the other unaffected, as shown for ET_A in primary astrocyte cultures in which ET_B remained unchanged.⁵³ In principle, ET_B can also undergo down-regulation.^{52,53} Down-regulation may originate at the mRNA level, extend to the expression of the receptor protein in the cell membrane, and could also consist of an internalization of the receptor-ligand complex, perhaps followed by receptor recycling.¹⁹¹⁻¹⁹³

As one aspect of autostimulatory phenomena, a negative correlation between ET-1 production and ET_A-receptor expression has been shown for smooth muscle cells and astrocytes.^{53,194,195} Interestingly, changes in the ratio of ET_A to ET_B receptor expression in human endometrium have been observed during the menstrual cycle, indicating a function-dependent shift in responsiveness to ET.¹⁹⁶

Removing or adding certain components may, despite the network's considerable plasticity, profoundly disturb vasomotor and autocrine balance. For instance, introducing ET-3 into the network would, considering the low affinity of ET-3 for ET_A receptors, result in a preferential stimulation of ET_B receptors. This in turn might lead to a temporary preponderance of vasodilating factors. On the other hand, reducing NOS activity, leading to impairment of NO production, would contribute to exaggerated vascular contraction.

DERANGEMENTS OF THE ET-NO NETWORK

Cerebral vasospasm—the functional narrowing of vessels—occurs in a number of conditions, including subarachnoid hemorrhage, cerebral trauma, and meningitis. It may result from increased activity of vasoconstricting agents or decreased vasorelaxing capacities, or both. In either case, a profound disturbance of the basal vasomotor balance in cerebral vessels would result. In a number of species, ETs can produce long-lasting spasm of cerebral vessels upon intracerebroventricular application.^{71,73,83,197-199} In patients with subarachnoid hemorrhage-induced vasospasm, elevated levels of immunoreactive ETs have been identified in ventricular cerebrospinal fluid in a temporal pattern paralleling the occurrence of clinically documented vasospasm.^{200,201} In addition, an increased sensitivity of cerebral vessels to ETs has been shown following experimental subarachnoid hemorrhage,²⁰² which may contribute

to the functional preponderance of vasoconstricting agents in this condition.

At the same time, vasorelaxing capacities seem to decrease considerably²⁰³⁻²⁰⁵; there is reduced production of NO,²⁰⁶ reduced NOS immunoreactivity at the adventitial side of cerebral vessels (possibly due to a loss of nitroergic innervation), and marked reduction in the level of cyclic guanosine monophosphate,^{207,208} which constitutes the effector pathway of NO. Taken together, these events may help to explain the powerful vasospastic reaction of the cerebral vasculature.

Ischemia-induced alterations may follow vasospastic reactions of various origins or may initiate or further enhance them. Both ETs and NO appear to be mediators involved in the pathophysiology of ischemia.^{77,209-213} The synthesis of NO is profoundly altered, as shown by transient peaks of NO release immediately after ischemia and, likewise, after reperfusion.²¹⁴ Furthermore, experimental ischemia leads to NOS induction.^{215,216} Similarly, ischemia has been shown in many ways to affect ET release as well as ET-receptor expression.^{41,212,217} Plasma ET levels have been found to be elevated in patients suffering from ischemic stroke.²¹⁸ Interestingly, this has also been reported in acute migraine attacks.²¹⁹

Discrete ischemic lesions have further been identified in HIV encephalopathy, characterized by abnormalities appearing early on single-photon emission computed tomography and positron-emission tomography.²²⁰⁻²²⁵ Macrophage-derived multinucleated giant cells in the brains of patients with acquired immunodeficiency syndrome were distinctly positively stained for ETs, as were astrocytes and endothelial cells in their vicinity.¹⁸² This may point to a concerted action *in vivo* of various cell types with respect to ET production in inflammatory conditions, and may be analogous to the autostimulatory amplification of ET levels shown *in vitro*.

APPROACHES TO THERAPY

Any disturbance of the ET-NO network, once recognized, could potentially be addressed by agents to restore the preexisting balance. Recently available ET antibodies, ET antagonists, and ECE inhibitors have been reported effective in counteracting cerebral vasospasm in a number of species.^{70,226-229} How treatment with ET antagonists will influence ET-receptor expression and, thus, tissue sensitivity to any ET-receptor ligand remains to be determined.

Such alterations will be of therapeutic significance.

The role of NO released during complete or incomplete ischemia as well as during reperfusion is still far from understood.²¹³ On one hand, vasodilation by NO may help to maintain regional blood flow above the critical threshold; on the other hand, NO may react with superoxide radicals to form peroxynitrite, a harmful free-radical species.^{230,231} Accordingly, no effect,²³² an increase,²³³ and a decrease²³⁴⁻²³⁶ in ischemic damage have all been observed after application of NOS inhibitors in different experimental models of brain ischemia. Further studies are needed to elucidate the involvement of NO in the pathophysiology of cerebral ischemia.

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SUMMARY

Accumulating evidence indicates an important role for ETs and NO in the regulation of cerebral perfusion. Attempts to understand the physiology and pathophysiology of the ET-NO network as delineated here have opened an exciting and promising area of clinical research with future therapeutic implications.

ACKNOWLEDGMENT

The author's work is supported by a grant from the DFG (Eh 133/1-2).

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