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INCREASED NITRIC OXIDE PRODUCTION MEDIATES ISCHEMIC BRAIN DAMAGE : DIRECT MEASUREMENT OF NITRIC OXIDE AND ENHANCED ACTIVITY OF NITRIC OXIDE SYNTHASE IN BRAIN MICROVESSELS AFTER CEREBRAL ISCHEMIA

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Types of encountered cerebral ischemia

Both clinical and experimental cerebral ischemia can be classified into two broad categories, i.e. global/forebrain and focal/regional ischemia. Cardiac arrest, for instance, induces typical global/forebrain ischemia since cerebral blood flow is completely dependent on the activity of the heart. This type of ischemia is further divided into two categories based on the grade of blood supply to the brain. One is termed "complete ischemia" in which no blood is supplied, while the other is termed "incomplete ischemia", and is characterized by a highly diminished global blood supply. Stenosis, thrombosis or occlusion of a specific artery causes the clinically typical syndromes of focal/regional ischemia related to that artery. A neurovascular syndrome resulting from occlusion of the middle cerebral artery (MCA), for example, includes contralateral hemiplegia, hemianesthesia and homonymous heminopia, as well as global aphasia in most patients afflicted with one side-lesions .

Ischemic brain damage incurred as a result of global ischemia is clearly different from that in MCA-occluded focal ischemia models. In the former, ischemia is usually very dense, and the insult is brief and transient. The damage incurred is conspicuously delayed. In the latter, the perifocal or "penumbra" zone of the MCA-related region has a more subtle deterioration of cellular energy metabolism than does the whole brain in clinical global ischemia. The pathogenetically important events are those occurring in the marginally ischemic penumbra zone. Thus, it is important to recognize the characteristic differences between these two types of cerebral ischemia and their symptomatic signs.

Glutamate neurotoxcity and nitric oxide synthase

Nitric oxide (NO) is one of the most ubiquitous and substantial biological mediators in mammalian cells. This freely diffusible and toxic gas is synthesized from the guanidino nitrogens of L-arginine by the enzyme NO synthase (NOS, E.C.1.14.13.39). At low concentrations, NO induces such physiological effects as vasorelaxation, inhibition of platelet aggregation and cell proliferation. At high concentration, however, NO leads to pathophysiological effects including target cell and target protein damage. Thus, NO appears to have double-edged roles in living systems.

It has become evident that during all types of global or focal cerebral ischemia there is an excessive accumulation of excitatory amino acids in the extracellular space, and subsequently an increased level of intracellular Ca^{2+}, predominantly by way of the *N*-methyl-D-aspartate (NMDA) class of glutamate receptor activation. These cellular events have a crucial triggering role in the elaboration of ischemic brain damage within the neurons. Intense efforts have been made to delineate the events activated by increased intracellular Ca^{2+} at the tissue, cellular and molecular levels. NOS in brain/neurons is one of the Ca^{2+}-dependent deoxygenases. The requirement of neuronal NOS for calmodulin (CaM), a Ca^{2+}-binding protein, exemplifies and facilitates elucidation of the relationship between this enzyme and the NMDA receptor.

To date, at least three distinct clones encoding independent NOS enzymes have been obtained. The deduced amino acid sequences of these NOS clones have predicted binding sites for NADPH, FMN, FAD and CaM. All of the NOS clones have been demonstrated to require L-arginine, molecular O_2 and NADPH as cosubstrates, and FMN, FAD, heme and

tetrahydrobiopterin as cofactors. Also, from a protein chemical or enzymological point of view, NOS enzymes are classifiable into three categories: *Type I* in brain/neurons (N-cNOS) (Mr = 150-160 kD) and *type III* in endothelial cells (E-cNOS) (Mr = 135 kD) are constitutively expressed and regulated by Ca^{2+} and CaM. *Type II* is Ca^{2+}-independent inducible NOS (iNOS, Mr = 130 kD) expressed on stimulation by endotoxins and/or cytokines in numerous cell types such as macrophages, glial cells and endothelial cells. Although cNOS in neurons and endothelial cells share common biological and features, they are structurally distinct. In contrast to E-cNOS which exists predominantly in the particulate fraction, the corresponding N-cNOS is found in both particulate and soluble fractions. Thus, at present, both genetically and in terms of protein chemistry, at least three distinct types of NOS isozymes have been found in mammalian cells.

Commonly recognized NOS inhibitors and their characteristics

As one of the substrates for NOS is L-arginine, it has been become evident that certain N^{G}-monosubstituted L-arginine analogs possess variable capabilities to suppress NOS activity. Among them, N^{G}-nitro-L-arginine (L-NNA), N^{G}-nitro-L-arginine methyl ester (L-NAME), N^{G}-monomethyl-L-arginine (L-NMMA), N^{G}-amino-L-arginine (L-AA), N^{G}-iminoethyl-L-ornithine (L-NIO), N^{G}-allyl-L-arginine (L-ALA), aminoguanidine (AG) have been extensively characterized.

However, no data has been presented that determines the potency and the selectivity of widespread NOS inhibitors on the three NOS isoforms based on the same method to measure NOS activities directly. To define this point, we investigated the concentrations of well-known NOS inhibitors required for 50 % inhibition (IC_{50}) by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline (6, 7). Rat brain cortical cytosolic fraction was used as the source of N-cNOS. Preliminary studies showed that the IC_{50} values of L-NNA, L-NAME and L-NMMA in this fraction were entirely similar to those in the brain cortical synaptosomes. In addition, Western blot analysis disclosed a single band corresponding to 155 kDa of N-cNOS in the rat brain cortical synaptosomes and cytosolic fractions. It seems reasonable, therefore, that the observed NOS activities in the cytosolic fraction mainly originate from neuronal NOS. Homogenates of cultured bovine pulmonary artery endothelial cells (CPAE) were employed to assess E-cNOS activity. For preparation of the iNOS source, rat lung cytosolic fraction was collected at 6 hr after i.p. injection of lipopolysaccharide (10 mg/kg). As a result, L-NNA was the most potent and AG the weakest inhibitor of N-cNOS among the compounds examined. However, L-NNA showed relatively high affinity for inhibition against E-cNOS. No inhibitor specific for only a certain type of NOS isoform was found among these compounds (7).

Effects of NOS inhibitors on global cerebral ischemia

Results of previous studies examining the effects of NOS inhibitors on global cerebral ischemia are inconsistent (7). Inasmuch as it is well accepted that NO synthesized by NOS in endothelial cells and/or smooth muscle cells plays a crucial role, at least in part, in maintaining physiological vascular tone and regulating regional cerebral blood flow (rCBF) or platelet aggregation, the dosage of NOS inhibitors would seem to be one vitally important factor in the interpretation of experimental results. In any event, when we consider the conflicting reports of the effects of NOS inhibitors on global ischemia, use-dependent studies of NOS inhibitors are required.

Therefore, we have made efforts to determine the therapeutic dosage of a NOS inhibitor in gerbils after transient forebrain ischemia (5, 7). To this end, L-NNA was used as the NOS inhibitor, because this compound was proven to have more potent inhibitory effects on brain cytosolic NOS activity than any other commercially available agent and it has been shown to cross the blood-brain barrier. L-NNA (0.3, 1, 3 and 10 mg/kg, i.p.) was administered to rats subjected to 10 min of left carotid artery occlusion seven times at 5 min, 3, 6, 24, 48, 72 and 96 hr after recirculation. Histopathological examination of the brain obtained 6 days after reperfusion showed that L-NNA ameliorates neuronal necrosis in the hippocampal CA1 with the optimal dose of 3 mg/kg. Treatment of ischemic animals with 10 mg/kg of L-NNA failed to elicit neuroprotective effects. This bell-shaped curve indicates that

the therapeutic window of NOS inhibitors against transient forebrain ischemia is critically small. Thus, the different doses of NOS inhibitors used in different studies may be at least partly responsible for the conflicting data obtained.

Effects of NOS inhibitors on focal cerebral ischemia

As in the case of global cerebral ischemia, studies of the effects of NOS inhibitors on focal ischemia in rodent models have yielded controversial results. The discrepancies in the previous data regarding the effects of NOS inhibitors on focal cerebral ischemia may originate in part from the differences in dosage.

To address this issue, we performed a dose-dependent study of the effects of L-NNA on brain edema formation at 48 hr post-MCA occlusion in rats (3, 4, 7). This study was undertaken because edema after cerebral ischemia is one of the most frequent problems encountered clinically. Treatment of ischemic animals with very low doses of L-NNA (0.03 mg/kg) at 5 min, 3, 6 and 24 hr after ischemia (total 0.12 mg/kg) surprisingly yielded the most profound ameliorative effects. Statistically significant protective effects were observed in the 0.01, 0.03, 0.1, 0.3 and 1 mg/kg groups. Treatment with 0.003, 3 and 10 mg/kg of L-NNA elicited no significant protective effects. This *U*-shaped response pattern strongly suggests that the therapeutic window of NOS inhibitors against focal cerebral ischemia is sharply restricted, as is the case also for their use in global ischemia. The experimental results discussed here provide a working rationale as to why puzzling and occasionally conflicting effects of NOS inhibitors on focal cerebral ischemia have been reported.

Direct measurement of nitric oxide during and after transient cerebral ischemia

No matter what the brain enzyme, if its inhibitor is proven to have ameliorative effects against ischemic brain damage, then it must be determined whether that enzyme is activated and/or the amount of its reaction products are increased by the ischemic insult. Therefore, we undertook an investigation into NO production during and after transient MCA occlusion in rats by means of a NO-sensitive microelectrode. A working electrode was made of Pt/Ir alloy 0.2 mm in diameter coated with a three-layered membrane consisting of KCl, NO-sensitive silicone resin, and normal silicone membranes, which made the electrode stronger and easier to manipulate than that developed previously. A reference electrode was made of carbon fiber. Although it is not possible to measure exactly the basal concentration of NO in the brain using this system, very interesting findings were obtained in our laboratories.

In summary, NO concentrations increased biphasically by 10 - 45 min and 1.5 - 4 hr after the induction of MCA occlusion in rats. The maximum NO concentrations corresponding to these peaks were about 1.7 and 0.5 μM, respectively. Restoration of blood supply following 2 hr MCA occlusion lead to an increase in NO concentration up to 1 μM by 1 to 2 hr after reperfusion. Administration of L-NNA (1 mg/kg, i.p.) at 5 min before and 3 hr after the onset of ischemia or at 5 min before the onset of ischemia and 1 hr after reflow caused a distinct inhibition of NO production during these periods and subsequently reduced brain infarct volume.

Enhancement of NOS activity in brain microvessels during and after transient cerebral ischemia

Now it has become clear that NO is indeed increased during and after transient cerebral ischemia, the next critical question concerns the cell types that possess the enhanced NOS activity and the types of NOS isozyme that are responsible for the increased concentration of NO. Our lab has been especially focused on brain microvessels for the following reasons. Firstly, the IC_{50} values of L-NNA for cNOS activity in brain cortical cytosolic fractions, brain cortical synaptosomes, and brain microvessels were almost equivalent each other. Secondly, Lineweaver-Burk plots showed that these three fractions possess a single binding site for L-[^{3}H]arginine with a similar Km value. Finally, L-NNA confers significant protection against vasogenic edema, in which disturbed endothelium function in brain microvessels is known to be involved (3, 4, 7).

It was shown that significant elevation (up to 906 % of the control) of Ca^{2+}-dependent cNOS activity in brain microvessels isolated from the ischemic hemisphere that manifested at 15 min returned to the control level at 4 hr after MCA occlusion in rats. Ca^{2+}-independent iNOS activity, on the other hand, invariably increased to 922 % and 920 % at 4 hr and 24 hr after ischemia, respectively (6). Reperfusion of the rCBF resulted in more than 5-fold increases in cNOS activity with no apparent alterations in iNOS activity (6, 7). These results appear to identify the sourse of the observed increases in NO concentrations at the acute phase of ischemia and reperfusion. Two major conclusions can be drawn from these results. Firstly, the cerebral microvessels possess two distinct types of NOS, i.e. Ca^{2+}-dependent cNOS and Ca^{2+}-independent iNOS, as does aortic endothelium and ventricular endothelium. Secondly, distinct types of NOS activity in brain microvessels were increased with different temporal patterns after MCA occlusion.

Possible mechanisms of nitric oxide-mediated ischemic brain injury

With regard to NO-mediated tissue damage during and after transient cerebral ischemia, three major cellular mechanisms have been proposed to date; formation of toxic free radical species, direct reaction with sulfhydryl groups and metal ions, and stimulation of neurotransmitter releases. Taking into account our data described above, we will try to summarize breifly (7).

Permanent ischemia, and reperfusion following transient ischemic insult cause cNOS activation in brain microvessels through agonist-dependent Ca^{2+}-entry and acidosis in the respective early phase. Thereafter, in the late phase of permanent ischemia, iNOS is also enhanced possibly by cytokine formation. This NO results in the disruption of microvascular function and barrier integrity in terms of the formation of toxic free radicals, $ONOO^-$, $OH\cdot$ and NO_2^- leading to edema formation. However, brain capillary endothelial cells are relatively resistant to these radicals compared to neurons and glial cells. Large amounts of NO produced in brain microvessels diffuse out and increase excitatory amino acid release via an inhibition of Na^+, K^+-ATPase (1, 2) from the adjacent nerve terminals. Abnormal accumulation of excitatory amino acids in the extracellular space induces overstimulation of glutamate receptors, allowing Ca^{2+}-influx both directly and indirectly. Once inside the cell Ca^{2+} binds to calmodulin, allowing it to enhance cNOS in neurons. In this way, NO invading directly from brain microvessels and that produced in target cells increases lipid peroxidation and decreases mitochondrial respiration, gycolysis and DNA replication, leading to neuronal cell death, and eventually infarction.

Conclusion remarks and future directions

Our continuing studies on the role of NO in cellular mechanisms of ischemic brain damage have raised the possibility that blockade of not only neuronal NOS but also microvascular NOS will come to be seen as a novel and powerful pharmacotherapeutic approach for the treatment of cerebral stroke. Further investigations into the temporal profiles of the activity itself, as well as of the mRNA expression of cNOS and iNOS following focal cerebral ischemia in neurons, glial and endothelial cells, will undoubtedly be necessary to fully elucidate the multiple mechanisms of this pathology. We are currently engaged in the design of experiments to address these questions.

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