NITRIC OXIDE IN CEREBRAL ISCHEMIA

Turgay Dalkara, M.D., Ph.D.,   Michael A. Moskowitz*, M.D.

Hacettepe University, Faculty of Medicine, Department of Neurology, Ankara, Turkey
*Massachusetts General Hospital and Harvard Medical School, Department of Neurology, Boston, MA, USA

Nitric oxide (NO) is synthesized from L-arginine and molecular oxygen by NO synthases (NOS). NOS has two constitutive, neuronal (Type 1) and endothelial (Type 3), and one inducible isoforms (Type 2) (1). In the brain, neuronal NOS (nNOS) is almost exclusively expressed in neurons and perivascular nerves, whereas endothelial isoform (eNOS) is mainly detected in endothelium of cerebral vessels with expression in small populations of neurons (2). The constitutive isoforms are calcium/calmodulin-dependent and activated by transient elevations in intracellular calcium (3). Small puffs of NO synthesized by constitutive NOS, regulates a wide variety of physiological functions such as blood pressure, vascular tone, permeability and neurotransmission. On the other hand, calmodulin is tightly bound to the inducible isoform (iNOS), and once expressed, iNOS is continuously active irrespective of intracellular calcium level and leads to a long-lasting, high output NO generation (4). iNOS can be induced in microglia, astrocytes, endothelium and vascular smooth muscle cells (5). High output NO synthesis by the inducible isoform is cytotoxic and mediates the inflammatory actions of NO (6). Like iNOS, nNOS can also generate high amounts of NO and cause cytotoxicity under pathological conditions due to an unregulated, persisting rise in intracellular calcium (7, 8). Although NOS positive neurons comprise only 1-2% of all the neurons in the cerebral cortex, corpus striatum and hippocampus, these neurons possess extensive axonal branching consistent with the idea that NOS positive cells kill neighboring neurons when NO or a reaction product is generated in excessive amounts (8, 9). Interestingly, these neurons

themselves are resistant to various insults including NMDA toxicity and ischemia, perhaps related to dismutation of superoxide and attendant inhibition of peroxynitrite anion formation, a potent cytotoxic mediator (10, 11). NO synthesized by nNOS has recently been implicated in many pathophysiological processes including cerebral ischemia and excitotoxicity (8). Using two strains of mutant mice that do not express the gene for the neuronal or the endothelial isoforms of NOS, we have documented the detrimental role of neuronal NO in cerebral ischemia and NMDA toxicity. The evidence will be reviewed below.

Knockout mice

Research over the last few decades has clearly documented the importance of gene deletion or overexpression as a promising alternative to pharmaceutical agents like enzyme inhibitors or receptor antagonists in elucidating the importance of a biomolecule. Several hundred mutant strains have been developed in the last decade (12, 13). Knockout mutant mice are generated by targeted disruption of one (sometimes two) of estimated 50-100.000 genes in the mouse genome by homologous reconstitution (14). The majority of the currently available mutants have "null" (or loss-of-function) alleles of the gene of interest. Null mutants have been developed to study the role of proteins during development, in adult life and disease states. The gene deletion was initially hoped to mimic specific and complete inhibition of the targeted protein. Surprisingly, many knockouts exhibited a normal phenotype due to redundancy of genes compensating for the deleted gene product (15). However, increasing evidence indicates that

despite an apparently normal phenotype, knockout animals may display conspicuous abnormalities in unanticipated tissues (15) and respond differently to various manipulations than wild type animals (16). They have been especially useful to confirm the pharmacological activity of drugs in whom selectivity is in question.

nNOS Mutant Mice :

Targeted disruption of nNOS gene was achieved by homologous recombination by substitution of a neomycin resistance gene for a critical exon (15). The mutant mouse develops normally and the endothelium of nNOS knockouts express eNOS immunoreactivity (16). In nNOS mutants, neuronal NOS expression and NADPH-diaphorase staining were markedly deficient, and in vitro NOS activity was significantly reduced to less than 5% of normal (15). Of interest, alternative splice variants of NOS have been reported which generate NO in vitro, but lack the PDZ-containing domain and potential coupling to the NMDA receptor (17, 18). However, the expressed splice variants do not compensate sufficiently as NOS activity in nNOS-/- brains is severely reduced. Consistent with this, low basal levels of (3H)L-NG-nitroarginine binding (19) and brain cGMP levels were found in mutants and no cGMP enhancement was observed during ischemia or hypercapnia, contrary to the robust increases in wild type mice (16, 20). Consistent results were also obtained by NMR spectroscopy as NO adducts were undetectable during cerebral ischemia as measured in nNOS null mice (Mullins et al, unpublished observations). Regional cerebral blood flow responses to hypercapnia (16) and whisker stimulation (21), and pial arterial dilation to topical ACh superfusion (22) were not significantly different from wild type mice. However, their responses to NOS inhibitors were markedly reduced.

Mechanisms of NO Mediated Neurotoxicity:

NO likely mediates neurotoxicity by several mechanisms including disruption of cellular metabolism (inhibition of aconitase, complex I and II of mitochondrial electron transport and glyceraldehyde-3-phosphate dehydrogenase) and of DNA synthesis (6, 7). NO also damages DNA structure (23). DNA damage may activate reparative enzymes such as poly-ADP ribose synthase (PARS), which can deplete the cell of ATP and nicotinamide dinucleotide, hence further compromising cellular energy metabolism (24). Inhibition of PARS activation or gene deletion confers protection to brain and possibly cardiac ischemia, apparently through an energy sparing mechanism (18, 25). One of the most attractive mechanisms involves peroxynitrite formation which is initiated via NMDA receptor activation, intracellular Ca^{++} increase, and augmented NO synthesis (26). Peroxynitrite (ONOO-) is formed by reaction of $NO^{.}$ with $O2^{.}$- (27, 28), and this complex rapidly decomposes into $NO2^{.}$ and hydroxyl radical ($OH^{.}$), or a reactive intermediate with $OH^{.}$-like activity (29). Hydroxyl radical is a highly reactive species which leads to oxidation of sulfhydryl groups, lipids, DNA, and proteins (30, 31). Peroxynitrite can directly inhibit glutamate transporters (32), or produce nitronium ions, causing irreversible nitration of tyrosine residues in proteins (31). Protein tyrosine nitration may contribute to $NO^{.}$ toxicity by reducing phosphorylation by tyrosine kinases (31) or targeting nitrated proteins for degradation (33).

NO was proposed as the neurotoxic agent mediating NMDA toxicity. Studies in dissociated cell cultures showed that NOS inhibitors effectively blocked NMDA-induced cell death whereas L-arginine depletion attenuated NMDA receptor-mediated toxicity (34). NMDA toxicity was also blocked by reducing NOS catalytic activity by calmodulin antagonists, flavoprotein inhibitors, inhibitors of NOS dephosphorylation, or by scavenging NO with hemoglobin or by increasing SOD activity to thereby reduce peroxynitrite anion formation (35). Based on these studies, it was hypothesized that an increase in intracellular calcium by ischemia-induced NMDA receptor overactivation increases NO. NO also reportedly inhibits glutamate uptake and mediates the sustained opening of NMDA receptor ion channels (36, 37).

NO and Cerebral Ischemia:

Cortical NO levels increase strikingly from approximately 10 nM to 2 ÊM within 3-24 minutes after middle cerebral artery (MCA) occlusion (38). Brain nitrite (stable NO metabolite) and cGMP (a product of NO-mediated guanylate cyclase activation) levels also rise within the first half hour of ischemia (39). These increases are effectively blocked by prior L-NG-nitroarginine

administration, indicating an enhanced NOS activity. It is likely that constitutive NOS activity increases during ischemia due to a rise in intracellular Ca++. However, constitutive NOS activity decreases shortly after its activation at the onset of ischemia possibly due to inactivation of the enzyme by unfavorable conditions in ischemic tissue.

nNOS Mutant Mice and Cerebral Ischemia:

The deficiency in neuronal NO production was associated with increased resistance to cerebral ischemia. The nNOS knockouts developed infarcts 38% smaller than the wild type mice when subjected to 24 hour permanent MCA occlusion (20). Infarct size was also reduced in mutants 3 to 4 days after permanent MCA occlusion. Neurological deficits were less in nNOS knockout mice. Since reductions in regional cerebral blood were similar within homologous ischemic regions after MCA occlusion in both groups, the observed group differences were attributed to the consequence of neuronal NOS deletion in brain tissue and not due to hemodynamic differences between strains.

The neuroprotective action obtained after deletion of neuronal NOS has also been demonstrated in mice subjected to 3 hours of ischemia and 24 hours of reperfusion. Infarcts were 69% smaller in nNOS knockouts than wild type mice (40). Brain protection was greater than after permanent occlusion possibly due to greater superoxide anion production during reversible occlusion (41, 42). Interestingly, quantitative (3H)L-NG-nitroarginine autoradiography demonstrated a significant increase (50-250%) in the density of (3H)L-NG-nitroarginine binding sites (Bmax) but not the dissociation constant (Kd), during transient focal ischemia and first 3 hours of reperfusion (43). nNOS mRNA was also increased as detected by reverse transcription polymerase chain reaction. Similar results were obtained after NMDA injection, with a more extended time course (12-48 hours). As noted above, (3H)L-NG-nitroarginine binding to nNOS protein was very low in nNOS mutant mice, and only a very small increase was observed after ischemia or NMDA excitotoxicity.

nNOS mutants were also protected against global ischemia (44). Fewer dead hippocampal neurons were counted 3 days after transient global ischemia induced by bilateral common carotid plus basilar artery occlusion for 5 or 10 mins. Not only were more hippocampal cells viable, but overall morbidity, weight loss, and neurological outcome were better in the mutant strain.

In addition to neurons, perivascular nerves and cerebrovascular endothelium may form NO during cerebral ischemia. A late but sustained increase in NO levels may also occur due to expression of inducible NOS within microglia and invading inflammatory cells 24-72 hours after the induction of ischemia (45). During the immediate period following ischemia, increased NO production in vascular endothelium or perivascular nerves may improve blood flow and be neuroprotective. Indeed, infusion of L-arginine, which increases NO production and dilates pial vessels via an NO-dependent mechanism, and increases rCBF in normal as well as in ischemic brain, reduces infarct size (46). CBF augmentation can lead to electrocorticogram recovery if blood flow enhancement exceeds the functional flow threshold of approximately 30% of pre-ischemic level (47). Blood flow may also be increased within ischemic tissue by intracarotid administration of NO donors and decreases infarct size in models of focal ischemia (48). In addition, endothelial NO production may also improve microcirculation by reducing platelet aggregation and leukocyte adhesion (49).

eNOS Mutant Mice :

Consistent with the above evidence larger infarcts developed in eNOS knockout mice after 24-hour permanent MCA occlusion (50). Deletion of eNOS rendered these mutants hypertensive (51). However, hypertension per se did not account for the increased susceptibility of eNOS mutants because infarct size did not decrease after blood pressure was reduced by hydralazine (50). eNOS mutants developed more pronounced rCBF reductions in corresponding brain regions after MCA occlusion and exhibited proportionally lower rCBFs at reduced perfusion pressures during controlled hemorrhagic hypotension (50). Dynamic CT scanning demonstrated that areas of hemodynamic penumbra were significantly smaller and core relatively larger in eNOS mutants (52). Hence, the susceptibility of eNOS mutants to ischemic injury may be due to its diminished capacity to adapt to reduced perfusion pressure

(i.e., dilate) at the margins of an ischemic lesion. This coupled to enhanced platelet and neutrophil adhesion, render eNOS mutants more susceptible to injury. Consistent with this notion, L-NG-nitroarginine administration increased infarct size in the nNOS knockout mouse, presumably due to inhibition of the constitutively expressed eNOS isoform (50).

Increases as well as reductions in the extent of tissue injury have been reported after L-NAME or L-NG-nitroarginine administration in models of ischemia with MCA occlusion, possibly because nonselective inhibition of NO synthesis within vessels and platelets may obscure neuroprotective effects of NOS inhibition in neurons during focal cerebral ischemia.(53). In fact, recent studies using selective nNOS inhibitors, 7-nitroindazole (54) or ARL17477 (55) showed neuroprotection.

NO-Induced Apoptotic Cell Death:

At least two mechanistically distinct forms of neuronal death have been identified. Severely injured neurons that do not immediately die by swelling and lysis may ultimately undergo apoptosis. Apoptotic neuronal death contributes to infarct formation in cerebral ischemia (56-59). NMDA, or concurrent generation of nitric oxide and superoxide can cause both necrosis and apoptosis, depending on the severity of the insult and resulting mitochondrial dysfunction (60, 61). Both NO and ONOO- have been linked to apoptosis (62-65). Since blockade of neuronal NO synthesis proves to be neuroprotective in focal and global cerebral ischemia and NMDA toxicity, in vivo, it is important to understand the extent to which NO-induced apoptosis contribute to neuronal death under these pathological conditions.

We performed TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling) and compared the number of apoptotic cells in wild type and nNOS mutant mice 6, 24 and 72 hours after permanent MCA occlusion by filament. TUNEL positive neurons were detected at 6 hours and continued to increase by 72 hours. These cells were detected in both penumbra and core regions but most of them were located on the inner boundary zone of the infarct. Numbers of apoptotic cells as well as their density were significantly lower in nNOS mutants, suggesting a selective decrease in the number of TUNEL positive cells. Such findings support the

notion that NO and its reaction products promote apoptosis as a mechanism of cytotoxicity as suggested by studies reporting apoptotic cell death after application of NO· or peroxynitrite (60, 62, 64-67). Precisely how the development of apoptosis in ischemia relates to caspase activation (40) and cleavage of PARP remains for further study.

REFERENCES

1. Griffith OW, Stuehr DJ : Nitric oxide synthases: Properties and catalytic mechanism. Ann Rev Physiol 1995; 57 : 707-736.
2. Iadecola C, Pelligrino DA, Moskowitz MA, Lassen NA : Nitric oxide synthase inhibition and cerebrovascular regulation. J Cereb Blood Flow Metab 1994; 14 : 175-192.
3. Knowles RG, Moncada S : Nitric oxide as a signal in blood vessels. Trends Biochem Sci 1992; 17 : 399-402.
4. Cho HJ, Xie QW, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Nathan C : Calmodulin as a tightly bound subunit of calcium-calmodulin independent nitric oxide synthase. J Exp Med 1992; 176 : 599-604.
5. Murphy S, Simmons ML, Agullo L, Garcia A, Feinstein DL, Galea E, Reis DJ, Minc-Golomb D, Schwartz JP : Synthesis of nitric oxide in CNS glial cells. Trends Neurosci 1993; 16 : 323-328.
6. Gross SS, Wolin MS : Nitric oxide: Pathophysiological mechanisms. Ann Rev Physiol 1995; 57 : 737-769.
7. Dawson TM, Dawson VL, Snyder SH : A novel neuronal messenger in brain: the free radical, nitric oxide. Ann Neurol 1992; 32 : 297-311.
8. Dawson TM, Dawson VL : Nitric oxide: Actions and pathological roles. The Neuroscientist 1995; 1 : 7-18.
9. Bredt DS, Glatt EC, Hwang PM, Fotuhi M, Dawson TM, Snyder SH : Nitric oxide synthase protein and mRNA discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. Neuron 1991; 7, 615-624.
10. Koh J, Choi DW : Vulnerability of cultured cortical neurons to damage by excitotoxins: differential susceptibility of neurons containing NADPH-diaphorase. J Neurosci 1988; 8 : 2153-2163
11. Uemura Y, Kowall NW, Beal MF : Selective sparing of NADPH-diaphorase-somatostatin-neuropeptide Y neurons in ischemic gerbil striatum. Ann Neurol 1990; 27 : 620-625.
12. Thomas KR : The knockout mouse: Six years old and growing stronger. Am J Respir Cell Mol Biol 1995; 12 : 461-463.

13. Rubin EM, Barsh GS : Biological insights through genomics: Mouse to man. J Clin Invest 1996; 97 : 275-280.

14. FÑssler R, Martin K, Forsberg E, Litzenburger T, Iglesias A : Knockout mice: How to make them and why. The immunological approach. Int Arch Allergy Immunol 1995; 106 : 323-334.

15. Huang PL, Dawson TM, Bredt DS, Snyder SH, Fishman, MC : Targeted disruption of neuronal nitric oxide synthase gene. Cell 1993; 75 : 1773-1286.

16. Irikura K, Huang PL, Ma J, Lee WS, Dalkara T, Fishman MC, Dawson TM, Snyder SH, Moskowitz MA : Cerebrovascular alterations in mice lacking neuronal nitric oxide synthase gene expression. Proc Natl Acad Sci USA 1995; 92 : 6823-6827.

17. Brenman JE, Chao DS, Gee SH, McGee AW, Craven SE, Santillano DR, Wu Z, Huang F, Xia H, Peters MF, Froehner SC, Bredt DS : Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and a1-syntrophin mediated by PDZ domains. Cell 1996; 84 : 757-767.

18. Eliasson MJL, Blackshaw S, Schell MJ, Snyder SH : Neuronal nitric oxide synthase alternatively spliced forms: prominent functional localizations in the brain. Proc Natl Acad Sci USA 1997; 94 : 3396-3401.

19. Hara H, Ayata C, Huang PL, Waeber C, Ayata G, Fujii M, Moskowitz MA : (3H)L-NG-nitroarginine binding after transient focal ischemia and NMDA-induced excitotoxicity in type I and type III nitric oxide synthase null mice. J Cereb Blood Flow Metab 1997; 17 : 515-526.

20. Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC, Moskowitz MA : Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science 1994; 265 : 1883-1885.

21. Ma J, Ayata C, Huang P, Fishman MC, Moskowitz MA : Regional cerebral blood flow response to vibrissal stimulation in mice lacking type I NOS gene expression. Am J Physiol 1996; 270 : H1085-H1090.

22. Meng W, Ma J, Ayata C, Hara H, Huang PL, Fishman MC, Moskowitz MA : ACh dilates pial arterioles in endothelial and neuronal NOS knockout mice by NO-dependent mechanisms. Am J Physiol 1996; 271 : H1145-H1150.

23. Liu RH, Hotchkiss JH : Potential genotoxicity of chronically elevated nitric oxide: a review. Mutation Res 1995; 339 : 73-89.

24. Zhang J, Dawson VL, Dawson TM, Snyder SH : Nitric oxide activation of poly (ADP-ribose) synthase in neurotoxicity. Science 1994; 263 : 686-689.

25. Hara H, Fink K, Endres M, Friedlander RM, Gagliardini V, Yuan J, Moskowitz MA : Attenuation of transient focal cerebral ischemic injury in transgenic mice expressing a mutant ICE inhibitory protein. J Cereb Blood Flow Metab 1997; 17 : 370-375.

26. Garthwaite J, Charles SL, Chess-Williams R : Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 1988; 336 : 385-388.

27. Lafon-Cazal M, Pietri S, Culcasi M, Bockaert J : NMDA-dependent superoxide production and neurotoxicity. Nature 1993; 364 : 535-537.

28. Dykens JA : Isolated cerebral and cerebellar mitochondria produce free radicals when exposed to elevated Ca2+ and Na+: Implications for neurodegeneration. J Neurochem 1994; 63 : 584-591.

29. Crow JP, Spruell C, Chen J, Gunn C, Ishciropoulos H, Tsai M, Smith CD, Radi R, Koppenol WH, Beckman JS : On the pH dependent yield of hydroxyl radical products from peroxynitrite. Free Rad Biol Med 1994; 16 : 331-338.

30. Beckman JS : Peroxynitrite versus hydroxyl radical: The role of nitric oxide in superoxide-dependent cerebral injury. Ann N Y Acad Sci 1994; 738 : 69-75.

31. Beckman JS, Y, YZ, Chen J, Conger KA : The interaction of nitric oxide with oxygen radicals and scavengers in cerebral ischemic injury. Adv Neurol 1996; 71 : 339-350.

32. Trotti D, Rossi D, Gjesdal O, Levy LM, Racagni G, Danbolt NC, Volterra A : Peroxynitrite inhibits glutamate transporter subtypes. J Biol Chem 1996; 271 : 5976-5979.

33. Gow AJ, Duran D, Malcolm S, Ischiropoulos H : Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation. FEBS letters 1996; 385: 63-66.

34. Dawson VL, Dawson TM, London ED, Bredt DS, Snyder SH : Nitric oxide mediate glutamate neurotoxicity in primary cortical culture. Proc Natl Acad Sci USA 1991; 88: 6368-6371.

35. Dawson VL, Dawson TM, Bartley DA, Uhl GR, Snyder SH : Mechanisms of nitric oxide mediated neurotoxicity in primary brain cultures. J Neurosci 1993; 13 : 2651-2661.

36. Pogun S, Dawson V, Kuhar MJ : Nitric oxide inhibits 3H-glutamate transport in synaptosomes. Synapse 1994; 18 : 21-26.

37. Akira T, Henry B, Wasterlain CG : Nitric oxide mediates the sustained opening of NMDA receptor-gated ionic channels which follows transient excitotoxic exposure in hippocampal slices. Brain Res 1994; 652 : 190-194.

38. Malinski T, Bailey F, Zhang ZG, Chopp M : Nitric oxide measured by a porphyrinic microsensor in rat brain after transient middle cerebral artery occlusion. J Cereb Blood Flow Metab 1993; 13 : 355-358.

39. Kader A, Frazzini VI, Solomon RA, Trifiletti RR : Nitric oxide production during focal cerebral ischemia. Stroke 1993; 24 : 1709-1716.

40. Hara H, Huang PL, Panahian N, Fishman MC, Moskowitz, MA : Reduced brain edema and infarction volume in mice lacking the neuronal isoform of nitric oxide synthase after reversible MCA occlusion. J Cereb Blood Flow Metab 1996; 16 : 605-611.

41. Chan PH, Kamii H, Yang G, Gafni J, Epstein CJ, Carlson E, Reola L : Brain infarction is not reduced in SOD-1 transgenic mice after a permanent focal cerebral ischemia. NeuroReport 1993; 5 : 293-296.

42. Yang G, Chan P, Chen J, Carlson E, Chen SF, Weinstein P, Epstein CJ, Kamii H : Human copper-zinc superoxide dismutase transgenic mice are highly resistant to reperfusion injury after focal cerebral ischemia. Stroke 1994; 25 : 165-170.

43. Hara H, Friedlander RM, Gagliardini V, Ayata C, Fink K, Huang Z, Shimizu-Sasamata M, Yuan J, Moskowitz MA : Inhibition of interleukin 1´-converting enzyme family proteases reduces ischemic and excitotoxic neuronal damage. Proc Natl Acad Sci USA 1997; 94 : 2007-2012.

44. Panahian N, Yoshida T, Huang PL, Hedley-Whyte ET, Dalkara T, Fishmon MC, Mokowitz MA : Attenvated hip-pocompol damage after global cerebral ischemia in mice mutant in neuronal nitric oxide synthase. Neurosci 1996; 72 : 343-354.

45. Iadecola C, Xu X, Zhang F, el-Fakahany EE, Ross ME : Marked induction of calcium-independent nitric oxide synthase activity after focal cerebral ischemia. J Cereb Blood Flow Metab 1995; 15 : 52-59.

46. Morikawa E, Moskowitz MA, Huang Z, Yoshida T, Irikura K, Dalkara T : L-arginine infusion promotes nitric oxide-dependent vasodilatation, increases regional cerebral blood flow, and reduces infarction volume in the rat. Stroke 1994; 25 : 429-435.

47. Dalkara T, Morikawa H, Moskowitz MA, Panahian N : Blood flow-dependent functional recovery in a rat model of cerebral ischemia. Am J Physiol 1994; 267 : H837-838.

48. Zhang F, White JG, Iadecola C : Nitric oxide donors increase blood flow and reduce brain damage in focal cerebral ischemia: evidence that nitric oxide is beneficial in the early stages of cerebral ischemia. J Cereb Blood Flow Metab 1994; 14 : 217-26.

49. Niu XF, Smith CW, Kubes P : Intracellular oxidative stress induced by nitric oxide synthesis inhibition increases endothelial cell adhesion to neutrophils. Circ Res 1994; 74 : 1133-1140.

50. Huang Z, Huang PL, Ma J, Meng W, Ayata C, Fishman MC, Moskowitz MA : Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine. J Cereb Blood Flow Metab 1996; 16 : 981-987.

51. Huang PL, Huang Z, Mashimo M, Bloch KD, Moskowitz MA, Bevan JS, Fishman MC : Hypertension in mice lacking the gene for nitric oxide synthase. Nature 1995; 377 : 239-242.

52. Lo EH, Hara H, Rogowska J, Trocha M, Pierce AR, Huang PL, Fishman MC, Wolf GL, Moskowitz MA : Temporal correlation mapping analysis of the hemodynamic penumbra in mutant mice deficient in endothelial nitric oxide synthase gene. Stroke 1996; 27 : 1381-1385.

53. Dalkara T, Moskowitz MA : Complex role of nitric oxide in cerebral ischemia. Brain Pathol 1994; 4 : 49-57.59.

54. Yoshida T, Limmroth V, Irikura K, Moskowitz M : The NOS inhibitor 7-nitroindazole decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. J Cereb Blood Flow Metab 1994; 14 : 924-929.

55. Zhang ZG, Reif D, Macdonald J, Tang WX, Kamp DK, Gentile RJ, Shakespeare WC, Murray RJ, Chopp M : ARL 17477, a potent and selective neuronal NOS inhibitor decreases infarct volume after transient middle cerebral artery occlusion in rats. J Cereb Blood Flow Metab 1996; 16 : 599-604.

56. Li Y, Chopp M, Jiang N, Yao F, Zaloga C : Temporal profile of in situ DNA fragmentation after middle cerebral artery occlusion in the rat. J Cereb Blood Flow Metab 1995; 15 : 389-397.

57. Li Y, Chopp M, Jiang N, Zhang ZG, Zaloga C : Induction of DNA fragmentationafter 10 to 120 minutes of focal cerebral ischemia in the rat. Stroke 1995; 26 : 1252-1258.

58. Li Y, Sharov VG, Jiang N, Zaloga C, Sabbah HN, Chopp M : Ultrastructural and light microscopic evidence of apoptosis after middle cerebral artery occlusion in the rat. Am J Pathol 1995; 146 : 1045-1051.

59. Charriaut-Marlangue C, Margaill I, Represa MA, Popovici T, Plotkine M, Ben-Ari Y : Apoptosis and necrosis after reversible focal ischemia: an in situ DNA fragmentation analysis. J Cereb Blood Flow Metab 1996; 16 : 186-195.

60. Bonfoco E, Krainc D, Ankarcrona M, Nicotera P, Lipton SA : Apoptosis and necrosis: Two distinct events induced, respectively, by mild and intense insults with N-methy-D-aspartate or nitric oxide/superoxide in cortical cell cultures. Proc Natl Acad Sci (USA) 1995; 92 : 7162-7166.

61. Ankarcrona M, Dypbukt JM, Bonfoco E, Zhivotovsky B., Orrenius S, Lipton SA, Nicotera P : Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. Neuron 1995; 15 : 961-973.

62. Albina JE, Cui S, Mateo RB, Reichner JS : Nitric oxide-mediated apoptosis in murine peritoneal macrophages. J Immunol 1993; 150 : 5080-5085.

63. Ankarcrona M, Dypbukt JM, Brune B, Nicotera P : Interleukin-1 beta-induced nitric oxide production activates apoptosis in pancreatic RINm5F cells. Exp Cell Res 1994; 213 : 172-177.

64. Estevez AG, Radi R, Barbeito L, Shin JT, Thompson JA, Beckman JS : Peroxynitrite-induced cytotoxicity in PC12 cells: evidence for an apoptotic mechanism differentially modulated by neurotrophic factors. J Neurochem 1995; 65: 1543-1550.

65. Lin K.T, Xue JY, Nomen M, Spur B, Wong PYK : Peroxynitrite-induced apoptosis in HL-60 cells. J Biol Chem 1995; 270 : 16487-16490.

66. Ratan RR, Murphy TH, Baraban JM : Oxidative stress induces apoptosis in embryonic cortical neurons. J Neurochem 1994; 62 : 376-379.

67. Palluy O, Rigaud M : Nitric oxide induces cultured cortical neuron apoptosis. Neurosci Lett 1996; 208 : 1-4.