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Review

The Role of Nitric Oxide in the Pathogenesis of Brain Lesions During the Development of Alzheimer's Disease

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Abstract. *Nitric oxide (NO) is a key bioregulatory active molecule in the cardiovascular, immune and nervous systems, synthesized through converting L-arginine to L-citrulline by NO synthase (NOS). Research exploration supports the theory that this molecule appears to be one of the key factors for the disruption of normal brain homeostasis, which causes the development of brain lesions and pathology such as in Alzheimer's disease (AD). Especially the vascular content of NO activity appears to be a major contributor to this pathology before the overexpression of NOS activity in other brain cellular compartments develop. We theorize that pharmacological intervention using NO donors and/or NO suppressors should delay or minimize brain lesion development and further progression of brain pathology and dementia.*

There are many common underlying risk factors that play key roles in the development of cardiovascular, cerebrovascular and neurodegenerative diseases [for review and references see (5,30,31)].

Nitric oxide (NO) is an important bioregulatory molecule in the nervous, immune and cardiovascular systems. NO is synthesized by the conversion of L-arginine to L-citrulline with the enzyme NO synthase [NOS (55)]. The three prototypical forms of NOS are neuronal, cytokine-inducible and endothelial (NOS1, NOS2 and NOS3, respectively). The three isoforms are derived from separate genes and are regulated by diverse signaling pathways (65). NO also inhibits enzymes in target cells and can interact with oxygen-derived radicals or free oxygen species (ROS) to produce other toxic substances. Thus, NO also plays a role in immunological host defense and in the pathophysiology of certain clinical conditions (55).

The role of NO in brain homeostasis has been extensively reviewed more recently (34). Experiments demonstrate that NO exerts several actions in the cerebral cortex. NO production is mediated by neuronal activity through at least two pathways: N-metylD-aspartate (NMDA) receptors and alpha-amino-3-hydroxy-5-metyl-4-isoxazoleproprionic acid (AMPA) receptors. NO can interact with synapses that are near the production site but not necessarily anatomically connected to the NO source by a conventional synaptic linkage by virtue of its diffusion in extracellular space. NOS's primary action is amplification of the release of the excitatory neurotransmitter, L-glutamate, thus effectively creating a positive feed-forward gain system. However, a number of effective brakes, presumably activated under physiological conditions, serve to limit the cascade (45). These include the ability of NO to inhibit NMDA receptors, its negative feedback on the rate-limiting NOS (59,61,62) and other inhibitory actions. In this review we outline the potential role of excess NO production and its oxidation products on the pathobiology of brain lesions as an accelerating factor for stimulating cerebrovascular diseases, especially Alzheimer's Disease (AD).

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The role of NO isoforms in regulation of cerebrovascular tone

The role of endothelial cells (EC) in the control of vascular tone is mediated by the synthesis and release of vasoactive substances such as the endothelium-derived vasodilator NO (53,56) and vasoconstrictor endothelin-1 (ET-1) (76). NO

regulates vascular tone, platelet aggregation, leukocyte adhesion, SMC proliferation, synaptic neurotransmission and cytotoxic/cytostatic actions of macrophages (24,25,27,28,54,56,71,74,75). This labile molecule may carry out important biological roles, both within the cell in which it is synthesized and by interacting with nearby cells and molecules (4).

Three distinct isoforms of NOS derived from different genes generate NO: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3) (2,53,56,63,65,71). These isoforms are similar in structure and function (2,53,65,70,71).

eNOS was first purified and cloned from vascular endothelium, but has also been discovered in cardiac myocytes, blood platelets, brain cells (2,54,56,57,65,75) and in subcellular compartments such as mitochondria (8). eNOS is unique among the NOS family of proteins due to the presence of an N-myristoylation consensus sequence elucidated from the cloning of its cDNA. Although eNOS was metabolically labelled with [^{3}H] myristic acid and mutation of glycine 2 in the N-myristoylation consensus sequence changed the particular localization of the enzyme to a cytosolic form, the definitive characterization of eNOS as an N-myristoylprotein has not been clarified (51). eNOS is regulated in part through specific protein interactions. Dynamin-2 is a large GTPase residing within similar membrane compartments as eNOS. It has been shown that dynamin-2 binds directly with eNOS thereby augmenting eNOS activity (21). Double label confocal immuno-fluorescence microscopy demonstrates colocalization of eNOS and dynamin in both Clone 9 cells cotransfected with green fluorescent protein-dynamin and eNOS, as well as in bovine aortic EC (BAEC) expressing both proteins endogenously, predominantly in a Golgi membrane distribution (21). Immunoprecipitation of eNOS from BAEC lysate coprecipitates dynamin and, conversely, immunoprecipitation of dynamin coprecipitates eNOS. Additionally, the calcium ionophore, a reagent that promotes NO release, enhances co-precipitation of dynamin with eNOS in BAEC, suggesting the interaction between the proteins can be regulated by intracellular signals (21). These events may have relevance for eNOS regulation and trafficking within vascular endothelium (21). Additionally, GST-dyn-2 PRD binds the *in vitro* transcribed ^{35}S-eNOS reductase domain but not the ^{35}S-eNOS oxygenase domain. Furthermore, GST-dyn-2 proline-rich domain (PRD) binds a ^{35}S-labelled eNOS reductase domain fragment (amino acids 645-850) that partially overlaps with the FAD binding domain of eNOS(22). A recombinant form of the SH^3-containing protein Fyn competes the binding of recombinant eNOS protein with dyn-2 PRD, thereby implicating the SH^3-like region contained within this reductase domain fragment as the dyn-2 binding region. Mammalian two-hybrid screen corroborates these interactions in cells as well. Functional studies demonstrate that dyn-2 PRD selectively potentiates eNOS activity in a concentration-dependent manner in an order of magnitude similar to that observed with dyn-2 full-length and in a manner that requires calmodulin. Although dyn-2 PRD does not influence eNOS oxygenase domain function or ferricyanide reduction, it does potentiate the ability of recombinant eNOS to reduce cytochrome c (COX), supporting an influence of dyn-2 PRD on electron transfer between FAD and FMN (22). These data indicate that the binding domains of dyn-2 and eNOS reside within the dyn-2 PRD domain and the FAD binding region of the eNOS reductase domains, respectively, and that dyn-2 PRD is sufficient to mediate dyn-2-dependent potentiation of eNOS activity, at least in part, by potentiating electron transfer (22).

NOS activity is a major determinant of vascular tone and blood pressure and is altered in diseases such as hypertension, diabetes, atherosclerosis, ischemia/reperfusion and AD (1,32). A dynamic balance of relaxing and constricting factors regulates cerebrovascular tone. Constitutively produced NO normally influences basal cerebral vascular tone and mediates vascular responses to diverse stimuli (33) and cerebral vasodilation (34). Impaired endothelium-dependent relaxation of cerebral blood vessels has been observed during chronic hypertension, diabetes, hypercholesterolemia, subarachnoid hemorrhage (SAH) and ischemia (2,34). Moreover, the accumulating body of evidence strongly supports the idea that NO is also involved in regulating the cerebral circulation during hypercapnia (40) and focal (10,19,20,25,64) or global brain ischemia (7,11,36,41-44,58). Iadecola (40) showed that arginine-derived NO mediates the powerful effects of CO_2 on cerebral circulation. NO, synthesized by the action of nNOS, participates in regulating basal cerebral blood flow (CBF) and is the major contributor to the hypercapnic CBF response (73). Chronic inhibition of constitutive NO production increases EC permeability during various vascular diseases (2). Due to its vascular effect, NO might improve tissue perfusion and exert a protective action. Chronic imbalance in NO activity plays a key role during the brain hypoperfusion and the consequences of this activity causes neurodegeneration such as AD (32). However, the regulation of vascular tone is influenced by other multiple vasoactive substances released by the endothelium such as endothelial-derived hyperpolarizing factors, prostacyclin and vasoconstrictor factors. There is also abundant evidence that these factors are altered by pathophysiologic states, although the mechanisms responsible are not as well understood as they seem to be for the NO system. It has been strongly debated that several endothelial-derived vasodilators, including hyperpolarizing factors, may exist.

One of these factors is almost certainly the cytochrome p450 metabolite of arachidonic acid, epoxyeicosatrienoic acid (EET) (38,38), whereas another is probably H_2O_2, which stimulates potassium channel opening in a fashion similar to EET (38). EET has anti-inflammatory properties, whereas H_2O_2 may potentially enhance inflammation and promote vascular hypertrophy. Thus, two factors released by the endothelium, with similar acute effects on the vascular smooth muscle, may have very different long-term consequences in terms of protecting against or promoting vascular disease.

During the past two decades, we have gained a substantial understanding of the L-arginine/eNOS/NO pathway and how this modulates vascular reactivity (29-32). Further, scientists and physicians are now aware that this process is altered by many risk factors for atherosclerosis as well as brain disorders and have begun to understand how these disorders alter NO production and bioavailability. These abnormalities are probably multifactorial and scientists are beginning to understand how they can be corrected. An exciting aspect of endothelial function is that it has prognostic significance above and beyond the traditional risk factors for atherosclerosis. Several studies have shown that individuals with intact endothelial function in either the forearm or the coronary circulation have a low incidence of events during follow-up periods, whereas those individuals with abnormal endothelial function have a high incidence of major cardiovascular events (6,38). Because of the complexity of abnormalities that underlie endothelial dysfunction, there are various therapeutic targets that may have to be addressed to improve endothelial function and ultimately improve prognosis in these individuals (38). Recent evidence indicates that NO and the mitogenic peptide angiotensin II (Ang II) have been implicated in endothelial cell growth. However, the putative relationship between these two opposing agents with respect to endothelial cell growth remains unknown. Bayraktutan and Ulker recently showed that treatments of coronary microvascular endothelial cells (CMEC) with Ang II or Ca^{2+} ionophore A23187 equally increased NO production without altering the fold-difference in the basal release of NO from proliferating *vs.* confluent CMEC. Valsartan abolished NO production in CMEC treated with Ang II but not Ca^{2+} ionophore A23187 (12). Treatments of endothelium-intact vascular rings with Ang II (from 1- to 10 micromol/l) plus valsartan or PD-123319, an Ang II type 2 (AT_2) receptor inhibitor, attenuated vascular responses to acetylcholine in an Ang II dose-dependent manner. In these rings, phenylephrine produced significant increases in contractile responses only at nmol/l concentrations of Ang II. In contrast, pharmacological and mechanical inactivation of endothelium enhanced contractile responses to phenylephrine at micromol/I concentrations of Ang II. These data demonstrate that Ang II stimulates NO production in CMEC in both an AT_1- and an AT_2 receptor-regulated manner, and that this stimulation of NO may be beneficial in counterbalancing the direct vasoconstrictor effect of Ang II on underlying smooth muscle cells (12).

The overproduction, either by activation of nNOS by excitatory aminoacids (35), or by induction of iNOS in glial, vascular or blood cells (7,41,42,44) during the ischemic episodes, might be deleterious. eNOS knock-out mice show that the NO synthesized by eNOS protects against ischemic damage by increasing blood flow, whereas NO produced by nNOS contributes to lesions (37,39). The inhibition of NO synthesis by EC leads to increased intracellular oxidative stress, which induces neutrophil-EC interactions (2,3,5) and may promote the development and progression of vascular diseases such as atherosclerosis (2) and ischemia/reperfusion injury (3). Shin and coauthors have investigated the expression of both constitutive and inducible forms of nitric oxide synthase (NOS) by immunohistochemical staining of formalin-fixed paraffin-embedded sections in normal and *Listeria monocytogenes*-infected brains of goats (66). Their findings suggest that normal caprine brain cells, including neurons, constitutively express iNOS and nNOS, and the expressions of these molecules are increased in *Listeria monocytogenes* infections. Furthermore, inflammatory cells, including macrophages, expressing both nNOS and iNOS, may play important roles in the pathogenesis of bacterial meningoencephalitis in goat (66). However, the overexpression of iNOS during the development and maturation of AD is still unknown.

Expression of nNOS in the entorhinal cortex and hippocampus is affected in AD (72). Dimethylargininase, primarily expressed in tissues containing the constitutive forms of NOS like brain, kidney and endothelium (17,46,47), regulates NO production by hydrolyzing free methylated arginine derivatives (effective endogenous inhibitors of NOS) (52). The expression of dimethylargininase is dramatically increased during AD (69). The presence of dimethylargininase abnormalities in the AD brain is not surprising since nitration, resulting from peroxynitrite or peroxynitrate, is increased in all neurons at risk of dying due to AD (13,14,16,67). However, the ultrastructural localization of dimethylargininase immunoreactivity in different cellular compartments of the AD brain or in transgenic animal models of AD has yet to be described.

Vascular endothelium constitutively generates NO in large vessels and induces a relaxation of smooth muscle cells. However, little is known about the production of NO in microvessels *via* the different isoforms of NOS, where smooth muscle layers are thin or absent. Kimura and coworkers demonstrated that constitutive production

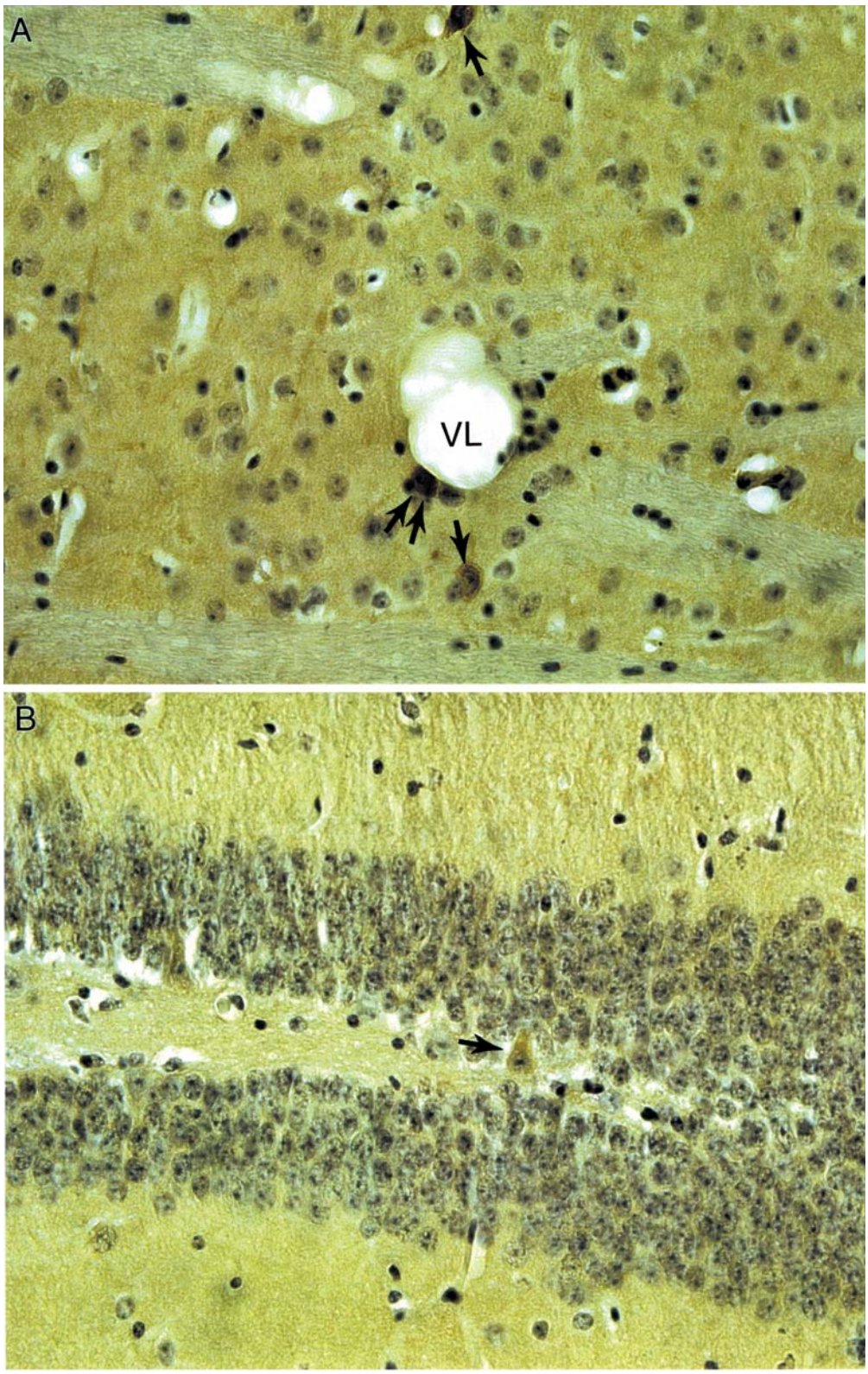

Figure 1. *The features of nNOS immunoreactivity in aged control wild mice brain. A- nNOS immunopositive reaction is associated with the neurons localized in cortical regions (single arrows). Positive immunoreactions were also seen in the matrix of perivascular cell (double arrow). Original magnification x30. B- The intensive nNOS immunopositive reaction was seen also in sub-cortical regions (single arrow). Original magnification x30. VL-Vessel lumen.*

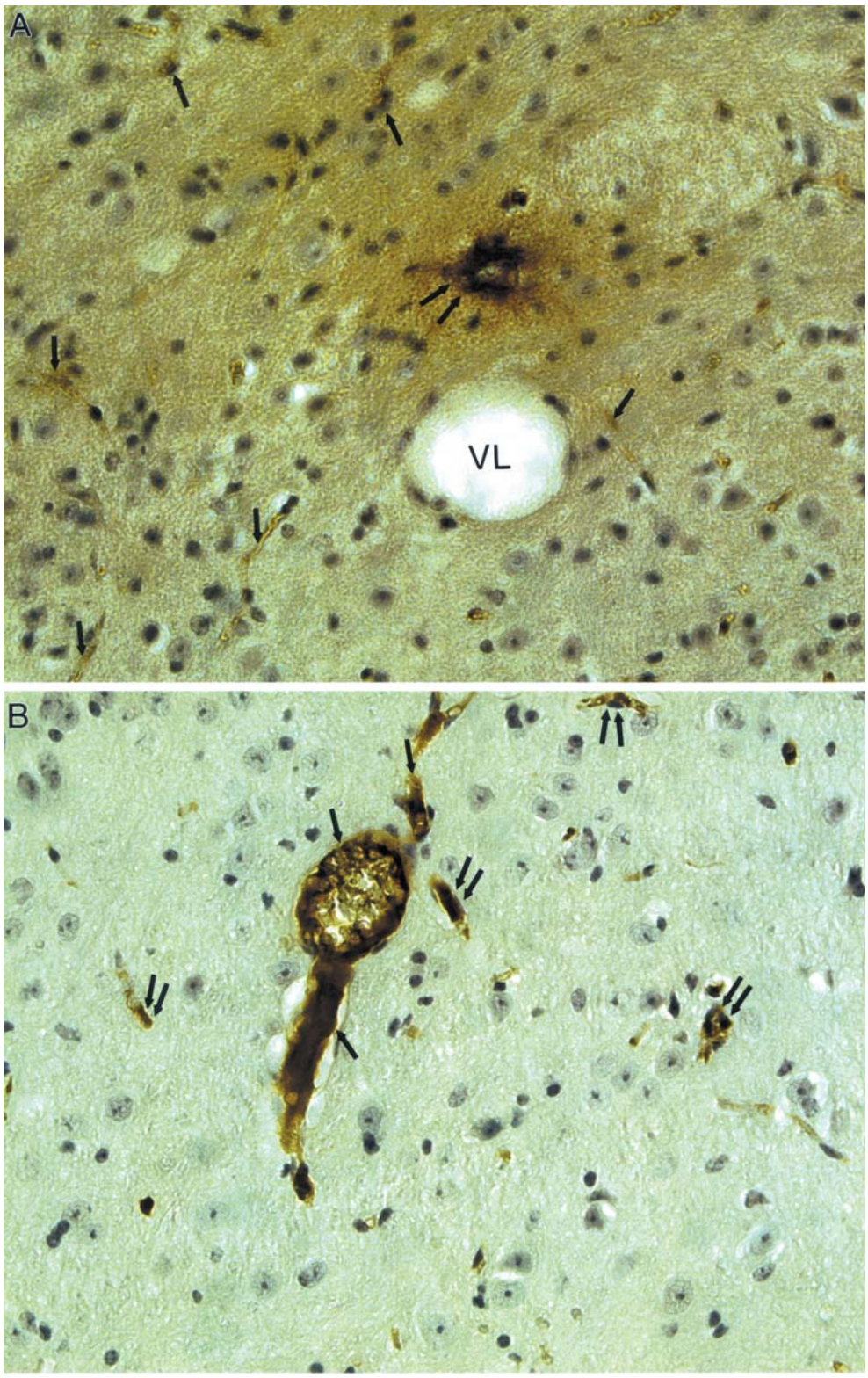

Figure 2. *The characteristics of iNOS staining from aged control and YAC AβPP transgenic mice. A-Age-matched control wild mice shows the iNOS positive astrocytes (double arrows) close to microvessels. The different intensity of immunopositive glial cells also seen throughout brain tissues (single arrows). Original magnification x30. B- Brain slices from YAC AβPP transgenic mice shows the cluster type of iNOS immunopositive reaction in vascular wall cells (single arrows). High intensity of immunopositive reaction was also seen in glial cells (double arrows). Original magnification x30. VL-Vessel lumen.*

of NO in bovine brain microvascular endothelial cells (BBEC) ATP, acetylcholine (ACh) and A23187 induced $Ca^{(2+)}$ transients both in BBEC and BAEC. In contrast, ATP and A23187 that evoked a similar degree of $[Ca^{(2+)}]$ increase failed to induce NO production in BBEC, as measured with an NO-sensitive fluorescent dye DAF-2, whereas BAEC showed an increase in DAF-2 fluorescence. Hypotonic stress induced ATP release and subsequent NO production in BAEC, whereas these were not observed in BBEC (48). Unfortunately, the effect of chronic oxidative stress such as hypoperfusion on NO production has not been studied yet.

Excess NO production is found during excitotoxicity, inflammation and ischemia-reperfusion injury (18), and the oxidation products of NO, namely peroxynitrite and peroxynitrate, are powerful oxidants. Also, $ONOO^-$ can generate the highly reactive OH^0, a more powerful oxidant than NO itself (13,18,27,67). Increased nitrotyrosine immunoreactivity in AD is found in the neuronal cytoplasm of the cerebral cortex within regions of neurodegeneration, yet it is undetectable in corresponding control regions (67). This distribution is essentially identical to that of free carbonyls (68). The widespread occurrence of nitrotyrosine immunoreactivity in neurons suggests that chronic oxidative damage is not restricted to long-lived polymers such as neurofibrillary tangles (NFTs), but, instead, reflects a generalized oxidative stress contributing to the pathogenesis of AD.

iNOS can be expressed in a variety of cells in response to lipopolysaccharides, certain cytokines and ROS generators (34,57). Because iNOS produces much greater amounts of NO than either eNOS or nNOS (2), it may be an important mediator of cytotoxicity in the brain (28). Recently, Iadecola and coworkers have proposed that iNOS makes a late contribution to ischemic brain damage (43,44). iNOS catalytic activity and iNOS mRNA expression were present in brain tissue after 2 h of transient focal ischemia or 1-2 days after permanent focal ischemia (43,44). Our extended study in aged control wild and yeast artificial chromosome (YAC) transgenic mice overexpressing amyloid beta precursor protein (AßPP) demonstrates that pathological changes such as vessel lesions, amyloid deposition, neuronal lesions especially mitochondria abnormalities including the mitochondrial DNA (mtDNA) deletions, were correlates with the degree of NOS overexpression. Control wild mice show that neuronal specific NOS overexpression mostly associates in the cortex rather than in other brain regions (Figure 1A). In addition, large neurons from hippocampus also show high overexpression of NOS immunoreactivity (Figure 1A). However, the intensity of the nNOS staining was very often high in the sub-cortical neurons in comparison with the cortical area (Figure 1B). In YAC AßPP transgenic mice the intensity of nNOS immunoreactivity was also much higher than that in control wild mice (data not shown). The vascular wall cells including perivascular pericytes also show high levels of nNOS immunoreactivity. This observation especially has been made in the cortical regions that develop AD-like pathology. Our extended study demonstrates that nNOS overexpression was seen throughout all brain regions of YAC AßPP transgenic mice. Neurons, glia and vascular wall cells from severely damaged regions show a very high intensity of nNOS immunoreactivity (data not shown).

In control wild mice the pattern of iNOS immunoreactivity was similar to the nNOS. iNOS immunoreactivity was seen in glial cells throughout the different brain regions. The highest intensity of iNOS immunopositive reaction was seen in astrocytes which closely associate with microvessels (Figure 2A). The pattern of iNOS immunoreactivity in YAC AßPP has a more heterogeneous distribution (Figure 2B). A highly immunopositive reaction was associated with all cellular compartments of the vessel wall (Figure 2B). The amyloid plaques also show a high intensity of iNOS immunopositive reaction (data not shown).

Immunocytochemistry against eNOS indicates that, in aged control wild mice, an immunopositive reaction always associates within vascular endothelium (data not shown). Occasionally some perivascular cells also show a very light immunopositive reaction (data not shown). Contrary to this observation, in aged YAC AßPP transgenic mice, high levels of eNOS immunoreactivity were seen in almost all cellular compartments of the brain. Vascular endothelium and eNOS immunopositive glial cells were often seen throughout these regions (data not shown). These observations indicate that a misbalance in the different isoforms of NOS occur before any visible amyloid deposition. Therefore pharmacological interventions using NO donor and/or NO suppressor may work to block these changes and development of brain lesions leading to amyloid deposition and dementia. The exact mechanism of the NO misbalance that occurs during the development and maturation of neurodegeneration needs to be elucidated.

NOS-positive neurons are present in neuron subgroups throughout many regions of the brain (27). Reduced NADPH-diaphorase, as well as nNOS and eNOS, are present in dendritic and axonal terminals that are closely associated with the middle cerebral artery and cerebral microvessels [for ref. see: (25-28)].

The presence of L-arginine in astrocytes *in vivo* suggests that glia may store this chemical for NO production in the brain (25,26,34). Moreover, glial cells exhibit an inflammatory response during disease, infection and ischemia. They also release pro-inflammatory cytokines and synthesize and release NO (34). The large amount of NO that is released from glial cells *via* the expression of iNOS after their stimulation is neurotoxic, because it induces oxidative stress, mitochondrial

dysfunction and excitotoxicity (10,26,75). Hypoxic brain injury (acute or chronic) is associated with the formation of both NO (9,14,15,23,27,34) and the superoxide anion, which may react to form free radicals (13) and cause neurotoxicity (14,15,26, 27,49,50,60,75). Therefore investigations to determine the exact ultrastructural localization of the different NOS isoforms in the brain vascular tree, neurons and glia in post-hypoxic and AD brain would be warranted.

Conclusion

NO as a liable molecule plays an important role in the vascular homeostasis of the brain. Disruption in the balance between the different isoforms of NO appears to be a key element during the development of different diseases including AD. In addition, NO overproduction occurs much earlier than amyloid deposition. Therefore pharmacological correction of these misbalances may open a new window for the prevention and/or treatment strategy for these diseases.

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References

1 Aliev G: Is non-genetic Alzheimer's disease a vascular disorder with neurodegenerative consequences? J Alzheimer's Disease *4(6)*: 513-6, 2002.

2 Aliev G and Burnstock G: Watanabe rabbits with heritable hypercholesterolaemia: a model of atherosclerosis. Histol Histopathol *13(3)*: 797-817, 1998.

3 Aliev G, Cirillo R, Salvatico E, Paro M and Prosdocimi M: Changes in vessel ultrastructure during ischemia and reperfusion of rabbit hindlimb: implications for therapeutic intervention. Microvasc Res *46(1)*: 65-76, 1993.

4 Aliev G, Ralevic V and Burnstock G: Depression of endothelial nitric oxide synthase but increased expression of endothelin-1 immunoreactivity in rat thoracic aortic endothelium associated with long-term, but not short-term, sympathectomy. Circ Res *79(2)*: 317-23, 1996.

5 Aliev G, Seyidova D, Lamb BT, Neal ML, Siedlak SL, Vinters H, LaManna JC, Friedland RP, Head E, Perry G and Cotman CW: Atherosclerotic lesions and mitochondria DNA deletions in brain microvessels as a central target for the development of human AD and AD-like pathology in aged YAC AßPP transgenic mice. Ann NY Acad Sci *977*: 45-64, 2002.

6 Aliev G, Seyidova D, Raina AK, Obrenovich ME, Neal ML, Siedlak SL, Lamb BT, Vinters H, LaManna JC, Smith MA and Perry G: Vascular hypoperfusion, mitochondria failure and oxidative stress in Alzheimer Disease. Proc Indian Nat Sci Academ *B69(2)*: 209-38, 2003.

7 Aliev G, Shi J, Perry G, Friedland RP and Lamanna JC: Decreased constitutive nitric oxide synthase, but increased inducible nitric oxide synthase and endothelin-1 immunoreactivity in aortic endothelial cells of Donryu rats on a cholesterol-enriched diet. Anat Rec *260(1)*: 16-25, 2000.

8 Aliev G, Smith MA, Seyidova D, Neal ML, Shi J, Loizidou M, Turmaine M, Friedland RP, Taylor I, Burnstock G, Perry G and Lamanna JC: Increased expression of NOS and ET-1 immunoreactivity in human colorectal metastatic liver tumours is associated with selective depression of constitutive NOS immunoreactivity in vessel endothelium. J Submicrosc Cytol Pathol *34(1)*: 37-50, 2002.

9 Aliev G, Smith MA, Vinters H, Johnson AB, Nunomura A and Perry G: Mitochondria abnormalities mark vulnerable neurons in Alzheimer's Disease. J Neuropathol Exp Neurol *58*: 511, 1999.

10 Almeida A, Bolanos JP and Medina JM: Nitric oxide mediates glutamate-induced mitochondrial depolarization in rat cortical neurons. Brain Res *816(2)*: 580-6, 1999.

11 Bates TE, Loesch A, Burnstock G and Clark JB: Mitochondrial nitric oxide synthase: a ubiquitous regulator of oxidative phosphorylation? Biochem Biophys Res Commun *218(1)*: 40-4, 1996.

12 Bayraktutan U and Ulker S: Effects of angiotensin II on nitric oxide generation in proliferating and quiescent rat coronary microvascular endothelial cells. Hypertens Res *26(9)*: 749-57, 2003.

13 Beal MF: Aging, energy and oxidative stress in neurodegenerative diseases. Ann Neurol *38(3)*: 357-66, 1995.

14 Beckman JS: The double-edged role of nitric oxide in brain function and superoxide-mediated injury. J Dev Physiol *15(1)*: 53-9, 1991.

15 Beckman JS, Beckman TW, Chen J, Marshall PA and Freeman BA: Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc Natl Acad Sci USA *87(4)*: 1620-4,1990.

16 Beckman JS, Carson M, Smith CD and Koppenol WH: ALS, SOD and peroxynitrite. Nature *364(6438)*: 584, 1993.

17 Bogumil R, Knipp M, Fundel SM and Vasak M: Characterization of dimethylargininase from bovine brain: evidence for a zinc binding site. Biochemistry *37(14)*: 4791-8, 1998.

18 Bredt DS and Snyder SH: Nitric oxide: a physiologic messenger molecule. Annu Rev Biochem *63*: 175-95, 1994.

19 Buisson A, Margaill I, Callebert J, Plotkine M and Boulu RG: Mechanisms involved in the neuroprotective activity of a nitric oxide synthase inhibitor during focal cerebral ischemia. J Neurochem *61(2)*: 690-6, 1993.

20 Buisson A, Plotkine M and Boulu RG: The neuroprotective effect of a nitric oxide inhibitor in a rat model of focal cerebral ischaemia. Br J Pharmacol *106(4)*: 766-7, 1992.

21 Cao S, Yao J, McCabe TJ, Yao Q, Katusic ZS, Sessa WC and Shah V: Direct interaction between endothelial nitric-oxide synthase and dynamin-2. Implications for nitric-oxide synthase function. J Biol Chem *276(17)*: 14249-56, 2001.

22 Cao S, Yao J and Shah V: The proline-rich domain of dynamin-2 is responsible for dynamin-dependent *in vitro* potentiation of endothelial nitric-oxide synthase activity *via* selective effects on reductase domain function. J Biol Chem *278(8)*: 5894-901, 2003.

23 Cazevieille C, Muller A, Meynier F and Bonne C: Superoxide and nitric oxide cooperation in hypoxia/reoxygenation-induced neuron injury. Free Radic Biol Med *14(4)*: 389-95, 1993.

24 Crow JP, Ye YZ, Strong M, Kirk M, Barnes S and Beckman JS: Superoxide dismutase catalyzes nitration of tyrosines by peroxynitrite in the rod and head domains of neurofilament-L. J Neurochem *69(5)*: 1945-53, 1997.

25 Dawson DA: Nitric oxide and focal cerebral ischemia: multiplicity of actions and diverse outcome. Cerebrovasc Brain Metab Rev *6(4)*: 299-324, 1994.

26 Dawson VL, Brahmbhatt HP, Mong JA and Dawson TM: Expression of inducible nitric oxide synthase causes delayed neurotoxicity in primary mixed neuronal-glial cortical cultures. Neuropharmacology *33(11)*: 1425-30, 1994.

27 Dawson VL and Dawson TM: Nitric oxide in neuronal degeneration. Proc Soc Exp Biol Med *211(1)*: 33-40, 1996.

28 Dawson VL, Dawson TM, London ED, Bredt DS and Snyder SH: Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc Natl Acad Sci USA *88(14)*: 6368-71, 1991.

29 de la Torre JC: Alzheimer's disease as a vascular disorder: nosological evidence. Stroke *33(4)*: 1152-62, 2002.

30 de la Torre JC: Alzheimer's disease: How does it start? J Alzheimer's Disease *4(6)*: 497-512, 2002.

31 de la Torre JC: Is Alzheimer's disease a neurodegenerative or a vascular disorder? Data, dogma and dialectics. Lancet Neurol *3(3)*: 184-90, 2004.

32 de la Torre JC and Stefano GB: Evidence that Alzheimer's disease is a microvascular disorder: the role of constitutive nitric oxide. Brain Res Brain Res Rev *34(3)*: 119-36, 2000.

33 Faraci FM: Role of endothelium-derived relaxing factor in cerebral circulation: large arteries *vs.* microcirculation. Am J Physiol *261(4 Pt 2)*: H1038-H1042, 1991.

34 Faraci FM and Brian JE: Nitric oxide and the cerebral circulation. Stroke *25(3)*: 692-703, 1994.

35 Garthwaite J and Beaumont PS: Excitatory amino acid receptors in the parallel fibre pathway in rat cerebellar slices. Neurosci Lett *107(1-3)*: 151-6, 1989.

36 Hamada Y, Hayakawa T, Hattori H and Mikawa H: Inhibitor of nitric oxide synthesis reduces hypoxic-ischemic brain damage in the neonatal rat. Pediatr Res *35(1)*: 10-4, 1994.

37 Hara H, Huang PL, Panahian N, Fishman MC and Moskowitz MA: Reduced brain edema and infarction volume in mice lacking the neuronal isoform of nitric oxide synthase after transient MCA occlusion. J Cereb Blood Flow Metab *16(4)*: 605-11, 1996.

38 Harrison DG and Cai H: Endothelial control of vasomotion and nitric oxide production. Cardiol Clin *21(3)*:289-302, 2003.

39 Huang Z, Huang PL, Panahian N, Dalkara T, Fishman MC and Moskowitz MA: Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science *265(5180)*: 1883-5, 1994.

40 Iadecola C: Does nitric oxide mediate the increases in cerebral blood flow elicited by hypercapnia? Proc Natl Acad Sci USA *89(9)*: 3913-6, 1992.

41 Iadecola C, Li J, Ebner TJ and Xu X: Nitric oxide contributes to functional hyperemia in cerebellar cortex. Am J Physiol *268(5 Pt 2)*: R1153-R1162, 1995.

42 Iadecola C, Zhang F, Casey R, Clark HB and Ross ME: Inducible nitric oxide synthase gene expression in vascular cells after transient focal cerebral ischemia. Stroke *27(8)*: 1373-80, 1996.

43 Iadecola C, Zhang F, Xu S, Casey R and Ross ME: Inducible nitric oxide synthase gene expression in brain following cerebral ischemia. J Cereb Blood Flow Metab *15(3)*: 378-84, 1995.

44 Iadecola C, Zhang F and Xu X: Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. Am J Physiol *268(1 Pt 2)*: R286-R292, 1995.

45 Kara P and Friedlander MJ: Dynamic modulation of cerebral cortex synaptic function by nitric oxide. Prog Brain Res *118*: 183-98, 1998.

46 Kimoto M, Tsuji H, Ogawa T and Sasaoka K: Detection of NG, NG-dimethylarginine dimethylaminohydrolase in the nitric oxide-generating systems of rats using monoclonal antibody. Arch Biochem Biophys *300(2)*: 657-62, 1993.

47 Kimoto M, Whitley GS, Tsuji H and Ogawa T: Detection of NG, NG-dimethylarginine dimethylaminohydrolase in human tissues using a monoclonal antibody. J Biochem (Tokyo) *117(2)*: 237-8, 1995.

48 Kimura C, Oike M, Ohnaka K, Nose Y and Ito Y: Constitutive nitric oxide production in bovine aortic and brain microvascular endothelial cells: a comparative study. J Physiol *554(Pt 3)*: 721-30, 2004.

49 Lafon-Cazal M, Culcasi M, Gaven F, Pietri S and Bockaert J: Nitric oxide, superoxide and peroxynitrite: putative mediators of NMDA-induced cell death in cerebellar granule cells. Neuropharmacology *32(11)*: 1259-66, 1993.

50 Lafon-Cazal M, Pietri S, Culcasi M and Bockaert J: NMDA-dependent superoxide production and neurotoxicity. Nature *364(6437)*: 535-7, 1993.

51 Liu J and Sessa WC: Identification of covalently bound amino-terminal myristic acid in endothelial nitric oxide synthase. J Biol Chem *269(16)*: 11691-4, 1994.

52 MacAllister RJ, Parry H, Kimoto M, Ogawa T, Russell RJ, Hodson H, Whitley GS and Vallance P: Regulation of nitric oxide synthesis by dimethylarginine dimethylaminohydrolase. Br J Pharmacol *119(8)*: 1533-40, 1996.

53 Marletta MA: Nitric oxide synthase: aspects concerning structure and catalysis. Cell *78(6)*: 927-30, 1994.

54 Michel T and Feron O: Nitric oxide synthases: which, where, how and why? J Clin Invest *100(9)*: 2146-52, 1997.

55 Moncada S and Higgs EA: Molecular mechanisms and therapeutic strategies related to nitric oxide. FASEB J *9(13)*: 1319-30, 1995.

56 Moncada S, Palmer RM and Higgs EA: Nitric oxide: physiology, pathophysiology and pharmacology. Pharmacol Rev *43(2)*: 109-42, 1991.

57 Morris SM and Billiar TR: New insights into the regulation of inducible nitric oxide synthesis. Am J Physiol *266(6 Pt 1)*: E829-E839, 1994.

58 Nakashima MN, Yamashita K, Kataoka Y, Yamashita YS and Niwa M: Time course of nitric oxide synthase activity in neuronal, glial and endothelial cells of rat striatum following focal cerebral ischemia. Cell Mol Neurobiol *15(3)*: 341-9, 1995.

59 Park CS, Pardhasaradhi K, Gianotti C, Villegas E and Krishna G: Human retina expresses both constitutive and inducible isoforms of nitric oxide synthase MRNA. Biochem Biophys Res Commun *205(1)*: 85-91, 1994.

60 Radi R, Beckman JS, Bush KM and Freeman BA: Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem *266(7)*: 4244-50, 1991.

61 Ravichandran LV, Johns RA and Rengasamy A: Direct and reversible inhibition of endothelial nitric oxide synthase by nitric oxide. Am J Physiol *268(6 Pt 2)*: H2216-H2223, 1995.
62 Rengasamy A and Johns RA: Inhibition of nitric oxide synthase by a superoxide generating system. J Pharmacol Exp Ther *267(3)*: 1024-7, 1993.
63 Robinson LJ, Weremowicz S, Morton CC and Michel T: Isolation and chromosomal localization of the human endothelial nitric oxide synthase (NOS3) gene. Genomics *19(2)*: 350-7, 1994.
64 Samadani AF, Dawson TM and Dawson VL: Nitric oxide synthase in model of focal ischemia. Stroke *28*: 1283-8, 1997.
65 Sessa WC: The nitric oxide synthase family of proteins. J Vasc Res *31(3)*: 131-43, 1994.
66 Shin T, Weinstock D, Castro MD, Acland H, Walter M, Kim HY and Purchase HG: Immunohistochemical study of constitutive neuronal and inducible nitric oxide synthase in the central nervous system of goat with natural listeriosis. J Vet Sci *1(2)*: 77-80, 2000.
67 Smith MA, Richey Harris PL, Sayre LM, Beckman JS and Perry G: Widespread peroxynitrite-mediated damage in Alzheimer's disease. J Neurosci *17(8)*: 2653-7, 1997.
68 Smith MA, Sayre LM and Perry G: Is Alzheimer's disease of oxidative stress? Alzheimer's Disease Rev *1*: 63-7, 1996.
69 Smith MA, Vasak M, Knipp M, Castellani RJ and Perry G: Dimethylargininase, a nitric oxide regulatory protein, in Alzheimer disease. Free Radic Biol Med *25(8)*: 898-902, 1998.
70 Stamler JS: Redox signaling: Nitrosylation and related target interactions of nitric oxide. Cell *78(6)*: 931-6, 1994.
71 Stuehr DJ: Structure-function aspects in the nitric oxide synthases. Annu Rev Pharmacol Toxicol *37(6)*: 339-59, 1997.
72 Thorns V, Hansen L and Masliah E: NNOS expressing neurons in the entorhinal cortex and hippocampus are affected in patients with Alzheimer's disease. Exp Neurol *150(1)*: 14-20, 1998.
73 Wang Q, Pelligrino DA, Baughman VL, Koenig HM and Albrecht RF: The role of neuronal nitric oxide synthase in regulation of cerebral blood flow in normocapnia and hypercapnia in rats. J Cereb Blood Flow Metab *15(5)*: 774-8, 1995.
74 Wever RMF, Luscher TF, Cosentino F and Rabelink TJ: Atherosclerosis and the two faces of endothelial nitric oxide synthase. Circulation *97*: 108-12, 1998.
75 Xia Y, Dawson VL, Dawson TM, Snyder SH and Zweier JL: Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci USA *93(13)*: 6770-4, 1996.
76 Yanagisawa M, Kurihara H, Kimura S, Goto K and Masaki T: A novel peptide vasoconstrictor, endothelin, is produced by vascular endothelium and modulates smooth muscle Ca^{2+} channels. J Hypertens Suppl *6(4)*: S188-S191, 1988.

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