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NITRIC OXIDE SYNTHASE ACTIVITY IS ELEVATED IN BRAIN MICROVESSELS IN ALZHEIMER'S DISEASE

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SUMMARY: The cerebral microcirculation undergoes specific biochemical changes in Alzheimer's disease. In this study, we have compared the nitric oxide synthase (NOS) activity of brain microvessels isolated from Alzheimer and control brains. L-[^{3}H]-citrulline, the stable co-product generated with nitric oxide (NO) from L-[^{3}H]-arginine, was measured as an indicator of NOS activity. The results indicated a significant increase in NOS activity in microvessels isolated from Alzheimer brains. In addition, using antibodies to both the endothelial and inducible NOS isoforms, we demonstrated a significant increase in enzyme level in Alzheimer-derived vessels. Elevated vascular production of NO, a potentially neurotoxic mediator in the CNS, may contribute to the susceptibility of neurons to injury and cell death in Alzheimer's disease. © 1994 Academic Press, Inc.

INTRODUCTION: There is considerable evidence that nitric oxide (NO) mediates diverse functions including vasodilation, macrophage toxicity, and neurotransmission (reviewed in 1). NO is formed by the enzyme, NO synthase (NOS), by oxidation of one of the guanidino nitrogens of L-arginine. Both constitutive and inducible isoforms of the enzyme have been identified in numerous cell types (1). Recent evidence suggests that NO is a neurotoxin (2) and that NO is an important agent in the pathogenesis of central nervous system damage following acute injury (e.g. ischemia/hypoxia) (3) as well as in chronic neurodegenerative diseases (4,5,6). The possible role of NO in neuronal cell loss observed in Alzheimer's disease (AD) has not been fully explored.

Vascular endothelial cells are a well characterized source of NO. In these cells, NO is produced by a particulate, constitutively expressed Ca^{2+}/calmodulin dependent NOS isoform, referred to as type III (7,8). Endothelial cells can also express inducible NOS

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(9). We believe an understanding of vascular NO production may be important in Alzheimer's based on the following: NO is a highly reactive compound with demonstrated neurotoxic properties; endothelial cells are a quantitatively important source of NO; and there appears to be a topographic association of capillaries with neuritic plaques (10). Therefore, the objective of this study was to examine NOS activity and expression in the cerebral microcirculation of Alzheimer and control patients.

METHODS: Brains from 9 patients that died as a result of AD (mean age 81.0 ± 4 years) and 8 controls (mean age 72.6 ± 8.1 years) whose neuropathology and clinical history showed no neurological and psychiatric disease were studied. The post-mortem interval was similar for both Alzheimer and control samples. The clinical diagnosis of primary degenerative dementia of Alzheimer type was confirmed by neuropathological examination and accepted quantitative criteria. Each case met with the diagnostic criteria established by the NIH Neuropathology Panel (11) and the Consortium to Establish a Registry for AD (12).

Microvessels were isolated from parietal, temporal and frontal cortices of AD and non-demented elderly controls as previously reported (13). Freshly isolated microvessels were resuspended in homogenization buffer (50 mM Tris pH 7.4, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% 2-ß-mercaptoethanol, 1 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin-A, 1 mM phenylmethylsulfonyl fluoride) and Dounce homogenized. Homogenates for iNOS were centrifuged at 200,000 x g for 30 min and supernatant collected. For ECNOS, homogenates were incubated with 10 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) for 20 min at 4°C with gentle agitation followed by centrifugation at 15,000 x g for 15 min at 4°C. Supernatants were immediately removed and frozen at -70°C until use. Protein concentrations were determined by the Bio-Rad assay with BSA as the standard.

NOS activity was assayed by measuring the conversion of [^{3}H] arginine to [^{3}H] citrulline (14) under the following conditions: supernatants (100-200 µg) were incubated with reaction buffer 10 µM L-arginine, 1 mM NADPH, 3 µM {6}- 5,6,7,8, tetrahydro-L-biopterin dihydrochloride (BH_4, Dr. B. Schircks Lab, Jona, Switzerland), 3 µM FAD, 2000 U/ml calmodulin, 2 mM $CaCl_2$, 10 mM (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (HEPES), pH 7.45, 2 µCi/ml L-[^{3}H]-arginine for 30 min at 37°C with agitation. Reactions were terminated with 1 ml stop buffer (20 mM HEPES, 2 mM EGTA, pH 5.5) and passed over columns of Dowex-50W (Na^+ form) equilibrated with stop buffer. Each column was washed with stop buffer and the column flow-through and wash were collected in scintillation vials.

For slot blot quantitation, immunodetection was performed using an anti-ECNOS antibody (H-32, 1:1000) and an anti-iNOS antibody (8196, 1:1000) followed by peroxidase labelled anti-mouse secondary antibody diluted in TBS with 1% nonfat dry milk (ECNOS 1:5000; iNOS 1:2000) and visualized by chemiluminescence. Band intensity was assessed by scanning densitometry.

The antibody to endothelial NOS was prepared against type III NOS isolated from cultured bovine aortic endothelial cells as previously described (7, 8). A polyclonal antibody to iNOS was raised against type II NOS isolated from induced RAW 264.7 macrophages as previously described (15). Positive controls were determined using

cultured bovine aortic endothelial cells for ECNOS and the monocyte line J744 for iNOS. Negative controls were performed without secondary antibody.

Statistical analyses were performed using Student's t-test.

RESULTS: NOS enzymatic activity was determined by measuring L-[^{3}H]-citrulline, the stable co-product of the conversion of L-[^{3}H]-arginine to NO. Confirmation that the production of citrulline was mediated specifically by NOS was demonstrated by the NOS inhibitor, N^GNitro-L-arginine methyl ester (10 μM) which markedly decreased the production of L-[^{3}H]-citrulline (44.7 ± 5.5 nmoles/min/mg protein versus 3.0 ± 2.2 nmoles/min/mg protein with inhibitor; *n = 3*). In addition, non-NOS activity was determined to be negligible by omitting NADPH, a necessary cofactor for NOS activity. Assays performed in the absence of NADPH yielded significantly ($p < 0.002$) less L-citrulline than samples containing NADPH (0.4 ± 2.2 nmoles\min\mg protein; *n = 3*). The measurement of citrulline in isolated microvessels from human brain indicated that the mean specific activity of NOS in AD-derived microvessels was significantly ($p < 0.001$) higher than that observed in microvessels from control brains (Table 1).

Slot blot analyses demonstrated a significantly ($p < 0.01$) higher level of endothelial cell and inducible ($p < 0.01$) NOS in AD-derived microvessels compared to controls. Scanning densitometry of chemiluminescent bands were analyzed and the data are shown in Figure 1. It should be noted that while the increase in ECNOS and iNOS over control levels of both enzymes were significantly elevated, the increase in iNOS was approximately 9 times higher than ECNOS.

DISCUSSION: Our results demonstrated that microvessels from AD patients have significantly higher NOS activity than vessels isolated from control brains. This elevated NOS activity reflects an increase in NOS protein expression. The increased L-citrulline production in control and AD-derived microvessels was due to NOS activity rather that

Table 1. NOS activity of control and AD microvessels

Tissue	Citruline formation (mean specific activity) nmol/min/mg*
Control	10.0 ± 2.4
AD	44.7 ± 5.5*

*Data are mean ± SEM of 3 control and 3 AD samples.
AD significantly different from control, *$p < 0.001$.

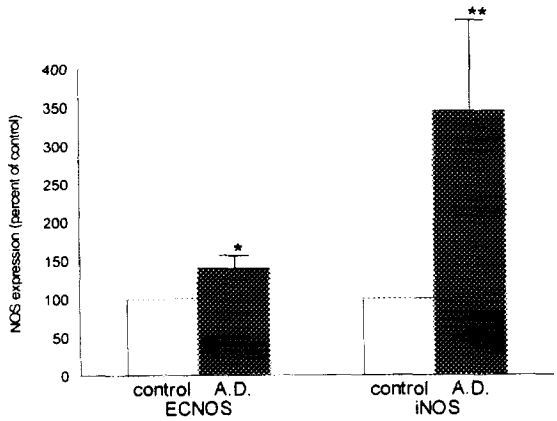

Fig. 1. Slot blot analyses of endothelial and inducible NOS proteins in control and AD-derived microvessel.

Equal quantities of AD (■) and control (□) microvessel protein samples were loaded per slot and applied to nitrocellulose. Bands were visualized by chemiluminescence and band intensity was assessed by scanning densitometry. Values from control-derived vessels (n=6) were expressed as 100%. AD-derived samples (n=6) are mean ± SEM, expressed as percent of control.

* $p < 0.01$ significantly different from ECNOS in control vessels.

** $p < 0.001$ significantly different from iNOS in control vessels.

the NOS-independent conversion of L-arginine to L-citrulline as demonstrated by the experimental conditions without NADPH. The slot blot analyses determined that there was a significant increase in both constitutive and inducible NOS protein in AD-derived microvessels. The circumstances under which the physiologic mediator NO becomes neurotoxic are unclear. It has been suggested that the cytotoxicity of NO may be related to the amount produced (16). In this regard, our demonstration of an elevation in iNOS is important since the iNOS enzyme generates significantly more NO over a more sustained period than the constitutive enzyme (17). It is possible that increased iNOS in AD may convert the microcirculation from a source of the tightly regulated messenger NO to a producer of a neurotoxic molecule.

NO, a well-characterized mediator in the vasculature, has recently been implicated as an important inter- and intra-cellular mediator in the brain. NO is believed to play an important role in controlling cerebral blood flow and may also serve to couple neural activity to blood flow (18). Although the physiologic and pathologic functions of NO in the CNS are controversial, considerable evidence suggests that NO contributes to neuronal dysfunction and death in ischemia/reperfusion injury (19,20,21). The mechanism of NO-mediated neuronal toxicity has yet to be elucidated. Recent studies have implicated both the free radical nature of NO and its interaction with other radical species. NO reacts

rapidly with superoxide to form the powerful oxidant peroxynitrite ($ONOO^-$) that can nitrate tyrosine residues. Nitration of proteins may be a general mechanism for chronic NO-mediated injury to cells (4,22). Another mechanism of NO-mediated toxicity has been shown to occur via ADP-ribosylation (23).

Our data suggest that in AD the cerebral microcirculation may be a source of a potentially neurotoxic mediator. Measurements of enzymatic activity in autopsy material can be complicated by the effects of post-mortem autolysis, however, our results on production of NO in control vessels (10 nmol/min/mg) are similar in magnitude to those reported for cultured bovine aortic endothelial cells (24) (15 nmol/min/mg) and considerably higher than data in human umbilical vein endothelial cells (pmol/min/mg) (25). The biochemical changes that occur in the cerebral microcirculation in AD have not been fully delineated. Selective changes in glucose transport (26), adrenergic receptors (13), cAMP (27) and protein kinase C (28) levels suggest that specific impairment of receptor-mediated signalling pathways occurs. Our previous demonstration of an elevation of cAMP in AD-derived vessels may be relevant in light of the demonstration that cAMP enhances NO formation in a neuronal cell line (29). Furthermore, cerebral blood vessels from AD patients are heavily invested with amyloid (30). Since ß- amyloid has been shown to increase intracellular Ca^{2+} (31,32), this might imply a relationship between ß-amyloid and Ca^{2+}-regulated constitutive NOS activity *in vivo*.

Although it is possible that a percentage of smooth muscle cells or glial processes could contribute to the NO production and NOS measurements performed on the isolated microvessels, our previous characterization of these vessels as primarily capillary (13) suggests that endothelial cells are the predominant cell type responsible. Since neurons, glia and microglia can produce NO it will be important to develop methods that allow quantitative comparisons of NO/NOS in each cell type, under basal conditions and in response to injury, to determine the contribution of the cerebral microcirculation to brain NO production.

Understanding the factors that influence the susceptibility of neurons to lethal injury is central to identifying the cellular mechanisms responsible for cell injury and death in AD. The results of these experiments suggest a new mechanism for brain injury and the neuronal cell loss which underlies the dementia characteristic of AD occurs. If elevated levels of vascular-derived NO mediate the neuronal damage observed in AD, this presents a novel therapeutic target for treatment of this neurodegenerative disease.

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