β-Amyloid Accumulation in Neurovascular Units Following Brain Embolism

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Received May 18, 2009; Accepted July 28, 2009

Abstract. Nitric oxide (NO) toxicity is in part mediated by generation of peroxynitrite with concomitant production of superoxide under pathological brain conditions such as ischemia and Alzheimer’s disease. The pathophysiologica relevance of endothelial nitric oxide synthase (eNOS) to brain embolism-induced neurovascular injury has not been documented. We found that microsphere embolism (ME)-induced aberrant eNOS expression in vascular endothelial cells likely mediates blood-brain barrier (BBB) disruption via peroxynitrite formation and in turn causes brain edema. We also demonstrated that a mild ME model was useful for investigating the sequential events of neurovascular injury followed by β-amyloid accumulation and tau hyperphosphorylation. Indeed, immunoblotting of purified brain microvessels revealed that β-amyloid accumulation significantly increased one week after ME induction and remained elevated for twelve weeks in those animals. Moreover, we also confirmed that peroxynitrite formation and eNOS uncoupling–mediated superoxide generation in microvessels are inhibited by a novel calmodulin inhibitor. Thus, peroxynitrite formation via elevated eNOS is associated with endothelial cell injury with concomitant β-amyloid accumulation in microvessels of aged rats. In this review, we focus on the detrimental effects of eNOS expression following brain embolism and introduce an attractive model representing progressive Alzheimer’s disease pathology in brain.

Keywords: microsphere embolism, nitric oxide (NO), calmodulin, endothelial nitric oxide synthase (eNOS), peroxynitrite, β-amyloid

Introduction

Ischemic stroke results in rapid loss of brain function due to significant neurovascular dysfunction, leading to cerebral hypoperfusion, endothelial cell degeneration, and eventually neuronal cell death (1). Emerging epidemiological and clinical evidence indicates that Alzheimer’s disease (AD) is also associated with vascular disorders that initiate pathology through cerebral microvascular abnormalities, including thinning and discontinuities within the vascular basement membrane, shrinkage of endothelial cells, pericyte degeneration, and luminal buckling (2, 3). These observations suggest that neurovascular dysfunction leads to the rapid cognitive decline associated with neurodegeneration in AD patients. For instance, hypoxia facilitates γ-secretase cleavage of amyloid precursor protein (APP) and β-amyloid deposition in APP transgenic mice. Investigators have also found that hippocampal nitric oxide elevation precedes β-amyloid accumulation in aged rats after permanent occlusion of both common carotid arteries (4 – 6). However, how microvascular injury initiates degeneration of neuronal and glial cells during AD progression remains unclear. In addition, the roles of vascular-related risk factors triggering β-amyloid accumulation in AD and vascular dementia have recently

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Published online in J-STAGE on September 26, 2009 (in advance)
doi: 10.1254/jphs.09R02CP

Invited article
attracted increased interest (7, 8). The neurovascular unit comprises neurons, microvessels, and supporting glial cells in the brain. The cerebral microvessels consist of endothelium, the basal lamina matrix, and the end-feet of astrocytes. Recent clinical therapeutics in brain attack and neurodegenerative disorders focus on the regeneration of the neurovascular unit to protect neurons from progressive neurodegeneration.

The nitric oxide synthase (NOS) family contains three members: two isoforms are constitutively expressed, calcium-dependent enzymes known as neuronal NOS (nNOS) and endothelial NOS (eNOS), and the third isoform is calcium-independent and inducible NOS (iNOS). All NOS proteins share 50% – 60% sequence homology. Human eNOS is encoded by a gene located on chromosome 7q35–36 and is a 135-kDa protein primarily expressed in the endothelium. Because of lack of specific nNOS and eNOS inhibitors, reports regarding the role of NOS in cerebral hypoxic-ischemic injury are often conflicting. Up-regulation of both nNOS and eNOS with concomitant increases in protein tyrosine nitration was reported in neonatal rat brain subjected to unilateral carotid artery occlusion (9). Significant increases in both nNOS and eNOS expression were also observed following brain embolism in the ipsilateral hemisphere of rats (10, 11). Suggestive of harmful effects of nNOS, it has been reported that nNOS-knockout mice show decreased infarct size in models of cerebral ischemia (12). Conversely, eNOS-transgenic knockout mice are more vulnerable to ischemic damage, an effect associated with reduced cerebral blood flow in the ischemic penumbra (13). Notably, aberrant expression of nNOS, eNOS, and iNOS in the AD brain is correlated with increased protein tyrosine nitration (14). Ca$^{2+}$-dependent NOS activity and iNOS immunoreactivity also increase in the cerebral cortex of amyloid precursor protein Tg2576-transgenic mice (15). Altogether, these findings suggest that increased expression of all NOS isoforms in various cell types contributes to peroxynitrite formation seen in neurodegeneration, thereby leading to cellular damage through protein tyrosine nitration. It is interesting to note that aberrant eNOS expression in brain microvessels was shown to account for increased protein tyrosine nitration, thereby disrupting blood-brain barrier (BBB) function (10). However, the precise molecular mechanisms underlying NO/peroxynitrite-induced neurovascular unit disruption are not fully understood. In this context, it is important to define the pathological role of NOS-mediated NO/peroxynitrite signaling during cerebral ischemia as well as in AD. From a pathophysiological standpoint, aberrant eNOS expression can contribute to development of vascular disease via two main mechanisms:

1) exposure of cells to oxidative stress by generation peroxynitrite and 2) conversion of eNOS to a superoxide-producing enzyme (uncoupled eNOS) (9, 10).

Endothelial cell injury in many diseases precedes, predicts, and predisposes patients to subsequent, more severe vascular alterations. As such, evidence presented in this review strongly suggests that cerebromicrovascular endothelial cells are potential targets of vasoprotection in neurodegenerative diseases. Thus we are particularly interested in the pathological role of eNOS. Although further studies are needed to examine the possibility that a novel vasoprotective agent could rescue cerebral microvascular endothelial cells from injury, candidate vasoprotective agents are also discussed in the present study.

**Microsphere embolism induces microvascular injury and neurovascular unit disruption**

Recent evidence suggests that the rat microsphere embolism (ME)–induced ischemia model resembles clinical features of human territorial stroke or brain vascular dementia (16, 17). ME in rats is an attractive model for evaluating microvascular injury following microcapillary embolism since injected microspheres of appropriate size and number can reach the microcirculation and specifically elicit microcapillary embolization rather than promote injury to larger arteries (10, 18). The characteristic property of microsphere embolism is the inhomogeneous disruption of microcirculation with very low flow rates in embolized vessels and high flow rates in non-embolized vessels, and this model causes permanent cerebral ischemia, leading to generation of multi-infarct areas (18). Findings derived from magnetic resonance imaging (MRI) demonstrate that recovery from acute cerebral embolism is dependent on the number of injected microspheres (19). Light microscopic analysis or triphenyltetrazolium chloride staining show widespread scattered infarct areas in the cerebral cortex, striatum, and hippocampus of the ipsilateral hemisphere after microsphere injection (11). Importantly, intra-arterial infusion of microspheres also leads to microvascular injury and/or BBB disruption, inducing severe brain edema (10, 19). Several investigators suggest the utility of this animal model, specifically to investigate the relationship between microvascular disorders and impairment of learning and memory seen in neurodegenerative diseases (19). Analysis of microsphere-embolized rats also reveals reduced function of forebrain cholinergic neurotransmission and other neurotransmitter systems, as well as impaired behavioral and cognitive function (20, 21).

Two-vessel occlusion is routinely applied to achieve
various degrees of cerebral hypoperfusion, but occlusion totally blocks carotid blood flow rather than eliciting the type of impairment seen in AD patients (22). We recently introduced a mild ME model by administering 500 microspheres (50 μm in diameter) into the left common carotid artery of aged rats (Fig. 1) (11). Compared to the severe neurological deficits resulting from injection of 1000 microspheres, the 500 microsphere–infused model promotes mild neurological deficits and smaller infarct size in the ipsilateral hemisphere of injected animals (Fig. 1: A and B). Likewise, BBB disruption as assessed by Evans Blue extravasation is also relatively mild compared to the 1000 ME model (Fig. 1C). The 500 ME model rats were apparently healthy, did not show progressive neurological deficits, and survived for at least 6 months after surgery. However, in these animals we did observe sequential events such as early microvascular injury by eNOS and peroxynitrite formation, secondary β-amyloid accumulation in microvessels and parenchyma, or glycogen synthase kinase-3β (GSK3β) activation, and hyperphosphorylation of tau proteins (11). It is worth noting that mild ME seen in aged rats is likely to serve as a unique AD model with vascular injury. Therefore, we propose that the mild ME model is appropriate for analyzing events associated with β-amyloid accumulation and neurofibrillary tangle formation characteristic of the AD brain and to evaluate novel therapeutic drugs for microvascular injury seen in these conditions.

**NO/peroxynitrite signaling contributes to neurovascular disruption**

Nitric oxide (NO, formula •N=O) is a pleiotropic messenger and effector molecule required for numerous brain functions (23). Given the extensive molecular interplay between NO signaling and the apoptosis cascade, it is not surprising that NO acts as both a signaling molecule and a neurotoxin, depending on dose, rate of flux, and the intracellular environment (24). Therefore, pharmacological modification of NO metabolism should be carefully monitored.

In contrast to the beneficial roles of physiological NO, evidence accumulated over the last two decades suggests that NO can be harmful, primarily under oxidative stress conditions, due to the oxidation and nitration of proteins functioning in pathological processes seen in cerebral diseases (25). Under conditions of cerebral ischemia, NO can be scavenged in a rapid reaction with superoxide to generate ONOO−, which has a half-life of seconds (25). Peroxynitrite is a major source in vivo of oxygen radicals, with 1000 times the oxidizing power of hydrogen peroxide. Peroxynitrite formation has been well documented in brain damage following ischemia.
and sustained hypoxia (26). Importantly, peroxynitrile modifies free tyrosine and tyrosine residues in proteins, which accounts for the effects of NO through oxidation, nitrosation, and nitrination reactions (26). Furthermore, up-regulation of eNOS and iNOS mRNA and protein levels accompanied by generation of nitrotyrosine during BBB disruption suggests that NO functions in that process (27). Consistently, we recently reported that NO and peroxynitrile formation is involved in pathological processes of cerebral ischemia, which may lead to cerebral edema by disrupting microvascular integrity (10, 28, 29). Of note, inhibition of NOS attenuates BBB disruption in cerebral ischemia or β-amyloid injected animals (10, 30). In addition, oxidative damage to neuronal DNA, RNA or proteins is the earliest biochemical event observed in both AD pathogenesis and Down’s syndrome in human subjects (31–33).

**Correlation between aberrant eNOS expression and β-amyloid deposition in brain embolism**

Rapid eNOS regulation is elicited by multiple stimuli, coinciding with enhanced calmodulin binding, which increases eNOS activity three-fold (34). Using a BBB disruption model, we recently reported that ME causes a marked increase in NO production with concomitant elevation of nNOS and eNOS activity 24–48 h after injection of rats with 1000 microsphere particles (10, 17). We observed a close relationship between BBB leakage and eNOS induction in ME-induced ischemia (10). The critical observation in our study was that aberrant eNOS induction and concomitant peroxynitrile formation in the microvascular endothelium precedes BBB leakage. Again, eNOS expression in the microvascular endothelium is closely associated with increased apoptotic signals, such as increased PARPp85 levels, in microvascular cells (10). We also found that eNOS expression was induced by forebrain ischemia in a gerbil model (28), in which increased eNOS expression peaked 48 h after transient ischemia. However, increased eNOS expression in the gerbil hippocampus was not associated with increased protein tyrosine nitration, which was transient and returned to basal levels within 48 h (35). It is interesting to note that the more pronounced increase in protein tyrosine nitration observed 24–48 h after transient ischemia in the ME model likely underlies injury to microvascular endothelium. Consistently, the protein tyrosine nitration in microvascular endothelium occurred at the regions with observed leakage of serum proteins, confirming that protein tyrosine nitration in endothelial cells is responsible for leakage of serum protein. In support of this idea, eNOS expression in the endothelium in ME permanent ischemia leads to protein tyrosine nitration and cell injury (10). One interpretation of this finding is that eNOS overexpression accounts for superoxide as well as NO generation, since eNOS is known to generate superoxide in the absence of substrates such as tetrahydrobiopterin (BH₄) and arginine. These intriguing results are evidence that aberrant eNOS expression in endothelial cells accounts for increased protein tyrosine nitration, leading to vascular endothelial dysfunction and neurotoxicity. In this context, it is worth noting that aberrantly expressed eNOS following brain embolism is less likely to elicit vasodilatory effects in microvessels, due to lacking of vascular smooth muscle in 50 μm microsphere–injured microvessels.

To evaluate the pathophysiological relevance of NOS activity in a mild ME model, we determined protein expression of all three NOS isoforms. In brain extracts derived from the ipsilateral hemisphere of injected ME rats, significant eNOS elevation was seen 6 h after injury and remained pronounced and elevated for 4–12 weeks. On the other hand, slightly elevated nNOS levels were seen at the late phase (4–12 weeks) after mild ME, whereas no change was seen in iNOS expression (Fig. 2). To evaluate pathological consequences of elevated eNOS expression in microvessels, we purified microvessels from ME brain at different time points after microsphere injection. Although eNOS expression and nitrotyrosine formation were coincidently elevated immediately after ME injection, elevated β-amyloid oligomer formation was elevated 1 week later (Fig. 2).

**Fig. 2.** Mild microsphere embolism–induced changes in nitric oxide synthase levels in aged rats. A: Representative immunoblotting image showing eNOS, nNOS, and iNOS expressions following ME (500) using cell extracts from the ipsilateral hemisphere in aged male rats (10-month-old). β-Tubulin serves as a loading control. B: Representative immunoblots probed with anti-β-amyloid antibodies. β-Amyloid oligomer was analyzed using purified microcapillaries obtained from the ipsilateral hemisphere in aged ME rats. Modified from Ref. 11 with permission.
Consistent with this biochemical observation (10), immunohistochemical analysis indicated that increased eNOS expression was predominant in brain microvessels of penumbra regions following ME (Fig. 3A). This observation is consistent with a report showing that NO elevation precedes β-amyloid accumulation in aged rat brain after permanent occlusion of both common carotid arteries (6). Consistently, strong β-amyloid immunoreactivity was observed both in eNOS-positive microvessel endothelium and the surrounding areas at the late phase of aged ME animals (Fig. 3A) (11). In addition to aberrant eNOS expression in microvessel endothelium, nNOS level also elevated one week after ME (Fig. 2A). The nNOS expression may also trigger the β-amyloid accumulation in neurons observed in the surrounding areas of injured microvessels (Fig. 3Ae). These findings clearly demonstrate that peroxynitrite formation via elevated eNOS is associated with endothelial cell injury assessed by nitrotyrosine formation in aged ME rats. Based on the above studies of pathological processes in the mild ME model, we could propose that the sequential events that follow brain embolism are similar to those seen in the AD model (Fig. 3B). For example, aberrant eNOS expression and peroxynitrite formation, as assessed by nitrotyrosine formation could be detected within 6 h and progressively elevated until 12 weeks. By contrast, β-amyloid accumulation markedly elevated 1 week after 500 microsphere injection. Notably, there is a delay between NO/superoxide production and β-amyloid accumulation. Furthermore, β-amyloid accumulation in microvessels coincides with GSK3β activation (11), whereas tau hyperphosphorylation is slightly delayed relative to GSK3β activation, suggesting that GSK3β may not be the only kinase upstream of tau hyperphosphorylation.

Increasing evidence also indicates that eNOS uncoupling is a potential mechanism underlying production of reactive oxygen associated with vascular endo-
Therapeutic perspective

Prior to our study (10, 11), several groups suggested that NO functions in BBB disruption, based on studies using NOS inhibitors. Ischemia-induced BBB permeability to [14C]sucrose was reduced by intravenous administration of aminoguanidine in a lipopolysaccharide-induced BBB damage model (7). In addition, intravenous administration of N\(\text{G}\)-nitro-L-arginine methyl ester (L-NAME) 3 h after ischemia significantly reduced infarct volume and blocked increased NO production after ischemia and ameliorated neurological dysfunction (41). Specifically, L-NAME treatment also significantly reduced cerebral vascular damage, as assessed by Evans Blue extravasation following cerebral ischemia (41, 42).

We also developed novel inhibitor of Ca\(^{2+}\)/calmodulin-dependent NOSs. 3-[(4-(3-Chloro-2-methylphenyl)-1-piperazinyl)ethyl]-5,6-dimethoxy-1-(4-imidazolylmethyl)-1H-indazole dihydrochloride 3.5 hydrate (DY-9760e), a novel calmodulin antagonist, is a potent inhibitor of calmodulin-dependent NOSs, including eNOS and nNOS (29, 43). DY-9760e significantly blocked increases in brain water content and extravasation of Evans Blue dye after ischemia (10, 44). MRI analysis of ischemic rat brain revealed that DY-9760e significantly prevented development of brain edema in the cortical region of the ipsilateral hemisphere. Trifluoperazine, a calmodulin antagonist structurally different from DY-9760e, also attenuated brain edema elicited by focal ischemia. Furthermore, DY-9760e also reduced TNF-induced increased permeability of inulin through a cultured brain microvascular endothelial cell monolayer. The data suggest the exciting possibility that calmodulin-dependent signaling plays a critical role in impairment of the brain microvascular barrier (44).

In the ME model, eNOS up-regulation predominantly occurs in vascular endothelial cells in rat brain, thereby eliciting protein tyrosine nitration in the same cells (10). Since eNOS activity depends on the binding of Ca\(^{2+}\)-calmodulin, which induces allosteric changes, we recently asked whether DY-9760e treatment inhibits NO generation and protein tyrosine nitration formation in a rat ME model (10). We found that there was a significant increase of Evans Blue extravasated from blood vessels into the area surrounding the brain parenchyma at 24 – 72 h after ME, whereas this leakage was partially inhibited by DY-9760e. Importantly, DY-9760e treatment preferentially inhibited protein tyrosine nitration induced by eNOS in endothelial cells of the same model. BBB integrity, which was preserved by DY-9760e treatment, was closely associated with decreased protein tyrosine nitration and PARP cleavage in the microvascular endothelium (10). Taken together with our previous observation that DY-9760e ameliorates BBB disruption, we proposed that the neuroprotective and vaso-protective effects of DY-9760e are in part mediated by inhibition of eNOS aberrantly expressed after ischemic insult.

We previously showed that DY-9760e inhibited nNOS and eNOS and reduced NO production in N1E-115 cells (43). Importantly, DY-9836, an N-de-alkylated form of DY-9760e and active metabolite, promoted neuroprotective and cardioprotective effects against cardiac hypertrophy induced by endothelin-1 and angiotensin II (36, 45). We also addressed an inhibitory effect of DY-9836 on uncoupling of eNOS and O\(^{2-}\) generation following ME-induced neurovascular injury. Of great interest, we found that eNOS uncoupling and superoxide generation 72 h after ME were blocked by incubation with DY-9836 in an in vitro study of isolated microvessels (46). These observations are potentially important because uncoupled eNOS generates superoxide. Thus, a novel calmodulin inhibitor such as DY-9760e (or DY-9836) could be an attractive vaso-protective drug exhibiting a potent protective action on the BBB in brain ischemia or in neurodegenerative disease.

Conclusion

In summary, current studies indicate that brain embolism–induced aberrant eNOS expression in microvessels could elevate local NO and O\(^{2-}\), thereby enhancing neuronal susceptibility to peroxynitrite-mediated apoptosis or \(\beta\)-amyloid accumulation in aged rats (Fig. 4). An attractive observation is that in a mild ME model, sequential and progressive neuronal events are reconstructed in the ME brain. The eNOS elevation and its uncoupling in microvessels triggers NO and super-
Further extensive studies are required to define the cause-and-effect relationship between these events, this model is useful for preclinical studies using inhibitors of \( \beta \)-amyloid accumulation.

**Acknowledgments**

This work was supported in part by Grants-in-Aid for Scientific Research and for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (19390150 and 20659008 to K.F.) and the Smoking Research Foundation (to K.F.).

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