Neurovascular Protective Function of Endothelial Nitric Oxide
– Recent Advances –

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In the central nervous system endothelial nitric oxide (NO) is an essential molecule responsible for the preservation of the functional integrity of the neurovascular unit. NO causes vasodilatation and is an important inhibitor of platelet aggregation, smooth muscle cell proliferation, and white blood cell adhesion. In addition, endothelium-derived NO exerts anti-inflammatory and pro-angiogenic effects. More recently, it has been recognized that endothelial NO modulates the expression and processing of amyloid precursor protein in cerebrovascular endothelium and neuronal tissue. Studies in endothelial NO synthase (eNOS) knockout mice indicate that endothelial NO functions as a neurovascular protective molecule during aging. Indeed, genetic inactivation of eNOS exacerbates the detrimental effects of aging on cerebrovascular, microglial, and neuronal functions as well as on cognition. These findings suggest that the preservation of healthy endothelium and normal function of eNOS might be important therapeutic targets. Because the beneficial effects of NO are mostly mediated by the activation of guanylate cyclase/cyclic GMP signaling, inhibitors of phosphodiesterase isoforms, or activation of this signaling with exercise, may offer therapeutic opportunities in the prevention and treatment of aging-induced cognitive decline and Alzheimer’s disease. Most recent advances in understanding the molecular mechanisms linking loss of endothelial NO with cognitive decline will be discussed in this review. (Circ J 2016; 80: 1499–1503)

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The production and release of nitric oxide (NO) from endothelial cells are considered fundamental regulatory mechanisms responsible for the maintenance of optimal cerebral blood flow. NO deficiency promotes vasoconstriction, platelet aggregation, smooth muscle cell proliferation, and white blood cell adhesion to the endothelium. Genetic inactivation of endothelial NO synthase (eNOS) causes hypertension, promotes the development of atherosclerosis and in the cerebral circulation increases vulnerability to stroke. Prior studies established that in cerebrovascular endothelium aging reduced the basal and stimulated production of NO, increased the chemical inactivation of NO by superoxide anion and accelerated the degradation of vascular cyclic GMP, thereby inhibiting the vasoprotective effects of endothelial NO. Aging is considered a major unmodifiable risk factor for cerebrovascular disease, in part because it significantly impairs the homeostatic function of endothelial NO. More recent findings increasingly support the concept that in the aging brain, endothelial NO participates in the control of 2 major mechanisms contributing to the pathogenesis of Alzheimer’s disease (AD); namely, metabolism of amyloid precursor protein (APP) and functional properties of the microglia. The amyloid hypothesis of AD postulates that deposition of APP cleavage products, amyloid β peptides (Aβ1–40 and Aβ1–42) initiates a cascade of events leading to inflammation, development of tau pathology, synaptic dysfunction, neuronal death, and impairment of cognitive function. Microglia, the phagocytic cells of the central nervous system, play an important role in the inflammatory response, but the exact contribution of these cells to the pathogenesis of AD is incompletely understood.

Recognition of the fact that chronic exposure to cardiovascular risk factors (including aging) increases the risk for development of AD implies that altered vascular function might increase brain vulnerability to AD pathology, thereby accelerating and/or exacerbating cognitive decline. To define the molecular mechanisms underlying the link between vascular dysfunction and the development of AD pathology, we focused on endothelial NO because it is one of the first molecular targets negatively affected by the oxidative stress and inflammation induced by cardiovascular risk factors. Recent progress in this area of research will be highlighted in this review.

Endothelial NO and Metabolism of APP

In the central nervous system, the metabolism of APP has been extensively studied during the past 3 decades. This
Accumulated knowledge (and failure of numerous clinical treatments of dementia) remains a major unmet medical need. The current lack of effective strategies in the prevention and treatment of AD was motivated by attempts to develop new therapies for AD within the framework of the amyloid hypothesis. However, the exact sequence of events and the molecular underpinning of the vascular contribution to the pathogenesis of AD remain to be defined.

APP is a complex single-pass transmembrane protein undergoing proteolytic cleavage resulting in the generation of several different products (Figure). APP is most abundantly expressed in the brain, kidney, and platelets. Notably, endothelial cells of the cerebral and peripheral arteries also express APP and the enzymes required for both non-amyloidogenic and amyloidogenic processing of APP (Figure). Three alternatively spliced isoforms of APP have been identified: APP695, APP751, and APP770 (number refers to the number of amino acids). Interestingly, higher expression of APP has been detected in human brain microvascular endothelial cells as compared with the expression in human umbilical vein endothelial cells. Strong evolutionary conservation of APP from invertebrates to humans suggests that the function of APP is critically important under physiological and pathological conditions. However, the function of APP remains poorly understood, particularly in tissues outside the central nervous system. Expansion of the knowledge base regarding the function of APP, especially in the cerebral circulation, is of major importance. Indeed, it is difficult to develop effective therapies targeting the metabolism of the APP without proper annotation of the physiological function(s) fulfilled by APP and its cleavage products. Despite relatively limited understanding of APP physiology, advances have been made in studies designed to determine the role of endothelial NO in the metabolism of APP. Studies in cultured human brain microvascular endothelial cells (BMECs) established that inhibition of eNOS activity increased the expression of APP and BACE1, thereby promoting amyloidogenic processing of APP. Moreover, loss of eNOS in human BMECs causes increased production and release of cytotoxic Aβ peptides. This effect can be prevented by treatment of the BMECs with the eNOS substrate L-arginine or by pharmacologic inhibition of phosphodiesterase (PDE). These findings support the concept that cyclic GMP signaling is responsible for the inhibitory effect of NO on the expression and function of APP and BACE1. Of note, inhibition of eNOS did not affect expression of α-secretase, γ-secretase or enzymes that participate in the degradation of Aβ peptides, including neprilysin, endothelin-converting enzyme 1, or insulin-degrading enzyme, thus suggesting that endothelial NO selectively modulates the amyloidogenic pathway in the metabolism of APP. The exact homeostatic reason for this interaction between NO and APP metabolism is unclear. One possibility is that upregulation of APP and BACE1 is an adaptive response to stress induced by the loss of NO. Indeed, APP is considered a stress-induced protein and it does exert protective effects in experimental models of ischemia, stroke and brain trauma. However, the mechanisms responsible for neuroprotection provided by APP are incompletely defined. In contrast, overexpression of human mutated APP in mice (a strategy used to create many models of AD) does not confer

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**Figure.** Amyloid precursor protein (APP) can be cleaved by a nonamyloidogenic pathway (A) or an amyloidogenic pathway (B). (A) In the nonamyloidogenic pathway, APP is cleaved by α-secretase within the Aβ domain and thus precludes the formation of Aβ. α-secretase cleavage releases the sAPPα ectodomain. Following α-secretase cleavage, γ-secretase processing results in the release of the p3 fragment and the APP intracellular domain (AICD). (B) In the amyloidogenic pathway, β-secretase (BACE1) cleaves APP and releases the sAPPβ ectodomain. Subsequent processing by γ-secretase results in the generation and release of the cytotoxic peptide, Aβ, as well as the AICD. (Reproduced with permission from De Strooper B, et al.)
Neurovascular Protection by NO

Effects of Aging

Because aging is a dominant risk factor for AD, it was of major interest to determine the effects of aging on the metabolism of APP in eNOS-knockout mice. Studies by our group were performed in late middle-aged male mice (14–15 months). This age was chosen to minimize the influence of the heart failure phenotype of eNOS-knockout mice that starts developing in male mice at approximately 15 months of age. Although elevated expression of APP and BACE1, as well as higher levels of Aβ peptides, persisted in brains derived from aged animals, the most striking difference between young and aged mice was detected in the microglia. Indeed, several microglial markers, including cluster of differentiation 68 (CD68), ionized calcium adaptor molecule 1 (Iba1), and major histocompatibility complex II (MHCII), were significantly upregulated in aged eNOS-knockout mice. In contrast, an astrocytic marker, glial fibrillary acidic protein, and a neuronal marker (neuronal nuclei) were not affected, thus demonstrating selectivity of alterations in the microglia. As microglia are an integral part of the immune system in the brain, these observations suggest that microglia were capable of detecting the loss of NO from vascular endothelium. Endothelial NO is known to possess anti-inflammatory properties and it is conceivable that NO deficiency might lead to activation of the microglia. Alternatively, activation of the microglia might be induced by elevated concentrations of Aβ peptides in the neuronal tissue of NO-deficient mice. To gain additional insights into possible inflammatory consequences of eNOS inactivation, a cytokine array coupled with ELISA assays was used. These studies demonstrated that granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1α (IL-1α), and macrophage inflammatory protein (MIP) 1β were significantly upregulated in the brains of aged eNOS-knockout mice.

In aggregate, these observations suggest that endothelial dysfunction caused by the loss of NO might affect the function of the microglia, thereby exacerbating the inflammatory response in brain tissue. It is generally accepted that the function of the microglia is tightly regulated because they have the potential to irreversibly damage neuronal cells. The exact sequence of events and molecular mechanisms responsible for alterations in microglial function are poorly understood and remain to be defined. In addition, we point out that the effects of genetic inactivation of eNOS on the function of aging astrocytes have not been systematically studied. Only one prior study using coculture of endothelium and astrocytes established that endothelial NO might affect the metabolic profile of astrocytes.

The results of that study suggest that high glycolytic activity in astrocytes is dependent on intact production and release of NO from cerebrovascular endothelium. Whether this mechanism is operative under in vivo conditions or affected by aging is unknown.

The importance of endothelial NO in the aging brain was further underscored by studies in aged heterozygous eNOS+/- mice. The phenotypic characteristics of 18-month-old eNOS+/- mice were quite remarkable. Angiography revealed multiple cortical/subcortical areas of nonperfusion in eNOS+/- mice, with a significantly higher number of lesions in aged mice. In contrast, there were no areas of nonperfusion in the brains of wild-type mice until the age of 24 months. Ischemic lesions were rarely found in brain sections without hippocampal involvement. This observation is consistent with the recognized vulnerability of the hippocampus during the initiation and progression of AD pathology.

Further analysis established that formation of intravascular in situ thrombi is responsible for these focal impairments of blood flow. Both immunohistochemistry and angiography demonstrated a higher microvascular density in the hippocampus of aged eNOS+/- mice as compared with wild-type age-matched control animals. This was interpreted as a compensatory angiogenic response of the ischemic brain.

Consistent with the increased BACE1 activity detected in the brains of eNOS-/- mice, examination of aged eNOS-/- mice revealed significant cerebral amyloid angiopathy (CAA) with dense deposits of Aβ in the vascular wall of pial and parenchymal arterioles in the cerebrum and hippocampus. Moreover, CAA was also detected in cerebral capillaries. In agreement with the findings of our group in eNOS-/- mice, a significant increase in soluble Aβ1-40 was detected in the forebrain of aged eNOS-/- mice, thereby demonstrating that loss of eNOS function promotes amyloidogenic processing of APP, thereby promoting selective processing of APP leading to formation of Aβ. Furthermore, this study showed that increased levels of Aβ peptides, whether induced by elevated concentrations of Aβ peptides or the loss of NO, promoted upregulation of Aβ peptides in the brains of aged eNOS-knockout mice. Indeed, several microglial markers, including cluster of differentiation 68 (CD68), ionized calcium adaptor molecule 1 (Iba1), and major histocompatibility complex II (MHCII), were significantly increased in brain tissue from eNOS-knockout mice. The differential effects of the wild-type and mutated APP were attributed to predominant amyloidogenic processing of mutated APP resulting in increased levels of cytotoxic Aβ peptides vs. predominant non-amyloidogenic processing of wild-type APP and production of neuroprotective soluble APPα (sAPPα).

Metabolism of APP in eNOS-Knockout Mice

Results regarding the role of NO in the metabolism of APP obtained in cultured human BMECs have been further validated under in vivo conditions in eNOS-knockout mice. Examination of the microvascular and neuronal tissues of eNOS-deficient mice confirmed that loss of NO was responsible for increased expression of APP and BACE1. Most importantly, levels of Aβ1-40 and Aβ1-42 were significantly increased in brain tissue from eNOS-knockout mice. Our group also examined the effects of eNOS inhibition on expression of APP and BACE1 in isolated human cerebral arteries and demonstrated that in intact human cerebral blood vessels, loss of NO promoted upregulation and amyloidogenic processing of APP (unpublished observation). In aggregate, these findings revealed a previously unrecognized role of endothelial NO in the modulation of APP expression and processing. Importantly, previous studies established that in mice, cerebral blood flow is not affected by inactivation of eNOS thereby suggesting that loss of NO per se rather than a decrease in cerebral blood flow is responsible for modulation of APP and BACE1. Besides identification of the molecular pathway possibly linking endothelial dysfunction with AD pathology, these observations pointed to preservation of eNOS/cyclic GMP signaling as a potentially important strategy in the prevention of excessive amyloidogenic processing of APP. Importantly, studies of exogenous NO in cultured neurons corroborated and extended this concept. Consistent with the findings of our group, treatment of cultured neurons with low concentrations of NO (10^-9 –10^-8 mol/L) suppressed expression of the BACE1 protein. Moreover, suppression of BACE1 transcription by NO was dependent on activation of guanylate cyclase and formation of cyclic GMP. Interestingly, in neuronal cells, higher concentrations of NO (10^-6 mol/L) cause S-nitrosylation of BACE1. S-nitrosylation does not affect BACE1 protein expression but inhibits BACE1 enzyme activity. Thus, both cyclic GMP and S-nitrosylation NO signaling exert inhibitory effects on the expression and activity, respectively, of BACE1. Inhibition of BACE1 reduces the production of Aβ peptides and amyloid load in both endothelial and neuronal cells.
processing of APP and elevation of Aβ peptides in neuronal tissue. Moreover, aged eNOS−/− mice exhibited cognitive deficits similar to aged eNOS−/− mice.7,37 These observations support the concept that healthy endothelium is essential for the preservation of cognitive function during aging. Most importantly, it appears that loss of endothelial NO is a major mechanism linking cardiovascular risk factors with cognitive decline and possibly the development of AD.46 This concept has important clinical implications. Indeed, strategies designed to protect the vascular endothelium might be helpful in the prevention and treatment of dementia, in particular loss of cognitive function induced by AD.

**Therapeutic Implications**

Despite the intensive effort and ever increasing number of publications, clinical trials, and innovative drug development, therapeutic options available to patients with cognitive impairment remain very limited. If indeed endothelial dysfunction caused by reduced eNOS activity or inactivation of NO derived from endothelium is an early event in the pathogenesis of cognitive impairment, then it is reasonable to expect that preservation of endothelial function and NO/cGMP signaling may help to prevent cognitive decline. It is of interest to point out that disruption of endothelial NO/cGMP signaling appears to be involved in the development of both amyloid-dependent and amyloid-independent AD pathology. Therefore, a number of preclinical studies have evaluated the therapeutic potential of selective PDE5 inhibitors in experimental models of AD. These drugs are designed to protect cGMP from degradation by PDE5, thereby preserving cGMP signaling.

Treatment with sildenafil, a PDE5 inhibitor currently used in treatment of erectile dysfunction, improved synaptic function and memory in APP/PS1 mice, a widely used model of AD.41 Moreover, sildenafil also decreased Aβ levels in the cortex of APP/PS1 mice.41 Studies in another APP transgenic model of AD, Tg2576 mice, also demonstrated the beneficial effects of sildenafil, manifested as significant improvement of cognitive function. Interestingly, sildenafil did not affect the brain amyloid burden in Tg2576 mice, but it reduced tau phosphorylation in the hippocampus.42 This was associated with reduced activity of both glycogen synthase kinase 3β (GSK3β) and cyclin-dependent kinase 5 (CDK5). Sildenafil also increased levels of brain derived neurotrophic factor (BDNF). Furthermore, the PDE5 inhibitor, tadalafil, exerts a beneficial effect on cognitive function in the J20 murine transgenic model of AD.43 Thus, preclinical studies suggest that PDE5 inhibitors may have potential in the prevention and treatment of cognitive impairment.

With regard to the effects of PDE5 inhibitors in the human brain, existing evidence suggests that sildenafil does not affect cerebral blood flow and does not modulate the hypercapnic response in healthy subjects.44,45 Interestingly, a study in patients with pulmonary hypertension suggests that sildenafil treatment improves neurovascular coupling.46 However, in patients with a history of stroke, sildenafil reduced local or regional perfusion in one or more areas of the brain, thereby suggesting that the safety of sildenafil could be problematic in certain populations of patients.47 Because a relatively small number of patients has been evaluated, the issue of sildenafil safety in stroke patients remains to be definitively determined. Of note, sildenafil did not affect cerebral blood flow in patients with hypertension or diabetes. It appears that the cerebrovascular effects of sildenafil may depend on the underlying pathology affecting vasomotor function in the cerebral circula-

**Endothelial NO, Exercise, and Cognitive Impairment**

It is well established that physical activity significantly reduces the risk of cerebrovascular disease, including stroke.48 Existing evidence also suggests that physical activity exerts a protective effect on cognitive function.49 The mechanisms underlying the protective effects of exercise in the cerebral circulation and neuronal tissue are largely unknown. Prior studies in mice established that exercise training increased resting cerebral blood flow in ischemic lesions and consequently this increase in blood flow was associated with improvement in functional and cognitive outcomes.49 Most notably, all these beneficial effects of physical activity were abolished in eNOS-deficient mice, thus demonstrating an essential role of endothelial NO in the observed protective effects of exercise. Exercise-induced increase in the shear stress imposed on the endothelium by circulating blood is a well-established stimulus responsible for the upregulation of eNOS and increased local production of NO.48 Despite strong autoregulation designed to maintain constant blood flow in the brain during changes in arterial blood pressure, existing evidence indicates that blood flow is increased in some regions of the brain during physical activity.48 Indeed, increased blood flow in the motor and sensory cortex, as well as the cerebellum, is the result of an exercise-induced increase in neuronal activity and subsequent vasodilation caused by neurovascular coupling. In this regard, highly relevant prior studies established that habitual physical training increases resting cerebral blood flow in healthy human subjects.51 Moreover, 6 months of aerobic exercise training improved cognitive function in 60–75-year-old human subjects.52 It is now generally accepted that exercise training exerts a number of beneficial effects on the cerebral circulation and cognitive function. Although the exact signaling mechanisms underlying the vasoprotective and neuroprotective effects of exercise remain to be defined, endothelial NO is emerging as a critical molecule linking exercise with increased cerebral blood flow, and improved cognitive function.

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