REVIEW

Paul M. Vanhoutte Lecture

Endothelial nitric oxide: protector of a healthy mind†

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Endothelial nitric oxide (NO) is generated by constitutively active endothelial nitric oxide synthase (eNOS), an essential enzyme responsible for cardiovascular homeostasis. Historically, endothelial NO was first recognized as a major vasodilator involved in control of vasomotor function and local blood flow. In this review, our attention is focused on the emerging role of endothelial NO in linking cerebrovascular function with cognition. We will discuss the recognized ability of endothelial NO to modulate processing of amyloid precursor protein (APP), influence functional status of microglia, and affect cognitive function. Existing evidence suggests that the loss of NO in cultured human cerebrovascular endothelium causes increased expression of APP and β-site APP-cleaving enzyme 1 (BACE1) thereby resulting in increased secretion of amyloid β peptides (Aβ1-40 and Aβ1-42). Furthermore, increased expression of APP and BACE1 as well as increased production of Aβ peptides was detected in the cerebral microvasculature and brain tissue of eNOS-deficient mice. Since Aβ peptides are considered major cytotoxic molecules responsible for the pathogenesis of Alzheimer's disease, these observations support the concept that a loss of endothelial NO might significantly contribute to the initiation and progression of cognitive decline. In addition, genetic inactivation of eNOS causes activation of microglia and promotes a pro-inflammatory phenotype in the brain. Behavioural analysis revealed that eNOS-deficient mice exhibit impaired cognitive performance thereby indicating that selective loss of endothelial NO has a detrimental effect on the function of neuronal cells. Together with findings from prior studies demonstrating the ability of endothelial NO to affect synaptic plasticity, mitochondrial biogenesis, and function of neuronal progenitor cells, it is becoming apparent that the role of endothelial NO in the control of central nervous system function is very complex. We propose that endothelial NO represents the key molecule linking cerebrovascular and neuronal function.

Keywords   Alzheimer's disease • Amyloid precursor protein • BACE1 • Cognitive impairment • Endothelium • Exercise • Hippocampus • Microglia • Neuronal progenitors • Nitric oxide • Mitochondria • Vascular dementia

Introduction

The vast medical, economic, and social burdens of dementia have stimulated the renewal of efforts to define the role of cerebrovascular disease in the pathogenesis of cognitive decline. Growing evidence continues to substantiate the link between vascular risk factors and higher incidence of cognitive impairment.[1] Based on these observations, it has been hypothesized that treatment of modifiable vascular risk factors could be an effective strategy to prevent cognitive decline. However, attempts to implement this approach in clinical practice resulted in inconsistent outcomes.[1] This problem is most likely the result of an incomplete understanding of specific molecular mechanisms underlying detrimental effects of different vascular risk factors

on cerebrovascular and neuronal function. In patients exposed to vascular risk factors, impaired endothelium-dependent relaxations are detectable before any morphological changes could be observed in the blood vessel wall. Most importantly, exposure to risk factors including hypertension, hypercholesterolaemia, diabetes, ageing, smoking, obesity, and sedentary life style results in identical outcome namely impairment of endothelial function. Therefore, endothelium has been recognized as an important therapeutic target for the prevention and treatment of vascular disease. However, each vascular bed has tissue-specific morphology, physiology, biochemistry, pathology, and pharmacology. Moreover, major differences between endothelial cells lining large conduit arteries, small resistant arteries, and microvessels within a single vascular

bed further increase the complexity of molecular mechanisms activated in the presence of vascular risk factors.

Nitric oxide in the cerebrovascular endothelium

Vascular endothelium has the capacity to produce and release vaso-active substances thereby actively participating in local control of blood vessel diameter and tissue perfusion. Production of NO in endothelial cells is considered the most important vasodilator mechanism responsible for the preservation of proper vasomotor function. Shortly after the discovery of NO and the identification of endothelial nitric oxide synthase (eNOS) enzymatic activity as a major source of NO, it was postulated that the loss of NO is the central mechanism in the pathogenesis of endothelial dysfunction. Indeed, both in cerebral and peripheral blood vessels, reduced availability of NO results in major detrimental alterations of vascular function, including propensity towards vasoconstriction, increase in arterial blood pressure, and development of atherosclerosis.[2] Loss of endothelial NO also promotes proliferation of smooth muscle cells, platelet aggregation, white blood cell adhesion, and inflammation thereby playing an essential role in the initiation and progression of vascular disease. In the cerebral circulation, preservation of endothelial NO production is an important strategy in the prevention of cerebrovascular disease and stroke. Activation of eNOS mediates protection from stroke by preserving cerebral blood flow, and by preventing inflammation, platelet aggregation, thrombosis, and apoptosis.[2] In addition, eNOS plays a critical role in the mobilization of progenitor cells and neovascularization.[3] Recognition of the local paracrine function of endothelium directly affecting neuronal function significantly expanded the concept of endothelial dysfunction.[4] The fact that the intact endothelium may confer a cytoprotective and homeostatic function in the brain raises an intriguing possibility that endothelial dysfunction in the cerebral circulation may contribute to, if not actually cause, neurologic disease. In this regard, the ability of NO released from the endothelium to directly affect function of neuronal cells is of major interest. This concept may help define molecular mechanisms underlying the link between vascular risk factors, loss of endothelial NO, and pathogenesis of neurological diseases.

Vascular risk factors and endothelial nitric oxide

Oxidative stress caused by excessive production of reactive oxygen species, primarily superoxide anion, is considered the most important mechanism by which risk factors deprive the endothelium of NO. The seminal observation by Rubanyi and Vanhoutte[5] regarding the ability of superoxide anions to chemically inactivate endothelium-derived relaxing factor/NO instigated an intense effort to determine endothelial sources of superoxide anions. Moreover, this observation provided a mechanistic framework for investigations of the role of oxidative stress in the pathogenesis of vascular disease. It is now well established that in endothelial cells, besides mitochondria, activity of NADPH oxidase, uncoupled eNOS, cyclooxygenase, and xanthine oxidase are important sources of superoxide anions.[6] Vascular risk factors promote the up-regulation of NADPH-oxidase activity, generation of superoxide anions, and subsequent chemical

inactivation of NO. This reaction generates a potent oxidant, peroxynitrite.[7] Oxidative stress induced by increased production of peroxynitrite may oxidize tetrahydrobiopterin (BH4), an essential co-factor required for the activity of eNOS. If the concentration of BH4 becomes suboptimal, eNOS becomes uncoupled thereby causing reduced endothelial production of NO, increased production of superoxide anions, and peroxynitrite.[8] In the context of this review, it is important to point out that in the cerebral circulation, increased local concentration of amyloid β (Aβ) peptide generated by sequential cleavages of the amyloid precursor protein (APP) [by activities of β-site APP-cleaving enzyme 1 (BACE1), and γ-secretase; *Figure 1*] stimulates production of superoxide anion.[9] Amyloid β represents a pivotal perpetrator of the neurovascular pathology that defines Alzheimer's disease (AD). It is also important to note that detrimental effects of Aβ on cerebral circulation precede cognitive decline thereby suggesting that impaired cerebrovascular function might play an important role in the pathogenesis of cognitive impairment.[10]

Endothelial nitric oxide in the pathogenesis of Alzheimer's disease

Historically, interest in the cerebrovascular effects of Aβ was initiated by formulation of amyloid hypothesis of AD by Selkoe and Hardy in 1991.[11,12] Over the last two decades, it became apparent that excessive amyloidogenic processing of APP and elevated local concentration of Aβ causes endothelial dysfunction in the cerebral blood vessels.[13] In experimental models of AD cerebrovascular endothelial function is impaired in very young animals long before development of AD pathology and appearance of detectable cognitive decline.[13] Mechanistically, endothelial dysfunction in AD models appears to be caused by increased formation of superoxide anion derived from NADPH-oxidase activity.[14] Genetic inactivation of the catalytic subunit Nox2 of NADPH-oxidase prevents endothelial dysfunction in young APP transgenic mice.[15] Additionally, pharmacological inhibition of NADPH-oxidase or genetic inactivation of Nox2 restored endothelial function in cerebral arteries derived from aged APP transgenic mice.[15] Activation of the scavenger receptor CD36 by Aβ1-40 is a critical step in signalling responsible for up-regulation of NADPH oxidase and the subsequent increase in production of superoxide anions in cerebral arteries.[16] CD36 also promotes cerebral amyloid angiopathy.[16] In contrast, genetic inactivation of CD36 in an experimental model of AD protects cerebral arterioles from the detrimental cerebrovascular effects of Aβ1-40. Most notably, deletion of CD36 also causes significant improvement in cognitive performance of AD mice.[16] These observations support the concept that CD36 is a key signalling molecule responsible for the pathogenesis of endothelial dysfunction in AD. It is of interest that in transgenic mice overexpressing mutated forms of APP, Aβ could be elevated in circulating blood and brain (as in Tg2576 mice[17,18]) or predominantly in brain (Tg-SwDI mice[17]). Although impairment of endothelium-dependent relaxations was observed in both models of AD, the impairment was significantly more pronounced in Tg2576 mice thereby suggesting that an increase in circulating Aβ significantly contributes to endothelial dysfunction.

Cerebral arteries do not have an external elastic lamina and therefore during progression of AD, aggregated forms of Aβ1-40 and
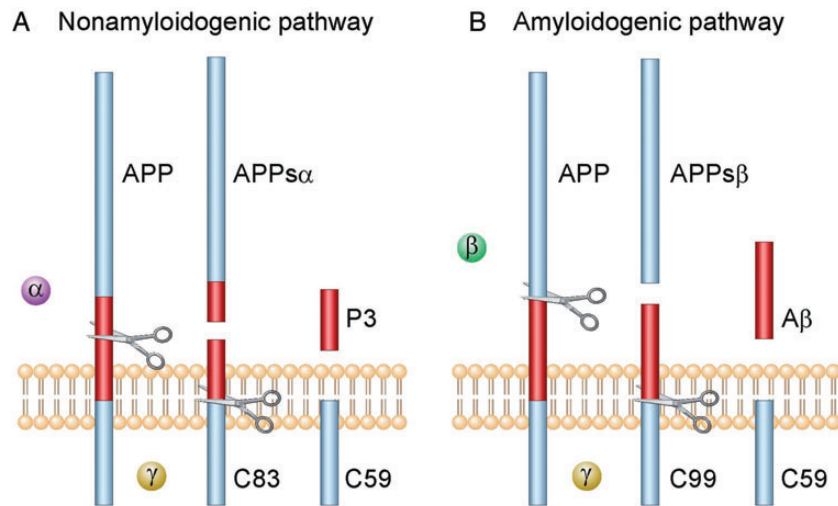
Figure 1 In the non-amyloidogenic pathway, (A) APP is first cleaved by α-secretase within the Aβ sequence thereby generating APPsα ectodomain. Further processing by γ-secretase results in the release of the p3 fragment. In the amyloidogenic pathway, (B) β-secretase (BACE1) cleaves APP and generates APPsβ ectodomain. Further processing by γ-secretase results in the release of cytotoxic Aβ (reproduced with permission from reference[50]).

Aβ1-42 (released from the neuronal tissue) accumulate within the vessel of small and medium size arteries, leading to cerebral amyloid angiopathy.[19] Circumferential bands of accumulated Aβ suggest that Aβ may also originate from the vascular smooth muscle cells.[19] These pathological changes in the smooth muscle layer are most likely responsible for the impairment of vasodilatation to hypercapnia and neurovascular coupling. Whether loss of endothelial NO may affect initiation and progression of cerebral amyloid angiopathy in humans is unknown and remains to be determined.

Given the fact that the brain does not have lymphatics and that the perivascular spaces serve as the lymphatic system,[20,21] Aβ produced by neurons and other cells is eliminated from the brain by perivascular drainage of interstitial fluid along the basement membranes in the walls of capillaries and arteries ultimately draining Aβ into the cervical lymph nodes.[21] Blood vessel pulsations are believed to stimulate flow of interstitial fluid along the vascular wall.[21] As arteries stiffen with age, it is conceivable that the amplitude of vascular pulsations is reduced thereby leading to impairment of this brain drainage system.[21] It is likely that the loss of endothelial NO may significantly contribute to stiffening of cerebral blood vessels thereby exerting a detrimental effect on clearance of Aβ. Indeed, previous studies demonstrated that reduced availability of endothelial NO increased arterial stiffening.[22]

Endothelial cells forming the blood-brain barrier (BBB) also actively participate in clearance of Aβ into the circulating blood and dysfunction of BBB clearance is considered an important mechanism in the pathogenesis of AD.[23] The receptor for advanced glycation end products (RAGE) mediates influx and reentry of circulating Aβ across the BBB into the brain. On the other hand, the low-density lipoprotein receptor-related protein-1 (LRP) mediates Aβ clearance from the brain to circulating blood.[23] The liver and kidneys are responsible for systemic clearance of free Aβ and of complexes between circulating soluble (s)LRP and Aβ. Whether a loss of

endothelial NO may affect mechanisms underlying BBB-dependent or systemic clearance of Aβ has not been studied. However, existing evidence indicates that there is a significant negative correlation between expression of eNOS in brain capillaries and Alzheimer lesion burden.[24] These findings imply that eNOS could be a direct modulator of the endothelial Aβ transport proteins, including LRP and RAGE. Alternatively, observed reduction in capillary eNOS expression might represent a secondary phenomenon resulting from vascular injury and loss of endothelial homeostasis caused by progression of AD.

Endothelial nitric oxide and synaptic plasticity

Existing evidence indicates that endothelial NO can influence synaptic plasticity in the cortex and striatum.[25,26] Current literature also strongly supports the major role of eNOS in modulation of synaptic function in the hippocampus which is the first and most severely affected brain region in the pathogenesis of AD.[27] Indeed, studies by Hopper and Garthwaite[28] demonstrated that tonic production of NO in vascular endothelium is a critical signal required for hippocampal synaptic function. Long-term potentiation is dependent on phasic release of NO caused by activation of neuronal NOS, as well as tonic release of NO caused by the activity of eNOS. This conclusion was based on the results generated by pharmacological and genetic inactivation of eNOS.[28] Enhancement of synaptic activity by NO is mediated by activation of soluble guanylyl cyclase and increased production of cyclic GMP.[29] It is important to emphasize that in the hippocampus, eNOS is exclusively expressed in vascular endothelium.[30] Notably, prior studies indicate that endothelium-derived NO can travel to ~100 μm distance from endothelial cells.[31,32] Distance between brain capillaries and neuronal cells is ~25 μm well within the range of NO diffusion from the

endothelium.[33] Consistent with these observations, studies in experimental animals including, rats, mice, fish, birds, and snails (reviewed by Bon and Garthwaite[29]) support the concept that intact NO/cGMP signalling is an essential mechanism required for memory formation. Thus, it is likely that the loss of endothelial NO caused by prolonged exposure to vascular risk factors might contribute to impairment of neuronal function and cognitive decline. Relevant to this concept, epidemiological studies established that midlife increase in arterial blood pressure significantly increases the risk for late-life AD.[34] In these studies, measurements of blood pressure were performed 10−20 years before diagnosis of AD. Although exact molecular mechanisms coupling elevated blood pressure with AD have not been defined, it is conceivable that the loss of endothelial NO and subsequent disruption of cGMP signalling caused by hypertension may contribute to development of AD. Therefore, control of arterial blood pressure and preservation of vascular health throughout the life span are currently considered important strategies in protecting the brain from cognitive decline.[35]

Endothelial nitric oxide and processing of amyloid precursor protein

Previous studies in eNOS-deficient mice established that the loss of endothelial NO production in the cerebral circulation does not affect basal cerebral blood flow.[36] However, when exposed to focal cerebral ischaemia, eNOS-null mice suffered from more severe haemodynamic deficit manifested by larger necrotic area and significantly reduced penumbra when compared with wild-type animals.[37] Further studies with genetically modified mice expressing constitutively active eNOS demonstrated that during focal cerebral ischaemia generation of NO in cerebrovascular endothelium is an essential protective mechanism designed to preserve normal cerebral blood flow and minimize loss of neuronal tissue.[37]

Relevant to the pathogenesis of AD, pharmacological or genetic inactivation of eNOS in cultured human brain microvascular endothelium (BMECs) increased expression of APP and BACE1 thus favouring amyloidogenic processing of APP.[38−40] Moreover, levels of Aβ1-40 and Aβ1-42 are significantly increased in cell culture media derived from NO-deprived BMECs[38] thereby suggesting that the loss of NO promotes production and release of cytotoxic Aβ peptides. These observations also suggest that NO plays an important role in modulating APP expression and processing. Examination of eNOS-deficient mice revealed that the modulatory role of NO is not limited to cerebral microvessels. Increased expression of APP and BACE1 as well as elevation of Aβ peptides concentration was also detected in the brain of eNOS-deficient mice[38−40] (*Figure 2*), thus demonstrating that endothelial NO is a critical modulator of APP processing *in vivo*. Of note, inactivation of eNOS also increased expression of APP and BACE1 in human cerebral microvessels (unpublished observation). Our group has also demonstrated that supplementation of NO by nitroglycerin suppresses expression of APP and BACE1 in cerebral microvessels of eNOS-null mice,[39] thus demonstrating that exogenous NO inhibits generation of Aβ peptides. In aggregate, these findings provide a novel mechanistic framework that may help define the relationship between vascular risk factors, endothelial NO, and cognitive impairment.

Endothelial nitric oxide and microglia

Microglia represent resident macrophages of the brain and spinal cord. These immunocompetent cells participate in the defence of neuronal tissue against different pathological agents and injury. Microglial cells constantly survey the brain environment and if faced with pathological changes or injured tissue may become activated.[41] Microglia are involved in the pathogenesis of AD, but the
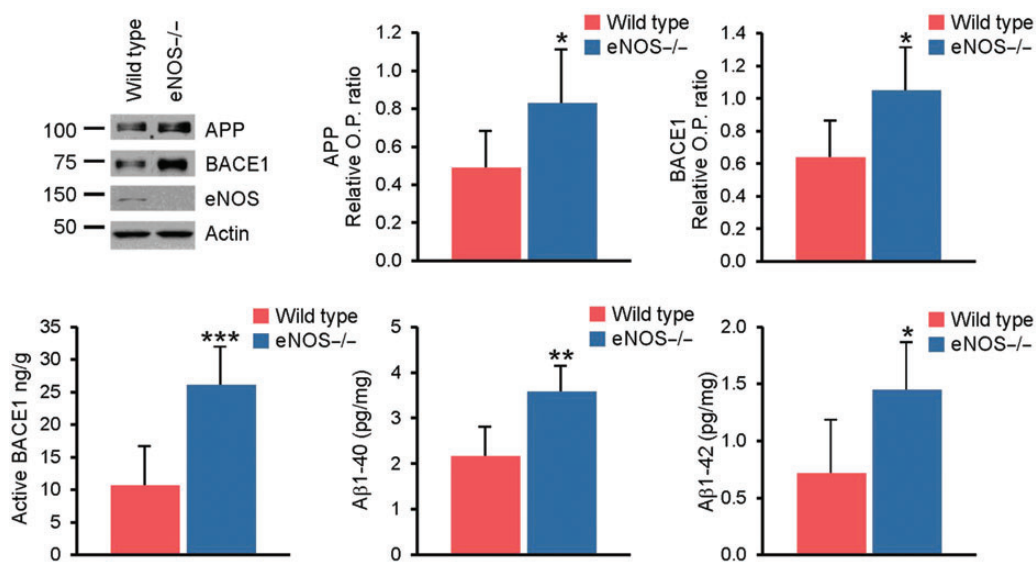

Figure 2 Loss of endothelial NO promotes amyloidogenic processing of APP in the brains of eNOS-deficient mice as demonstrated by increased levels of APP, BACE1, and Aβ peptides (reproduced with permission from reference[38]).

exact role is poorly understood. Microglia may exert both beneficial and detrimental effects during the progression of AD.[42] In this regard, it is of interest that mice deficient in eNOS have elevated expression of microglial markers cluster of differentiation (CD) 68, ionized calcium-binding adaptor molecule (Iba) 1, and major histocompatibility complex (MHC II).[40] These observations suggest that microglia can detect loss of NO from vascular endothelium, although the exact mechanism responsible for microglial activation in eNOS null mice remains to be determined. In addition, genetic inactivation of eNOS also causes increase in brain levels of granulocyte macrophage colony stimulating factor, interleukin-1α, and macrophage inflammatory protein-1β.[40] The exact source of these cytokines has not been identified; however, it is apparent that lack of NO production in endothelium promotes a pro-inflammatory phenotype in the brain. Since under certain circumstances, inflammation might have stimulatory effect on regeneration of brain tissue, it is unclear whether detected changes in the brain of eNOS knockout mice represent an adaptive response designed to protect neuronal tissue. In contrast, it is possible that the loss of NO initiates an inflammatory response that may exert detrimental effects on neuronal tissue thereby contributing to development and progression of AD. Notably, eNOS deficient mice exhibit spatial memory deficit, suggesting that observed impairment of cGMP signalling, enhanced amyloidogenic processing of APP, and resulting inflammatory environment might represent important components contributing to cognitive decline.[40] Most importantly, these findings provide evidence that endothelial NO may play a much more complex role in the pathogenesis of dementia than previously thought. Indeed, it appears that besides regulation of cerebral blood flow, endothelial NO affects function of neuronal and microglial cells (Figure 3). It is therefore conceivable that prolonged exposure to vascular risk factors might reduce the biological activity of endothelial NO thereby significantly increasing vulnerability of the brain for the development of neurodegenerative diseases, including AD.

Endothelial nitric oxide and mitochondrial function

Mitochondrial dysfunction is believed to be an important contributor to the pathogenesis of AD. Prior studies established that endothelial NO stimulates mitochondrial biogenesis.[43] The brain, kidney, liver, heart, and gastrocnemius muscle derived from eNOS-deficient mice display significantly reduced mitochondrial content associated with lower oxygen consumption and ATP content.[44] The stimulatory effect of NO on mitochondrial biogenesis is mediated by activation of soluble guanylyl cyclase and increased formation of cyclic GMP. Moreover, calorie restriction, an intervention known to extend the life span, promotes mitochondrial biogenesis by inducing the expression of eNOS.[45] The stimulatory effect of calorie restriction on mitochondrial biogenesis is abolished in eNOS-null mice thereby suggesting that preservation of normal endothelial production of NO is an essential regulator of tissue metabolism. Indeed, eNOS-deficient mice exhibit insulin resistance, dyslipidaemia, and hypertension which are typical features of metabolic syndrome.[46] Thus, preservation of healthy endothelium and normal bioavailability of NO is an important target in prevention of not only cardiovascular
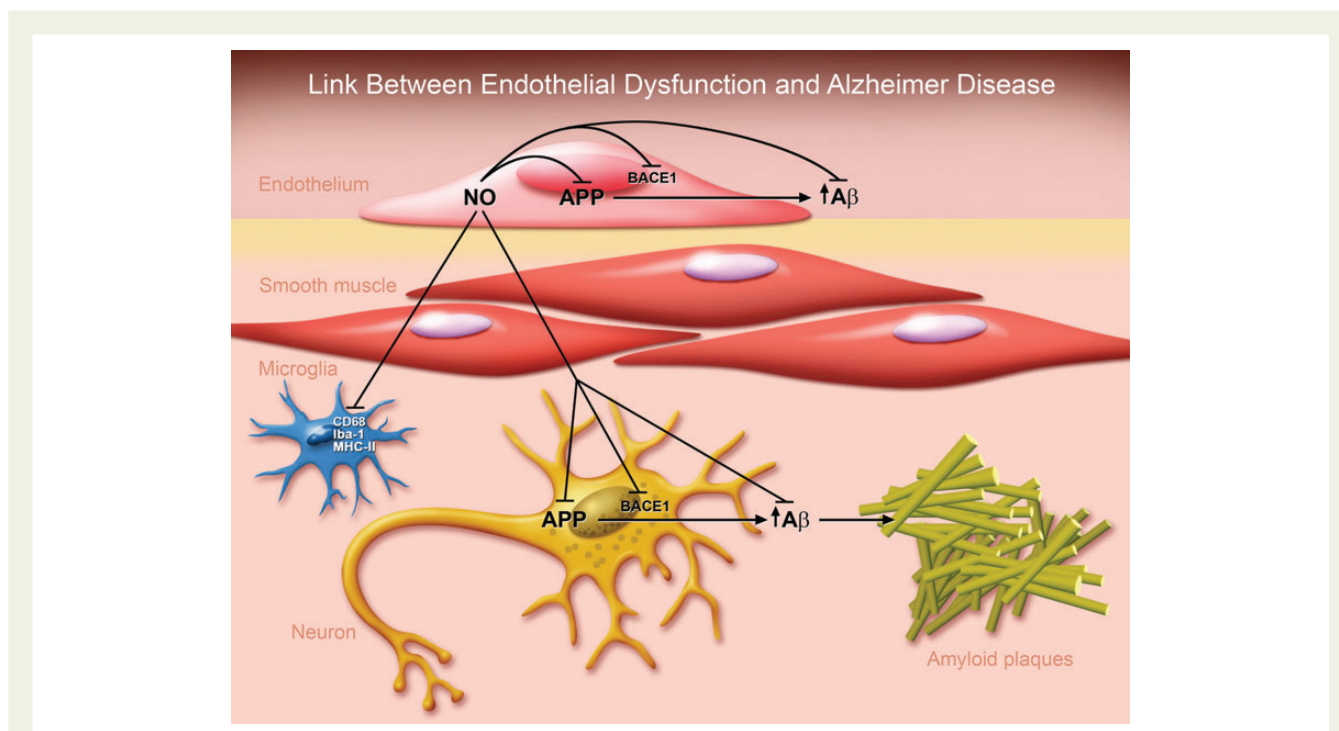

Figure 3 Schematic representation of the inhibitory effects of endothelial NO on APP, BACE1, and Aβ in the cerebrovascular endothelium and neuronal cells. In addition, inhibitory effects of endothelial NO on expression of CD68, Iba-1, and MHC-II in microglia are shown (reproduced with permission from reference[40]).

and cerebrovascular disease but also in the maintenance of homeostatic function in tissue parenchyma.

In contrast to findings obtained in eNOS-deficient mice, analysis of physiological concentration and targets affected by NO derived from cerebrovascular endothelium indicate that under physiological conditions NO does not affect mitochondrial respiration in brain tissue.[47] Activation of eNOS by acetylcholine, bradykinin, or by phosphorylation and subsequent increase in production of endogenous NO did not reach a high enough concentration to inhibit cytochrome c oxidase and oxygen consumption.[47] Notably, levels of cyclic GMP were significantly increased, thereby suggesting that the concentration of NO was sufficient to activate cyclic GMP signalling but not to inhibit cytochrome c oxidase. The authors performed extensive studies designed to model predicted NO concentrations *in vivo* and concluded that physiological NO concentrations released from endothelium were too low to affect brain mitochondrial respiration. The exact reasons for the discrepancy between this conclusion and observations obtained in studies on eNOS null mice are unclear.

Endothelial nitric oxide and neuronal progenitor cells

In the adult brain, the subventricular zone contains neural stem and progenitor cells capable of differentiating into neuronal and glial cells.[48] Existing evidence suggests that the brain has regenerative capacity and that neural progenitor cells contribute to repair of injured brain tissue after ischaemic injury.[48] Studies in eNOS-deficient mice demonstrated that endothelium-derived NO is an important regulator of brain regenerative function.[48] Indeed, loss of NO production in endothelium impairs proliferation and migration of neuronal progenitors.[48] Moreover, expression of brain-derived neurotrophic factor (BDNF) was significantly reduced in the ischaemic brain of eNOS-deficient mice. This may provide mechanistic explanation for reduced neurogenesis in eNOS-deficient mice after stroke.[48] Most importantly, loss of ability to generate BDNF may increase vulnerability of neuronal tissue and exacerbate neurodegenerative changes, including the development of AD.[49] The exact role of neurogenesis in the development of cognitive impairment has not been defined. However, it is likely that impaired regenerative function might contribute to cognitive decline.

In summary, current understanding of the relationship between cerebrovascular and neuronal function increasingly argues for therapeutic and lifestyle interventions designed to protect production and biological activity of endothelial NO. Preservation of healthy endothelium appears to be a prerequisite for the preservation of a healthy mind.

Funding

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