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Author manuscript

*Stroke*. Author manuscript; available in PMC 2024 March 01.

Published in final edited form as:

*Stroke*. 2023 March ; 54(3): 686–696. doi:10.1161/STROKEAHA.122.041444.

## Emerging roles of endothelial nitric oxide synthase in preservation of cognitive health

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### Abstract

Endothelial nitric oxide synthase (eNOS) is critically important enzyme responsible for regulation of cardiovascular homeostasis. Under physiological conditions, constitutive eNOS activity and production of endothelial nitric oxide (NO) exert essential neurovascular protective functions. In this review, we first discuss the roles of endothelial NO in prevention of neuronal amyloid accumulation and formation of neurofibrillary tangles, hallmarks of Alzheimer's disease pathology. Next, we review existing evidence suggesting that NO released from endothelium prevents activation of microglia, stimulates glycolysis in astrocytes, and increases biogenesis of mitochondria. We also address major risk factors for cognitive impairment including aging and apolipoprotein 4 (ApoE4) genotype with focus on their detrimental effects on eNOS/NO signaling. Relevant to this review, recent studies suggested that aged eNOS heterozygous mice are unique model of spontaneous cerebral small vessel disease. In this regard, we review contribution of dysfunctional eNOS to deposition of amyloid- $\beta$  (A $\beta$ ) into blood vessel wall leading to development of cerebral amyloid angiopathy (CAA). We conclude that endothelial dysfunction manifested by the loss of neurovascular protective functions of NO may significantly contribute to development of cognitive impairment.

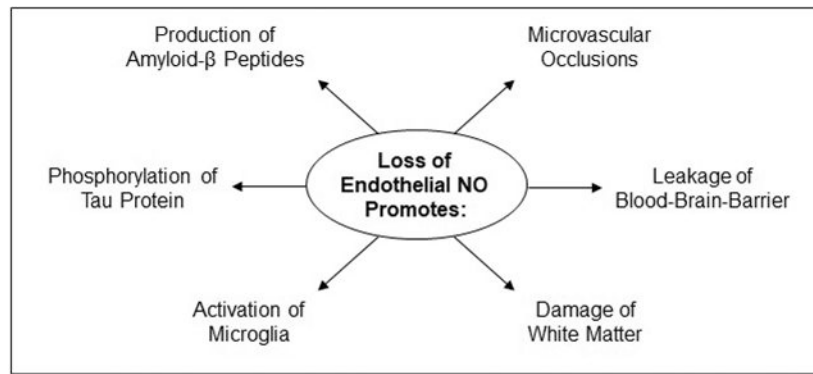
### Graphical Abstract

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Disclosures

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: Dr. Katusic served as a consultant to Ironwood Pharmaceuticals (Massachusetts, USA).



Detrimental effects of endothelial nitric oxide (NO) deficiency contributing to impairment of cognitive function. Exact longitudinal sequence of described functional alterations remain to be determined.

## Keywords

BACE1; ADAM10; amyloid; tau protein; microglia; astrocyte; mitochondria

## Introduction

According to the World Health Organization, currently, more than 55 million people suffer from dementia.<sup>1</sup> This difficult problem is aggravated by the lack of effective therapeutic interventions. Indeed, effective therapy for dementia is major unmet need in modern medicine. Prior studies established that loss of neuroprotective function of endothelium significantly contributes to vulnerability of brain to neurodegeneration and dementia.<sup>2</sup> More precisely, impaired endothelial nitric oxide synthase (eNOS)/nitric oxide (NO) signaling appears to be an essential molecular mechanism linking cardiovascular risk factors with altered brain function and development of cognitive impairment.<sup>3,4</sup> The focus of this review is on mechanisms underlying contribution of impaired vascular eNOS/NO signaling to initiation and progression of cognitive impairment.

## Nitric oxide in the cerebrovascular endothelium

Extensive studies in experimental models and human subjects documented critically important role of eNOS in control of vascular function.<sup>5,6</sup> Under pathological conditions, impaired production and/or biological activity of endothelial NO promotes vasoconstriction, platelets aggregation, upregulation of white blood cells adhesion molecules, and proliferation of vascular smooth muscle cells thereby significantly contributing to pathogenesis of hypertension, atherosclerosis, and vascular complications of diabetes.<sup>6</sup> The importance of vascular protective functions of eNOS is further underscored by observations demonstrating that human subjects with a genetic predisposition to enhanced endothelial NO signaling have reduced risk of stroke.<sup>7</sup> Whether this protective effect of eNOS polymorphisms translates into reduced risk of cognitive impairment and dementia is unknown.

Because capillaries are by far the longest segment of cerebrovascular tree, majority of eNOS in the brain is localized in capillary endothelium. Importantly, the number of endothelial cells in the brain closely matches number of neurons.<sup>8</sup> Moreover, brain capillaries are located less than 15  $\mu\text{m}$  from the nearest neuronal cell body<sup>9</sup>, and therefore “cloud of NO” released from the capillary endothelium may affect function of surrounding parenchymal cells.<sup>10–13</sup> Indeed, prior studies have demonstrated that release of NO from cerebrovascular endothelium and diffusion of NO into surrounding environment enables communication between brain vasculature and neuronal cells.<sup>14–16</sup> Furthermore, evidence continues to accumulate that under pathological conditions, dysfunctional communication between vascular and neuronal compartments in the brain is an important component of complex pathogenesis of neurodegeneration.<sup>17</sup>

## The role of endothelial NO in development of amyloid pathology

Amyloid precursor protein (APP) is highly expressed in human and murine cerebrovascular endothelium.<sup>12</sup> Interestingly, expression of APP is significantly higher in endothelium of cerebral blood vessels compared with endothelium of systemic blood vessels.<sup>18</sup> High expression and strong evolutionary conservation of APP implies important functional relevance. Studies in cultured human brain endothelial cells indicate that under physiological conditions, endothelial APP is for the most part cleaved by  $\alpha$ -secretase thus resulting in the release of soluble APP $\alpha$  (sAPP $\alpha$ ) from endothelium into local environment (Figure 1).<sup>19</sup> Importantly,  $\alpha$ -secretase cleaves APP within A $\beta$ -sequence thereby contributing to low formation of A $\beta$  peptides in healthy endothelium.<sup>19,20</sup> However, in human brain endothelial cells, genetic or pharmacologic inactivation of eNOS function increases expression of APP and  $\beta$ -site APP-cleaving enzyme 1 (BACE1) thereby shifting APP processing towards  $\beta$ -cleavage and increased production of A $\beta$  peptides (Figure 2).<sup>12,21</sup> Reduced activity of soluble guanylate cyclase (sGC) and reduction of cyclic guanosine monophosphate (cGMP) levels are responsible for increased amyloidogenic processing of APP.<sup>12,22</sup> Resulting high local concentration of A $\beta$  may exert detrimental effects on endothelial function mediated by increased formation of reactive oxygen species.<sup>23</sup> Moreover, the results of prior studies suggest that elevated production of A $\beta$  may also adversely affect endothelium-mediated A $\beta$  clearance.<sup>24</sup>

Importantly, genetic inactivation of eNOS in mice (eNOS<sup>-/-</sup> mice) increases expression of APP and BACE1 as well as levels of A $\beta$  peptides in the brain parenchyma.<sup>12</sup> Of note, genetic inactivation of endothelial NO in mice does not affect expression of  $\alpha$ -, and  $\gamma$ -secretase proteins, or A $\beta$  degradation enzymes thereby demonstrating selectivity of endothelial NO modulatory effect for  $\beta$ -processing of APP.<sup>12</sup> Agreement between data generated in brain blood vessels derived from humans and mice, demonstrates that observed endothelial NO modulation of APP and BACE1 expression is evolutionary conserved mechanism. Importantly, prior studies in eNOS<sup>-/-</sup> mice established that the loss of endothelial NO production in the cerebral circulation does not affect global cerebral blood flow.<sup>25,26</sup> Based on these observations, we speculate that in eNOS<sup>-/-</sup> mice, increased expression of APP and BACE1 might be the primary effect of impaired endothelial NO production rather than secondary effect caused by global hypoperfusion of the brain. However, even though cerebral blood flow is not affected, eNOS<sup>-/-</sup> mice suffer from

localized microvascular occlusions that apparently contribute to development of cerebral amyloid angiopathy.<sup>27</sup> How much microvascular occlusions may contribute to development of amyloid pathology in neuronal tissue is unclear at the present time.

Because aging is a major risk factor for cognitive impairment, studies in aged eNOS<sup>-/-</sup> mice are critically important for understanding vascular and cognitive mechanisms impaired by joint loss of eNOS and aging. Indeed, new insights emerged from studies of 14–15 month [late middle aged (LMA)] eNOS<sup>-/-</sup> mice. Levels of APP, BACE1, and A $\beta$  are significantly higher in the brains of LMA eNOS<sup>-/-</sup> mice as compared with LMA wild type mice. In addition, increased levels of APP and A $\beta$ <sub>1–40</sub> were detected in hippocampus of aged-matched eNOS<sup>-/-</sup> mice.<sup>28</sup> Most notably, brain tissue derived from LMA eNOS<sup>-/-</sup> mice demonstrated significantly higher levels of microglial markers, cluster of differentiation 68 (CD68), ionized calcium-binding adaptor molecule 1 (Iba1), and major histocompatibility complex II (MHC II).<sup>28</sup> Further analysis of brain tissue identified elevation of pro-inflammatory cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1 $\alpha$  (IL-1 $\alpha$ ), and macrophage inflammatory protein-1 $\beta$  (MIP-1 $\beta$ ). These observations were confirmed with individual ELISA essays for GM-CSF, IL-1 $\alpha$ , and MIP-1 $\beta$  thereby proving that loss of endothelial NO in aged mice is associated with microglial activation. We also wish to point out that LMA eNOS<sup>-/-</sup> mice suffered from spatial memory deficit consistent with prior reports demonstrating importance of NO pathway in formation of long-term potentiation (LTP) and normal function of hippocampus.<sup>15,29</sup> Thus, studies in LMA eNOS<sup>-/-</sup> mice revealed previously unrecognized mechanism whereby loss of endothelial NO promotes amyloid pathology and activation of microglia.

It is important to note that LMA eNOS<sup>-/-</sup> mice displayed a phenotype consistent with metabolic syndrome (elevated blood pressure, high circulating total cholesterol, density lipoprotein, triglycerides, and glucose detected in LMA eNOS<sup>-/-</sup> mice).<sup>28</sup> The exact role of metabolic changes in development of amyloid pathology and microglial activation is unclear. However, it is unlikely that only metabolic factors and/or hypertension are responsible for development of amyloid pathology in LMA eNOS<sup>-/-</sup> mice. Indeed, pharmacological inhibition or genetic deletion of eNOS in cultured human brain microvascular endothelial cells increased expression of APP, BACE1, and A $\beta$  levels independently of alterations of blood pressure and/or altered metabolic factors.<sup>12</sup> In agreement with these findings, increased brain APP, BACE1, and A $\beta$ , were detected in young eNOS<sup>-/-</sup> mice which displayed normal metabolic profile thus suggesting that loss of direct NO effects on endothelium is critical mechanism underlying alterations in cerebrovascular and neuronal expression and processing of APP.

Interestingly, several studies reported increased expression of eNOS protein in brains of AD patients and experimental animals with AD pathology.<sup>30–35</sup> However, studies in murine model of AD indicate that despite significant upregulation of eNOS protein, eNOS enzyme activity is uncoupled from production of NO.<sup>35</sup> Instead of NO, uncoupled eNOS generates superoxide anion and peroxynitrite thereby leading to dysfunctional NO signaling. Indeed, in cerebral microvessels of AD mice, uncoupling of eNOS results in significantly reduced

levels of cGMP.<sup>35</sup> As we have already noted, impairment of NO/cGMP signaling pathway promotes development of amyloid pathology.

Aging, a major non-modifiable risk factor for cardiovascular disease and AD, increases expression of eNOS mRNA in large cerebral arteries and brain capillaries<sup>36,37</sup> but does not affect expression of eNOS protein.<sup>38,39</sup> However, aging causes shift in eNOS protein phosphorylation patterns thereby promoting inhibition of eNOS activity.<sup>38</sup> Importantly, aging also causes inactivation of endothelial NO by superoxide anion generated by upregulated NADPH oxidase and uncoupled eNOS.<sup>40,41</sup> Similarly, increased production of superoxide anion by NADPH oxidase and uncoupled eNOS impairs NO signaling in endothelium exposed to cardiovascular risk factors including hypertension, diabetes, and hyperlipidemia.<sup>42</sup> In aggregate, these observations suggest that loss of endothelial NO signaling in the brain may be a molecular mechanism linking cardiovascular risk factors with higher risk for development of AD pathology and cognitive impairment.<sup>43</sup>

Ongoing efforts to develop new drugs for prevention and treatment of cognitive impairment resulted in discovery of the first central nervous system (CNS)-penetrant sGC stimulators which are positive allosteric modulators of sGC.<sup>44,45</sup> They can sensitize the sGC enzyme to NO, thereby preserving and amplifying spatiotemporal control of endogenous NO signaling.<sup>46</sup> Preclinical studies have established that the CNS-penetrant sGC stimulators exert beneficial effects in experimental models of neuroinflammation and neurodegeneration.<sup>44,45</sup> Currently, clinical trial with CNS-penetrant sGC stimulator, CY6463, is enrolling patients with Alzheimer's disease (AD) and associated vascular pathology ([ClinicalTrials.gov Identifier: NCT04798989](https://clinicaltrials.gov/ct2/show/study/NCT04798989)). Of note, single-center, proof of concept study reported that treatment of AD patients with three medications, simvastatin, L-arginine, and tetrahydrobiopterin (known to stimulate eNOS/NO signaling) was well-tolerated and resulted in modest increase in cerebral blood flow and improved cognitive function ([ClinicalTrials.gov Identifier: NCT01439555](https://clinicaltrials.gov/ct2/show/study/NCT01439555)).<sup>47</sup>

Over many years, targeting cGMP/NO signaling with phosphodiesterase inhibitors have been extensively investigated in preclinical models and in patients with cognitive impairment and/or dementia including AD. While several phosphodiesterase inhibitors have shown some promising beneficial effects, none of tested phosphodiesterase inhibitors have been approved for prevention or treatment of cognitive impairment and dementia. Recently, Tropea and colleagues (2022) comprehensively reviewed therapeutic potential of phosphodiesterase inhibitors as future therapeutic approach to impaired cognition.<sup>48</sup>

## The role of endothelial NO in development of tau pathology

In neuronal cells, dissociation of hyperphosphorylated tau protein from the microtubules causes formation of neurofibrillary tangles, a hallmark of AD pathology.<sup>49</sup> Excessive phosphorylation of tau and formation of tau aggregates are critically important steps in development of cognitive impairment in AD. Existing literature supports the concept that impairment of endothelial NO production and signaling promotes tau phosphorylation thereby contributing to pathogenesis of AD.<sup>50-52</sup> Indeed, genetic inactivation of eNOS in murine model of AD (APP/PS1/eNOS<sup>-/-</sup> mice) increases phosphorylation of tau in

neuronal cells.<sup>50</sup> This effect is driven by increased cyclin-dependent kinase 5 (Cdk5) activator, p25, and increased activity of Cdk5, enzyme responsible for phosphorylation of tau.<sup>50</sup> These observations suggest that impaired production of NO in cerebrovascular endothelium may initiate and/or exacerbate tau pathology. Indeed, extensive analyses of vascular and cognitive function in mouse model of endothelial dysfunction induced by high salt diet, provided several new mechanistic insights into the role of eNOS in tau phosphorylation.<sup>51,52</sup> Historically, brain hypoperfusion has been considered major mechanism by which impaired vascular function contributes to cognitive impairment.<sup>2</sup> However, more recent findings indicate that high salt diet causes endothelial dysfunction by phosphorylation of eNOS at Threonine 495 thereby inhibiting eNOS activity and reducing endothelial production of NO.<sup>51,52</sup> Most importantly, in this model, suppression of eNOS activity increases phosphorylation of tau thus leading to impairment of cognitive function. Comprehensive analysis of vascular and cognitive functions reinforced the conclusion that tau phosphorylation rather than cerebral hypoperfusion contributes to the cognitive dysfunction induced by high salt diet.<sup>52</sup> More precisely, it was demonstrated that loss of endothelial NO reduces neuronal calpain nitrosylation thereby elevating levels of Cdk5 activator, p25 (Figure 3). Thus, loss of endothelial NO promotes phosphorylation of tau protein and formation of neurofibrillary tangles in murine model of endothelial dysfunction. Whether this mechanism contributes to pathogenesis of cognitive impairment in humans remains to be determined.

### **Loss of endothelial NO, chronic mild hypoperfusion, and white matter damage**

Recent study by Dr Liao's group confirmed that eNOS deficiency does not reduce cerebral blood flow<sup>26</sup>, however, genetic inactivation of eNOS causes microvascular occlusions apparently leading to localized mild brain hypoperfusion.<sup>26,53</sup> Moreover, the authors also provided evidence that loss of endothelial NO impairs mitochondrial function, increases permeability of blood-brain-barrier (BBB) and causes damage of the brain white matter.<sup>26,53</sup> These observations significantly expanded understanding of cerebrovascular mechanisms contributing to pathogenesis of vascular cognitive impairment and dementia (VCID).<sup>54</sup> Most notably, treatment of eNOS-deficient mice with sodium nitrate (supplementation with exogenous NO) completely prevented microvascular occlusions, leakage of BBB, damage of white matter, and normalized gait performance indicating that loss of endothelial NO is major perpetrator of vascular and central nervous system pathology.<sup>26</sup> Impairment of BBB is entirely in agreement with reported increased expression of BACE1 in eNOS-deficient human and murine cerebrovascular endothelium.<sup>12,55</sup> Indeed, most recent findings demonstrate that BACE1 cleaves occludin, a critically important protein responsible for preservation of intact BBB<sup>56</sup>. This observation establishes link between eNOS-deficiency and loss of BBB integrity (another major mechanism of endothelial dysfunction). It is also important to note that elevation of BACE1 exerts detrimental effect on expression and function of eNOS and therefore may further exacerbate loss of endothelial NO, leakage of BBB, and attendant cerebrovascular and neuronal pathology.<sup>56,57</sup>



## Endothelial NO and A $\beta$ cytotoxicity

Formulation of amyloid hypothesis,<sup>58,59</sup> provided foundation for the studies designed to define exact role of expression and processing of amyloid precursor protein (APP) in pathogenesis of AD. The recognition of cytotoxic characteristics of amyloid- $\beta$  (A $\beta$ ) peptides (A $\beta$ 1–40 and A $\beta$ 1–42) was followed by the studies demonstrating detrimental effects of A $\beta$  peptides on cerebrovascular function.<sup>60–64</sup> The mechanisms underlying these detrimental effects were recently reviewed by Cortes-Conteli and Iadecola, 2020.<sup>65</sup> Briefly, A $\beta$  peptides increase expression of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-2 (NOX-2) in perivascular and meningeal macrophages<sup>66</sup>. Enzyme activity of NOX-2 is major source of superoxide anion. Chemical reaction between superoxide anion and NO generates powerful oxidant, peroxynitrite,<sup>67</sup> thereby significantly reducing local concentration of NO thus leading to impairment of endothelium-dependent vasodilatation.<sup>66</sup> Most importantly, loss of endothelial NO increases sensitivity of endothelial cells to apoptosis.<sup>68,69</sup> Indeed, more recent studies suggest that cerebrovascular tree is the primary target of pro-apoptotic effects of A $\beta$  peptides in patients with AD.<sup>40,70</sup> Moreover, and in contrast to parenchymal cells<sup>71,72</sup>, extensive loss of vascular cells including endothelium was detected in brains of patients with AD.<sup>73</sup> Only about half as many endothelial cells were isolated from AD brain as compared to number of cells isolated from normal brain.<sup>73</sup> Disappearance of endothelial cells in AD brain is consistent with vascular regression reported by Religa and colleagues (2013).<sup>47</sup> We would like to emphasize that endothelial NO is a major anti-apoptotic molecule and endothelial cell survival factor.<sup>68</sup> This vascular protective effect of endothelial NO is impaired by aging.<sup>69</sup> Aging also impairs stimulatory effect of shear stress on expression of eNOS thereby increasing sensitivity of endothelium to apoptosis.<sup>69</sup> The anti-apoptotic effect of shear stress is restored by overexpression of eNOS in senescent endothelial cells.<sup>40,69</sup> Consistent with loss of eNOS signaling, patients with AD have lower levels of cyclic GMP in their cerebrospinal fluid and higher expression of phosphodiesterase 5 (PDE5) in the temporal cortex than healthy, age-matched controls.<sup>74,75</sup> As mentioned earlier, eNOS/sGC/cGMP signaling is therapeutic target for central nervous system (CNS)-penetrant sGC stimulator, CY6463. This compound is currently being evaluated in clinical trial designed to improve cognitive function in patients with AD.

## The role of endothelial NO in control of glycolysis in astrocytes

Prior studies established that NO enhances glycolysis in astrocytes but not in neurons, however, exact cellular source of NO was not identified.<sup>76,77</sup> Relevant to this review, Brix and colleagues (2012)<sup>13</sup> demonstrated that NO produced in endothelium enhances aerobic glycolysis in astrocytes. This observation was confirmed and extended to show that nanomolar concentration of NO is capable of modulating energy metabolism in astrocytes with the speed, reversibility, and potency of physiological signal.<sup>78</sup> Notably, the stimulatory effect of NO on aerobic glycolysis is not mediated by activation of soluble guanylate cyclase and production of cyclic-GMP.<sup>13,76</sup> Further analysis of signal transduction pathways demonstrated that co-culturing of astrocytes with primary brain vascular endothelial cells caused stabilization of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and enhancement of glucose transporter-1, hexokinase-1 and monocarboxylate transporter-4 expression (MCT4).<sup>13</sup> Most importantly, endothelium derived NO stimulated glycolysis and production of lactate in

astrocytes.<sup>13</sup> Lactate is then exported from astrocytes to neurons *via* MCT4 and it serves as an important substrate for production of pyruvate and adenosine triphosphate (ATP) in neurons. Moreover, lactate is signaling molecule in the brain involved in regulation of wide range of cellular and physiological homeostatic mechanisms.<sup>79</sup> Whether impairment of endothelial NO production may reduce glycolysis in astrocytes is unknown. Of note, expression of glial fibrillary acidic protein (GFAP), marker for astrocytes, is not altered in aged eNOS<sup>-/-</sup> mice.<sup>28</sup> The effects of eNOS deficiency on expression of glycolytic enzymes and production of lactate in aged eNOS<sup>-/-</sup> mice remain to be determined.

## The role of endothelial NO in mitochondrial biogenesis

Dysfunctional mitochondria are believed to play a major role in pathogenesis of AD.<sup>80-82</sup> Existing evidence indicate that endothelial NO activates the transcriptional mechanisms driving mitochondrial biogenesis (the process designed to increase cellular mitochondrial mass).<sup>83</sup> Notably, the brain, kidney, liver, heart, and gastrocnemius muscle isolated from eNOS<sup>-/-</sup> mice display significantly reduced mitochondrial content associated with lower oxygen consumption and reduced levels of adenosine triphosphate (ATP).<sup>84</sup> This effect is mediated by impairment of NO/sGC/cyclic GMP signaling pathway leading to downregulation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ).<sup>84</sup> Importantly, the stimulatory effect of calorie restriction on mitochondrial biogenesis is abolished in eNOS<sup>-/-</sup> mice thereby demonstrating that intact eNOS function is critically important for mitochondrial adaptation to low calorie intake.<sup>85</sup> The NO-dependent signaling responsible for mitochondrial biogenesis are not very well defined, however, stimulatory effects of NO on PGC-1 $\alpha$ , cyclic AMP-responsive element-binding protein-1 (CREB), and nuclear respiratory factor-1 (NRF-1), and are believed to be of major importance.<sup>86</sup> Moreover, mitochondria from hepatocytes of eNOS<sup>-/-</sup> mice have decreased markers of mitochondrial biogenesis PGC-1 $\alpha$ , mitochondrial transcription factor A, and autophagy/mitophagy [BCL-2-interacting protein-3 (BNIP3), 1A/1Blight chain 3B (LC3)], suggesting decreased mitochondrial turnover rate.<sup>87</sup>

The stimulatory effect of endothelial NO on mitochondrial biogenesis has also been described in human cells.<sup>88</sup> More precisely, genetic inactivation of eNOS blocks mitochondrial biogenesis and adipogenesis in human mesenchymal stem cells (hMSCs). Remarkably, the transfer of mitochondria from normal hMSCs to eNOS-deficient hMSCs restored adipogenesis thus showing that mitochondrial remodeling can be employed in treating abnormalities in energy metabolism caused by dysfunctional eNOS.<sup>88</sup>

## Detrimental effects of aging and apolipoprotein 4 (ApoE4) on eNOS function

### Aging –

In cerebral blood vessels, aging impairs endothelium-dependent relaxations mediated by activation of eNOS (recently reviewed by De Silva and Faraci).<sup>40</sup> Several mechanisms have been implicated in aging-induced dysregulation of eNOS/NO signaling. The renin-angiotensin-system is responsible for production of angiotensin II, and activation of NOX-2-



containing NADPH oxidase leading to increased formation of superoxide anion.<sup>89</sup> Chemical reaction between superoxide anion NO decreases local concentration of NO and results in formation of powerful oxidant, peroxynitrite. In addition, the adaptor protein, p66<sup>Sch</sup> and activated Rho kinase exert inhibitory effects on eNOS in aged blood vessels.<sup>90,91</sup> Interestingly, aging causes significant increase in vascular expression of APP protein.<sup>92</sup> Increased expression of APP in aged blood vessels is associated with elevation of circulating levels of sAPP $\alpha$ .<sup>92</sup> In contrast,  $\beta$ -processing of APP is not affected.<sup>92</sup> Since APP and sAPP $\alpha$  are considered vascular protective molecules,<sup>92,93</sup> upregulation of APP is most likely adaptive response to aging, designed to protect and preserve normal vascular function. In contrast, in cerebral microvessels derived from aged heterozygous eNOS<sup>+/-</sup> mice, impaired production of sAPP $\alpha$  has been detected in cerebral microvessels. Most importantly, proteolytic cleavage of APP is shifted towards  $\beta$ -processing and increased production of A $\beta$ 1–40.<sup>94</sup> Thus, it appears that in mice, healthy aging of blood vessel wall is associated with increased vascular protective function of sAPP $\alpha$ . However, partial loss of eNOS impairs production of sAPP $\alpha$  and increases vascular production of cytotoxic A $\beta$  peptides in aged arteries.<sup>94</sup> We therefore speculate that long-lasting impairment of eNOS function associated with chronic increase in amyloidogenic processing of APP, may help explain epidemiological findings demonstrating that mid-life exposure to cardiovascular risk factors increases vulnerability to AD.<sup>95</sup>

Aging-induced impairment of eNOS signaling contributes to increased arterial stiffening,<sup>96–100</sup> increase in pulse pressure, and mechanical stress on capillaries thereby damaging the capillary wall of strongly perfused organs such as brain, heart, and kidneys.<sup>101</sup> Moreover, increased stiffness itself suppresses eNOS signaling thus further exacerbating endothelial dysfunction.<sup>101,102</sup> Importantly, existing literature supports relevance of aging-induced increase in arterial stiffness to development of cognitive decline and AD.<sup>101,103–105</sup>

#### APOE4 –

Three different ApoE isoforms (ApoE2, ApoE3, and ApoE4) confer different risk for AD.<sup>106,107</sup> Prior studies established that carriers of ApoE4 alleles have increased risk for AD as compared to the carriers of more common ApoE3 variant, whereas ApoE2 is protective against AD.<sup>107</sup> Importantly, ApoE4 is considered the strongest genetic risk for late-onset AD.<sup>107</sup> Despite intensive investigation, the exact mechanisms underlying contribution of ApoE4 to pathogenesis of AD remain incompletely defined.

In the context of this review, we wish to draw attention to the study demonstrating that circulating ApoE4 inhibits enzyme activity of eNOS.<sup>108</sup> In addition to direct inhibitory effect on eNOS activity, ApoE4 exerts dominant-negative effect against ApoE3 thus preventing ApoE3-induced stimulation of eNOS activity in endothelium. These detrimental vascular effects of ApoE4 are mediated by binding of ApoE4 to endothelial apolipoprotein receptor 2 (ApoER2 encoded by *LRP8* gene).<sup>108</sup> Moreover, ApoE4 and genetic variant of ApoER2-R952Q attenuate eNOS activity thereby leading to impairment of reparative and anti-inflammatory capacity of endothelium.<sup>108</sup> Since human brain endothelial cells express ApoER2,<sup>73</sup> it is likely that circulating ApoE4 may bind to endothelial ApoER2 thereby inhibiting activity of cerebrovascular eNOS.

Prior studies in systemic arteries established that cyclophilin A (CypA), a protein secreted in response to inflammatory stimuli, promotes atherosclerosis in aorta of ApoE-deficient mice.<sup>109</sup> This effect is mediated by CypA-induced impairment of eNOS protein expression.<sup>109</sup> Existing evidence support the concept that ApoE4-induces activation of CypA-matrix metalloproteinase-9 signaling in cerebrovascular pericytes, thus leading to blood brain barrier (BBB) breakdown, impairment of cerebral blood flow, neurodegeneration, and cognitive dysfunction.<sup>110,111</sup> Notably, these detrimental effects are independent of A $\beta$  production. Given the results of prior studies demonstrating that CypA mRNA and protein are expressed in endothelium of cerebral and systemic blood vessels,<sup>73,109,112</sup> it appears likely that circulating ApoE4 might contribute to impairment of eNOS function by increasing cerebrovascular endothelial production of CypA. Consistent with this hypothesis are the results of previous study demonstrating elevated levels of CypA in endothelial cells of ApoE4 carriers with AD.<sup>113</sup>

### Endothelial NO and cerebral amyloid angiopathy (CAA)

Sporadic CAA is small vessel brain disease caused by cerebrovascular deposition of A $\beta$ .<sup>114</sup> CAA is common in elderly, and it is an important contributor to age-related cognitive decline.<sup>114</sup> Importantly, CAA is detected in 90% of patients with AD. The brain injury caused by CAA is the result of cerebrovascular dysfunction leading to reduced brain blood supply and ischemia. Moreover, CAA may cause large symptomatic intracerebral hemorrhages and small (mostly asymptomatic) cerebral microbleeds.<sup>115</sup> It is important to note that CAA makes an independent contribution to AD dementia. Indeed, AD patients suffer more severe cognitive impairment in the presence of CAA.<sup>115</sup> Currently, there is no disease-specific treatment available to patients with CAA.

Studies on aged eNOS<sup>+/-</sup> mice provided new insights into contributions of dysfunctional eNOS to pathogenesis of cerebral small vessel disease.<sup>27,53</sup> Traditional understanding of CAA pathogenesis is based on evidence demonstrating that A $\beta$  deposition and accumulation in the blood vessel wall is largely driven by impaired clearance of A $\beta$  from the brain interstitial fluid.<sup>24,116,117</sup> More recent discoveries suggest that partial loss of endothelial NO production in aged (18-month-old) heterozygous eNOS<sup>+/-</sup> mice significantly contributes to development of CAA pathology.<sup>27,53</sup> Indeed, aged heterozygous eNOS<sup>+/-</sup> mice exhibit CAA pathology including intracerebral hemorrhage and siderosis.<sup>27,53</sup> Aged eNOS<sup>+/-</sup> also suffer from microinfarcts, increased permeability of blood-brain-barrier, and white matter pathology.<sup>53</sup> In fact, aged eNOS<sup>+/-</sup> mice are currently considered a model of age-dependent, spontaneous cerebral small vessel disease including CAA.<sup>53</sup>

Increased production of A $\beta$  in cerebral microvessels of aged eNOS<sup>+/-</sup> mice appears to be very early event in pathogenesis of CAA.<sup>94</sup> A $\beta$ 1–40 levels are increased in microvascular tissue of aged eNOS<sup>+/-</sup> mice, and production and secretion of A $\beta$ 1–40 from cerebral microvessels is elevated before any changes in A $\beta$  concentration could be detected in neuronal tissue.<sup>94</sup> This suggests that cerebrovascular alterations of A $\beta$  may occur independently of A $\beta$  changes within the brain, and that cerebral blood vessels are more vulnerable than neurons to injury imposed by partial deficiency of eNOS. We also wish to point out that partial loss of endothelial NO is a clinically relevant as complete loss of

endothelial NO does not occur in human disease.<sup>94</sup> It is also important to emphasize that metabolic parameters body weight, glucose, total and HDL cholesterol of 18-month-old eNOS<sup>+/-</sup> mice are comparable to their littermate wild type control mice.<sup>94</sup> In addition, arterial blood pressure is not elevated in aged eNOS<sup>+/-</sup> mice.<sup>94</sup> These findings rule out hypertension and alterations in glucose and lipid metabolism as mechanisms responsible for increased amyloidogenic processing of APP observed in cerebral microvessels of 18-month-old eNOS<sup>+/-</sup> mice. Indeed, these findings confirm the importance of reduced local concentration of NO in blood vessel wall (rather than systemic metabolic or hemodynamic dysfunction) as an important early event in development of CAA.

Notably, adaptive responses were identified in cerebral microvessels of 18-month-old eNOS<sup>+/-</sup> mice. Expression of insulin degrading enzyme (IDE) and low-density lipoprotein receptor-related protein 1 (LRP1) are significantly upregulated in cerebral microvessels of aged eNOS<sup>-/-</sup> mice.<sup>94</sup> This is most likely, adaptive response designed to lower elevated local concentration of A $\beta$  in the brain blood vessel wall. In addition, significant upregulation of copper-zinc superoxide dismutase (CuZnSOD) and catalase was also present in cerebral microvessels of aged eNOS<sup>+/-</sup> mice. This upregulation of ant-oxidant enzymes system also appears to be adaptive response to oxidative stress imposed by increased A $\beta$ .<sup>94</sup> Of note, increased expression of IDE was detected in brain tissue in the absence of increased brain A $\beta$  levels. These findings reinforce the concept that partial eNOS-deficiency first causes elevation of A $\beta$  within vasculature and not in brain parenchyma.<sup>94</sup>

The importance of endothelial expression and processing of APP in pathogenesis of CAA is further supported by recent findings demonstrating that endothelium-specific expression of human APP [with the Swedish (KM670/671NL) mutation] in mice resulted in increased production of A $\beta$ .<sup>118</sup> Interestingly, this increased production of A $\beta$  in endothelium did not result in A $\beta$  deposition in the cortical blood vessels. However, crossing of these animals with AD model mice (APP<sup>NL-F/NF-F</sup> mice overexpressing APP in neuronal tissue) markedly exacerbated CAA pathology in APP<sup>NL-F/NF-F</sup> mice.<sup>118</sup> This observation is particularly noteworthy finding because it suggests that production of A $\beta$  in endothelium may significantly exacerbate cerebrovascular deposition of A $\beta$  in patients with AD. Thus, inhibition of endothelial production of A $\beta$  may offer a viable therapeutic strategy designed to reduce A $\beta$  accumulation in the cerebral blood vessel wall.

## Conclusions

Loss of endothelial NO signaling promotes amyloidogenic processing of APP in the brain endothelial and neuronal cells. Moreover, inactivation of eNOS stimulates neuronal tau phosphorylation thereby suggesting that dysfunctional eNOS may promote formation of neurofibrillary tangles. Existing evidence also suggests that loss of endothelial NO function activates microglia and creates pro-inflammatory environment in the brain. Furthermore, loss of endothelial NO causes microvascular occlusions, leading to localized mild hypoperfusion, aberrant mitochondrial function, increased permeability of BBB, and white matter damage. In addition, it appears that endothelial NO significantly contributes to control of glycolysis in astrocytes and neuronal biogenesis of mitochondria. More recent findings support the hypothesis that partial loss of cerebrovascular NO signaling may

contribute to initiation and progression of CAA. Growing recognition of dysfunctional endothelial NO signaling contribution to development of cognitive impairment provides basis for further efforts to develop, optimize, and refine therapies designed to preserve healthy cognition. Activation of NO/cGMP pathway may offer cerebrovascular protective therapy that will minimize vascular contribution to cognitive impairment. For instance, if this approach may prevent microvascular occlusions, reduce amyloid deposition in cerebral blood vessels, and protect BBB, improved vascular function may translate into significantly less severe symptoms of CAA and cognitive impairment.

## Sources of Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by National Institutes on Aging AG071190 and by the Mayo Foundation.

## Nonstandard Abbreviations and Acronyms

<b>A<math>\beta</math></b>	amyloid- $\beta$
<b>AD</b>	Alzheimer's disease
<b>ApoE</b>	apolipoprotein
<b>AAA</b>	amyloid precursor protein
<b>BACE1</b>	$\beta$ -site APP-cleaving enzyme 1
<b>BBB</b>	blood-brain-barrier
<b>CAA</b>	cerebral amyloid angiopathy
<b>CD68</b>	cluster of differentiation 68
<b>Cdk5</b>	cyclin-dependent kinase 5
<b>CNS</b>	central nervous system
<b>ELISA</b>	enzyme-linked immunosorbent assay
<b>eNOS</b>	endothelial nitric oxide synthase
<b>GMCSF</b>	granulocyte-macrophage colony stimulating factor
<b>cGMP</b>	cyclic guanosine monophosphate
<b>HDL</b>	high density lipoprotein
<b>hMSCs</b>	human mesenchymal stem cells
<b>Iba1</b>	ionized calcium-binding adaptor 1
<b>IDE</b>	insulin degrading enzyme
<b>IL-1<math>\alpha</math></b>	interleukin-1 $\alpha$

<b>LMA</b>	late middle aged
<b>LTP</b>	long-term potentiation
<b>MHC II</b>	major histocompatibility complex II
<b>MIP-1<math>\beta</math></b>	macrophage inflammatory protein-1 $\beta$
<b>NO</b>	nitric oxide
<b>NOX2</b>	nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-2
<b>sAPP<math>\alpha</math></b>	soluble amyloid precursor protein- $\alpha$
<b>sGC</b>	soluble guanylate cyclase
<b>tau</b>	tubulin associated unit

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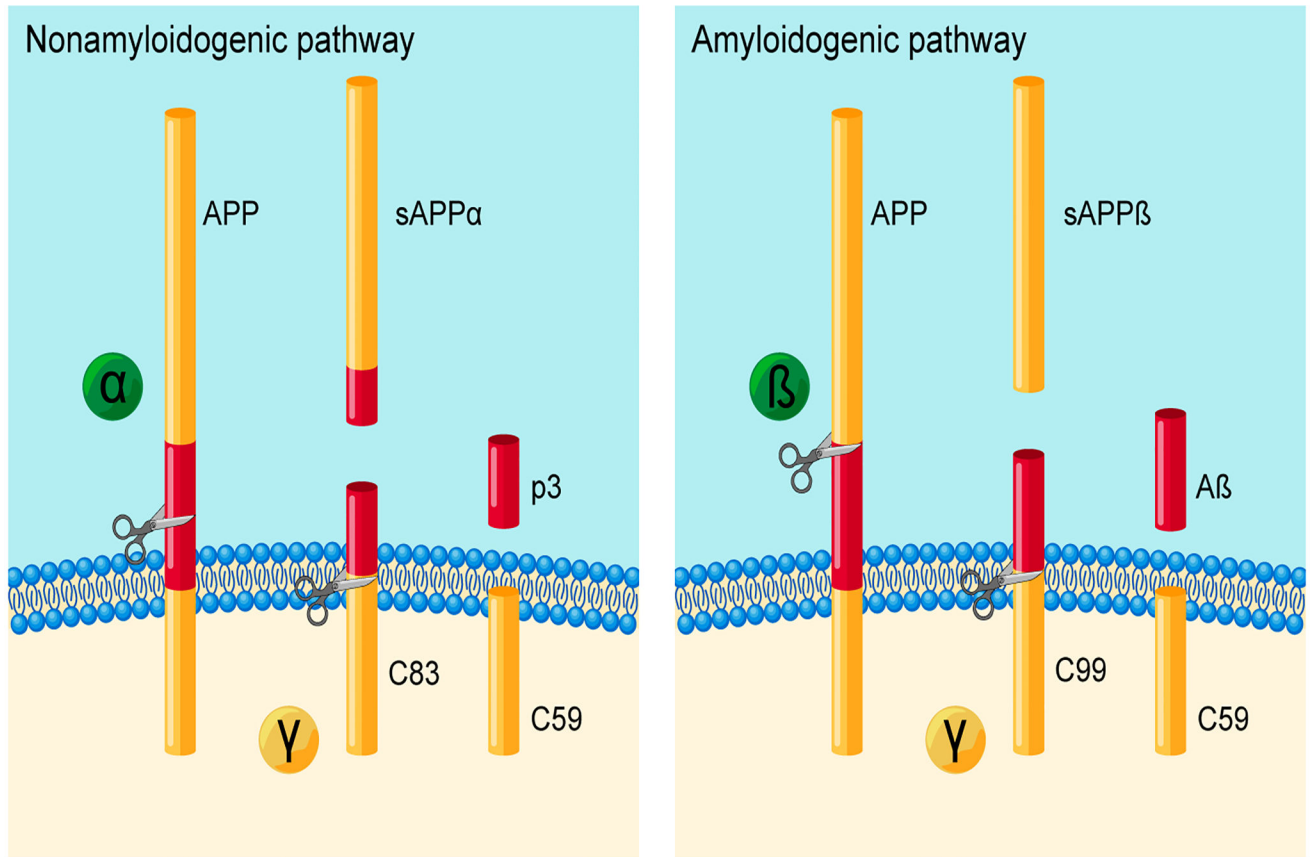
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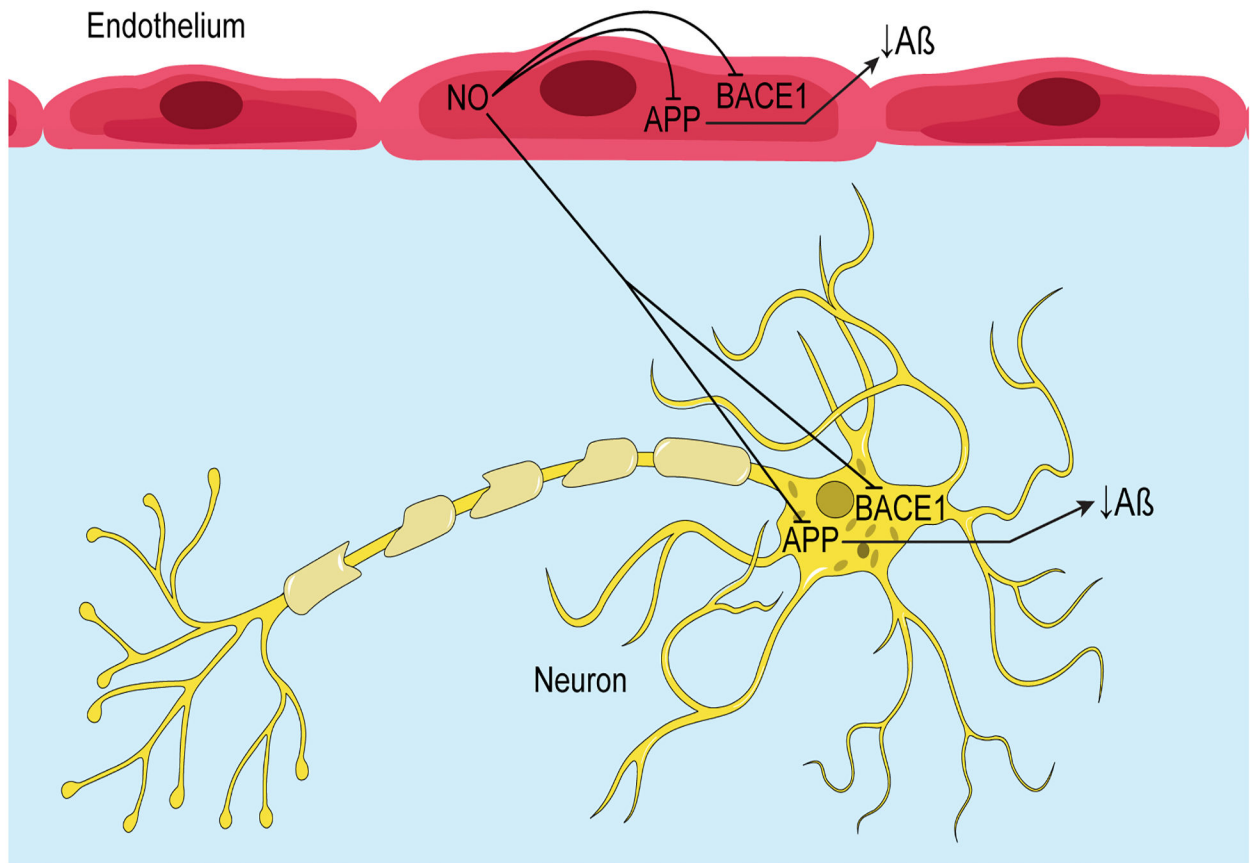
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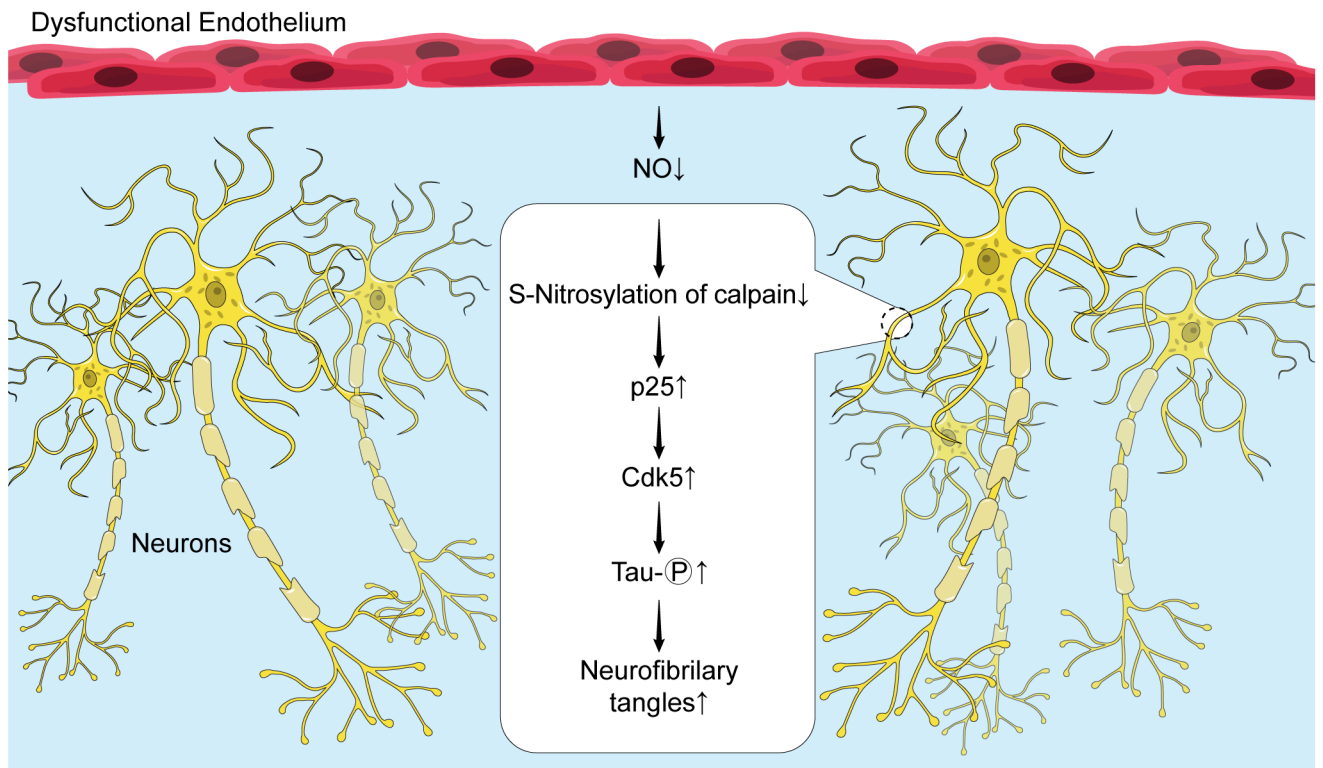


**Figure 1.**

Proteolytic cleavage of amyloid precursor protein (APP) by a nonamyloidogenic pathway (left) or an amyloidogenic pathway (right). In the nonamyloidogenic pathway, APP is cleaved by  $\alpha$ -secretase thereby releasing the sAPP $\alpha$  ectodomain. Following  $\alpha$ -secretase cleavage,  $\gamma$ -secretase releases the p3 fragment and the APP intracellular domain C59. In the amyloidogenic pathway,  $\beta$ -secretase (BACE1) cleaves APP and releases the sAPP $\beta$  ectodomain. Subsequent processing by  $\gamma$ -secretase generates the cytotoxic peptide, A $\beta$ , as well as the C59.



**Figure 2.** Schematic representation of the inhibitory effects of endothelial NO on expression of APP and BACE1, and production of A $\beta$  in endothelial and neuronal cells.



**Figure 3.** Schematic representation of the role of endothelial NO deficiency in stimulation of tau protein phosphorylation and formation of neurofibrillary tangles in neuronal cells.