Short Communication

Endothelial Nitric Oxide Modulates Expression and Processing of Amyloid Precursor Protein

Susan A. Austin, Anantha V. Santhanam, Zvonimir S. Katusic

Rationale: The exact etiology of sporadic Alzheimer disease (AD) is unclear, but it is interesting that several cardiovascular risk factors are associated with higher incidence of AD. The link between these risk factors and AD has yet to be identified; however, a common feature is endothelial dysfunction, specifically, decreased bioavailability of nitric oxide (NO).

Objective: To determine the relationship between endothelial derived NO and the expression and processing of amyloid precursor protein (APP).

Methods and Results: We used human brain microvascular endothelial cells to examine the role of NO in modulating APP expression and processing in vitro. Inhibition of endothelial nitric oxide synthase (eNOS) with the specific NOS inhibitor L-NAME (N\textsuperscript{G}-nitro-L-arginine methyl ester) led to increased APP and BACE1 (β-site APP-cleaving enzyme1) protein levels, as well as increased secretion of the amyloidogenic peptide amyloid β (A\textsubscript{β}) (control 10.93±0.70 pg/mL; L-NAME 168.21±27.38 pg/mL; P<0.001). To examine the role of NO in modulation of APP expression and processing in vivo, we used brain and cerebral microvessels from eNOS-deficient (eNOS\textsuperscript{-/-}) mice. Brain tissue from eNOS\textsuperscript{-/-} mice had statistically higher APP and BACE1 protein levels, as well as increased BACE1 enzyme activity and A\textsubscript{β} (A\textsubscript{β}1-42 wild-type control, 0.737 pg/mg; eNOS\textsuperscript{-/-}, 1.475 pg/mg; P<0.05), compared with wild-type controls (n=6 to 8 animals per background). Brain microvessels from eNOS\textsuperscript{-/-} mice also showed statistically higher BACE1 protein levels as compared with wild-type control.

Conclusions: Our data suggest that endothelial NO plays an important role in modulating APP expression and processing within the brain and cerebrovasculature. The NO/cGMP pathway may be an important therapeutic target in preventing and treating mild cognitive impairment, as well as AD. (Circ Res. 2010;107:1498-1502.)

Key Words: endothelium • amyloid precursor protein • Alzheimer’s disease • cerebrovascular biology • β amyloid

Alzheimer’s disease (AD) is a chronic neurodegenerative disease affecting more than 5 million persons in the United States and more than 20 million worldwide. AD is characterized by progressive loss of neurons, cognitive decline, and 2 defining histopathologies: extracellular amyloid plaques and intracellular tangles composed primarily of amyloid β (A\textsubscript{β}) peptide and hyperphosphorylated tau, respectively. Furthermore, AD is often accompanied by cerebrovascular dysfunction, as well as amyloid deposition within the cerebral vessels, termed cerebral amyloid angiopathy.

A\textsubscript{β} is generated from sequential cleavages of its parent molecule, the amyloid precursor protein (APP), by the activities of β-site APP-cleaving enzyme (BACE1) and γ secretase. Importantly, A\textsubscript{β} has been shown to exert a plethora of effects on endothelial phenotype, including angiogenesis, proliferation, adhesion, and responsiveness to vasoactive molecules. Moreover, it has been suggested that APP has functional roles in coagulation, adhesion, and inflammation. The exact etiology of sporadic AD is unclear, but it is interesting that cardiovascular risk factors including hypertension, hypercholesterolemia, diabetes mellitus, aging, and sedentary lifestyle are associated with higher incidence of AD. The link between cardiovascular risk factors and AD has yet to be identified; however, a common feature is endothelial dysfunction, specifically, decreased bioavailability of nitric oxide (NO). In the cerebral circulation, endothelial NO is generated by endothelial nitric oxide synthase (eNOS), which, under basal conditions, is expressed exclusively in endothelial cells. NO is an extremely important signaling molecule responsible for maintaining vascular homeostasis by promoting vasodilatation, inhibiting platelet aggregation and leukocyte adhesion. Taken together, these data suggest that NO availability may be a common link between cardiovascular risk factors and the development of AD; therefore, we examine here the role of endothelial-derived NO in modulating brain and microvascular APP expression and processing and generation of the amyloidogenic fragment A\textsubscript{β}.
Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org and includes detailed information regarding animals, tissue collection, cerebral microvessel isolation, human brain microvascular endothelial cell (BMEC) culture, confocal microscopy, eNOS, guanylyl cyclase and phosphodiesterase 5 inhibition, small interfering RNA transfection, Western blotting, ELISA for Aβ, NOx measurements, BACE1 enzyme activity assay, and statistical analysis.

Results

eNOS-Derived NO and APP Expression and Processing in Human BMECs
Inhibition of eNOS led to increased APP and BACE1 protein levels after 3 days of treatment in BMECs (Figure 1A and 1B). Addition of L-arginine, the stereoisomer-specific substrate of eNOS, was able to reverse the L-NAME–induced increases in APP and BACE1 protein levels (Figure 1B). Furthermore, genetic knockdown of eNOS led to similar increases in APP and BACE1 protein levels (Figure 1D). Levels of the amyloidogenic peptide Aβ were significantly higher following eNOS inhibition and attenuated with the addition of L-arginine (Figure 1C; P<0.001 from control).

cGMP Pathway and APP Expression and Processing
The effects of NO are commonly mediated by activation of guanylyl cyclase, which then generates cGMP.13 BMECs treated with 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), a highly selective inhibitor of soluble guanylyl cyclase, had statistically higher protein levels of APP and BACE1 (Figure 2A). Furthermore, when we treated BMECs with sildenafil, a selective inhibitor of cGMP-specific phosphodiesterase type 5, to increase cGMP levels, APP and BACE1 protein levels were significantly reduced (Figure 2B). Taken together, these data suggest that basal NO levels, provided by the activity of eNOS, suppress APP and BACE1 protein levels via the guanylyl cyclase pathway.

Non-standard Abbreviations and Acronyms

- Aβ: amyloid β
- AD: Alzheimer disease
- APP: amyloid precursor protein
- BACE1: β-site amyloid precursor protein–cleaving enzyme 1
- BMEC: brain microvascular endothelial cell
- eNOS: endothelial nitric oxide synthase
- iNOS: inducible nitric oxide synthase
- L-NAM: Nω-nitro-L-arginine methyl ester
- nNOS: neuronal nitric oxide synthase
- NOx: nitrate/nitrite
- NOS: nitric oxide synthase
- ODQ: 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one

Figure 1. Inhibitory effect of eNOS-derived NO on APP and BACE1 protein expression and Aβ generation in human BMECs. A, BMECs were cultured in the absence or presence of 0.3 mmol/L L-NAME for 1, 3, and 5 days; medium was changed daily. Representative image from 3 to 5 independent experiments and densitometric analysis are shown. Data are presented as means±SD. *P<0.05 from control; **P<0.01 from control. BMECs were cultured in the absence or presence of 1 mmol/L L-arginine or D-arginine and 0.3 mmol/L L-NAME for 4 days; medium was changed daily. B, Representative image from 3 to 5 independent experiments and densitometric analysis are shown. Data are presented as means±SD. *P<0.05 from control; **P<0.01 from control. C, Cell supernatant was collected and secreted Aβ1-40 and Aβ1-42 was measured using a commercially available colorimetric ELISA. Data are represented as means±SD. ***P<0.001 from control; $P<0.001 from L-NAME; SP<0.01 from L-NAME. D, BMECs were transfected with 30 mmol/L eNOS or control small interfering RNA (siRNA). Cells were lysed 3 days after transfections and used for Western analyses. Representative image from 3 to 5 independent experiments and densitometric analysis are shown. Data are presented as means±SD. *P<0.05 from untreated; &P<0.05 from control siRNA. Statistical analysis was performed using 1-way ANOVA and Tukey–Kramer post hoc comparison.
NOx and BACE1 Levels in Cerebral Microvessels From eNOS<sup>−/−</sup> Mice

Microvessels from eNOS<sup>−/−</sup> mice displayed an approximately 50% reduction in the levels of nitrate/nitrite (NOx) as compared with wild-type controls (Figure 3A). Whereas overall NOx levels were decreased in the microvessels of eNOS<sup>−/−</sup> mice, protein expression of inducible (i)NOS were unchanged and neuronal (n)NOS was undetectable in the microvessels from both mice (Figure 3B).

APP and BACE1 protein levels from the microvascular fraction of both wild-type and eNOS<sup>−/−</sup> animals were studied. Whereas APP protein levels were not significantly different between the mice, BACE1 levels were statistically higher in the microvascular fraction from the eNOS<sup>−/−</sup> animals as compared with wild type (Figure 3C). Because the majority of Aβ produced by the endothelial cells would have been secreted into the vessel lumen, we were unable to detect Aβ levels from the microvascular fraction.

We did not detect any alterations in other key enzymes involved in APP processing or Aβ degradation (Online Figures I and II).<sup>14</sup>

APP, BACE1, and Aβ in the Brains of eNOS<sup>−/−</sup> Mice

Levels of brain NOx were unchanged between eNOS<sup>−/−</sup> and wild-type control mice (Figure 4A). Furthermore, protein levels of iNOS and nNOS were unaltered in the brains of eNOS<sup>−/−</sup> mice as compared with wild-type control (Figure 2B and 2C). These results suggest that the deficiency in eNOS-derived NO is restricted to the microvessels.

APP and BACE1 protein levels were significantly higher in the brains of eNOS<sup>−/−</sup> animals as compared with control (Figure 4D and 4E). Levels of active BACE1 enzyme were also increased in the brains of eNOS<sup>−/−</sup> mice as compared with wild type (Figure 4E).

Figure 2. NO-mediated suppression of APP and BACE1 protein expression is mediated by the guanylyl cyclase/cGMP pathway. A, BMECs were cultured in the absence or presence of DMSO vehicle or 1 μmol/L ODQ, a soluble guanylyl cyclase inhibitor, for 4 days; medium was changed daily. Representative image from 3 to 5 independent experiments and densitometric analysis are shown. Data are presented as means±SD. *P<0.05 from control; **P<0.01 from control; &P<0.05 from vehicle; $P<0.01 from vehicle. B, BMECs were cultured in the absence or presence of 1 μmol/L sildenafil citrate for 4 days; medium was changed daily. Representative image from 3 to 5 independent experiments and densitometric analysis are shown. Data are presented as means±SD. *P<0.05 from control; &P<0.05 from vehicle.

Figure 3. Levels of NO were decreased, whereas BACE1 protein levels were increased, in the microvessels of eNOS<sup>−/−</sup> mice as compared with age-matched wild-type control animals. A, Brain microvascular tissue from eNOS<sup>−/−</sup> and wild-type animals was collected and analyzed for levels of NOx using a commercially available kit. Data are presented as mean NOx levels expressed in pmol/g brain tissue (6 to 8 animals per background; *P<0.05 from wild-type control). B and C, Brain microvascular tissue from eNOS<sup>−/−</sup> and wild-type control animals was Western blotted using anti-eNOS, anti-iNOS, anti-nNOS, and anti-actin (loading control) antibodies (B) or anti-APP, anti-BACE1, anti-eNOS, and anti-actin (loading control) antibodies (C). Representative Western blot and densitometric analysis are shown (6 to 8 animals). Data are presented as relative mean optical density (O.D.):±SD. *P<0.05, ***P<0.001 from wild-type control.
statistically higher in the brains of eNOS^{-/-} animals as compared with wild-type control mice (Figure 4F; *P < 0.001). Furthermore, brain lysates from eNOS^{-/-} animals demonstrated significantly higher levels of both Aβ_{1-42} and Aβ_{1-40} as compared with wild type (Figure 4G and 4H; *P < 0.01, **P < 0.05, respectively). Importantly, this demonstrates that loss of endothelial NO is sufficient to increase protein levels of APP, BACE1, and the amyloidogenic Aβ peptide in brain tissue.

We observed no alteration in the α or γ secretase proteins or Aβ degradation enzymes (Online Figures III and IV).

**Discussion**

Our results support the concept that the relationship between endothelial dysfunction and the development of AD is at least in part mediated by the loss of basal eNOS-generated NO and subsequent upregulation of expression and amyloidogenic processing of APP. We have observed this both in vitro and in vivo. Our in vitro data suggest that this NO-dependent mechanism is mediated by activation of guanylyl cyclase, leading to increases in cGMP levels, because inhibition of guanylyl cyclase also led to increases in APP and BACE1 protein expression, whereas phosphodiesterase type 5 lowered APP and BACE1 expression. Consistent with our observations, Pak et al^{15} demonstrated that in human neuroblastoma cells, NO downregulated β secretase, suggesting that NO might suppress Aβ levels in the brain. Our in vivo observations demonstrate that endothelial-derived NO modulates APP and BACE1 expression, not only within the vessels themselves, but also in brain tissue. Most importantly, endothelial NO also seems to inhibit Aβ generation and secretion. Our findings also suggest that the effect of NO is selective for BACE1 because we did not detect alterations in expression of α-secretases, γ-secretases, or Aβ degradation enzymes.

It is important to note that, phenotypically, eNOS^{-/-} animals have a higher resting mean arterial blood pressure and are prone to insulin resistance as compared with wild-type animals.^{16} Although additional studies are required to determine the exact contribution of hypertension and insulin resistance to the elevation of Aβ, our in vitro data indicate that loss of endothelial NO is sufficient to increase APP, BACE1, and Aβ levels in the absence of any hemodynamic forces or insulin resistance. In addition, existing evidence suggests that hypoperfusion can lead to alterations in brain APP and Aβ levels; however, prior reports demonstrated that cerebral blood flow is normal in eNOS^{-/-} animals.^{17,18}

The results from the present study demonstrate that the NO/cGMP pathway plays an important role in maintaining APP,
BACE1, and Aβ levels within the brain and cerebral microvascularity and that this may provide a therapeutic target in treating and preventing mild cognitive impairment, as well as AD. Indeed, in humans, physical exercise has been shown to be protective against developing AD. Moreover, elevated shear stress imposed on the endothelium by exercise-induced increased blood flow is known to upregulate eNOS. Taken together, our findings offer novel insights into previously unrecognized mechanisms underlying the beneficial effects of eNOS-derived NO on prevention of Aβ elevation in cerebrovascular and neuronal tissue.

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Disclosures
None.

References

Novelty and Significance
What Is Known?
• Amyloid precursor protein (APP) is the parent molecule that when sequentially cleaved by β-site APP cleaving enzyme 1 (BACE1) and γ-secretase generates the amyloid β (Aβ) peptide which is the primary component of Alzheimer’s disease (AD) plaques.
• Cardiovascular risk factors such as hypertension, hypercholesterolemia, diabetes mellitus, aging and a sedentary lifestyle are associated with a higher incidence of AD.
• Endothelial dysfunction caused by decreased bioavailability of nitric oxide (NO) is a common feature among cardiovascular risk factors.

What New Information Does This Article Contribute?
• Endothelial-derived NO suppresses APP, BACE1 and Aβ by increasing cyclic guanosine monophosphate (cGMP) in brain microvascular endothelial cells (BMECs).
• Endothelial nitric oxide synthase (eNOS) deficient (eNOS−/−) mice display increased APP, BACE1, and Aβ peptide in brain tissue and increased BACE1 protein levels in the cerebral microvascularity when compared with wild-type control mice.
• NO-mediated effects were specific to APP and BACE1 as there were no differences in the other secretase enzymes or Aβ-degradation enzymes between eNOS−/− and wild-type mice.

Several cardiovascular risk factors are associated with a higher incidence of AD. We show for the first time that loss of endothelial derived NO generated by eNOS leads to an increase in APP, BACE1 and Aβ peptide both in cultured BMECs and in the brains of eNOS−/− mice. Our data suggest that endothelial NO plays an important role in suppressing APP, BACE1 and Aβ levels within the brain and cerebral vasculature. These findings identify a previously unrecognized mechanism linking endothelial dysfunction with amyloidogenic processing of APP. Our results suggest that preservation of the NO/cGMP signaling pathway may be an important therapeutic strategy for preventing and treating mild cognitive impairment as well as AD.

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Endothelial nitric oxide synthase modulates expression and processing of amyloid precursor protein

Susan A. Austin, Ph.D., Anantha V. Santhanam, Ph.D., and Zvonimir S. Katusic, M.D., Ph.D.

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Supplementary Methods

Animals
Nos3<sup>TmUnc</sup>/J (eNOS<sup>−/−</sup>) and C57BL/6 (wild-type) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were provided food and water ad libitum and housed in a 12 hr. light/dark cycle. Mice were sacrificed at 2-4 months of age by a lethal dose of pentobarbital. 6-8 animals were used per background per experiment. All animal care and use were approved by Mayo Institutional Animal Care and Use Committee.

Tissue collection
Brains were carefully removed and immediately placed in ice cold modified Krebs-Ringer bicarbonate solution containing 118.6 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25.1 mM NaHCO<sub>3</sub>, 0.026 mM EDTA, 10.1 mM glucose plus protease inhibitors. Basilar and cerebral arteries were carefully removed prior to homogenization.

Cerebral Microvessel Isolation
Cerebral microvessels were isolated from the remaining brain tissue as previously described by Zhang et al., with minor modifications. Brain tissue, devoid of large vessels, was homogenized in ice cold PBS with Dounce homogenizer and centrifuged twice at 2000g at 4°C. The supernatant, containing the parenchymal tissue, was discarded. The pellet was resuspended in PBS and centrifuged as described above. The resulting pellet was resuspended and layered over 15% Dextran (in PBS) (Sigma, St. Louis, MO) and centrifuged at 4500g for 30 minutes at 4°C. The top layer was aspirated and discarded and the remaining pellet resuspended in 15% Dextran and centrifuged. The final pellet was resuspended in 1% bovine serum albumin (BSA), the suspension was then passed through a 40 µm nylon mesh (BD Falcon). Microvessels retained on the mesh were washed with BSA/PBS and collected by centrifugation at 900g for 10 minutes at 4°C. Microvessels were resuspended in appropriate buffer according to assay being performed.

Tissue Culture
Human brain microvascular endothelial cells (BMECs) were purchased from Applied Cell Biology Research Institute (Kirkland, WA). BMECs were grown in endothelial growth medium 2 (EGM2; Lonza, Basel, Switzerland) which contained: endothelial basal media 2 (EBM-2; Lonza, Basel, Switzerland) supplemented with 2% fetal bovine serum, fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor, epidermal growth factor, ascorbic acid, hydrocortisone, and heparin. Culture media was changed every other day. Cells were used between passages 4-9.

Confocal Microscopy
Mice were killed by an overdose of pentobarbital and perfused with 4% paraformaldehyde. Brains were dissected and fixed in a 4% paraformaldehyde solution and frozen in OCT under isopentane on dry ice and stored at -80°C. Histological examinations were performed on sagittal sections (5 µm) cut using a Leica CM3000 cryostat and mounted onto glass slides. Tissue was permeabilized using 0.1% Triton X-100 in 10% normal goat serum. Sections were incubated with anti-APP (Invitrogen) or anti-BACE1 (Santa Cruz Biotech) antibodies. Sections were incubated with Cy5-conjugated secondary antibody (Jackson Immuno Research). 4',6'-diamiddino-2-
phenylindole dilactate (DAPI) to visualize nuclei. Sections were visualized using a Zeiss LSM 510 laser scanning confocal microscope.

**eNOS Inhibition**
BMEC were treated with or without L-Arg (1 mM), D-Arg (1 mM) and N(G)-Nitro-L-Arginine Methyl Ester (L-NAME, 0.3 mM) (Sigma-Aldrich, St. Louis, MO), a specific inhibitor of NOS, for up to 5 days with media changed daily as previously described².

**Guanylyl Cyclase Inhibition**
In order to inhibit soluble guanylyl cyclase activity, BMEC were treated with 1 μM 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) or vehicle (dimethylsulfoxide; DMSO) (Sigma-Aldrich, St. Louis, MO) for 4 days with media changed every day³.

**Phosphodiesterase 5 (PDE5) Inhibition**
Inhibition of PDE5 was achieved by treating BMEC with or without 1 μM sildenafil citrate or vehicle (DMSO) (Sigma-Aldrich, St. Louis, MO) for 4 days with media changed daily.

**siRNA Transfection**
BMEC were transfected at 50% confluency with 30 nM of eNOS siRNA or Control siRNA (Santa Cruz Biotechnology) by use of Lipofectamine 2000 (Invitrogen) in serum free medium (EBM-2) according to manufacturer’s instructions. The target sequence of eNOS-siRNA (gene accession: NM_000603) was 5’- CAGCACAAGAGUUAUAAGA-3’. The target sequence of the negative control siRNA duplex oligonucleotides (Control-siRNA) was 5’-UUCUCCGAACGUGUCAGU-3’. Fresh EGM-2 was added 6.5 hours after transfection and cells were analyzed 72 hours after transfection as previously described⁴.

**Western blotting**
To perform Western blot analyses, tissue homogenates or cells were collected, and lysed in ice cold Triton lysis buffer (10 mM Hepes, 50 mM NaF, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 100 μM Na₃VO₄, 50 mM Na pyrophosphate and 1% Triton X-100). Equal protein amounts were resolved by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. Blots were probed with APP, PS2, Neprilysin (Millipore, Billerica, MA), BACE1, nNOS, ADAM9 (Cell Signaling, Danvers, MA), eNOS, iNOS (BD Transduction Laboratories, San Jose, CA.), ECE1, IDE (Abcam, Cambridge, MA), ADAM17, Aph1, Pen2, PS1, Nicastrin (ProSci Incorporated, Poway, CA), ADAM10 (Chemicon (Millipore), Billerica, MA), and Actin (Sigma-Aldrich, St. Louis, MO) (loading control) specific primary antibodies.

**Aβ ELISA**
Secreted Aβ1-40 and Aβ1-42 from cell supernatant was measured using a commercially available colorimetric ELISA kit per manufacturer’s instructions (Invitrogen, Camarillo, CA). Aβ1-40 and Aβ1-42 from brain tissue lysates was measured using a commercially available colorimetric ELISA kit following manufacturer’s instructions (Covance, Princeton, NJ).

**Nitrate/Nitrite (NOx) Fluorometric Assay**
Levels of NOx were measured using a commercially available fluorometric Nitrate/Nitrite assay kit per manufacturer’s instructions (Cayman Chemical Co., Ann Arbor, MI). Briefly, brain and brain microvascular tissue were isolated as described above. Tissue was homogenized in ice cold PBS and centrifuged at 10,000 x g, at 4°C for 20 minutes. The
supernatant was then ultrafiltered using a 10KD cutoff ultrafilter (Millipore, Billerica, MA) according to manufacturer’s instructions. The resulting filtrate was assayed for Nitrate/Nitrite.

**BACE1 activity assay**
Active BACE1 was measured from eNOS−/− and wild type brain tissue using a commercially available BACE1 activity assay kit (Sensizyme BACE1 activity colorimetric assay kit, Sigma Aldrich, St. Louis, MO). Brain tissue was collected as described above and homogenized in ice cold kit wash buffer. Tissue homogenates were centrifuged at 10,000 x g for 10 minutes and resulting supernatant assayed for active BACE1 enzyme according to kit instructions.

**Statistical analysis**
Data are represented as mean ± SD. Statistical analysis performed using one-way ANOVA with Tukey-Kramer post hoc comparison or the unpaired Student’s t-test.

**Supplemental References**

Supplemental Figure I. Proteins involved in alpha and gamma secretase cleavage of APP were unaltered in the microvessels of eNOS<sup>−/−</sup> and wild type control animals. A, Brain microvascular tissue from eNOS<sup>−/−</sup> and wild type control animals was lysed, quantified, resolved by 10% SDS-PAGE and Western blotted using anti-ADAM9, anti-ADAM10, anti-ADAM17 and anti-Actin (loading control) antibodies or B, anti-Aph1, anti-Pen2, anti-PS1, anti-PS2, anti-Nicastrin and anti-Actin (loading control) antibodies. Representative Western blot and densitometric protein analysis is shown. Relative optical density (O.D.) of protein bands from 6-8 animal samples were calculated. Data is presented as relative mean O.D. ± SD.
Supplemental Figure II. Proteins involved in the degradation of Aβ were unaltered in the microvessels of eNOS<sup>−/−</sup> and wild type control animals. A, Brain microvascular tissue from eNOS<sup>−/−</sup> and wild type control animals was lysed, quantified, resolved by 10% SDS-PAGE and Western blotted using anti-ECE1, anti-IDE, anti-Neprilysin, and anti-Actin (loading control) antibodies. Representative Western blot and densitometric protein analysis is shown. Relative optical density (O.D.) of protein bands from 6-8 animal samples were calculated. Data is presented as relative mean O.D. ± SD.
**Supplemental Figure III.** Proteins involved in alpha and gamma secretase cleavage of APP were unaltered in the brains of eNOS−/− and wild type control animals. **A,** Brain tissue from eNOS−/− and wild type control animals was lysed, quantified, resolved by 10% SDS-PAGE and Western blotted using anti-ADAM9, anti-ADAM10, anti-ADAM17 and anti-Actin (loading control) antibodies or **B,** anti-Aph1, anti-Pen2, anti-PS1, anti-PS2, anti-Nicastrin and anti-Actin (loading control) antibodies. Representative Western blot and densitometric protein analysis is shown. Relative optical density (O.D.) of protein bands from 6-8 animal samples were calculated. Data is presented as relative mean O.D. ± SD.
Supplemental Figure IV. Proteins involved in the degradation of Aβ were unaltered in the brains of eNOS−/− and wild type control animals. A, Brain tissue from eNOS−/− and wild type control animals was lysed, quantified, resolved by 10% SDS-PAGE and Western blotted using anti-ECE1, anti-IDE, anti-Neprilysin, and anti-Actin (loading control) antibodies. Representative Western blot and densitometric protein analysis is shown. Relative optical density (O.D.) of protein bands from 6-8 animal samples were calculated. Data is presented as relative mean O.D. ± SD.