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

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### Abstract

**Rationale**—The exact etiology of sporadic 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 utilized human brain microvascular endothelial cells (BMECs) 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, N(G)-Nitro-L-Arginine Methyl Ester (L-NAME), led to increased APP and  $\beta$ -site APP cleaving enzyme 1 (BACE1) protein levels as well as increased secretion of the amyloidogenic, amyloid beta (A $\beta$ ), peptide (control  $10.93 \pm 0.70$  pg/mL; L-NAME  $168.21 \pm 27.38$  pg/mL,  $P < 0.001$ ). To examine the role of NO in modulation of APP expression and processing in vivo, we utilized brain and cerebral microvessels from the eNOS<sup>-/-</sup> mice. Brain tissue from eNOS deficient (eNOS<sup>-/-</sup>) mice had statistically higher APP and BACE1 protein levels as well as increased BACE1 enzyme activity and A $\beta$  (A $\beta$ <sub>1-42</sub> wild-type control 0.737pg/mg; eNOS<sup>-/-</sup> 1.475pg/mg,  $P < 0.05$ ), compared to wild-type controls (n=6–8 animals per background). Brain microvessels from eNOS<sup>-/-</sup> mice also showed statistically higher BACE1 protein levels as compared to 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.

### Keywords

Endothelium; Amyloid precursor protein; Alzheimer's disease; cerebrovascular biology; beta amyloid

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**Disclosures**  
None.

Alzheimer's disease (AD) is a chronic neurodegenerative disease affecting over 5 million persons in the US and over 20 million worldwide<sup>1</sup>. AD is characterized by progressive loss of neurons, cognitive decline and two defining histopathologies: extracellular amyloid plaques and intracellular tangles composed primarily of amyloid beta ( $A\beta$ ) peptide and hyperphosphorylated tau, respectively<sup>2</sup>. Furthermore, AD is often accompanied by cerebrovascular dysfunction as well as amyloid deposition within the cerebral vessels, termed cerebral amyloid angiopathy (CAA)<sup>3</sup>.

$A\beta$  is generated from sequential cleavages of its parent molecule, the amyloid precursor protein (APP), by the activities of beta-site APP-cleaving enzyme 1 (BACE1) and gamma secretase<sup>4, 5</sup>. Importantly,  $A\beta$ , has been shown to exert a plethora of effects on endothelial phenotype, including: angiogenesis, proliferation, adhesion, and responsiveness to vasoactive molecules<sup>6, 7</sup>. Moreover, it has been suggested that APP has functional roles in coagulation, adhesion and inflammation<sup>8</sup>.

The exact etiology of sporadic AD is unclear but it is interesting that cardiovascular risk factors including hypertension, hypercholesterolemia, diabetes mellitus, aging and sedentary life style are associated with higher incidence of AD<sup>9</sup>. 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)<sup>10</sup>. In the cerebral circulation, endothelial NO is generated by endothelial nitric oxide synthase (eNOS) which under basal conditions is expressed exclusively in endothelial cells<sup>11</sup>. NO is an extremely important signaling molecule responsible for maintaining vascular homeostasis by promoting vasodilatation, inhibiting platelet aggregation and leukocyte adhesion<sup>12</sup>. 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  $A\beta$ .

## Methods

A detailed methods section is provided online at <http://circres.ahajournals.org> in the Online Supplement and includes detailed methods regarding: animals, tissue collection, cerebral microvessel isolation, human brain microvascular endothelial cells (BMECs) culture, confocal microscopy, eNOS, guanylyl cyclase and phosphodiesterase 5 inhibition, small interfering (si)RNA transfection, Western blotting, ELISA for  $A\beta$ , 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 (Fig. 1A–B). 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 (Fig. 1B). Furthermore, genetic knockdown of eNOS led to similar increases in APP and BACE1 protein levels (Fig. 1D). Levels of the amyloidogenic peptide,  $A\beta$ , were significantly higher following eNOS inhibition and attenuated with the addition of L-Arginine ( $P < 0.001$  from control).

### cGMP pathway and APP expression and processing

The effects of NO are commonly mediated by activation of guanylyl cyclase which then generates cyclic guanosine monophosphate (cGMP)<sup>13</sup>. BMECs treated with 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), highly selective inhibitor of soluble

guanylyl cyclase had statistically higher protein levels of APP and BACE1 (Fig. 2A). Furthermore, when we treated BMECs with Sildenafil, a selective inhibitor of cGMP specific phosphodiesterase type 5 (PDE5), to increase cGMP levels, APP and BACE1 protein levels were significantly reduced (Fig. 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.

### **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 to wild type controls (Fig 3A). While overall NOx levels were decreased in the microvessels of eNOS<sup>-/-</sup> mice protein expression of inducible NOS (iNOS) were unchanged and neuronal NOS (nNOS) undetectable in the microvessels from both mice (Fig 3B).

APP and BACE1 protein levels from the microvascular fraction of both wild type and eNOS<sup>-/-</sup> animals. While APP protein levels were not significantly different between the two, BACE1 levels were statistically higher in the microvascular fraction from the eNOS<sup>-/-</sup> animals as compared to wild type (Fig. 3C). As the majority of A $\beta$  produced by the endothelial cells would have been secreted into the vessel lumen, we were unable to detect A $\beta$  levels from the microvascular fraction.

We did not detect any alterations in other key enzymes involved in APP processing or A $\beta$  degradation<sup>14</sup> (Supplemental Fig. I and II).

### **APP, BACE1 and A $\beta$ in the brains of eNOS<sup>-/-</sup> mice**

Levels of brain NOx were unchanged between eNOS<sup>-/-</sup> and wild type control mice (Fig 4A). Furthermore, protein levels of iNOS and nNOS were unaltered in the brains of eNOS<sup>-/-</sup> mice as compared to wild type control (Fig 2B–C). 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 to control (Fig 4D–E). Levels of active BACE1 enzyme were also statistically higher in the brains of eNOS<sup>-/-</sup> animals as compared to wild type control mice (Fig 4F,  $P < 0.001$ ). Furthermore, brain lysates from eNOS<sup>-/-</sup> animals demonstrated significantly higher levels of both A $\beta$ 1–40 and A $\beta$ 1–42 as compared to wild type (Fig. 4G–H,  $P < 0.01$ ,  $P < 0.05$ , respectively). Importantly, this demonstrates that loss of endothelial NO is sufficient to increase protein levels of APP, BACE1, and the amyloidogenic A $\beta$  peptide in brain tissue.

We observed no alteration in the alpha or gamma secretase proteins or A $\beta$  degradation enzymes (Supplemental Fig. III-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 suggests that this NO-dependent mechanism is mediated by activation of guanylyl cyclase leading to increases in cGMP levels as inhibition of guanylyl cyclase also led to increases in APP and BACE1 protein expression while PDE5 lowered APP and BACE1 expression. Consistent with our observations, Pak et al<sup>15</sup> demonstrated that in human neuroblastoma cells, NO downregulated  $\beta$  secretase suggesting that NO might suppress A $\beta$  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 A $\beta$  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  $\alpha$ -secretases,  $\gamma$ -secretases or A $\beta$  degradation enzymes.

It is important to note that phenotypically, eNOS<sup>-/-</sup> animals have a higher resting mean arterial blood pressure and are prone to insulin resistance as compared to wild type animals<sup>16</sup>. While additional studies are required to determine the exact contribution of hypertension and insulin resistance to the elevation of A $\beta$  our in vitro data indicate that loss of endothelial NO is sufficient to increase APP, BACE1 and A $\beta$  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 A $\beta$  levels; however, prior reports demonstrated that cerebral blood flow is normal in eNOS<sup>-/-</sup> animals<sup>17, 18</sup>.

The results from the present study demonstrate that the NO/cGMP pathway plays an important role in maintaining APP, BACE1 and A $\beta$  levels within the brain and cerebral microvasculature 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<sup>19</sup>. Moreover, elevated shear stress imposed on the endothelium by exercise-induced increased blood flow is known to upregulate eNOS<sup>20</sup>. Taken together, our findings offer novel insights into previously unrecognized mechanisms underlying the beneficial effects of eNOS-derived NO on prevention of A $\beta$  elevation in cerebrovascular and neuronal tissue.

#### Novelty and Significance

##### What Is Known?

- Amyloid precursor protein (APP) is the parent molecule that when sequentially cleaved by  $\beta$ -site APP cleaving enzyme 1 (BACE1) and  $\gamma$ -secretase generates the amyloid beta (A $\beta$ ) 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 A $\beta$  by increasing cyclic guanosine monophosphate (cGMP) in brain microvascular endothelial cells (BMECs).
- Endothelial nitric oxide synthase (eNOS) deficient (eNOS<sup>-/-</sup>) mice display increased APP, BACE1, and A $\beta$  peptide in brain tissue and increased BACE1 protein levels in the cerebral microvasculature 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 A $\beta$ -degradation enzymes between eNOS<sup>-/-</sup> and wild-type I mice.

#### Summary

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 A $\beta$  peptide both in cultured BMECs and in the brains of eNOS<sup>-/-</sup> mice. Our data suggest that endothelial NO plays an important role in suppressing APP, BACE1 and A $\beta$  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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Non-standard Abbreviations and Acronyms

<b>A<math>\beta</math></b>	amyloid beta
<b>AD</b>	Alzheimer's disease
<b>APP</b>	amyloid precursor protein
<b>NO</b>	nitric oxide
<b>BMECs</b>	brain microvascular endothelial cells
<b>NOS</b>	nitric oxide synthase
<b>eNOS</b>	endothelial nitric oxide synthase
<b>iNOS</b>	inducible nitric oxide synthase
<b>nNOS</b>	neuronal nitric oxide synthase
<b>L-NAME</b>	N(G)-Nitro-L-Arginine Methyl Ester
<b>eNOS<sup>-/-</sup></b>	eNOS deficient
<b>BACE1</b>	$\beta$ -site APP cleaving enzyme 1
<b>cGMP</b>	cyclic guanosine monophosphate
<b>CAA</b>	cerebral amyloid angiopathy
<b>siRNA</b>	small interfering RNA
<b>ODQ</b>	[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one
<b>PDE5</b>	phosphodiesterase type 5
<b>NO<sub>x</sub></b>	nitrate/nitrite
<b>ADAM</b>	a disintegrin and metalloprotease
<b>Aph1</b>	anterior defective-1
<b>Pen2</b>	presenilin enhancer 2
<b>PS</b>	presenilin
<b>ECE</b>	endothelin converting enzyme
<b>IDE</b>	insulin degrading enzyme

## Acknowledgments

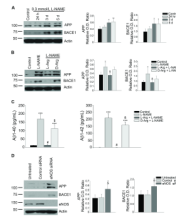
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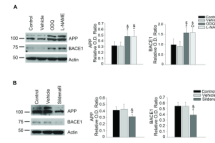
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**Figure 1.**

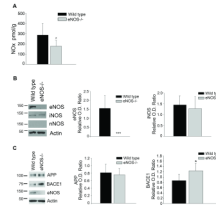
Inhibitory effect of eNOS-derived NO on APP and BACE1 protein expression and A $\beta$  generation in human BMEC. **A**, BMEC were cultured in the absence or presence of 0.3 mM L-NAME for 1, 3, and 5 days; medium was changed daily. Representative image from 3–5 independent experiments and densitometric analysis is shown. Data is presented as mean  $\pm$ SD (\*P<0.05 from control, \*\*P<0.01 from control). BMEC were cultured in the absence or presence of 1 mM L-Arginine or D-Arginine and 0.3 mM L-NAME for 4 days; medium was changed daily. **B**, Representative image from 3–5 independent experiments and densitometric analysis shown. Data is presented as mean  $\pm$ SD (\*P<0.05 from control, &P<0.05 from L-NAME, \$P<0.01 from L-NAME). **C**, Cell supernatant was collected and secreted A $\beta$ <sub>1–40</sub> and A $\beta$ <sub>1–42</sub> was measured using a commercially available colorimetric ELISA. Data is represented as mean  $\pm$  SD (\*\*P<0.001 from control, #P<0.001 from L-NAME, \$P<0.01 from L-NAME, &P<0.05 from L-NAME). **D**, BMEC were transfected with 30 nM of eNOS or Control siRNA. Cells were lysed 3 days after transfections and used for Western analyses. Representative image from 3–5 independent experiments and densitometric analysis is shown. Data is presented as mean  $\pm$ SD (\*P<0.05 from untreated, &P<0.05 from Control siRNA). Statistical analysis was performed using one-way ANOVA and Tukey-Kramer post hoc comparison.





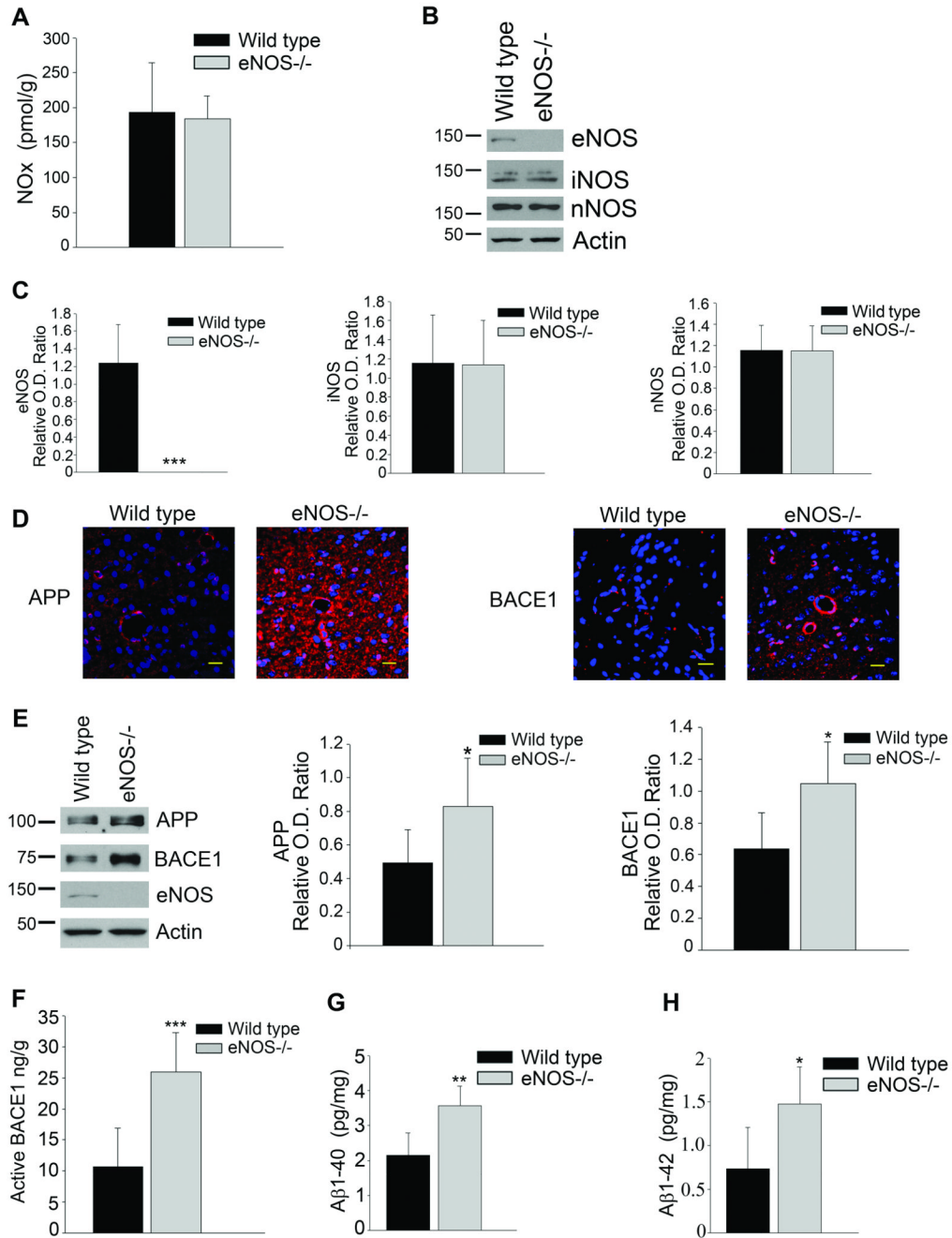
**Figure 2.**

NO-mediated suppression of APP and BACE1 protein expression is mediated by the guanylyl cyclase/cGMP pathway. **A**, BMEC were cultured in the absence or presence of DMSO vehicle or 1  $\mu$ M ODQ, a soluble guanylyl cyclase inhibitor, for 4 days, medium was changed daily. Representative image from 3–5 independent experiments and densitometric analysis is shown. Data is presented as mean  $\pm$ SD (\*P<0.05 from control, \*\*P<0.01 from control, &P<0.05 from vehicle, \$P<0.01 from vehicle). **B**, BMEC were cultured in the absence or presence of 1  $\mu$ M sildenafil citrate, for 4 days, medium was changed daily. Representative image from 3–5 independent experiments and densitometric analysis is shown. Data is presented as mean  $\pm$ SD (\*P<0.05 from control, &P<0.05 from vehicle).



**Figure 3.**

Levels of NO were decreased while BACE1 protein levels were increased in the microvessels of eNOS<sup>-/-</sup> mice as compared to 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 is presented as mean NOx levels expressed in pmol/g brain tissue (6–8 animals per background; \*P<0.05 from wild type control). **B**, 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 or **C**, anti-APP, anti-BACE1, anti-eNOS, and anti-Actin (loading control) antibodies. Representative Western blot and densitometric analysis is shown (6–8 animals). Data is presented as relative mean O.D. ± SD (\*P<0.05, \*\*\*P<0.001 from wild type control).

**Figure 4.**

Levels of NO were unaltered while APP, BACE1 and A $\beta$  levels were increased in the brains of eNOS<sup>-/-</sup> mice as compared to age-matched wild type control animals. **A**, Brain tissue from eNOS<sup>-/-</sup> and wild type animals was analyzed for levels of NOx using a commercially available kit. Data is presented as mean NOx levels expressed in pmol/g brain tissue (6–8 animals per background). **B**, **C**, Brain tissue from eNOS<sup>-/-</sup> and wild-type animals was Western blotted using anti-eNOS, anti-iNOS, anti-nNOS, and anti-Actin (loading control) antibodies. Representative image and densitometric analysis is shown. Data is presented as mean  $\pm$ SD (\*\*\*) $P$ <0.001 from wild type control). **D**, Fixed tissue sections from the brains of wild type and eNOS<sup>-/-</sup> animals were immunolabeled with anti-APP or anti-BACE1

antibodies. Representative images of the cortex are shown. Magnification 40× bar is representative of 20 μm. **E**, Brain tissue from eNOS<sup>-/-</sup> and wild-type animals was Western blotted using anti-APP, anti-BACE1, anti-eNOS, and anti-Actin (loading control) antibodies. Representative blot and densitometric analysis is shown (6–8 animals). Data is presented as relative mean O.D. ± SD (\*P<0.05). **F**, BACE1 activity was measured from the brain tissue of eNOS<sup>-/-</sup> and wild type animals (6–8 animals per background) using a commercially available BACE1 activity kit. Data is represented as mean active BACE1 enzyme (ng/g protein) ±SD (\*\*P<0.001 from wild type control). **G**, Aβ<sub>1-40</sub> and Aβ<sub>1-42</sub> levels from 6–8 individual brain lysates (500 μg) from eNOS<sup>-/-</sup> and wild type control were analyzed via commercially available ELISA kits. Data is represented as mean ± SD (\*\*P<0.01, \*P<0.05 from wild type control).