Inactivation of BACE1 increases expression of endothelial nitric oxide synthase in cerebrovascular endothelium

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Running headline: BACE1 and eNOS

Supplemental material

Mice

Male BACE$^{-/-}$ mice (3-5 months old) were used for assessment of body weight, arterial blood pressure, and metabolic parameters.

Female BACE1$^{-/-}$ mice (3-6 months old) were used to determine body weight, blood glucose, lipid profile, and eNOS protein expression in brain microvessels.

Assessment of vascular structure on basilar arteries of male eBACE1$^{-/-}$ mice (3-5.5 months old): Basilar artery was dissected free from surrounding tissue in cold modified Krebs-Ringer bicarbonate solution under a microscope. Internal and external diameters of basilar arteries were recorded under ex-vivo conditions with transmural pressure of 30 mmHg, using a video dimension analyzer system (Living Systems Instrumentation, Burlington, VT). Medial thickness was calculated by substraction of the internal diameter from the external diameter and divided by two (2).

Western blot antibodies

Mouse anti-catalase (cat# C0979, dilution 1:250) and mouse anti-nitrotyrosine (clone 1A6, cat# 05-233, dilution 1:250) were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-CuZn superoxide dismutase (CuZnSOD, cat# ADI-SOD-100-F, dilution 1:500), and rabbit anti-manganese superoxide dismutase (MnSOD, cat# ADI-SOD-110-F, dilution 1:500) were purchased from Enzo Life Sciences (Farmingdale, NY).

Detection of intracellular levels of superoxide anion

Intracellular superoxide anions were quantified using a high-performance liquid chromatography/fluorescence assay that uses dihydroethidium as a probe, as described in previous study with modifications (1). Briefly, after cells were treated with BACE1siRNA or CtsiRNA for 2 days, cells in culture dishes were incubated with dihydroethidium (10 μM) in phenol red-free EBM2 at 37 ^{0}C for 15 min. The culture dishes were then washed to remove the free probe and cells were incubated with phenol red-free EBM2 for 1 hour at 37°C. After dishes were washed with PBS, 300 μl 100% chilled methanol was added to the dishes. Cells were harvested and homogenized by sonication for 5 second, 3 times. After centrifugation at 12,000rpm for 10 min, the supernatant was collected and analyzed by high-performance liquid chromatography/fluorescence (Beckman Coulter) in 37.0% acetonitrile in 0.1% trifluoroacetic acid aqueous solution. Data were quantified using 2-hydroxyethidium standard from the reaction between dihydroethidium and Fremy salt as described and normalized against cell protein levels.

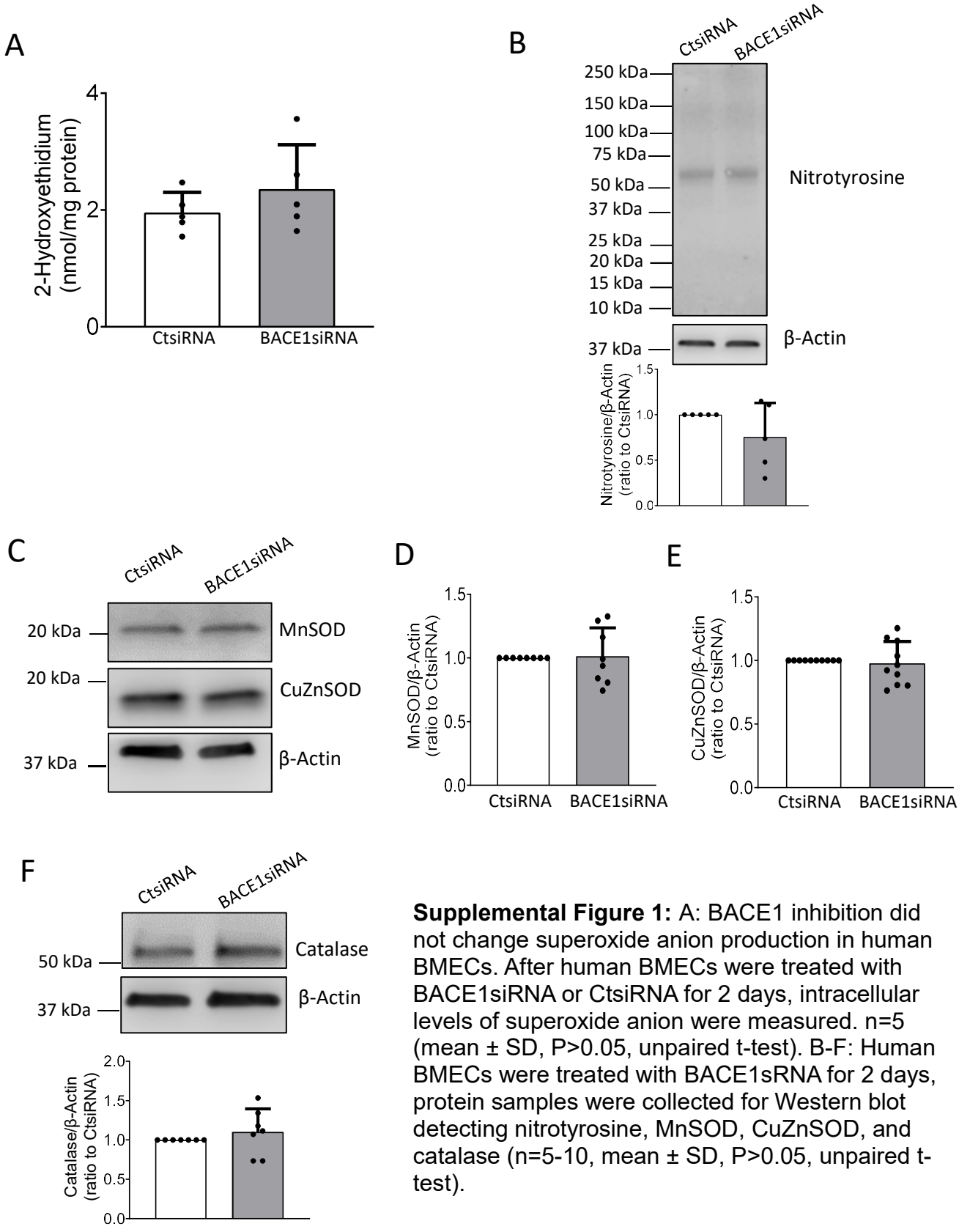
Acute treatment with Aβ1-40 and Aβ1-42

Human Aβ1-40 peptides or human Aβ1-42 peptides (Anaspec, Inc, Fremont CA) were reconstituted with 1.0% NH4OH (1mg/70 µl), then further diluted with PBS, according to the manufacturer's protocol. Reconstituted peptides were aliquoted and stored at -20^{0}C, according to manufacturer's recommendation. Human BMECs were treated with Aβ1-40 or Aβ1-42 (10^{-12}, 10^{-9}, or 10^{-5} M) for 24 hours, the cells were collected for Western blot.

References:

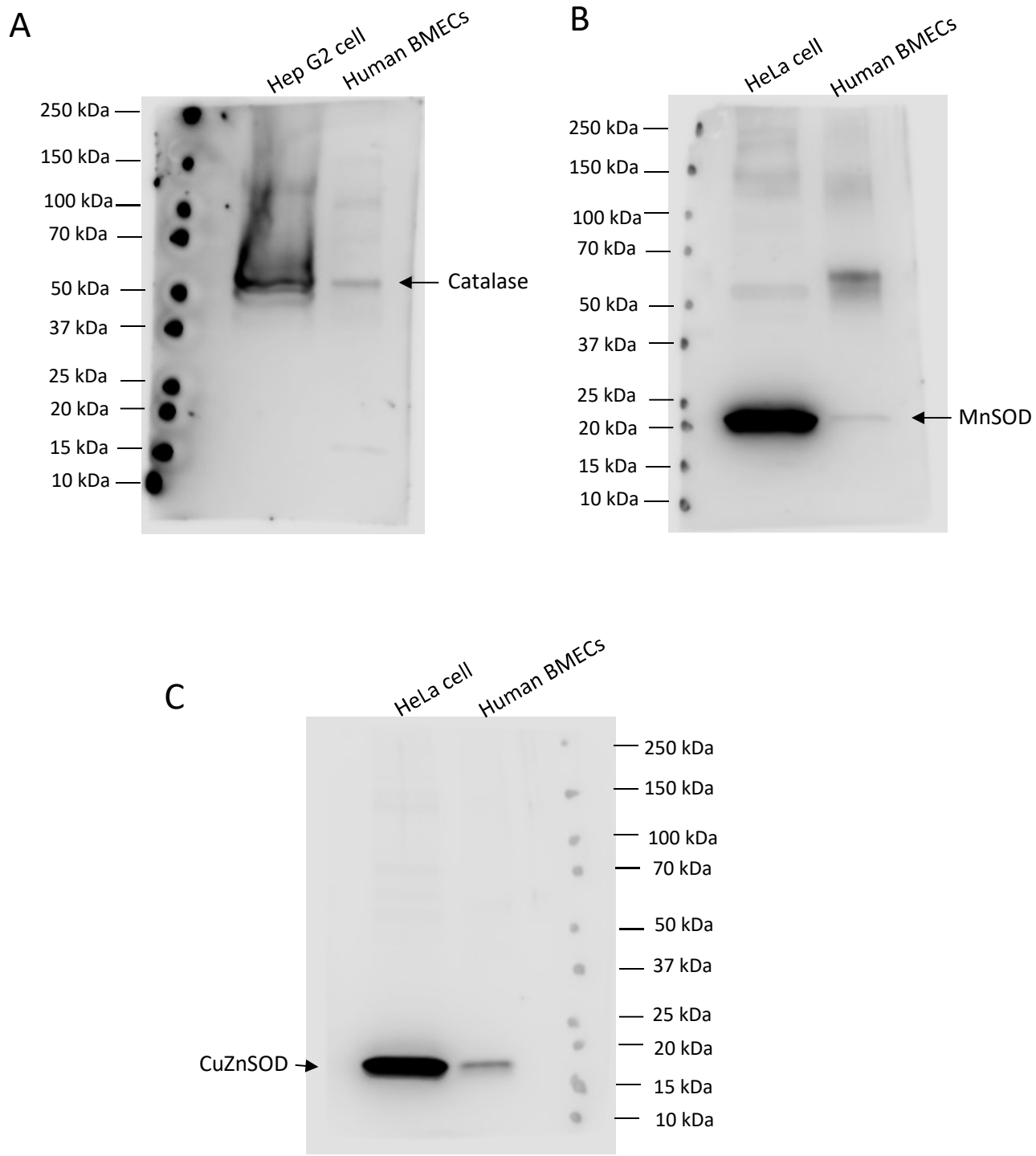
1. d'Uscio LV, Smith LA, Katusic ZS. Erythropoietin increases expression and function of vascular copper- and zinc-containing superoxide dismutase. Hypertension. 2010 Apr;55(4):998-1004.
2. d'Uscio LV, He T, Santhanam AV, et al. Endothelium-specific amyloid precursor protein deficiency causes endothelial dysfunction in cerebral arteries. J Cereb Blood Flow Metab 2018; 38: 1715-1726.

Supplemental Figure 1



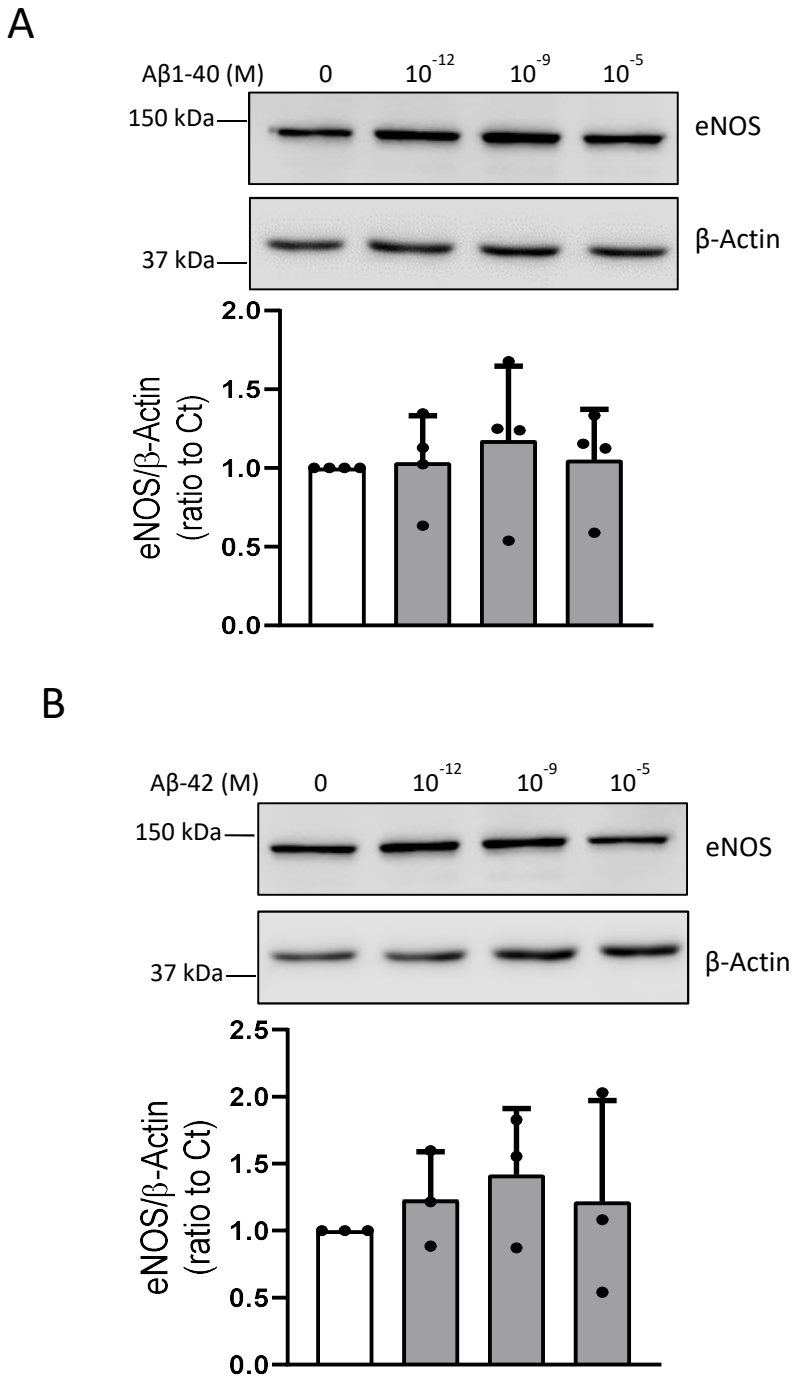
Supplemental Figure 1: A: BACE1 inhibition did not change superoxide anion production in human BMECs. After human BMECs were treated with BACE1siRNA or CtsiRNA for 2 days, intracellular levels of superoxide anion were measured. n=5 (mean ± SD, P>0.05, unpaired t-test). B-F: Human BMECs were treated with BACE1sRNA for 2 days, protein samples were collected for Western blot detecting nitrotyrosine, MnSOD, CuZnSOD, and catalase (n=5-10, mean ± SD, P>0.05, unpaired t-test).

Supplemental Figure 2



Supplemental Figure 2: Verification of antibodies. Positive controls for antibodies were used according to recommendations by manufacturers from which antibodies were purchased.

Supplemental Figure 3



Supplemental Figure 3: Aβ peptides did not change eNOS expression in human BMECs. Cells were treated with Aβ1-40 or Aβ1-42 for 24 hours. Data are presented as mean ± SD, P>0.05; n=3-4. For non-parametric data analysis of multiple groups, Kruskal-Wallis test was used. $P < 0.05$ was considered statistically significant.

Supplemental Table 1. Characteristics of male wild-type littermates and BACE1$^{-/-}$ mice.

Parameters	**WT**	**BACE1$^{-/-}$**
Body weight (g)	31±3 (15)	26±3 (15) *
SBP (mmHg)	116±5 (9)	109±9 (9)
MBP (mmHg)	89±5 (9)	85±7 (9)
DBP (mmHg)	76±6 (9)	73±6 (9)
Glucose (mg/dL)	166±35 (11)	177±23 (11)
Cholesterol (mg/dL)	59±15 (9)	69±10 (9)
HDL (mg/dL)	47±13 (9)	56±8 (9)
Triglyceride (mg/dL)	88±43 (9)	112±41 (9)
Aβ1-40 (pg/mL)	136±62 (17)	46±32 (17) *

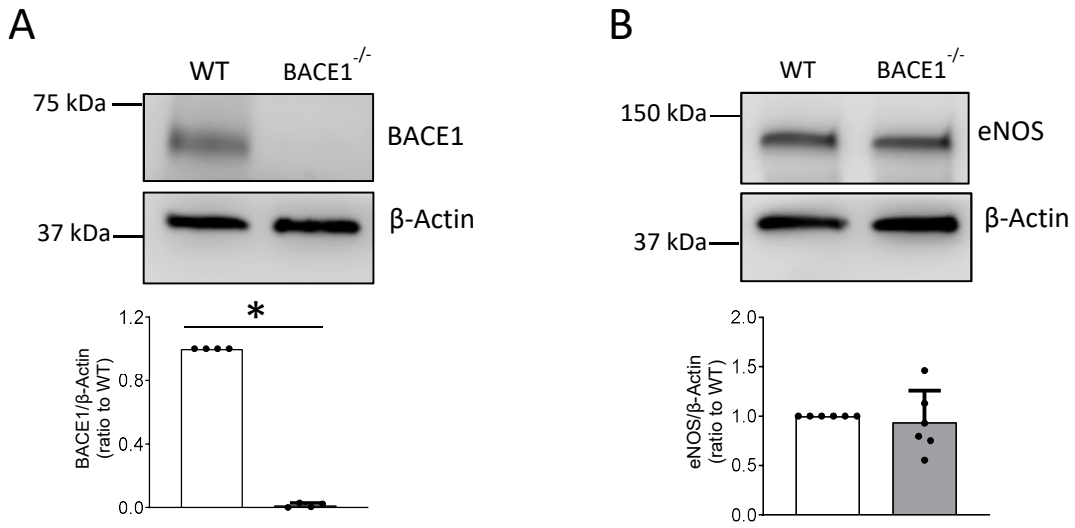
SBP indicates systolic blood pressure; MBP, mean blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; Aβ, amyloid-β; WT, wild-type. Data are mean ± SD and the numbers of mice are indicated in the parentheses. * $P<0.05$ vs. wild-type (WT) littermates (unpaired t-test).

Supplemental Table 2. Characteristics of female wild-type littermates and BACE1$^{-/-}$ mice.

Parameters	**WT**	**BACE1$^{-/-}$**
Body weight (g)	25±2 (6)	22±2 (6) *
Glucose (mg/dL)	177±15 (6)	157±20 (5)
Cholesterol (mg/dL)	51±7 (5)	44±13 (5)
HDL (mg/dL)	38±7 (5)	33±11 (5)
Triglyceride (mg/dL)	67±19 (5)	85±22 (5)

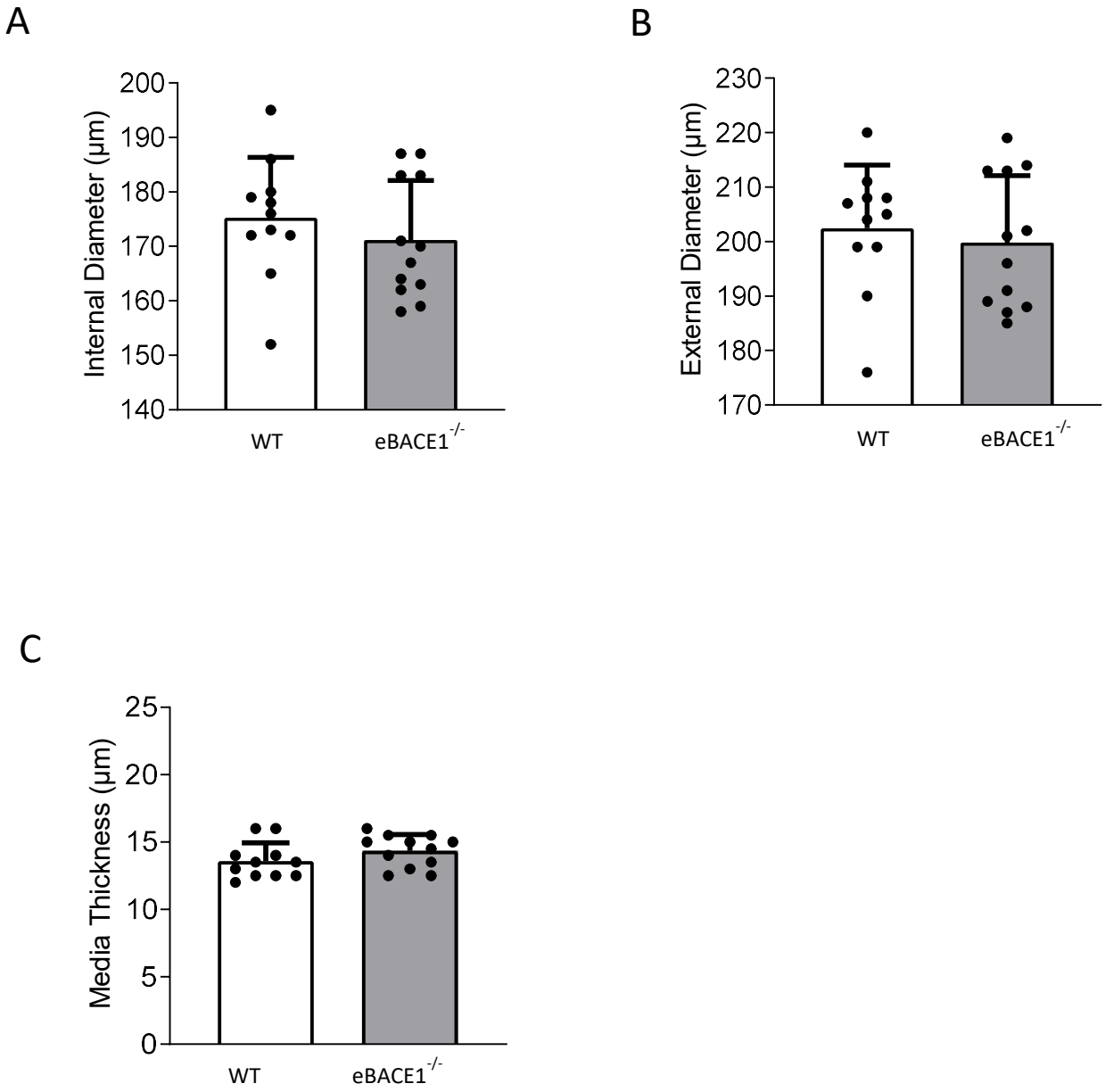
HDL indicates high-density lipoprotein; WT, wild-type. Data are means ± SD and the numbers of mice are indicated in the parentheses. * $P<0.05$ vs. wild-type (WT) littermates (unpaired t-test).

Supplemental Figure 4



Supplemental Figure 4: Expressions of eNOS was not affected in brain microvessels of female BACE1$^{-/-}$ mice. A: BACE1 expression was abolished in cerebral microvessels (n=4, *P<0.05). B: Microvascular protein levels of eNOS were not significantly changed (n=6).

Supplemental Figure 5



Supplemental Figure 5: Vascular structure of basilar arteries were not significantly different between male eBACE1$^{-/-}$ mice and WT mice. n=11-12 (mean ± SD, P>0.05, unpaired t-test).