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BACE2 deficiency impairs expression and function of endothelial nitric oxide synthase in brain endothelial cells

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Abstract

Beta-site amyloid precursor protein (APP)-cleaving enzyme 2 (BACE2) is highly expressed in cerebrovascular endothelium. Notably, BACE2 is one of the most downregulated genes in cerebrovascular endothelium derived from patients with Alzheimer's disease. The present study was designed to determine the role of BACE2 in control of expression and function of endothelial nitric oxide synthase (eNOS). Genetic downregulation of BACE2 in human brain microvascular endothelial cells (BMECs) with small interfering RNA (BACE2siRNA) significantly decreased expression of eNOS and elevated levels of eNOS phosphorylated at threonine residue Thr495, thus leading to reduced production of nitric oxide (NO). BACE2siRNA also suppressed expression of APP, and decreased production and release of soluble APPα (sAPPα). In contrast, adenovirus-mediated overexpression of APP increased expression of eNOS. Consistent with these observations, nanomolar concentrations of sAPPα and APP 17mer peptide (derived from sAPPα) augmented eNOS expression. Further analysis established that γ-aminobutyric acid type B receptor subunit 1 and Küppel-like factor 2 may function as downstream molecular targets significantly contributing to BACE2/APP/sAPPα-induced up regulation of eNOS. In agreement with studies on cultured human endothelium, endothelium-dependent relaxations to acetylcholine and basal production of cyclic GMP were impaired in cerebral arteries of BACE2-deficient mice. We propose that in brain vessels, BACE2 may function as a vascular protective protein.

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AUTHOR CONTRIBUTIONS

TH designed and carried out experiments, collected and interpreted data, and wrote manuscript. LU designed and performed *in vivo* studies, interpreted data, and wrote manuscript. ZK designed experiments, interpretated data, and wrote manuscript.

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1. INTRODUCTION

Existing literature suggests that impairment of nitric oxide (NO) release from cerebrovascular endothelium increases expression of amyloid precursor protein (APP) and β-site APP-cleaving enzyme 1 (BACE1), thereby promoting generation of amyloid-β (Aβ) peptides and development of Alzheimer's disease (AD) pathology (Austin et al., 2010, 2013a, 2013b, 2016, 2020; Chen et al., 2022; Liao et al., 2021; Tan et al., 2015; Katusic et al., 2023). In 1999, soon after discovery of BACE1, BACE2 was identified as a close homologue of BACE1 (Hussain et all., 1999). Several studies have reported that BACE2 cleaves APP at β site in carriers of the Flemish missense mutation of APP and AD-associated mutations that disrupt the juxtamembrane helix of APP (Farzan et al., 2000; Wang et al., 2019). However, other studies have provided evidence that BACE2 cleaves APP at theta (θ) site, adjacent to the α-secretase cleavage site within the Aβ domain, thus functioning as an alternative α-secretase (Abdul-Hay et al., 2012; Fluhrer et al., 2002; Basi et al., 2003; Sun et al., 2005, 2006; Yan et al., 2001). Importantly, this α-secretase like activity of BACE2 exerts neuroprotective effects (Alić et al., 2021; Luo et al., 2022).

BACE2 mRNA expression in human brain capillaries is about 7.5-fold higher than in neurons (Yang et al., 2022). Moreover, in human cerebral capillaries, BACE2 mRNA expression is about 47-fold higher than expression of BACE1 mRNA (Yang et al., 2022). High BACE2 expression has also been detected in human cerebral arteries and veins (Yang et al., 2022). Remarkably, BACE2 expression is downregulated in cerebral vasculatures of AD patients (Yang et al., 2022). Interestingly, BACE2 downregulation is strikingly noticeable in cerebral capillaries (Yang et al., 2022), yet vascular function of BACE2 is unknown. Relevant to our study, reduced eNOS expression in capillaries of AD brain (Jeynes et al., 2009; Yang et al., 2022) may make AD patients more vulnerable to further deterioration of cerebrovascular function. In the present study we advance the hypothesis that in brain endothelial cells, BACE2 plays an important role in preservation of endothelial nitric oxide synthase (eNOS) function. We also provide entirely novel insights into the mechanisms underlying the vascular protective role of brain endothelial BACE2.

2. MATERIALS AND METHODS

2.1 Cell culture

Primary human brain cortex microvascular endothelial cells [BMECs, from a single donor (24 years old, sex unknown)] were purchased from Applied Cell Biology Research Institute (Cell Systems, Kirkland, WA; catalog number ACBRI 376V). The cell line used in this study is not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee. Cells were grown in endothelial growth medium 2 (EGM2; Lonza) containing endothelial basal medium 2 (EBM2; cat. No. cc-3156) supplemented with EGM2 bullet kit (cat. No. cc-3162, containing 2% fetal bovine serum, fibroblast growth

factor, vascular endothelial growth factor, insulin-like growth factor, epidermal growth factor, ascorbic acid, hydrocortisone, bac-off and heparin). Passages 4-6 were used. In some experiments, cells were treated with human soluble APP alpha (sAPPα, Sigma-Aldrich, cat. No. s9564) 7 nM for 3 days (treatment was refreshed every 24 hours). Protein samples were then collected for Western blot.

Human neurons were purchased from ScienCell Research Laboratories (cat. No. 1520-5) and cultured in Neuronal Medium (ScienCell Research Laboratories, cat. No. 1521). The cell line used in this study is not listed as a commonly misidentified cell line by the International Cell Line Authentication Committee.

2.2 Mice

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Mayo Clinic (protocol number A00004948-19). Experimental protocols complied with the National Institute of Health and the ARRIVE guidelines.

Heterozygous BACE2-deficient (BACE2$^{-/-}$) mice (B6;129P2-Bace2^{tm1Bdes}/J) on C57BL/6J background (Strain #005618) were obtained from the Jackson Laboratory and were used to generate wild type (WT) littermates and BACE2$^{-/-}$ off springs. All mice were maintained on 12h/12h light/dark cycle, with free access to drinking water and on standard chow. PCR was performed to identify the genotype by using primers (forward: 5'GCTATAGAGACCAAAGCCCACAAATC3'; reverse:5'GCCCGAATAACAAGAGCATCAC3'), designed by the Jackson Laboratory and purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa). The following PCR conditions were used: 94°C for 30 seconds, 53°C for 1 minute, 72°C for 1.5 minutes (35 cycles). Male mice (3-4 months old) were used in the experiments. The study was not pre-registered with any official preregistration sites comparable to clinicaltrials.gov for clinical studies. No randomization and blinding were performed in this study. No exclusion criteria were pre-determined.

2.3 Mouse plasma Glucose and Lipid profile

Mice were overdosed with pentobarbital (200-250 mg/kg BW, intraperitoneal). Blood samples were collected by right ventricle puncture and transferred to a tube containing EDTA. Blood glucose levels were measured immediately with ACCU-CHEK (Roche Diagnostics; cat. No. 2030365). For measuring plasma lipid profile, blood samples were centrifuged at 2000 rpm at 4°C for 10 min, then the supernatants were collected and stored at −80°C. Levels of cholesterol, HDL, and triglyceride were measured on the Hitachi 912 chemistry analyzer (Roche Diagnostics).

2.4 Overexpression of human APP751 and human APP695

Human BMECs were incubated with replication-deficient adenoviral constructs containing human APP751 (Ad-APP751; Vector Biolabs, cat. No. 1472) or APP695 (Ad-APP695; Vector Biolabs, product name: Ad-CMV-hAPP695) at 5 multiplicities of infection (MOI) in 1.5 mL of EBM-2 for 8 hours. Cells were then recovered in EGM-2 for 2 days. For controls,

cells were transduced with 5 MOI Adeno-CMV-Null adenovirus (Ad-Null, Vector Biolabs, cat. No. 1300).

2.5 Genetic downregulation of BACE2, γ-aminobutyric acid type B receptor subunit 1 (GABBR1), Krüppel-like factor 2 (KLF2), or GABBR2 by small interfering RNA (siRNA)

Experiments were performed by use of Lipofectamine 2000 (Invitrogen) as described in our previous study (He et al., 2022). BACE2siRNA targeting human BACE2 mRNA (ON-TARGETplus human BACE2 siRNA #14; cat. No. J-003802-14), GABBR1siRNA targeting human GABBR1 mRNA (ON-TARGETplus human GABBR1siRNA mix of 4 siRNAs; cat. No. LQ-00509-01-0002) (please note that $GABA_BR1$ refers to γ-aminobutyric acid type B receptor subunit 1 protein, while GABBR1 refers to the gene encoding $GABA_BR1$), KLF2siRNA (ON-TARGETplus human KLF2 siRNA #8; cat, No. J-006928-08), GABBR2siRNA (ON-TARGETplus human GABBR2siRNA #8, cat. No. J-005579-08), and Control-siRNA (ON-TARGETplus Non-targeting siRNA #1; cat. No. D-001810-01) were obtained from Dharmacon, Horizon Discovery. Human BMECs were transfected with siRNA 30-60 nM for 2-3 days. RNA samples were isolated for PCR experiments, or protein samples were then collected for Western blot.

2.6 APP 17mer treatment

APP 17mer and scrambled 17mer peptides were synthesized at Mayo Clinic Proteomics Core Facility, with ≥98% purity, according to previously published peptide sequences [APP 17mer (204-220AA of APP695): acetyl-DDSDVWWGGADTDYADG-amide and Scrambled 17mer: acetyl-DWGADTVSGDGYDAWDD-amide (Rice et al., 2019)]. Human BMECs were treated with APP 17mer or scrambled 17mer (5, 10, or 30 nM) for 2 days (treatment was refreshed every 24 hours). Protein samples were collected for Western blot. In some experiments, cells were treated with APP 17mer or scrambled 17mer for 40 hours (treatment was refreshed after 24 hours), and mRNA samples were collected.

2.7 Western blot analysis

Western blot analysis was conducted as described in our previous studies (He et al., 2019, 2022). Human BMECs were homogenized (by sonication) in lysis buffer (containing 50 mmol/L NaCl, 50 mmol/L NaF, 50 mmol/L sodium pyrophosphate, 5 mmol/L EDTA, 5 mmol/L EGTA, 0.1 mmol/L Na_3VO_4, 1% Triton X-100, 10 mmol/L HEPES, pH 7.4) and protease inhibitor cocktail (Sigma-Aldrich; cat. No. P8340). For Western Blot analysis of *in vivo* experiments, large cerebral arteries (basilar arteries, middle cerebral, anterior cerebral, and posterior cerebral arteries) were removed from the brain and dissected free from surrounding tissue in the cold (4 °C) Krebs–Ringer bicarbonate solution (in mmol/L: NaCl 118.6; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25.1; glucose 10.1; EDTA 0.026), and then were homogenized in lysis buffer.

The protein samples were then subjected to Western blot using 4-15% mini-protein TGX gels for *in vitro* experiment samples (Bio-Rad Laboratories; cat. No. 4561084), or 7.5% and 10% mini protein TGX gels for *in vivo* experiment samples (Bio-Rad Laboratories, cat. No. 4561024, and 4561034, respectively). Nitrocellulose membrane (0.2μM; Bio-Rad; cat. No. 1620112) was used for protein transferring. After transferring protein to membranes, the

membranes were blocked with 5% blotting-grade Blocker (Bio-Rad; cat. No. 1706404) in Tris-buffered salt (TBS) solution for 1 hour, followed by incubation with primary antibody in Blocker/TBS for 1-2 days at 4°C. Membranes were then washed with 0.1% Tween-20 in TBS (TBS-T) for 3 times, then were incubated with secondary antibody for 3 hours at room temperature. After washed with TBS-T for 3 times, the blots were imaged with LI-COR digital imaging analysis system (Odyssey Fc, Model 2800; LI-COR, Inc.) and analyzed using Image Studio Software Version 5.0) (LI-COR, Inc.). Following primary antibodies were used: mouse anti-eNOS (BD Transduction Laboratories; cat. No. 610297); mouse anti-phosphorylated eNOS at Ser1177 [p-eNOS(S1177); BD Transduction Laboratories; cat. No. 612393]; mouse anti-phosphorylated eNOS at Thr495 [p-eNOS(T495); BD Transduction Laboratories; cat. No. 612706]; mouse anti-BACE2 (Santa Cruz Biotechnology; cat. No. sc-271212); rabbit anti-APP (Thermo Scientific; cat. No. 51-2700); rabbit anti-BACE1 (Cell Signaling Technology; cat. No. 5606); rabbit anti-a disintegrin and metalloproteinase 10 (ADAM10; EMD Millipore Corporation; cat. No. AB19026). Blots probed with mouse anti-β-actin (Sigma-Aldrich, cat. No. A5316) were used as loading controls. Protein expression was normalized to β-actin. For α-secretase, the protein levels of mature ADAM10 were quantified. The ratio of p-eNOS(S1177)/eNOS of each sample was calculated after p-eNOS(S1177) or eNOS was normalized to β-actin. The ratio of p-eNOS(T495)/eNOS of each sample was also calculated after p-eNOS(T495) or eNOS was normalized to β-actin.

2.8 Real Time-quantitative PCR (RT-qPCR) and PCR

RT-qPCR was performed as described in our previous study (He et al., 2011, 2022). Total RNA was isolated using RNeasy Plus Mini kit (Qiagen, cat. No. 74134). Superscript III First-Strand Synthesis System kit (Invitrogen by Life Technologies; cat. No. 18080-051) was used to reverse transcribe RNA to cDNA. For quantification of human eNOS, BACE2, APP, GABBR1, GABBR2 and KLF2 mRNA we used Bio-Rad CFX Connect Real-Time System and following primers (Bio-Rad): PrimPCR SYBR Green Assay human eNOS (cat. No. qHsaCID0015042), BACE2 (cat. No. qHsaCID0012156), APP (cat. No. qHsaCID0010429), GABBR1(cat. No. qHsaCID0038341), GABBR2 (cat. No. qHsaCID15507), KLF2 (cat. No. qHsaCED005139), and GAPDFI (qHsaCED0038674, a loading control), according to manufacturer's instruction.

We used the primers amplifying sequence from exon 2 through exon 6 of GABBR1 (NM_001470.4) to identify GABBR1a (containing 2 sushi domains on exon 3 and 4) and GABBR1c (containing 1 sushi domain on exon 3), according to previous studies (Bettler et al., 2004; Lee et al., 2010; Martin et al., 2001). Primers for human GABBR1a (product size 563 bp) and GABBR1c (product size 377bp) were forward 5'CAGATCATACACCCGCCCTG3' and reverse 5'CTGTCGTGGTGGATGAGCTT3'. Primers for human GAPDH (NM_001289745.3, product size 200 bp) (a loading control) were forward 5' GAGCCAAAAGGGTCATCATCTC3' and reverse 5' GATGGCATGGACTGTGGTCATG3'. Veriti Thermal Cycler (AB Applied Biosystems) was used for PCR (reaction cycles: 95°C for 1 min, 57°C for 1 min, and 72°C for 1 min, for 27 cycles). mRNA isolated from cultured human neurons was reverse transcribed to cDNA that was used as a positive control. ImageJ (NIH) was used to analyze the PCR product bands on the gel. GABBR1a or GABBR1c mRNA expression was normalized to GAPDH.

2.9 Measurement of nitric oxide (NO) production

Human BMECs were treated with BACE2siRNA for 2 days, then cultured in EBM2 without phenol red (Lonza; cat. No. cc3129) supplemented with EGM2 bullet kit without serum for 24 hours. Conditioned media were collected after 24 hours incubation. Total nitrite and nitrate (NO_2+NO_3) levels in conditioned media were measured using a commercially available Nitrate/nitrate Fluorometric Assay Kit, according to manufacturer's instructions (Cayman Chemical Co; cat. No. 780051). In some experiments, human BMECs were treated with APP 17mer 30 nM (treatment with scrambled 17mer as a control) for 24 hours, then cells were treated with APP 17mer or scrambled 17mer in phenol red-free EGM2 for 24 hours. The supernatants were collected for NO_2+NO_3 measurement.

2.10 Detection of sAPPα and Aβ1-40 release from human BMECs

After Human BMECs were treated with BACE2siRNA for 2 days, cells were incubated in EGM2 containing 0.2% serum for 24 hours. The conditioned media of 24 hour-incubation were collected for measuring sAPPα, using a Human sAPPα (highly sensitive) Assay Kit (Immuno-Biological Laboratories; cat. No. 27734), according to manufacturer's instructions. For detection of Aβ1-40 in conditioned media, two days after cells were transfected with BACE2siRNA, conditioned media were collected after additional culturing for 24 hours. Levels of Aβ1-40 in conditional media were measured using Aβ 40 human ELISA kit (Invitrogen; cat. No. KHB3481), according to the manufacturer's protocols.

2.11 Vasomotor function of isolated basilar artery

Basilar artery was dissected free from surrounding tissues in cold Krebs solution under a microscope and was transferred to small vessel chamber filled with 37°C Krebs solution [composition (in mmol/L) NaCl 118.6; KCl 4.7; $CaCl_2$ 2.5; $MgSO_4$ 1.2; KH_2PO_4 1.2; $NaHCO_3$ 25.1; glucose 10.1; EDTA 0.026]. Vascular reactivity was studied in-vitro under pressurized conditions using a video dimension analyzer system (Living Systems Instrumentation, Burlington, VT) as described elsewhere (Santhanam et al., 2015). Concentration-dependent contractions to 9,11-dideoxy-9α, 11α-methanoepoxy prostaglandin $F_{2\alpha}$ (U46619; 10^{-9} – 10^{-6} mol/L; Cayman Chemical) were obtained. Endothelium-dependent relaxations to acetylcholine (10^{-9} – 10^{-5} mol/L; Sigma) and endothelium-independent relaxations to diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEA-NONOate; 10^{-9} – 10^{-5} mol/L) were cumulatively obtained during submaximal contractions to U46619.

2.12 Measurements of cGMP/cAMP

Large cerebral arteries (middle cerebral, anterior cerebral, posterior cerebral arteries, and basilar arteries) were removed from the brain and dissected free from surrounding tissue in cold (4°C) Krebs solution. cGMP and cAMP levels were determined using ELISA colorimetric kits (Cell Biolabs; cat. No. SAT-505, and SAT-500, respectively).

2.13 Statistical analysis

Data are presented as mean ± standard deviation. n = number of independent cell culture preparations, and denotes the number of mice from which tissue samples were collected. All

data sets were tested for normality using Shapiro-Wilk normality test and QQ plot analysis. Differences between mean values of two groups were compared using two-tailed unpaired t-test. Comparisons between two groups with non-normal distributed data were analyzed by the Mann-Whitney U-test for non-parametric data (GraphPad Prism 9 software). For non-parametric data analysis of multiple groups, Kruskal-Wallis test followed by Mann-Whitney U-test were performed. $P < 0.05$ was considered statistically significant (two-tailed). The concentration-response curves obtained in isolated mouse arteries were compared by two-way repeated-measures ANOVA. Formal prior power calculations were not performed. Sample size determination was based on previous publications (He et al., 2019, 2022). No data deletion such as outliers were performed.

3. RESULTS

3.1 Effects of genetic inactivation of BACE2 on eNOS expression and APP processing

We used BACE2siRNA to inhibit BACE2 expression (Figure 1a and b). Reduced BACE2 expression led to decrease in mRNA and protein levels of eNOS, compared to those of CtsiRNA-treated cells (Figure 1c, d, and e). Prior studies established that phosphorylation of eNOS at serine residue Ser1177 (p-eNOS/S1177) activates eNOS enzyme activity (Fulton et al., 1999; Dimmeler et al., 1999). In contrast, phosphorylation of eNOS at threonine residue Thr495 (p-eNOS/T495) inhibits activity of eNOS (Chen et al., 1999). In the present study genetic knockdown of BACE2 did not change levels of eNOS phosphorylation at serine residue Ser1177 [calculated as p-eNOS(S1177)/eNOS] (Figure 1d and f). In contrast, levels of eNOS phosphorylation at threonine residue Thr495 [calculated as p-eNOS(T495)/eNOS] was elevated (Figure 1d and g). Importantly, NO production (concentration of NO_2 + NO_3 in conditioned media) was significantly decreased in BACE2 deficient human BMECs (Figure 1h).

In the next series of experiments, we determined the effects of BACE2 deficiency on APP processing in human BMECs. BACE2siRNA treatment decreased protein and mRNA expressions of APP, increased BACE1 protein levels, but had no significant effect on α-secretase ADAM10 expression (Figure 2a–e). Inhibition of BACE2 expression significantly reduced sAPPα release from human BMECs (Figure 2f). In contrast, the production of Aβ1-40 by human BMECs was low (as compared to production of sAPPα) and was not significantly affected by genetic knockdown of BACE2 (Figure 2g).

3.2 Stimulatory effect of sAPPα on eNOS expression

We have previously reported that expression of eNOS was reduced in APPsiRNA-treated human BMECs, and in cerebral arteries of global APP knockout mice or endothelial specific APP knockout mice (d'Uscio et al., 2018). We also reported that treatment with Aβ-40 or Aβ-42 did not change eNOS expression in cultured human BMECs (He et al., 2022), thus it is unlikely that Aβ peptides contribute to regulation of eNOS expression. To determine the effects of sAPPα on eNOS protein levels we incubated human BMECs with sAPPα for 3 days. As shown in Figure 3a, treatment with sAPPα significantly elevated protein levels of eNOS. Furthermore, overexpression of recombinant APP695 (expressed predominantly in neuronal tissue) (Donev et al., 2007; Kitazume et al., 2012) or APP751 (ubiquitously

expressed) (Donev et al., 2007) *via* adenovirus-mediated transduction of cells with either Ad-hAPP695 or Ad-APP751, respectively, augmented eNOS protein levels (Figure 3b–e).

3.3 Stimulatory effect of APP 17mer on eNOS expression

To define cell signaling mechanisms responsible for the effects of sAPPα we focused on $GABA_BR1$. The rationale for this approach was based on previous studies demonstrating that sAPPα and APP 17mer peptide derived from 204-220 amino acid of human APP695 (the sequence is shared among human APP695, APP751, and APP770, and is also contained within sAPPθ sequence), bind to extracellular sushi 1 domain of $GABA_BR1$ a thereby suppressing neuronal synaptic vesicle release (Dawkins et al., 2014; Rice et al., 2019). Furthermore, examination of the existing literature indicated that activation of $GABA_B$ receptors expressed in human aortic endothelial cells may play a role in translocation of eNOS (Wang et al., 2014).

RT-qPCR analysis revealed that BACE2siRNA treatment did not significantly affect mRNA levels of GABBR1 (BACE2siRNA treated cells 0.0091 ± 0.0024 relative unite vs. CtsiRNA treated cells 0.0095 ± 0.0004 relative unite, n=3, P=0.7812, t=0.297, df=4, two-tailed unpaired t test). We further examined the effects of BACE2siRNA on mRNA expression of isoforms of GABBR1, GABBR1a and GABBR1c (both isoforms express sushi 1 domain). As shown on Supplemental Figure 1, BACE2siRNA did not significantly affect GABBR1a or GABBR1c mRNA expression. These findings ruled out inhibitory effect of BACE2 deficiency on expression of GABBR1a and GABBR1c isoforms.

Next, we examined the effect APP 17mer on expression of eNOS. Incubation of human BMECs with APP 17mer for 2 days significantly increased eNOS expression (Figure 4a–d) at concentrations of 10 and 30 nM. Treatment with APP 17mer also suppressed eNOS phosphorylation at Thr495 residue (Figure 4c and e) and increased eNOS mRNA levels (Figure 4f). As a result, NO production was augmented in response to APP 17mer treatment (Figure 4g). Importantly, suppression of GABBR1 gene expression by GABBR1siRNA significantly decreased mRNA and protein levels of eNOS (Figure 4h–j). In addition, GABBR1siRNA treatment prevented APP 17mer-induced elevation of eNOS expression (Figure 4k).

Furthermore, although knockdown of BACE2 decreased GABBR2 mRNA expression in human BMECs (Suppl. Figure 2a), GABBR2siRNA treatment did not change eNOS expression (Suppl. Figure 2b–d). Thus, the effect of BACE2 deficiency on expression of eNOS appears to be mediated by $GABA_BR1$ receptors.

3.4 BACE2 deficiency exerts inhibitory effects on expression of KLF2

Since KLF2 is a critically important transcription factor responsible for activation of eNOS gene expression (SenBanerjee et al., 2004), we explored the role of KLF2 in BACE2siRNA-induced inhibition of eNOS expression. After incubation of human BMECs with BACE2siRNA (30nM) for 2 days, KLF2 mRNA expression was significantly reduced (Figure 5a). As anticipated, and consistent with well-established function of KLF2 in control of eNOS expression, genetic inactivation of KLF2 significantly decreased protein levels of eNOS (Figure 5b and c). Importantly, GABBR1siRNA treatment reduced KLF2

expression (Figure 5d), while APP 17mer treatment augmented KLF2 mRNA levels (Figure 5e). In aggregate, these results suggest that KLF2 may be an important mediator of BACE2-dependent preservation of eNOS expression.

3.5 Studies on cerebral arteries derived form BACE2-deficient mice

Genotype of BACE2$^{-/-}$ mice was confirmed by PCR experiments (Figure 6a). BACE2$^{-/-}$ mice had similar body weight, blood pressure and blood glucose levels as WT mice (Suppl. Table1). Lipid profile analysis revealed that circulating levels of cholesterol and HDL of BACE2$^{-/-}$ mice were significantly lower than those of WT mice (Suppl. Table1).

Resting lumen diameter and wall media thickness of isolated cerebral basilar arteries were not different between WT and BACE2$^{-/-}$ mice [diameter: 162 ± 21 μm versus 157 ± 14 μm, respectively, n=5, P=0.833, sum of ranks (26 and 29), U=11, Mann Whitney U-test; media thickness: 11.1 ± 1.4 μm versus 11.7 ± 1.0 μm, respectively, n=5, P=0.4609, t=0.775, df=8, two-tailed unpaired t test]. Contractions of basilar arteries to U46619 were not affected by genetic inactivation of BACE2 (Figure 6b). Importantly, endothelium-dependent relaxations to acetylcholine were significantly impaired in BACE2$^{-/-}$ mice (Figures 6c). In contrast, endothelium-independent relaxations to DEA-NONOate were significantly augmented (Figure 6d). Western blot revealed that eNOS protein expression was not changed in large cerebral arteries of BACE2$^{-/-}$ mice (Figure 6e and f). However, protein levels of p-eNOS(T495) [calculated as ratio of p-eNOS(T495)/eNOS] were significantly increased in large cerebral arteries of BACE2$^{-/-}$ mice (Figure 6e and g). Furthermore, cGMP concentrations were significantly lower in large cerebral arteries of BACE2$^{-/-}$ mice (Figure 6h) while cAMP concentrations were not changed (Figure 6i)

4. DISCUSSION

This is the first study designed to determine cerebrovascular function of BACE2. We report several novel findings generated in cultured human BMECs: 1) genetic inactivation of BACE2 decreases eNOS expression, increases phosphorylation of eNOS at Thr495, and suppresses NO production, 2) this phenomenon appears to be dependent on impaired expression of APP and reduced sAPPα production, 3) stimulatory effects of sAPPα and APP 17mer on eNOS expression are mediated by activation of $GABA_BR1$, and 4) KLF2 expression is impaired in BACE2- or $GABA_BR1$-deficient cells thereby suggesting that BACE2 and $GABA_BR1$ play important role in preservation of KLF2/eNOS signaling. In addition, in basilar arteries of BACE2$^{-/-}$ mice, endothelium-dependent relaxations to acetylcholine are impaired. This is accompanied by increased inhibitory phosphorylation of eNOS at Thr495 and decreased cerebrovascular levels of cGMP. In aggregate, these findings support the concept that BACE2 may serve as a vascular protective protein. In the context of previously reported dramatic downregulation of BACE2 in brain capillaries derived from AD patients (Yang et al., 2022), our findings may help explain widespread vascular loss caused by AD. Indeed, eNOS and KLF2 are critically important pro-survival factors in vascular endothelium (SenBanerjee et al., 2004; Sweet et al., 2018).

Several BACE1 inhibitors have been tested as therapies for prevention and treatment of AD (Voytyuk et al., 2022). The exact reasons for disappointing performance of BACE1

inhibitors in clinical arena remain to be determined. However, it is important to note that BACE1 inhibitors may also inhibit BACE2 (Voytyuk et al., 2022). Aberrant expression of BACE2 may impair expression of eNOS thereby possibly weakening vasoprotective functions of eNOS including vasodilation, inhibition of platelet aggregation, and prevention of vascular inflammation (Forstermann and Sessa, 2012). Moreover, loss of eNOS may promote development of cerebrovascular and neuronal AD pathology (Katusic et al., 2016, 2023).

To gain additional understanding of molecular mechanisms underlying BACE2-dependent regulation of eNOS, we first focused on the role of BACE2 in endothelial APP processing. BACE2 has (α-secretase like) θ-secretase and (conditional) β-secretase properties in neurons, however, the role of BACE2 in endothelial APP processing has not been studied. We observed that in human BMECs genetic inactivation of BACE2 down-regulated expression of APP, did not affect expression of major α-secretase enzyme ADAM10, but increased expression of BACE1. Importantly, we detected significant reduction of sAPPα release from BACE2 deficient cells. This finding suggests that genetic inactivation of BACE2 reduces (α-secretase like) θ secretase processing of APP. It is important to note that θ cleavage product of APP has only 3 extra amino acids on C-terminal of sAPPα (Wang et al., 2019). We contacted manufacturer of assay kit for human sAPPα to determine whether this assay may also detect θ cleavage product. Currently available information suggests that we cannot exclude the possibility that sAPPα assay used in our study also detects the product of θ cleavage of APP (communication provided by manufacturer). Therefore, observed reduction of sAPPα release might reflect loss of α- and/or θ-secretase cleavage products. Since APP is substrate needed for production of sAPPα, it is important to note that observed lowering APP also contribute to impaired production of sAPPα. The mechanism of APP downregulation caused by genetic inactivation of BACE2 is unknown and thus remains to be investigated.

In contrast to sAPPα, levels of Aβ were not affected. This could be explained by elevation of BACE1 expression in BACE2 deficient cells. Higher expression of BACE1 may compensate for the loss of substrate (APP) thus maintaining production of Aβ at normal levels. Importantly, these observations also suggest that in endothelium, BACE2 does not play a significant role in β-processing of APP. In addition, our previous *in vitro* study (He et al., 2022) demonstrated that Aβ1-40 or Aβ1-42 did not affect eNOS expression in cultured human BMECs. These observations suggest that Aβ peptides are unlikely mediators of eNOS downregulation thus pointing to loss of APP and sAPPα as the explanation for dysfunction of eNOS in BACE2 deficient cells.

In $GABA_BRs$ family, $GABA_BR1a$ or $GABA_BR1b$ subunit combines with $GABA_BR2$ subunit to generate heterodimeric $GABA_BR1a/2$ or $GABA_BR1b/2$ receptors (Fritzius et al., 2020). Importantly, $GABA_BR2$ subunit is responsible for coupling to and activation of G-protein signaling (Fritzius et al., 2020). Relevant to further analysis of signaling pathway activated by APP and/or sAPPα, prior studies established that both APP and sAPPα bind to the N-terminal sushi 1 domain of $GABA_BR1a$ (Dinamarca et al. 2019; Rem et al., 2023; Rice et al. 2019). In the brain, binding of sAPPα or APP 17mer to $GABA_BR1a$ suppresses synaptic transmission and spontaneous neuronal activity (Rice et al. 2019). However, ability

of sAPPα and APP 17mer to behave as functional ligands for $GABA_BR1a$ is controversial (Rem et al., 2023; Rive et al., 2019). Recent study by Rem and colleagues (2023) suggests that although APP 17mer binds to $GABA_BR1a$, APP 17mer does not behave as functional ligand when evaluated in human embryonic kidney 293T cells (Rem et al., 2023). In the present study, we detected expression of GABBR1 (and GABBR1a and GABBR1c isoforms), as well as GABBR2 in human BMECs. Knockdown of $GABA_BR1$ decreased eNOS expression and prevented stimulatory effect of APP 17mer on eNOS expression. In contrast, genetic inhibition of $GABA_BR2$ expression did not affect expression of eNOS. These observations suggest that in endothelium, the inhibitory effect of BACE2 deficiency on eNOS expression is independent of $GABA_BR2$ subunit. Moreover, it is unlikely that activation of classical heterodimeric $GABA_B$ receptors and subsequent signaling via G protein plays a role in the BACE2-regulated eNOS expression. Further studies are needed to characterize downstream signaling pathways activated by APP 17mer binding to sushi domain of $GABA_BR1$ isoform(s). We recognize that findings of the present study cannot rule out the possibility that APP 17mer may also activate other currently unknown receptors for sAPPα.

Our previous study demonstrated that APP deficiency suppressed eNOS protein levels *in vitro* and *in vivo* (d'Uscio et al., 2018). In agreement with these findings, adenovirus-mediated overexpression of recombinant APP isoforms enhanced eNOS expression in the present study. Thus, APP contributes to preservation of eNOS expression. This conclusion is reinforced by observations demonstrating that treatment with nanomolar concentrations of APP 17mer (Rice et al. 2019; Rem et al., 2023), increased eNOS expression. Since APP localized on cell membrane binds to sushi domain of $GABA_BR1a$ receptor (Dinamarca et al., 2019), it is possible that in BACE2-deficient cells, down-regulation of APP and resulting reduction of APP binding to $GABA_BR1a$ may contribute to decreased expression of eNOS.

We provide evidence that in human BMECs, BACE2 knockdown enhanced phosphorylation of eNOS at Thr495 residue, while APP 17mer treatment reduced Thr495 phosphorylation of eNOS thus demonstrating that α and/or θ proteolytic cleavage of APP inhibited by BACE2 knockdown regulates activity of eNOS. Several signaling pathways have been reported to regulate phosphorylation of eNOS (Shu et al., 2015). The exact signalling pathway that mediates sAPPα or APP 17mer-induced inhibition of eNOS phosphorylation at Thr495 residue remains to be defined.

Our results obtained in human BMECs were validated by analysis of endothelial function of cerebral arteries derived from $BACE2^{-/-}$ mice. Consistent with dysregulation of eNOS caused by loss of BACE2 in human BMECs, we demonstrated that endothelium-dependent relaxations to acetylcholine were impaired in BACE2-deficient basilar arteries. This impairment is most likely the result of elevated inhibitory phosphorylation of eNOS at Thr495. In agreement with loss of endothelial NO signaling, levels of cyclic GMP were significantly reduced in BACE2-deficient arteries. Moreover, impaired production of cyclic GMP resulted in increased sensitivity of smooth muscle cells to NO thereby enhancing relaxations in response to NO-donor, DEA-NONOate (Brandes et al., 2000). Why eNOS protein expression was not affected by genetic inactivation of BACE2 in mice is unknown but could be related to species or methodological differences (cultured human

endothelium versus intact murine cerebral arteries). We also wish to point out that body weight, arterial blood pressure and blood glucose levels were not affected by deficiency of BACE2. However, circulating levels of cholesterol and HDL were significantly decreased. Whether the changes of cholesterol and HDL detected in the present study may affect eNOS expression is unknown and thus remains to be investigated. In aggregate, our findings support the concept that BACE2 plays an important role in preservation of cerebral vascular eNOS function thereby exerting an important cerebrovascular protective function.

Finally, additional analysis of cell signaling mechanisms revealed that expression of major endothelial transcription factor KLF2 (SenBanerjee et al., 2004) is dependent on intact expression of BACE2. In agreement with this observation, genetic inhibition of GABBR1 suppressed, while treatment with APP 17mer increased expression of KLF2. These novel findings reinforce our conclusion that BACE2 is vascular protective gene. Indeed, under physiological conditions, KLF2 plays critically important role in maintenance of eNOS function and preservation of healthy endothelium (SenBanerjee et al., 2004).

We provide several novel insights into the role of BACE2 in guarding homeostasis of human vascular endothelium. Intact expression of BACE2 protects expression of eNOS. This beneficial effect of BACE2 appears to be mediated by sAPPα(sAPPθ)/$GABA_BR1$/KLF2 signaling. To avoid possible adverse cerebrovascular effects of BACE2 inhibition, highly selective BACE1 inhibitors should be used in future clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Abbreviations:

Aβ	beta-amyloid
AD	Alzheimer’s disease
ADAM10	a disintegrin and metalloproteinase domain-containing protein 10
Ad-APP751	adenoviral construct containing human APP751
APP	amyloid precursor protein
BACE 1	β-secretase 1
BMECs	brain microvascular endothelial cells

EBM2	endothelial basal medium 2
EGM2	endothelial growth medium 2
eNOS	endothelial nitic oxide synthase
$GABA_BR1a$	γ-aminobutyric acid type B receptor subunit 1a (protein)
GABBR1a	γ-aminobutyric acid type B receptor subunit 1a (gene)
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
KLF2	Krüppel-like Factor 2
MOI	multiplicity of infection
NO	nitric oxide
sAPPα	soluble APP alpha
siRNA	small interfering RNA

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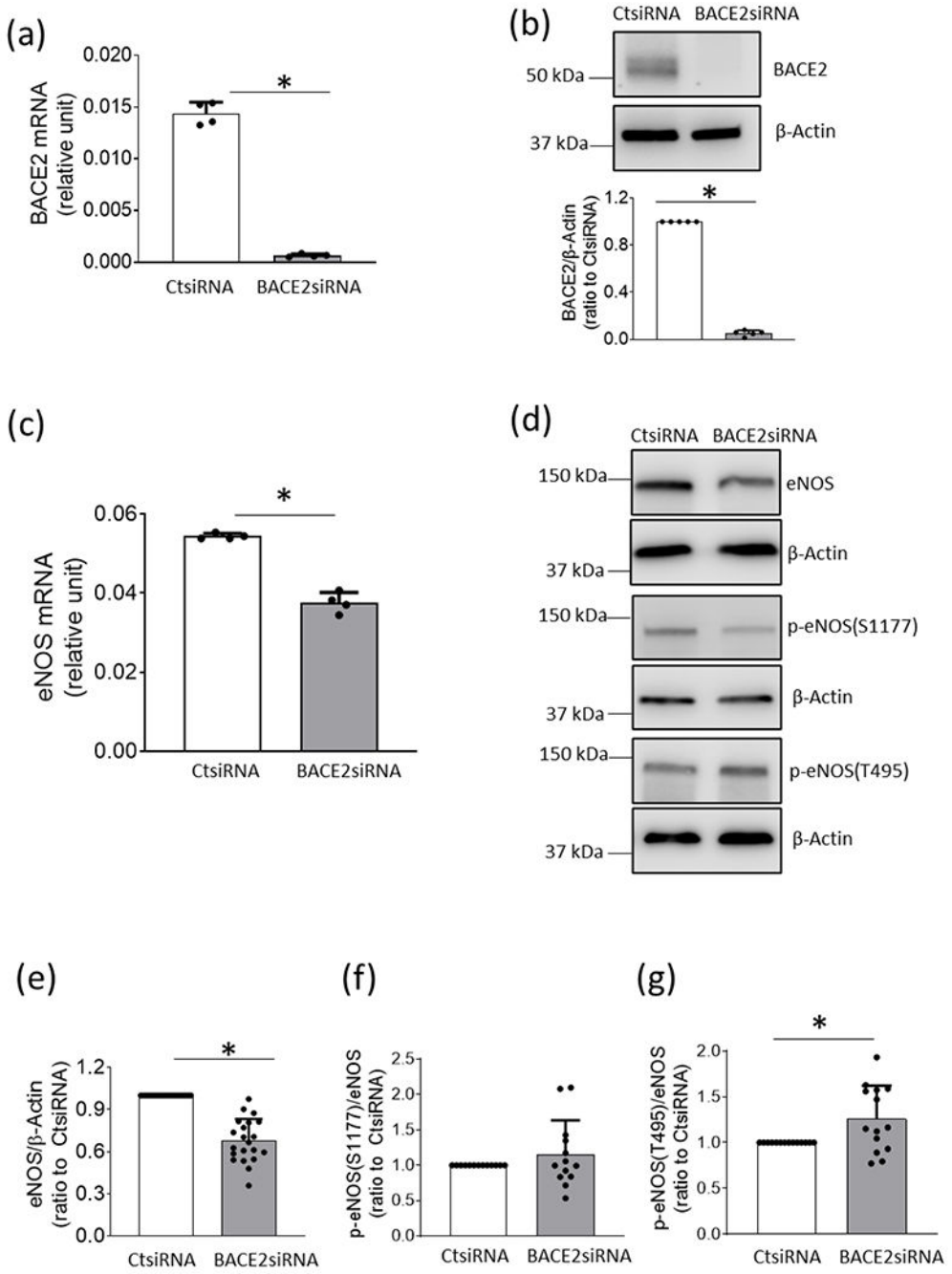


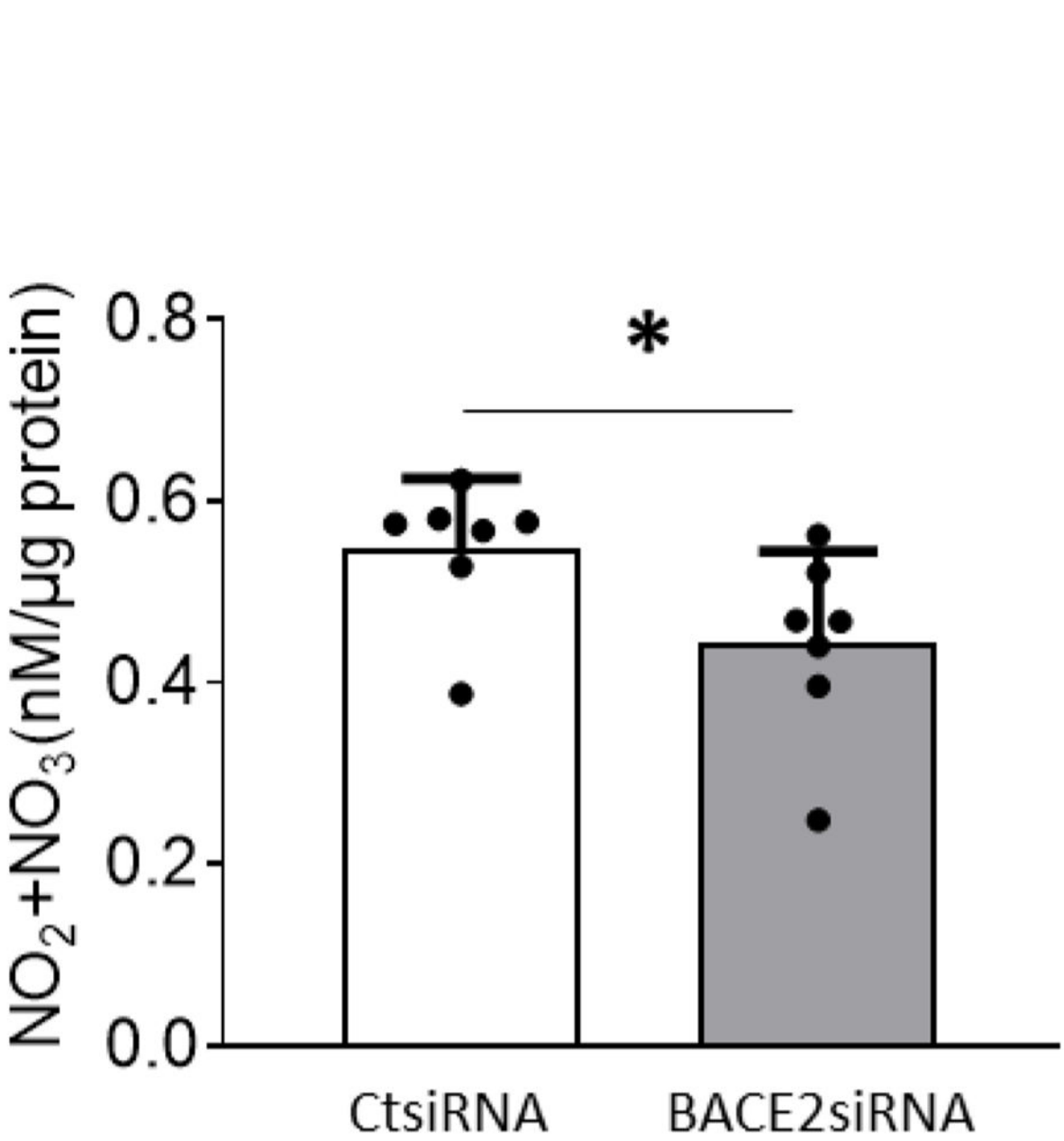


FIGURE 1.

Knockdown of BACE2 in human BMECs decreased eNOS expression, increased p-eNOS(T495) levels and reduced NO production. (a)-(g): Human BMECs were treated with BACE2siRNA (30nM) for 65 hours (a and c) or 3 days (b, d, e, f and g), RNA samples or protein samples were collected, respectively. (a), n=4, *P<0.0001, t=24.67, df=6 (two-tailed unpaired t-test); (b), n=5, *P=0.0079, sum of ranks are 40 and 15, U=0 (Mann-Whitney U-test); (c), n=4, *P<0.0001, t=12.49, df=6 (two-tailed unpaired t-test); (e), n=21, *P<0.0001, sum of ranks are 672 and 231, U=0 (Mann-Whitney U-test); (f), n=13, P=0.7442, sum of ranks are 182 and 169, U=78 (Mann-Whitney U-test); (g), n=14, *P=0.0385, sum of ranks are 161 and 245, U=56 (Mann-Whitney U-test). (h): Human BMECs were incubated with

BACE2siRNA for 48 hours. After cells were cultured in EGM2 without serum for 24 hours. Conditioned media were collected for NO_2 + NO_3 measurements. n=7, *P=0.0262, sum of ranks are 70 and 35, U=7 (Mann-Whitney U-test).

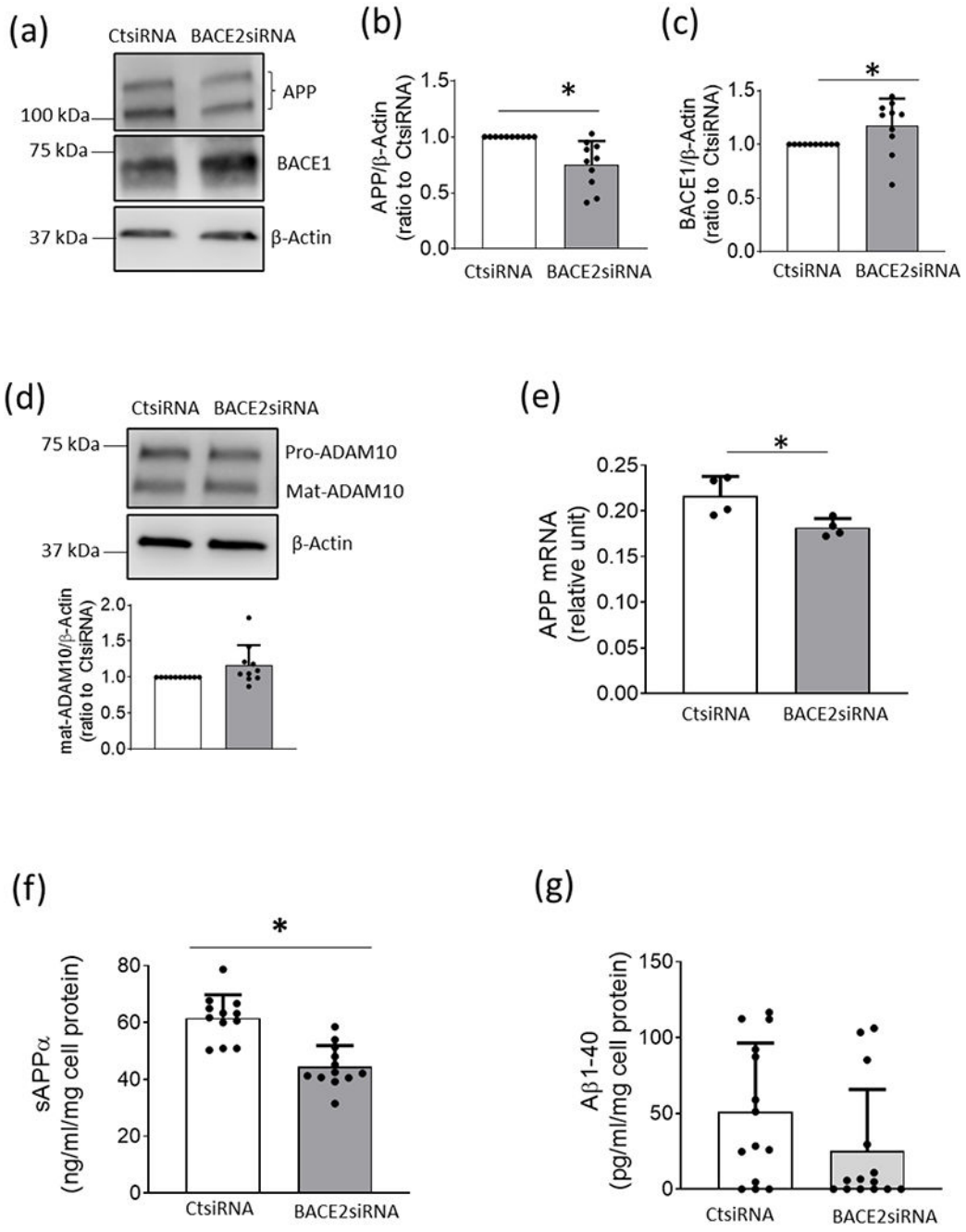
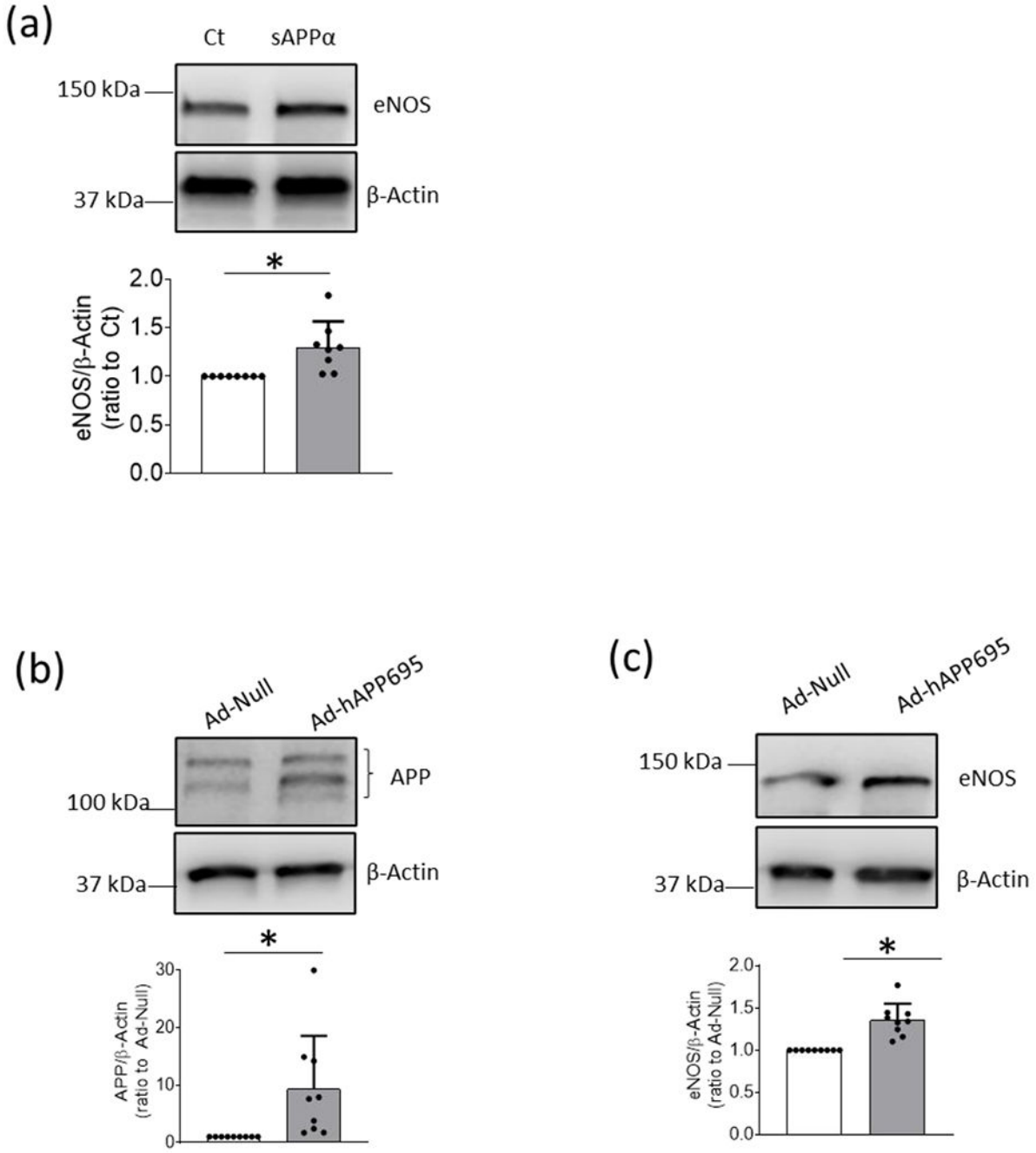


FIGURE 2.

BACE2siRNA treatment decreased release of sAPPα from human BMECs. (a)-(d): Cells were treated with BACE2siRNA (30nM) for 3 days, protein samples were collected for Western blots. (b), n=10, *P=0.0006, sum of ranks are 145 and 65, U=10 (Mann-Whitney U-test); (c), n=10, *P=0.0135, sum of ranks are 75 and 135, U=20 (Mann-Whitney U-test); (d), n=10, P=0.1138, sum of ranks are 85, 125, U=30 (Mann-Whitney U-test). (e): Cells were treated with BACE2siRNA (30nM) for 2.5 days, mRNA samples were collected for real-time PCR (n=4, *P=0.0242, t=2.995, df=6, two-tailed unpaired t-test). (f) and (g):

After BACE2 was knockdown by BACE2siRNA treatment for 2 days, conditioned media (24 h incubation) were collected and levels of sAPPα (f, n=12, *P<0.0001, t=5.37, df=22, two-tailed unpaired t-test) or Aβ1-40 (g, n=14, P=0.1077, sum of ranks are 238 and 168, U=63, Mann-Whitney U-test) in conditioned media were measured



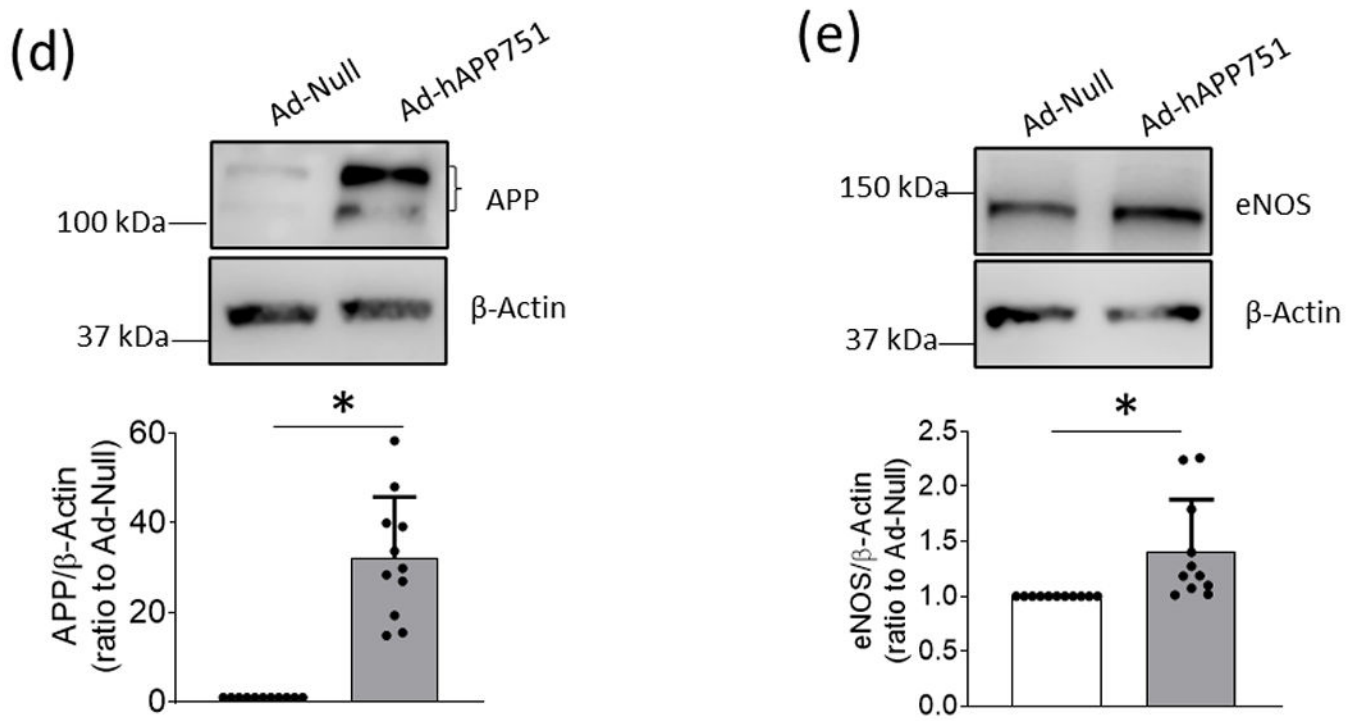
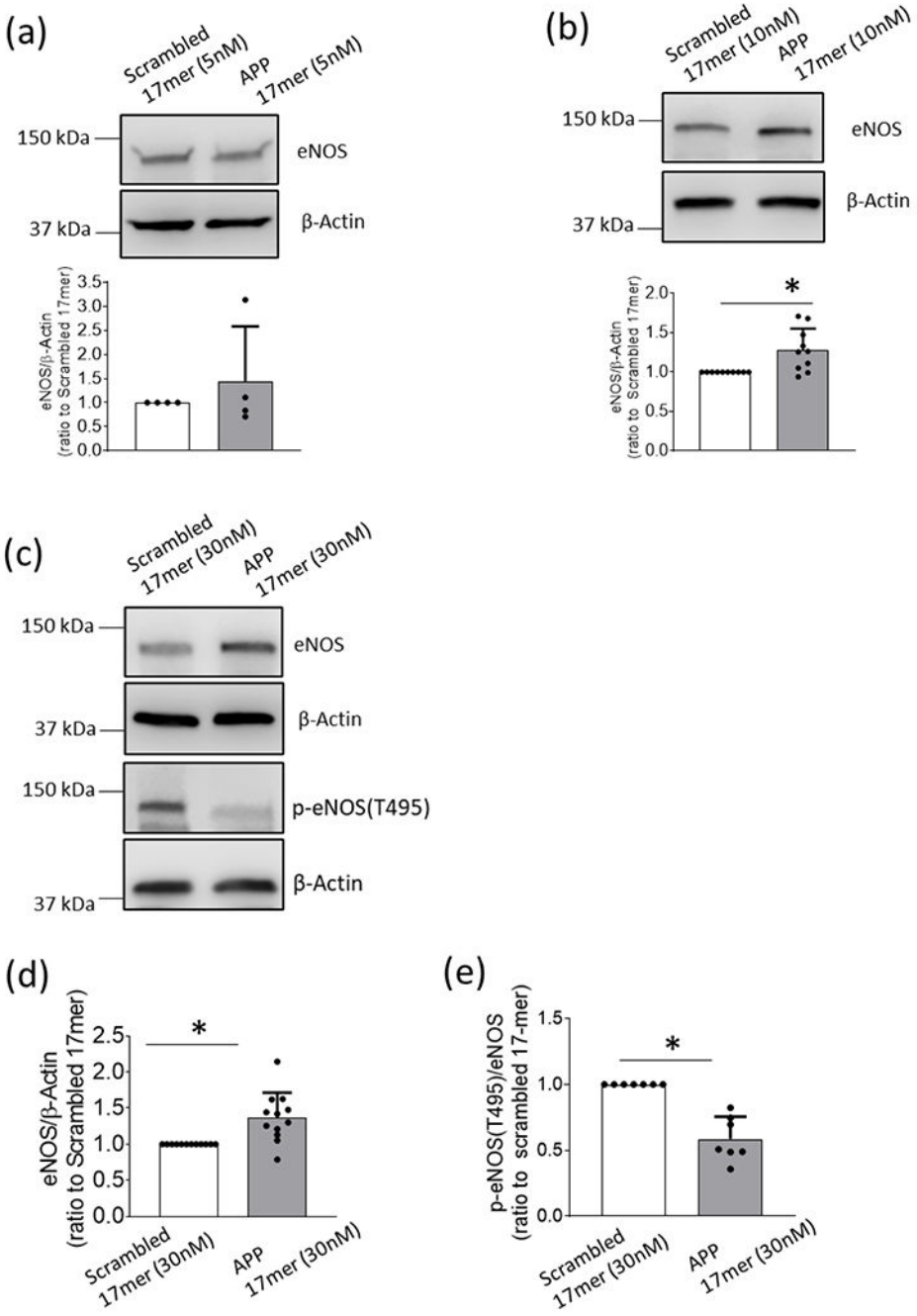
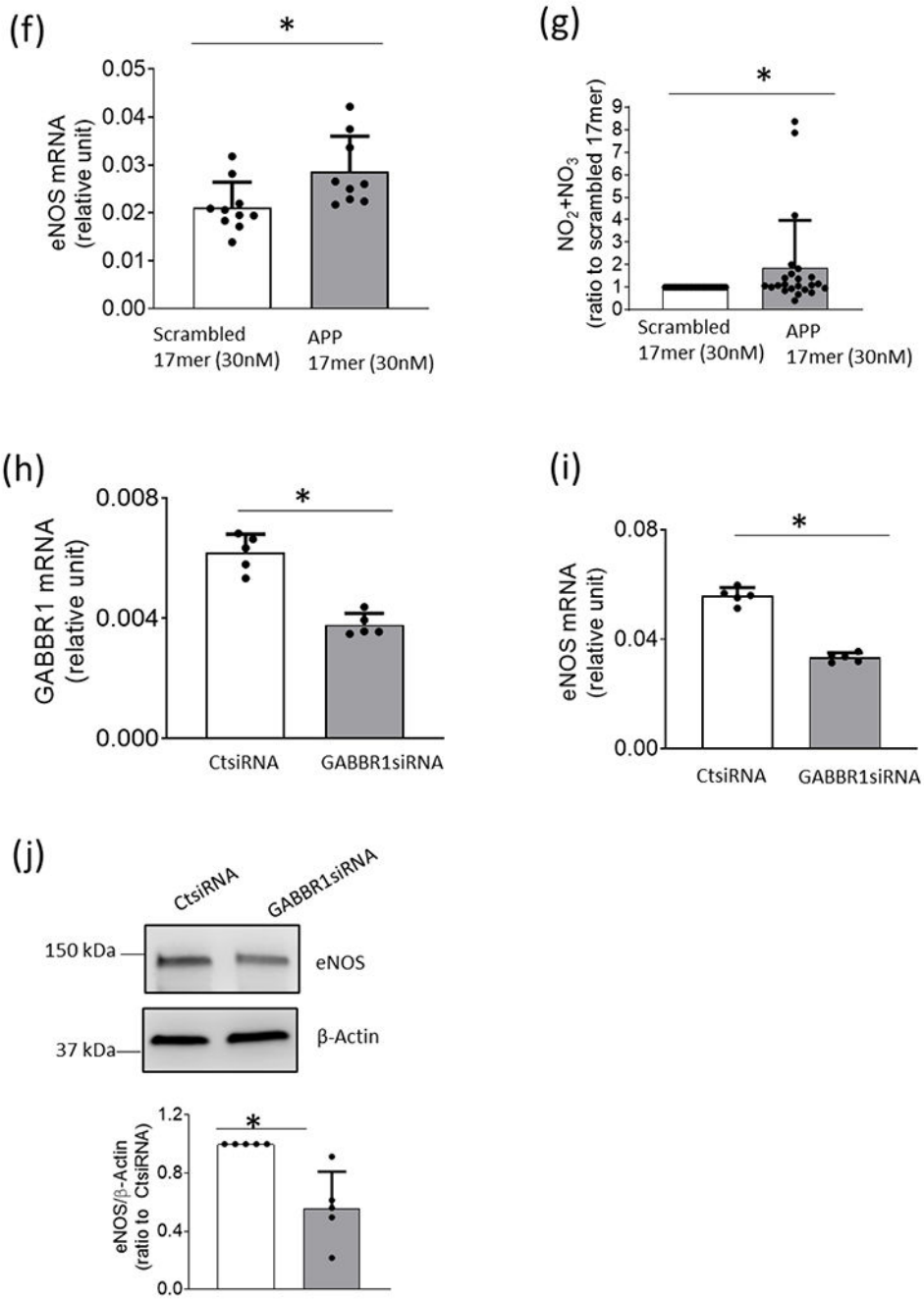


FIGURE 3.

sAPPα stimulated eNOS expression in human BMECs. (a): Cells were treated with human sAPPα (7 nM) for 3 days, protein samples were collected for Western blots. n=8, *P=0.0002, sum of ranks are 36 and 100, U=0 (Mann-Whitney U-test). (b)-(e): human BMECs were transduced with Ad-hAPP695 (5 MOI; b and c) or Ad-hAPP751 (5 MOI; d and e) for 2 days, Western blots were performed. (b), n=9, *P<0.0001, sum of ranks are 45 and 126, U=0 (Mann-Whitney U-test); (c), n=9, *P<0.0001, sum of ranks are 45 and 126, U=0 (Mann-Whitney U-test); (d), n=11, *P<0.0001, sum of ranks are 66 and 187, U=0 (Mann-Whitney U-test); (e), n=11, *P<0.0001, sum of ranks are 66 and 187, U=0 (Mann-Whitney U-test).





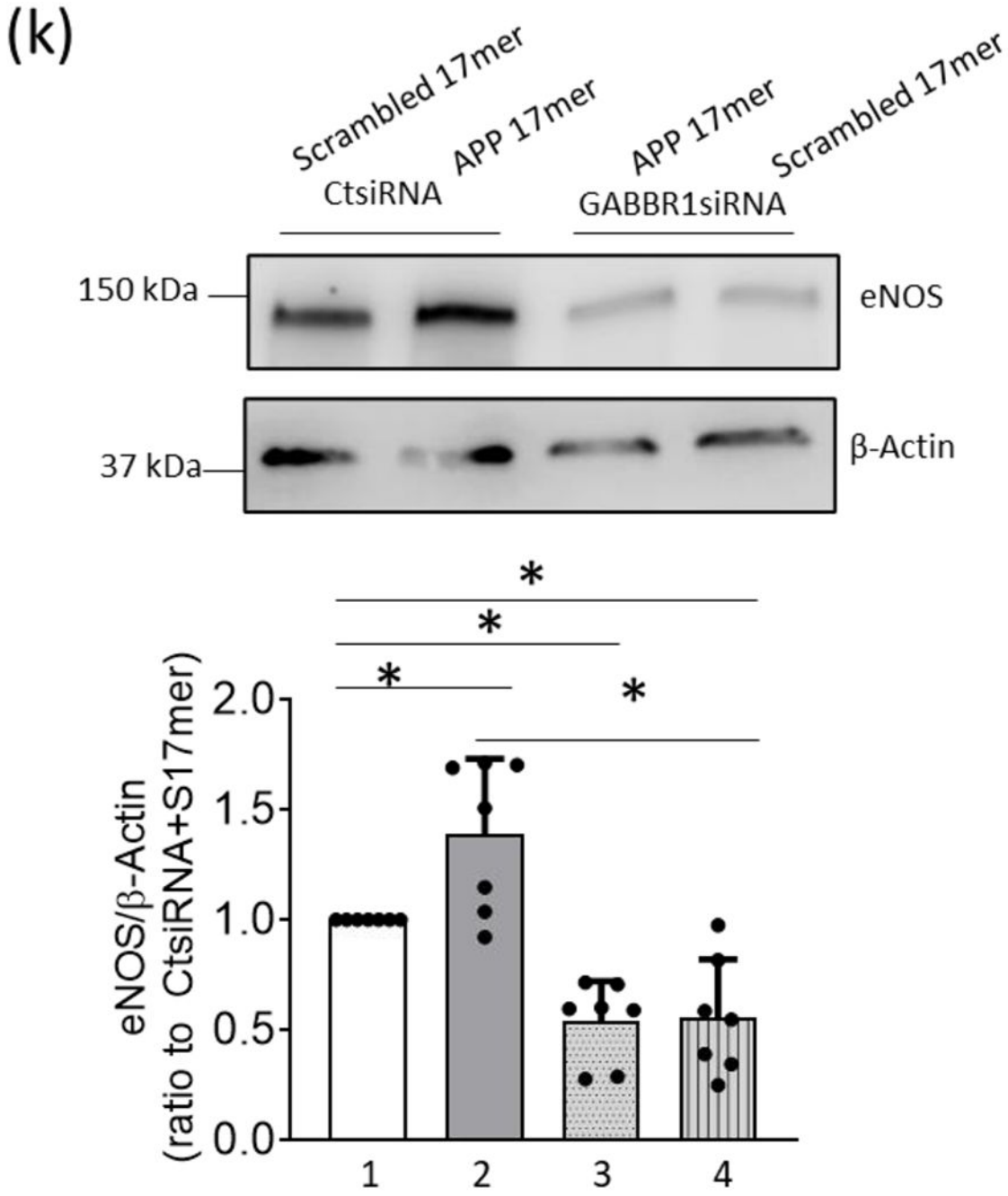


FIGURE 4.

$GABA_BR1$ mediated APP 17mer-induced eNOS expression in human BMECs. (a)-(f): Cells were treated with 5, 10, or 30nM of 17mer for 2 days (a-e) or 40 hours (f), eNOS protein expression (a-d), levels of p-eNOS(T495) (c and e), or mRNA levels (f) were measured. (a), n= 4, P=0.9999, sum of ranks are 18 and 18, U=8 (Mann-Whitney U-test); (b), n=10, P=0.0135, sum of ranks are 75 and 135, U=20 (Mann-Whitney U-test); (d), n=12, *P<0.0001, sum of ranks are 90 and 210, U=12 (Mann-Whitney U-test); (e), n=7, P=0.0006, sum of ranks are 77 and 28, U=0 (Mann-Whitney U-test); (f), n=9-10. *P=0.0196, t=2.58,

df=17 (two-tailed unpaired t-test). (g): NO production in response to APP 17mer treatment for 2 days (conditioned media were collected after 24 hours incubation). n=23, *P=0.0143, sum of ranks are 437 and 644, U=161 (Mann-Whitney U-test). (h)-(j): After cells were treated with GABBR1siRNA (45nM) for 2 days (h and i) or 3 days (j), RNA samples (h and i) or protein samples (j) were collected. (h), n=5, *P<0.0001, t=7.45, df=8 (two-tailed unpaired t-test); (i), n=5, *P<0.0001, t=14, df=8 (two-tailed unpaired t-test); (j), n=5, P=0.0079, sum of ranks are 40 and 15, U=0 (Mann-Whitney U-test). (k): Cells were treated with GABBR1siRNA (60nM) for 1 day, then were supplemented with 17mer (25nm) for 2 days. n=7, Kruskal-Wallis test P<0.0001, Kruskal-Wallis statistic=21.43, df=3; Column 1 vs. column 2, *P=0.0169, sum of ranks are 35 and 70, U=7 (Mann-Whitney U-test); column 1 vs. column 3, *P=0.0006, sum of ranks are 77 and 28, U=0; Column 1 vs. column 4, *P=0.0006, sum of ranks are 77 and 28, U=0 (Mann-Whitney U-test); Column 2 vs. column 4, *P=0.0003, t=5.08, df=12 (two-tailed unpaired t-test); Column 3 vs. column4, P=0.8048, sum of ranks are 55 and 50, U=22 (Mann-Whitney U-test).

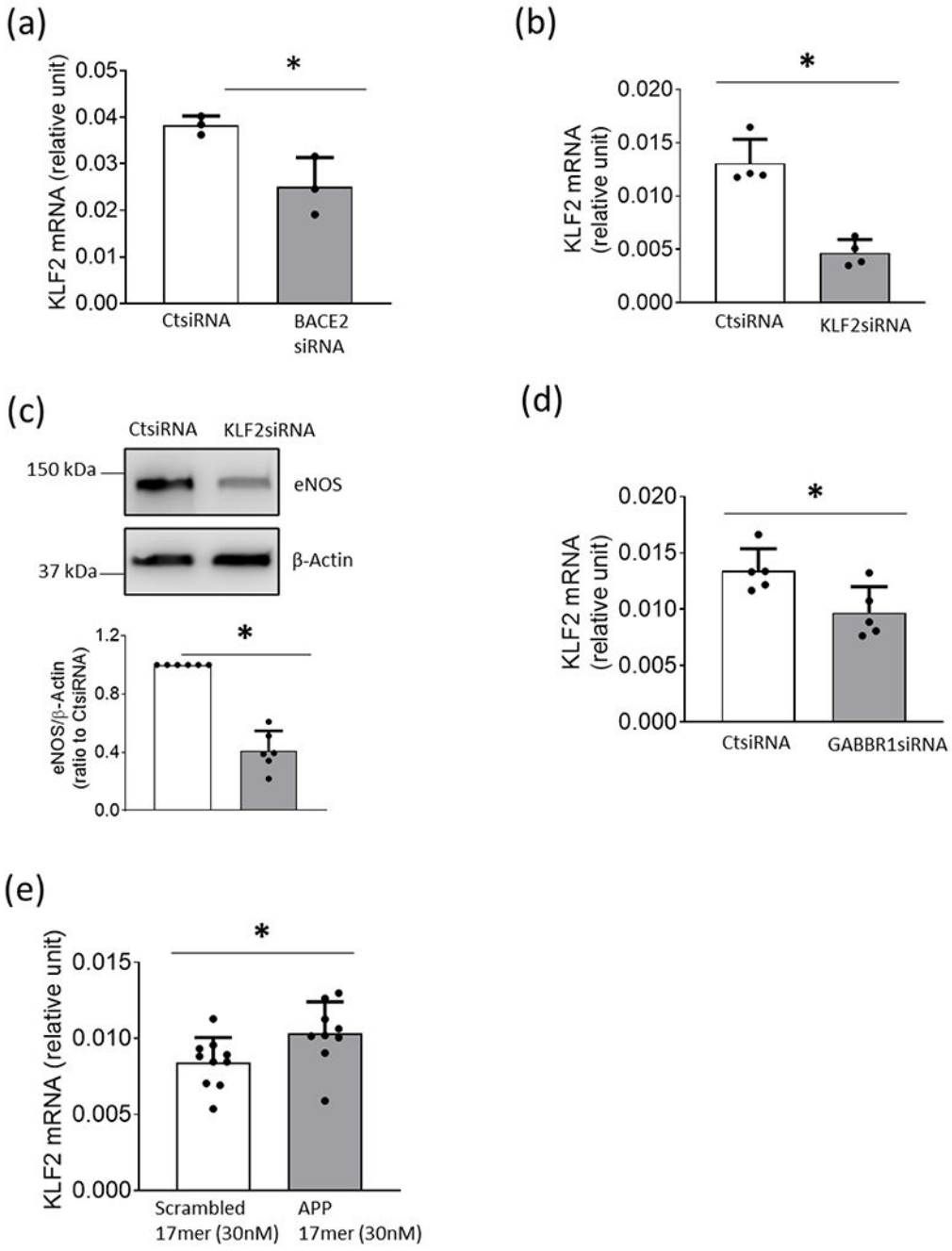
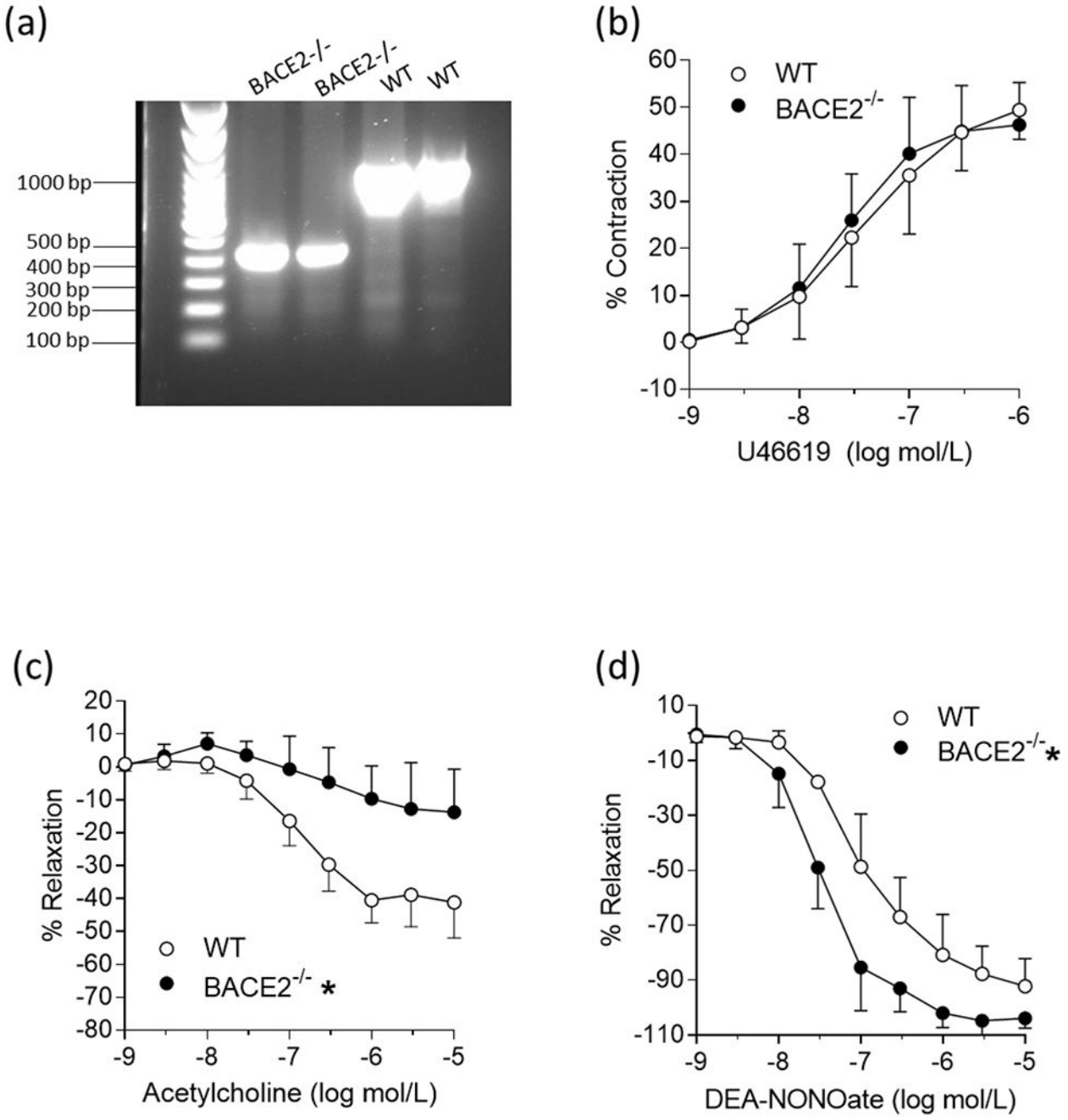


FIGURE 5.

Role of KLF2 in BACE2 regulated eNOS expression in human BMECs. (a): Cells were treated with BACE2siRNA (30nM) for 2 days. mRNA samples were collected for detection of KLF2 mRNA, n=3, *P=0.025, t=3.49, df=4 (two-tailed unpaired t-test); (b) and (c): Cells were incubated with KLF2siRNA (30nM) for 2 days, mRNA and protein samples were collected. (b), n=4, *P=0.0286, sum of ranks are 26 and 10, U=0 (Mann-Whitney U-test); (c), n=6, *P=0.0022, sum of rank are 57 and 21, U=0 (Mann-Whitney U-test). (d): Cells were treated with GABBR1siRNA (45nM) for 2 days. n=5, *P=0.0242, t=2.77, df=8

(two-tailed unpaired t-test); (e): Cells were incubated with APP 17mer and scrambled 17mer for 40 hours. n=9-10, *P=0.0402, t=2.22, df=17 (two-tailed unpaired t-test).



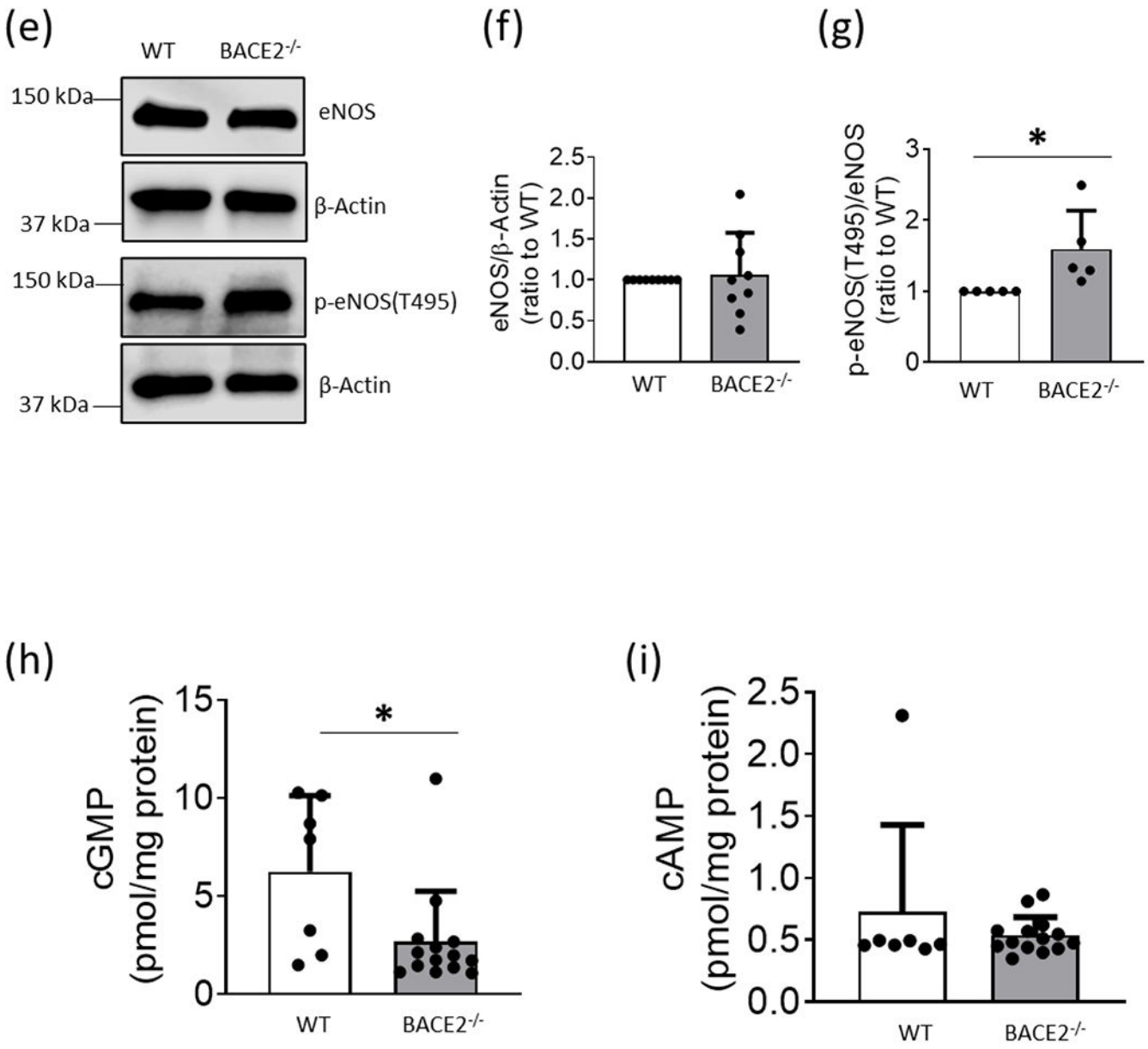


FIGURE 6.

Studies on cerebral arteries derived from BACE2$^{-/-}$ mice. (a): Genotyping of WT and BACE2$^{-/-}$ mice (PCR product size: WT, 1010bp; BACE2$^{-/-}$ 450 bp). (b): Concentration-dependent contractions to U46619 (p=0.8078; F(1,8)=0.063; n=5; two-way ANOVA). (c): Endothelium-dependent relaxations to acetylcholine (*p=0.0028; F(1,8)=18.04; n=5; two-way ANOVA). (d): Endothelium-independent relaxations to DEA-NONOate (*p<0.008; F(1,7)=13.41; n=4-5; two-way ANOVA). (e-g): Protein levels of eNOS and p-eNOS(T495) in large cerebral arteries. (f), n=9, P=0.709; sum of ranks are 90 and 81), U =36 (Mann Whitney U-test); (g), n=5, *P=0.0079, sum of ranks are 15 and 40, U=0 (Mann Whitney U-test). (h): cGMP levels in large cerebral arteries (n=7-14, *P=0.0379, sum of ranks are

105 and 126, U =21, Mann Whitney U-test). (i): cAMP levels in large cerebral arteries (n=7-14, P=0.7990, sum of ranks are 73 and 158), U=45, Mann Whitney U-test).