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SARS-CoV-2 spike S1 subunit protein-mediated increase of beta-secretase 1 (BACE1) impairs human brain vessel cells

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ABSTRACT

Increasing evidence suggests incomplete recovery of COVID-19 patients, who continue to suffer from cardiovascular diseases, including cerebral vascular disorders (CVD) and neurological symptoms. Recent findings indicate that some of the damaging effects of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, especially in the brain, may be induced by the spike protein, leading to the disruption of the initial blood-brain barrier (BBB). SARS-CoV-2-infected cells and animals exhibit age-dependent pathogenesis. In this study, we identified endothelial BACE1 as a critical mediator of BBB disruption and cellular senescence induced by the SARS-CoV-2 spike S1 subunit protein. Increased BACE1 in human brain microvascular endothelial cells (HBMVEC) decreases the levels of tight junction proteins, including ZO-1, occludin, and claudins. Moreover, BACE1 overexpression leads to the accumulation of p16 and p21, typical hallmarks of cellular senescence. Our findings show that the SARS-CoV-2 spike S1 subunit protein upregulated BACE1 expression in HBMVECs, causing endothelial leakage. In addition, the SARS-CoV-2 spike S1 subunit protein induced p16 and p21 expression, indicating BACE1-mediated cellular senescence, confirmed by β-Gal staining in HBMVECs. In conclusion, this study demonstrated that BACE1-mediated endothelial cell damage and senescence may be linked to CVD after COVID-19 infection.

1. Introduction

Recent studies show that many patients infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) experience fatigue and cognitive impairment or develop cardiovascular disease including cerebral vascular disorders (CVD), collectively termed long-COVID, after they recover from their initial illness. Some patients show such symptoms even 7 months post-infection and continue suffering from long COVID [1], which is associated with persistent residual SARS-CoV-2 antigen including the S1 subunit of the spike, full-length spike, and nucleocapsid [2]. A recent study demonstrates that cognitive impairment due to long COVID is equivalent to accelerated aging, the equivalent of 20 years [3,4]. Age-associated pathogenesis has been observed in SARS-CoV-2 infected animals [5] and COVID-19 patients [6]. Clinical studies show that approximately 30–50% of COVID-19 patients experience long COVID symptoms, which are common with increasing age and female sex [7].

SARS-CoV-2 infects cells through its S1 subunit, which binds to ACE2 on target cells [8]. ACE2 is expressed in several organs including the lungs, heart, kidneys, brain, and intestines, leading to detection of viral elements in the lungs and multiple extrapulmonary organs, including the brain of COVID-19 patients [9]. Histological data from several organs of COVID-19 patients provide evidence that SARS-CoV-2 directly infects the endothelial cell [10]. Recent studies have suggested that SARS-CoV-2 spike S1 protein translocate to the brain parenchyma by crossing the blood-brain barrier (BBB) [11], inducing the BBB disruption; however, the mechanism involved in BBB dysfunction has not been fully explored.

BACE1, an aspartic acid protease, is a transmembrane protein with two active sites for aspartate residues in the extracellular domain. It is also well known as a β-secretase that works with γ-

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secretase to produce amyloid-beta (Aβ) through the cleavage of amyloid precursor protein (APP), which accumulates in the brains of patients with Alzheimer's disease (AD). Growing evidence shows that BACE1 induces the loss of tight junction integrity in cerebrovascular endothelial cells [12]. BACE1 elevation is observed in the cerebral microvessels of postmortem human brains with cerebral amyloid angiopathy (CAA) [12]. Aberrant endothelial BACE1 activity induces endothelial dysfunction and memory deficits [13]. Therefore, endothelial BACE1 upregulation may be associated with BBB disruption through loss of tight junction proteins.

In the present study, we observed that the SARS-CoV-2 spike S1 subunit protein collapsed the integrity of tight junction proteins in human brain vessels, which had BACE1 upregulation. BACE1 inhibition blocked SARS-CoV-2 spike S1 subunit protein-induced leakage of endothelial cells. Moreover, the SARS-CoV-2 spike S1 subunit protein-mediated BACE1 upregulation triggered the accumulation of β-gal, p62, and p21, which are related to cellular senescence. Our findings collectively demonstrated that SARS-CoV-2 spike S1 subunit protein-induced endothelial BACE1 mediates vascular leakage and endothelial senescence, which weakens the function of brain vessels and may consequently initiate CVD, including stroke and hemorrhage.

2. Materials and methods

2.1. Reagents

Anti-BACE1 (CS-5606) antibody and senescence β-galactosidase staining kit (CS-9860) were obtained from Cell Signaling Technology (Beverly, MA, USA), anti-p16 (sc-1207) and anti-p21 (sc-756) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). anti-occludin (ab31721) antibody was from Abcam (Cambridge, MA, USA). Anti-ZO-1 (61–7300), anti-claudin-1 (37–4900), and anti-claudin-5 (35–2500) antibodies were purchased from Invitrogen (Carlsbad, CA, USA). Anti-β-actin (A5316) antibody was obtained from Sigma (Saint Louis, MO, USA). SARS-CoV-2 spike S1 subunit protein (10522-CV) was purchased from R&D Systems (Minneapolis, MN, USA). β-secretase inhibitor IV (BI-IV; 565788) and vascular permeability assay kit (ECM644) were purchased from EMD Millipore (Temecula, CA, USA).

2.2. Cell culture

Human brain microvascular endothelial cells (HBMVECs) were purchased from Cell Systems (Kirkland, WA, USA). HBMVECs were grown in M199 medium (Gibco, Waltham, MA, USA) supplemented with 20% FBS, 3 ng/mL recombinant human fibroblast growth factor-basic (FGF-b; Millipore, Temecula, CA, USA), 5 U/mL heparin, penicillin (100 U/mL), and streptomycin (100 μg/mL). Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD, USA). HUVECs were grown in cell growth medium (EGM-2, Lonza) with full supplements (EGM-2 bullet kit, Lonza). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Immunoblotting

Cells grown in plates were washed with phosphate-buffered saline (PBS) and lysed in RIPA buffer. The cell lysate was centrifuged (13,000 rpm, 30 min, 4 °C) and measured with Bradford protein assays following the manufacturer's protocol for concentration. Proteins were separated by 4–12% NuPAGE gel (Invitrogen) and transferred onto nitrocellulose membranes. Membranes were blocked in tris-buffered saline with 5% skim milk and 0.1% Tween 20 and incubated with the primary antibody overnight at 4 °C. Following washes, membranes were incubated with secondary antibody. Proteins were detected chemiluminescent kit (Amersham Pharmacia Biotech, Buckinghamshire, UK, USA). The following primary antibodies were used; anti-ZO-1 (1:1000), anti-p16 (1:1000), anti-p21 (1:1000), anti-occludin (1:1000), anti-claudin-5 (1:1000), anti-claudin-1 (1:1000), anti-BACE1 (1:1000), and anti-β-actin (1:5000).

2.4. Cell transfection

Cells were transiently transfected with the human BACE1 cloned into the pCMV-Myc vector. The Lipofectamine LTX reagent and Opti-MEM medium (Life Technology, Grand Island, NY, USA) were used for transfection according to the manufacturer's instructions.

2.5. Vascular permeability assay

A vascular permeability assay kit was used for the permeability assay following the product instructions. Briefly, HBMVECs were seeded on 1 μm-pore size membrane inserts in 24 *trans*-well plates and transferred to a 24 *trans*-well plate filled with 500 μl complete media. Upon confluency (100%), HBMVECs were treated with SARS-CoV-2 spike S1 subunit protein for 24 h. Then, cells were supplemented with a complete medium containing a high molecular weight FITC-Dextran. The FITC-Dextran migrated across the membrane of the insert were collected in 24 *trans*-well plates for 20 min and 100 μl volume of the medium containing transferring FITC-dextran was placed in a clean 96 well plate for fluorescence measurement at 485 nm.

2.6. Senescence-associated beta-galactosidase assay

Senescent HBMVECs were detected by senescence β-galactosidase (SA-β-gal) staining kit (Cell signaling) according to the manufacturer's instructions. Briefly, HBMVECs incubated with SARS-CoV-2 spike S1 subunit protein and fixed in 0.2% glutaraldehyde for 15 min at room temperature and then incubated overnight in β-gal staining solution at 37 °C. Senescent cells were identified by their blue staining of the β-gal solution under a standard light microscope (Olympus, Tokyo, Japan). To quantify positive staining, >100 cells were counted for each sample in several fields of view to obtain a standard deviation [14,15].

2.7. Immunofluorescence assay

Cells were quickly rinsed with PBS and immediately fixed with 4% paraformaldehyde in PBS for 15 min at 37 °C and washed three times with PBS. Cells were permeabilized with 100% ice-cold methanol for 10 min at room temperature. Cells rewashed with PBS and blocked with 4% bovine serum albumin for 1 h and incubated with the primary antibodies (anti-ZO-1, 1:200) overnight. After overnight incubation, fluorophore-conjugated secondary antibody was applied for 45 min and sealed with cover glass. The stains were reviewed using a fluorescence microscope (Zeiss, Jena, Germany).

2.8. Statistical analysis

Results are expressed as mean ± S.D. A student's *t*-test was applied for the analysis of significant differences between groups.

3. Results

3.1. SARS-CoV-2 spike S1 subunit protein dysregulates human brain microvessels

We evaluated the expression of ZO-1, occludin, and claudins,

essential components for BBB formation, in HBMVEC exposed to SARS-CoV-2 spike S1 subunit protein for 24 h to investigate its effect on the integrity of brain vessels. We found that the SARS-CoV-2 spike S1 subunit protein reduced the expression of ZO-1, occludin, and claudin-5 in HBMVEC (Fig. 1A). Immunofluorescence staining revealed that ZO-1 positive cells disappeared in the presence of SARS-CoV-2 spike S1 subunit protein (Fig. 1B). Moreover, exposure to the SARS-CoV-2 spike S1 subunit protein for 24 h increased vascular permeability (Fig. 1C), suggesting human brain microvessel dysregulation.

3.2. SARS-CoV-2 spike S1 subunit protein-induced BACE1 regulates brain vessels' permeability

Increased BACE1 expression induces endothelial dysfunction [12]. We first observed that BACE1 overexpression reduced ZO-1, occludin, claudin-1, and claudin-5 levels in HBMVECs (Fig. 2A and B). Notably, a 24 h treatment with SARS-CoV-2 spike S1 subunit protein upregulated endothelial BACE1 expression (Fig. 3A). BACE1 inhibitor, BI-IV effectively blocked the loss of tight junction proteins by the SARS-CoV-2 spike S1 subunit protein (Fig. 3B), thereby protecting the endothelial cells from leakage (Fig. 3C). Immunofluorescence staining further supported that the BACE1 inhibitor, BI-IV recovered the reduction of ZO-1 positive cells following SARS-CoV-2 spike S1 subunit protein treatment (Fig. 3D). These results suggested a SARS-CoV-2 spike S1 subunit protein-mediated increase in endothelial BACE1 expression, which may regulate BBB permeability.

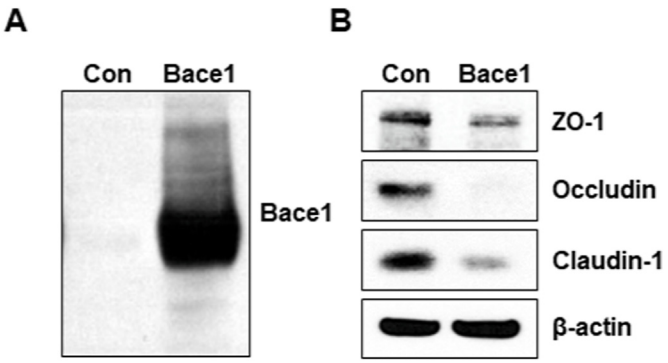

Fig. 2. BACE1 overexpression downregulated the expression of tight junction proteins. HUVECs were transiently transfected with pCMV-control (Con) or pCMV-myc-BACE1 (BACE1) as described in Section 2.4. (A) Confirmation of transfection efficiency by BACE1 overexpression. (B) Results were normalized to β-actin and expressed relative to tight junction protein level from control (Con).

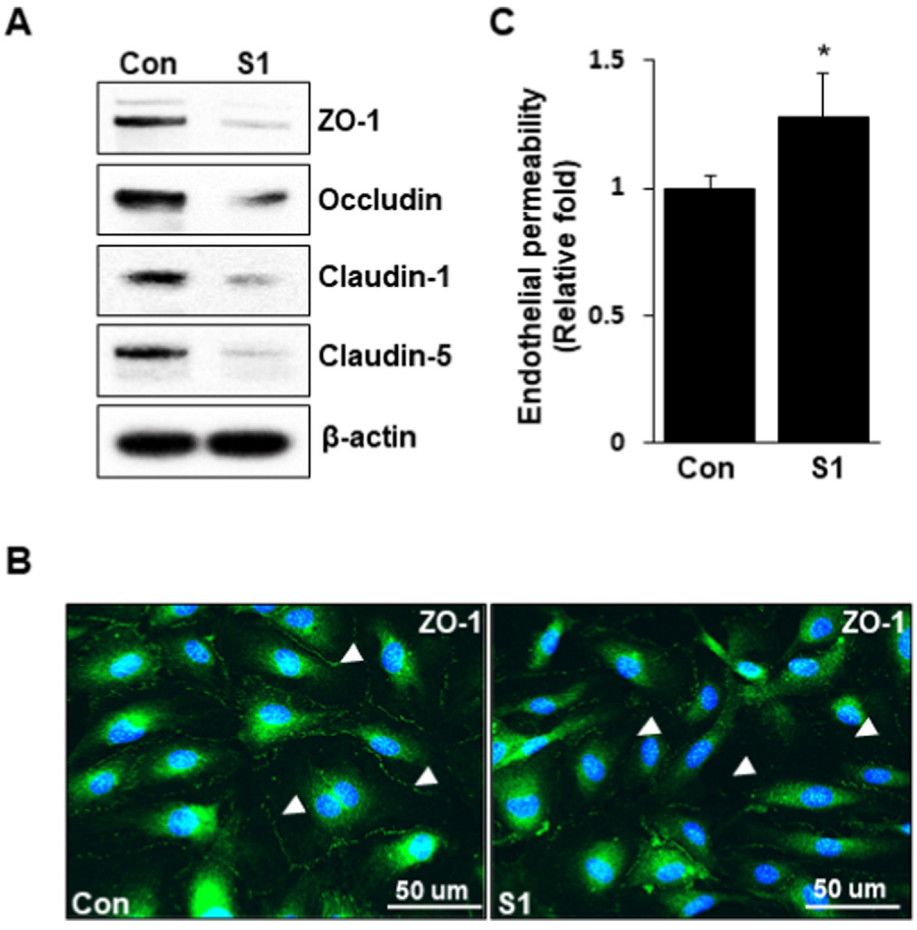

Fig. 1. SARS-CoV-2 spike S1 subunit protein exposure increased endothelial permeability.(A) HBMVECs were treated with 1.0 μg/ml SARS-CoV-2 spike S1 subunit protein (S1) for 24 h. The protein level of endothelial tight junctions, including ZO-1, occludin, claudin-1, and -5 was observed by immunoblotting as described in Section 2.3. β-actin was used as a loading control. (B) HBMVECs with or without SARS-CoV-2 spike S1 subunit protein (S1) treatment were fixed and stained with an anti-ZO-1 antibody as described in Section 2.7. Arrows indicate the ZO-1 in normal control (Con); disappeared in SARS-CoV-2 spike S1 subunit protein (S1) treatment. Horizontal white bars indicate 50 μm. (C) Endothelial permeability was quantified in vitro using FITC-dextran upon exposure to the SARS-CoV-2 spike S1 subunit protein (S1). Data are presented as the mean ± S.D. of three independent experiments (*p < 0.05 versus Con).

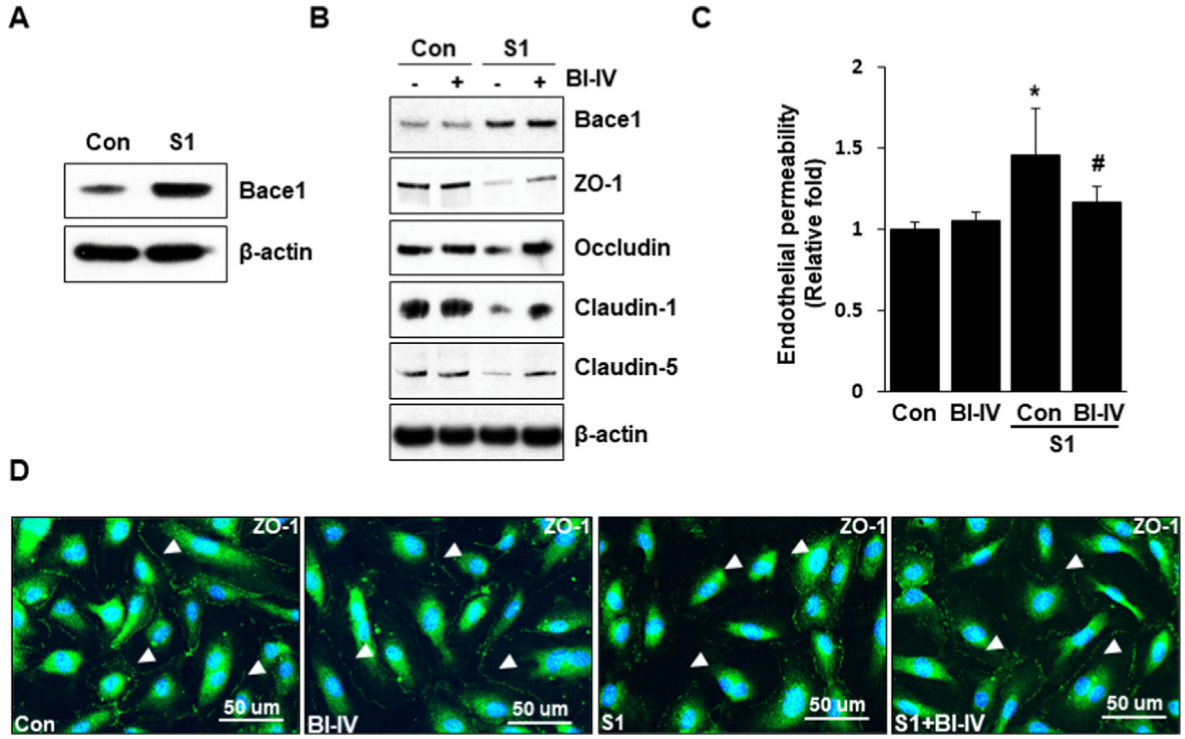

Fig. 3. BACE1 mediated SARS-CoV-2 spike S1 subunit protein-induced endothelial junction disruption. (A) BACE1 protein level. Quantification was performed using densitometry (Image J software). (B) HBMVECs were pretreated with 2.5 μM of BI-IV for 24 h before treatment of SARS-CoV-2 spike S1 subunit protein (S1). (C) Endothelial permeability was quantified in vitro FITC-dextran upon SARS-CoV-2 spike S1 subunit protein (S1) exposure with BI-IV. Data are presented as the mean ± S.D. in three independent experiments (*$p < 0.05$, versus Con; #$p < 0.05$, versus S1). (D) The ZO-1 was visualized by an immunofluorescence image (scale bar = 50 μm).

3.3. SARS-CoV-2 spike S1 subunit protein responsible for BACE1 induces cellular senescence

Exogenous stimuli inducing persistent endothelial cell damage aggravate aging. A recent study showed increased aging-related pathogenic molecules in rodents infected with SARS-CoV-2 [5]. Here, we investigated whether exposure to the SARS-CoV-2 spike S1 subunit protein affects cellular senescence, a key phenomenon of aging. Senescent phenotypes include increased cell size, expression of senescence-associated β-galactosidase activity (SA-β-gal), and numerous changes in gene expression associated with the senescence-associated secretory phenotype (SASP). Thus, we estimated the expression of SA-β-gal, p16, and p21 in HBMVEC exposed to the SARS-CoV-2 spike S1 subunit protein. Fig. 4A–C shows increased β-gal-positive cell numbers, and the upregulation of senescence-associated markers such as p16 and p21 in HBMVEC exposed to the SARS-CoV-2 spike S1 subunit protein, suggesting regulation of cellular senescence by SARS-CoV-2 spike S1 subunit protein. Treatment with a BACE1 inhibitor, BI-IV reversed this effect, indicating the specific involvement of BACE1 in SARS-CoV-2 spike S1 subunit protein-induced cellular senescence (Fig. 4D–F). SARS-CoV-2 spike S1 subunit protein-mediated BACE1 overexpression and the consequent induction of p16 and p21 expression (Fig. 4G) suggested a disruption of the function of brain vessels.

4. Discussion

Our data demonstrated that the SARS-CoV-2 spike S1 subunit protein responsible for BACE1 upregulation collapses the integrity of tight junction proteins and triggers endothelial senescence in brain vessels. Although detailed clinical studies using SARS-CoV-2 infected postmortem brain are needed for a conclusive claim, our data highlight a molecular mechanism underlying the endothelial dysfunction in COVID-19.

The ACE2 receptor mediates cellular entry of SARS-CoV-2. In our previous study, we found ACE2 expression in endothelial cells, which increased CVD with high-risk factors for COVID-19 infection, such as diabetes and smoking [16]. Notably, the expression of ACE2 in endothelial cells and perivascular pericytes in the brain suggests a brain-infecting potential of SARS-CoV-2. A clinical study showed that SARS-CoV-2-infected endothelium is observed in the postmortem organs of patients with COVID-19 [10], which might be responsible for endothelial dysfunction. SARS-CoV-2 can directly infect human blood vessel organoids in vitro [17]. Recently, increasing evidence has suggested that SARS-CoV-2 impairs brain vessels [18] that may be linked to CVD [19].

Encephalitis, ischemia, hemorrhage, microstructural and functional changes, and CVD have been observed in up to 36% of COVID-19 patients [20,21]. Recent clinical cohort data showed a risk of incident cardiovascular problems, including blood clots, abnormal heart rhythms, and stroke 1 year after SARS-CoV-2 infection [22]. The SARS-CoV-2 antigens, including the S1 subunit of the spike, full-length spike, and nucleocapsid are present in 65% of patients with long covid [2]. It is becoming increasingly clear that SARS-CoV-2-induced cardiovascular impairment and neurological disorders are not restricted to individuals with severe COVID. Patients with mild disease due to SARS-CoV-2 infection appear to be at a higher risk of cardiovascular disease and neurological disorders.

It remains unknown why some people continue to be at an increased risk of cardiovascular diseases and neurological disorders after SARS-CoV-2 infection. Many studies have attempted to understand the mechanisms underlying heart diseases or neurological disorders in COVID-19 patients. Recent studies suggest that the spike regions of SARS-CoV-2 inflict various damages after COVID-19

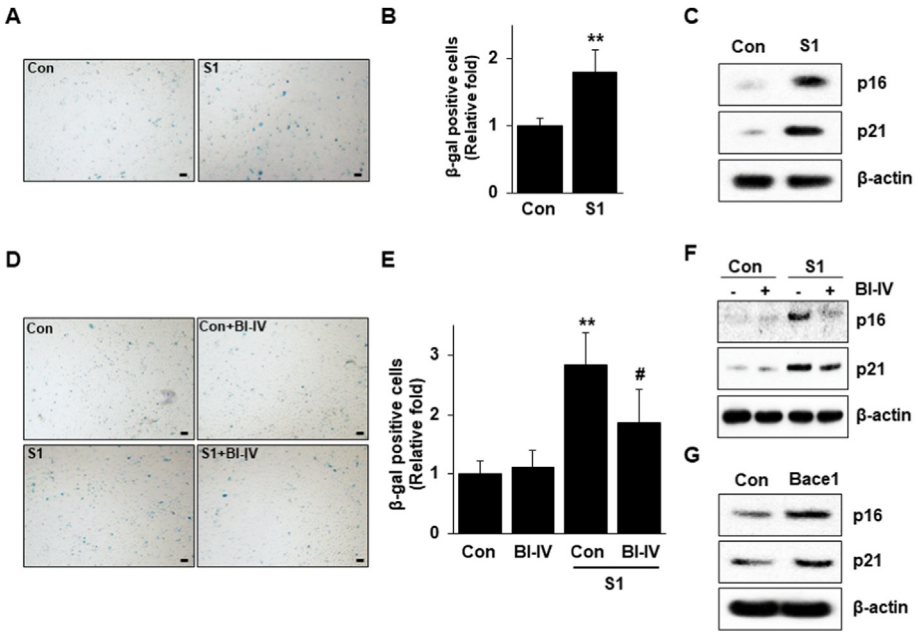

Fig. 4. BACE1 mediated SARS-CoV-2 spike S1 subunit protein-induced endothelial cell senescence. (A) Senescence in HBMVECs following SARS-CoV-2 spike S1 subunit protein (S1) was detected by SA-β-gal staining (scale bar = 200 μm). (B) The graph represents the fold-change of SA-β-gal-positive cell numbers (**p < 0.01 versus Con). (C) The protein level of p16 and p21 was observed by immunoblotting as described in Section 2.3. (D) The SA-β-gal staining (scale bar = 200 μm). HBMVECs were pretreated with 2.5 μM of BI-IV for 24 h before treatment with SARS-CoV-2 spike S1 subunit protein (S1). (E) The graph represents fold-change SA-β-gal-positive cell numbers (**p < 0.01, versus Con; #p < 0.05, versus S1). (F) p16 and p21 protein levels. Results were normalized to β-actin and expressed relative to p16 and p21 protein levels from normal control (Con), respectively. (G) HUVECs were transiently transfected with either control (Con) or myc-tagged BACE1. Data are presented as the mean ± S.D. in three independent experiments.

infection [23]. Accumulating evidence supports that the SARS-CoV-2 spike S1 subunit protein induces endothelial dysfunction in the brain through endothelial injury [24,25] or cellular senescence [6], which could initiate neurological disorders including CVD. For example, the spike protein of SARS-CoV-2 administration in healthy mouse cerebral arteries leads to the loss of junctional proteins including VE-cadherin, PECAM-1, JAM-A, and Connexin-43 [25]. The loss of endothelium integrity in several organs was observed postmortem in COVID-19 [10]. Rodent and endothelial cells exposed to the SARS-CoV-2 spike protein exhibited aging-related pathogenic molecules such as p16, p21, and SA-β-gal, suggesting the induction of cellular senescence [26,27]. Corroborating the role of cellular senescence in COVID19 pathology, a recent study demonstrated that cognitive impairment due to long COVID is equivalent to aging by 20 years [4]. Based on these results, we suggest that CVD development in COVID-19 patients and stroke post-COVID-19 might be closely related to endothelial dysfunction. Consistent with these observations, we showed that the SARS-CoV-2 spike S1 subunit protein collapsed the integrity of tight junctions in primary human brain vessel cells, causing vascular leakage, and also induced cellular senescence. We found that the effect of the SARS-CoV-2 spike S1 subunit protein on brain vessels is regulated by BACE1. These results implicate the crucial role of BACE1 in endothelial dysfunction in critical COVID-19 infections and its therapeutic implications.

It has been reported that endothelial BACE1, which is increased in the brain vessels of patients with CAA linked to hemorrhage, degrades occluding [12]. Our data show that endothelial BACE1 overexpression reduced the expression levels of claudin-1,-5, ZO-1, and occludin. In addition, for the first time, we found that BACE1-overexpressing brain vessel cells have senescent phenotypes, including the expression of β-gal, p16, and p21, suggesting that BACE1 is a novel mediator of SASP in brain microvessels. Although BACE1 is not known to be directly associated with SARS-CoV-2 infection or replication, it is noteworthy that the SARS-CoV-2 spike S1 subunit protein upregulates BACE1 expression, weakening brain vessels through the loss of junction proteins and aging via a general mechanism.

In conclusion, we demonstrated that the SARS-CoV-2 spike S1 subunit protein induces endothelial dysfunction, mediated by BACE1. We found that BACE1 acts as the uppermost signaling molecule in the loss of endothelial integrity and triggers senescence by SARS-CoV-2 spike S1 subunit protein; therefore, BACE1 inhibitors may protect against post-COVID symptoms such as cardiovascular problems and CVD. Finally, although the in vivo relevance of our data needs to be verified using animal and human populations, caution should be warranted in COVID-19 patients and such a line of therapy may be considered in the future.

Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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