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Altered COVID-19 receptor ACE2 expression in a higher risk group for cerebrovascular disease and ischemic stroke

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ABSTRACT

Coronavirus disease 2019 (COVID-19) is a worldwide pandemic. It has a high transmission rate among humans, and is a threat to global public health. However, there are no effective prophylactics or therapeutics available. It is necessary to identify vulnerable and susceptible groups for adequate protection and care against this disease. Recent studies have reported that COVID-19 has angiotensin-converting enzyme 2 (ACE2) as a functional receptor, which may lead to the development of severe cerebrovascular diseases (CVD), including strokes, in patients with risk factors for CVD such as diabetes and smoking. Thus, the World Health Organization (WHO) advised caution against COVID-19 for smokers and patients with underlying clinical symptoms, including cardiovascular diseases. Here, we observed ACE2 expression in the brain of rat middle cerebral artery occlusion (MCAO) model and evaluated the effects of cigarette smoke extract (CSE) and diabetes on ACE2 expression in vessels. We showed that the levels of ACE2 expression was increased in the cortex penumbra after ischemic injuries. CSE treatment significantly elevated ACE2 expression in human brain vessels. We found that ACE2 expression was upregulated in primary cultured human blood vessels with diabetes compared to healthy controls. This study demonstrates that ACE2 expression is increased in ischemic brains and vessels exposed to diabetes or smoking, makes them vulnerable to COVID-19 infection.

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1. Introduction

The *Coronaviridae* family has several members, which continuously circulate among humans and lead to mild respiratory diseases [1]. In contrast, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV) cause severe respiratory diseases. SARS-CoV was first reported in Guang-dong, China, in 2002–2003. MERS-CoV was reported in Saudi Arabia in June 2012. In December 2019, a novel SARS-CoV emerged in Wuhan, China, from patients with pneumonia, which was identified as a SARS pathogen. This virus was denoted severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), while the disease is denoted COVID-19. The typical symptoms of COVID-19 at illness onset are fever, dry cough, myalgia, and dyspnea [2]. Some patients might suffer from headaches, dizziness,

diarrhea, nausea, and vomiting. However, people with underlying diseases such as hypertension, diabetes, and cardiovascular disease may develop severe neurological disorders, including acute cerebrovascular disease [3–5]. Based on these clinical data, the WHO advised caution against COVID-19 infection among smokers and patients with underlying clinical symptoms, including cardiovascular disease [6].

Zhou et al. reported that SARS-CoV-2 shares the same receptor, ACE2, with SARS-CoV [7]. However, it does not use another coronavirus receptor, dipeptidyl peptidase 4 (DPP4), whereas MERS-CoV does [8]. Increasing evidence supports the idea that the S-protein of SARS-CoV-2 binds to ACE2. These studies suggest that the cellular entry of SARS-CoV-2 is mediated through ACE2. Since SARS-CoV-2 infection causes severe lung injury, the SARS-CoV-2 virus may use ACE2 expressed by pneumocytes in the epithelial alveolar lining to infect subjects. However, increasing clinical studies have shown that SARS-CoV-2 is not only observed in organs with endothelial dysfunction [9] but also in the postmortem brain [10]. Since cells that express ACE2 are potentially at risk for SARS-

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CoV-2 infection, ACE2 expression profiling under various conditions in the brain can help understand the process of COVID-19 and cardiovascular complications, including neurological diseases.

Among patients with COVID-19, new-onset CVD increases in individuals who have risk factors, including smoking and diabetes. The Chinese Center for Disease Control and Prevention reported that COVID-19 patients with diabetes had higher mortality [11]. In South Korea, the KCDC reported that as of April 30, 247 deaths occurred, of which 244 are deaths with underlying disease. Among them, the mortality rate of COVID-19 patients with the underlying disease with a metabolic disease or cardiovascular diseases, such as diabetes, stroke, and hypertension is high [12]. Clinical data characterizing patients with COVID-19 give evidence that CVD risk factors, including smoking and diabetes, are likely associated with negative progression and adverse outcomes of COVID-19 [13]. Recently, a high level of ACE2 has been observed in the brains of smokers [14]. Hence, we consider that smoking and diabetes might increase the ability of SARS-CoV-2 to enter and infect the brain based on the high expression of ACE2.

In the present study, we investigated the alteration of ACE2 expression in the brains of ischemic stroke, as well as the effect of CVD risk factors, including CSE and diabetes, on ACE2 expression. We showed that ACE2 expression was altered in the cortex penumbra of ischemic injuries. In addition, ACE2 expression was highly increased in brain microvessels exposed to CSE, and in endothelial cells derived from patients with diabetes.

2. Materials and methods

2.1. Reagents

The anti-ACE2 antibody (NBP2-90854) was purchased from Novus (Littleton, CO, USA). Heparin, dimethyl sulfoxide (DMSO), bicinchoninic acid, and all chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Diabetes mice

C57BL/KsJ *db/db* male mice were used as type 2 diabetes mellitus model mice, while C57BL/KsJ male *m⁺/db* mice were used as control mice. The mice were obtained from SLC (Hamamatsu, Japan). All animal experimental procedures were conducted according to the institutional guidelines for the care, and the use of laboratory animals, and permission was granted by the KCDC-Institutional Animal Care and Use Committee (KCDC-IACUC; Approval Number KCDC-148-14-1A). The mice were 5 weeks old; they were housed in a temperature- and humidity-controlled environment under a 12 h light/dark cycle, and allowed free access to water and standard mouse chow. After 6 weeks, 11-week-old male C57BL/KsJ *db/db* and *m⁺/db* mice were sacrificed. After the mice were anesthetized, each tissue was weighed, immediately snap-frozen with liquid nitrogen, and stored at -80°C before analysis.

2.3. Cell culture

Primary human brain microvascular endothelial cells (HBMVECs) were obtained from Cell Systems (Kirkland, WA, USA; Table 1) and maintained in CSC complete medium with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), and 1% penicillin-streptomycin (Gibco) and CultureBoost (Cell Systems). All primary HBMVEC cultures were used between passages 4 and 9. Cells were grown on attachment factor-coated plates in CSC complete serum-free medium (Cell Systems), or M199 medium supplemented with 20% FBS, 3 ng/mL recombinant human fibroblast growth factor-

basic (FGF-b; Millipore, Temecula, CA, USA), 5 U/mL heparin, and 1% penicillin-streptomycin in a humidified atmosphere of 5% CO_2 at 37°C .

Healthy and diseased human aortic endothelial cells (HAECs) with type I and II diabetes were obtained from Lonza (Walkersville, MD, USA; Table 1). Cells were grown on attachment factor-coated plates in cell growth medium (EGM-2, Lonza), with full supplements (EGM-2 bullet kit: 2% FBS, 0.4% hFGF-2, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% hEGF, 0.04% hydrocortisone, 0.1% ascorbic acid, 0.1% heparin, and 0.1% GA-100), in a humidified atmosphere of 5% CO_2 at 37°C . The C6 astrocyte cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco), supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were maintained at 37°C in a humidified atmosphere with 95% air and 5% CO_2 .

2.4. Preparation of cigarette smoke extracts (CSE)

CSE was generated as described previously [15]. Total particulate matter (TPM) was collected on Cambridge filter pads. The final concentration of 10 mg/mL TPM was extracted with DMSO by shaking for 20 min. The solution was then filtered and stored at -80°C .

2.5. Surgical procedure used for MCAO

Male Sprague-Dawley rats were housed under diurnal lighting conditions and allowed food and tap water *ad libitum*. All animal studies were carried out in strict accordance with the recommendations made in the Guide for the Care and Use of Laboratory Animals, published by the National Institute of Health (2013), and ARRIVE guidelines (<http://www.nc3rs.org/ARRIVE>). The animal protocol used was reviewed and approved by the KCDC-Institutional Animal Care and Use Committee (Approval Number KCDC-144-19-2A). Eight-week-old male Sprague-Dawley rats (250–300g) were subjected to MCAO. Rats were anesthetized with a ketamine (50 mg/kg) and xylazine (6 mg/kg of body weight) mixture during surgery. Occlusion of the right middle carotid artery was induced for 1h by advancing a nylon suture (4-0; AILEE, Busan, Korea) with a heat-induced bulb at its tip (~ 0.3 mm in diameter) along the internal carotid artery, for 20–22 mm, from its bifurcation with the external carotid artery. This was followed by reperfusion. A thermoregulated heating pad and a heating lamp were used to maintain a rectal temperature of $37 \pm 0.5^{\circ}\text{C}$ during surgery. Animals were randomly allocated to sham and MCAO groups, as follows: 1) the sham control group: animals underwent surgery but were not subjected to MCAO ($n = 5$); 2) MCAO groups: animals were subjected to MCAO and sacrificed at 1, 4, 7, 14, and 21 days post-MCAO ($n = 22$).

2.6. Total RNA-Seq

MCAO group animals were sacrificed at 4, 7, 14, and 21 days post-MCAO, and brain tissues were immediately extracted. Brain tissues containing cortex penumbra from two animals were used per sample. Total RNA was purified using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The total mRNA-seq was commercially commissioned to EBIOGEN (EBIOGEN, Seoul, South Korea). In brief, the data were generated by HiSeq X10 through NGS, in 4G, read as paired-end. After sequencing, BBDuk was run to obtain read data in a fastq format. Then, TopHat was used to map reads against the rn6 reference transcript and genome. We counted each sample's reads mapped to each gene in rn6, and analyzed the results of the differential expression of genes through ExDEGA (EBIOGEN, Seoul, South Korea).

Table 1

The list of primary human endothelial cells.

Primary Cell types	Disease	Company	Catalog number
Human brain microvascular endothelial cells	Normal	Cell Systems	ACBRI 376
Human aortic endothelial cells	Normal	Lonza	CC-2535
Human aortic endothelial cells	Diabetes type I	Lonza	CC-2919
Human aortic endothelial cells	Diabetes type II	Lonza	CC-2920

2.7. Immunoblotting

Cells in plates were washed with phosphate-buffered saline (PBS) and lysed in radioimmunoprecipitation (RIPA) buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na₃VO₄, and 100 µg/mL of leupeptin). The cell lysate was collected by centrifugation (15,000 rpm, 15 min, 4 °C), and the protein concentrations were determined using the bicinchoninic acid method. Equivalent amounts of protein were separated on NuPAGE (4%–12%; Invitrogen, Carlsbad, CA, USA) gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% skim milk in Tris-buffered saline (TBS) with 0.1% tween-20, and incubated with the primary antibody overnight at 4 °C. Immune complexes were then detected using an enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

2.8. Statistical analysis

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

3. Result

3.1. Upregulation of the *Ace2* gene in the ischemic brain

To analyze the gene expression pattern after cerebral ischemic damage, we performed total mRNA-seq in the cortical penumbra of a rat transient MCAO model (Fig. 1A). Of the 17,048 mapped genes in the cortical penumbra of the MCAO groups (4, 7, 14, 21 days post MCAO) or sham control, we identified 11,078 differentially expressed genes (DEGs, fold change 1.5) between the sham control and each MCAO group, including 5,568 up-, 4,537 down-, and 975 contra-regulated genes (Fig. 1B). Comparing each gene of the sham control to the MCAO groups (7 or 21 days post MCAO), the gene expressions of *Ace2* or *Dpp4* in the cerebral ischemic brain were upregulated after the MCAO (Fig. 1C).

The heat map of cluster analysis of DEGs, representing virus receptor activity, showed that *Ace2* gene expression gradually increased after 4 days of ischemic injury, and this increase peaked in the post-stroke subacute phase (7 days; 1.8 folds). The upregulation of *Ace2* was observed after 21 days. The expression level of the *Dpp4* gene was already increased 4 days after ischemic injury, and was maintained for 14 days (1.6 fold). However, increased *Dpp4* levels were decreased to the basal level (1.2 fold) of sham controls 21 days after the MCAO.

The alteration of *Ace2* mRNA expression by ischemic injury was validated by an immunoblotting analysis. As shown in Fig. 2C, the increase in ACE2 protein levels began 7 days after the ischemic injury, and the maximum increase was observed at 21 days.

3.2. Effect of CSE, one of CVD risk factors, on ACE2 expression

Our previous study found that CSE induces brain inflammation

[15]. In the brain, ACE2 is mainly expressed in microvessels and astrocytes [16]. Since a recent study showed high expression of ACE2 in smoker groups, we investigated the effect of CSE on the expression of ACE2 in human brain microvessels and astrocytes. Cells were treated with CSE for the indicated times (0, 3, and 6 h). As shown in Fig. 3A, ACE2 protein expression was significantly increased by CSE in HBMVEC. This increase was also observed in astrocytes treated with CSE (Fig. 3B). Furthermore, CSE elevated the level of ICAM1, a risk factor for blood-brain barrier (BBB) disruption in HBMVEC.

3.3. Effect of diabetes on ACE2 expression

Blood vessel damage has been found in patients with type I and II diabetes [17]. Here, we wondered whether ACE2 expression is altered in blood vessels of patients with diabetes. We compared the ACE2 levels between the endothelial cells of healthy humans, and subjects with type I and II diabetes. As shown in Fig. 4, we found that ACE2 protein levels were slightly but significantly increased in both endothelial cells of patients with type I or II diabetes compared with those of healthy controls. Using type II diabetes (*db/db*) mice, we confirmed that ACE2 expression was increased in the brains of mice with type II diabetes.

4. Discussion

Our data demonstrate that ACE2, a functional receptor of SARS-CoV-2, was upregulated in the brain of CVD, and its risk factors were diabetes and cigarette smoke. Although detailed clinical studies using large populations are needed before making a conclusive claim, diabetes patients, stroke patients, and smokers should be identified as groups that are susceptible to SARS-CoV-2 infection, in order to provide them adequate protection and care.

ACE2 is expressed in all tissues, including the lungs, arteries, heart, kidneys, intestines, and brain. It regulates blood pressure through the hydrolysis of angiotensin II (vasoconstrictor) into angiotensin1-7 (vasodilator). Excessive angiotensin II activates diverse cellular signaling pathways related to inflammation, free radical generation, and recruitment of inflammatory cells. Thus, the deficiency of ACE2 causes an increase in angiotensin, and leads to organ injury through aberrant production of reactive oxidative stress and inflammation [18]. ACE2 deficiency has been associated with exacerbation of hypertension and cardiac hypertrophy induced by angiotensin II [19]. Several studies have shown that the loss of ACE2 is involved in the progression of strokes, Alzheimer's disease (AD) [16], and hypertension [20]. Conversely, the overexpression of ACE2 blocks the pro-inflammatory responsiveness related to the pathogenesis of AD. Therefore, the deficiency of ACE2 in the progression of the disease may cause unregulated cytokine production.

The WHO suggests that smoking and diabetes are high-risk factors for COVID-19 infection, which led us to investigate ACE2 expression patterns in CVD in these high-risk groups. Our present data showed that ACE2 mRNA and protein levels gradually

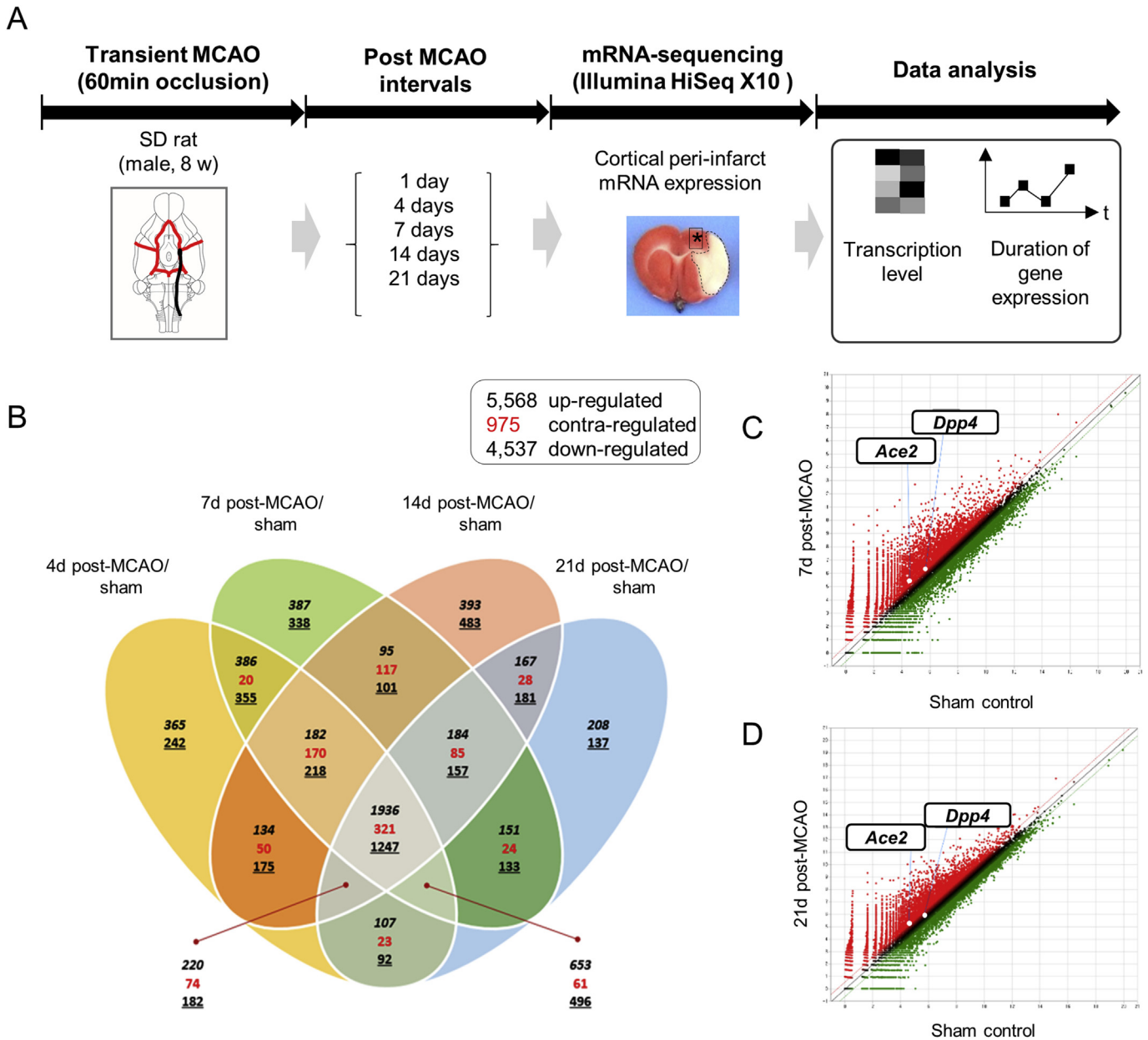


Fig. 1. Total mRNA seq analysis in the cerebral ischemic brain.

(A) Experimental scheme of total mRNA-seq in sham control or MCAO groups and subsequent data analysis. Brain tissues for mRNA-seq and immunoblotting were obtained from the cortex penumbra of the ischemic hemispheres (region indicated with *). (B) A Venn diagram shows the differentially expressed and overlapped DEGs among the four comparisons: 4 d-, 7 d-, 14 d-, or 21 d post MCAO/sham control. (C–D) A scatter plot shows the \log_2 values for each gene in the 7 or 21 days post MCAO groups (Y-axis) versus the sham control (X-axis). The receptors for COVID-19 (*Ace2*) or MERS-CoV (*Dpp4*) are marked in bold. Green indicates downregulated genes, red indicates up-regulated genes, and black indicates no difference between the expressions in sham control vs. MCAO groups.

increased during reoxygenation after a stroke, and the protein levels were significantly increased 21 days after ischemic injury, compared to the sham control (Fig. 2). In the study supporting our data, the ACE2/angiotensin1-7/mas receptor signaling axis is activated after an ischemic stroke in which ACE2 expression is increased [21], which may play a therapeutic role in vasodilatation and anti-inflammatory responses [22]. Administration of Ang 1–7, derived from ACE2, reduced improve neurological deficits following ischemic stroke through reducing oxidative stress and inflammation [23,24]. Thus, in our present data, increased ACE2 at the subacute and chronic stages after an ischemic injury is likely to

have beneficial effects beyond repairing injured cells and lowering blood pressure. In other words, it indicates that ACE2 levels may be elevated in the brains of stroke patients, and in individuals who have previously experienced a stroke. However, this induced ACE2 expression that follows ischemic injuries may lead to COVID-19 susceptibility. In an *in vivo* study, the regulation of ACE2 expression by SARS-CoV-mediated internalization was predicted to worsen lung failure [25]. Hence, stroke patients might need to take extra caution during the COVID-19 pandemic.

COVID-19 causes new-onset CVD in individuals with risk factors such as smoking and diabetes. However, to date, there is not

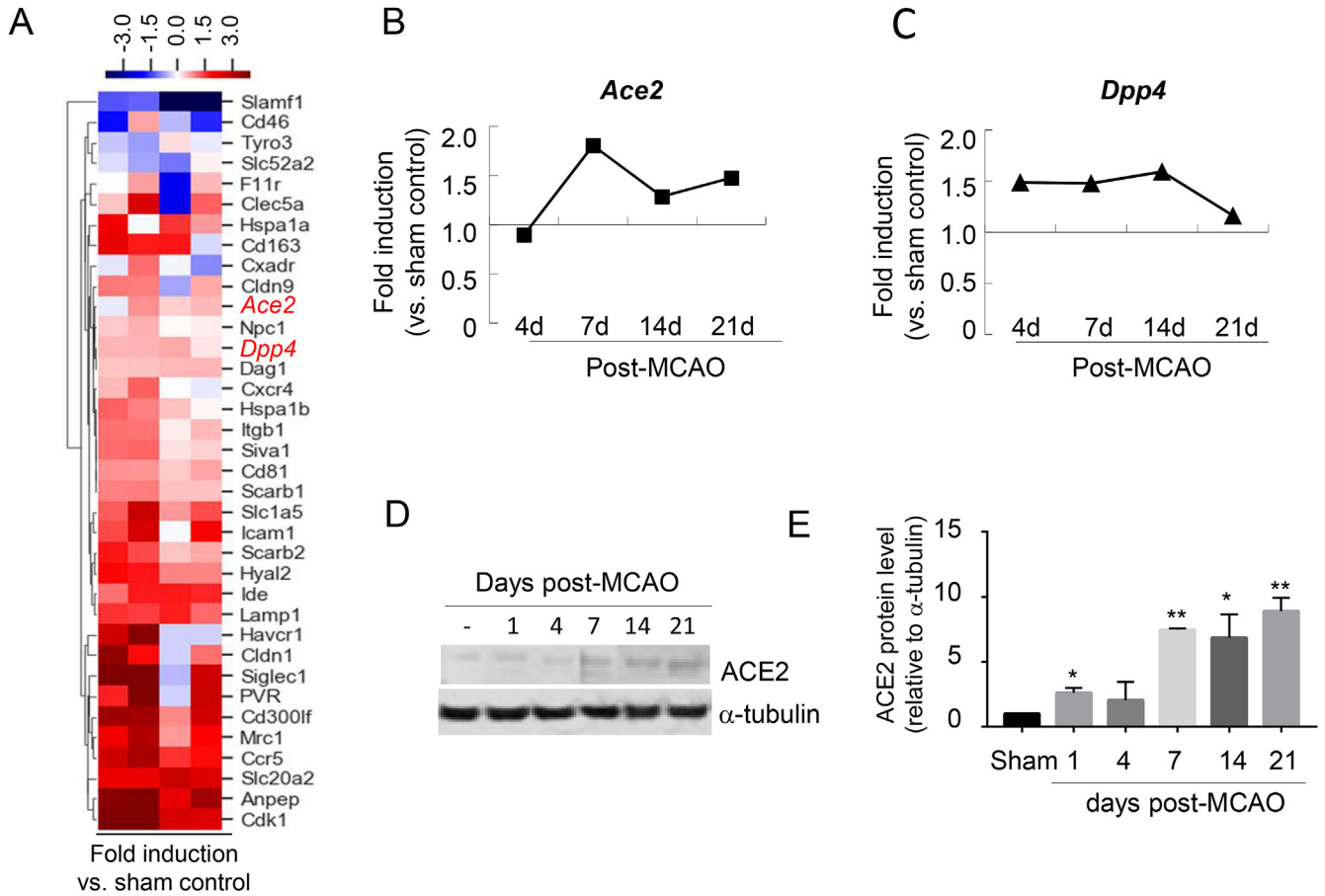


Fig. 2. Temporal changes of *Ace2* expression in the cortex penumbra after ischemic stroke. (A) A heat map of representative genes for virus receptor activity from the mRNA-seq results. Each column represented a fold induction of each gene under MCAO that was normalized to the sham control in the cortex penumbra at 4, 7, 14, and 21 days post-MCAO. *Ace2* and *Dpp4* are indicated by red letters. (B–C) The temporal gene expression changes of *Ace2* and *Dpp4* were obtained by RNA-seq at the indicated time points and normalized to the sham control. (D–E) Changes in *ACE2* protein levels were examined by immunoblotting at 1, 4, 7, 14, and 21 days after MCAO. α -Tubulin was used as a control. Tissue lysates were prepared from asterisked regions (Fig. 1A). The results are representative of three independent experiments. $**p < 0.01$, $*p < 0.05$ vs. the sham control.

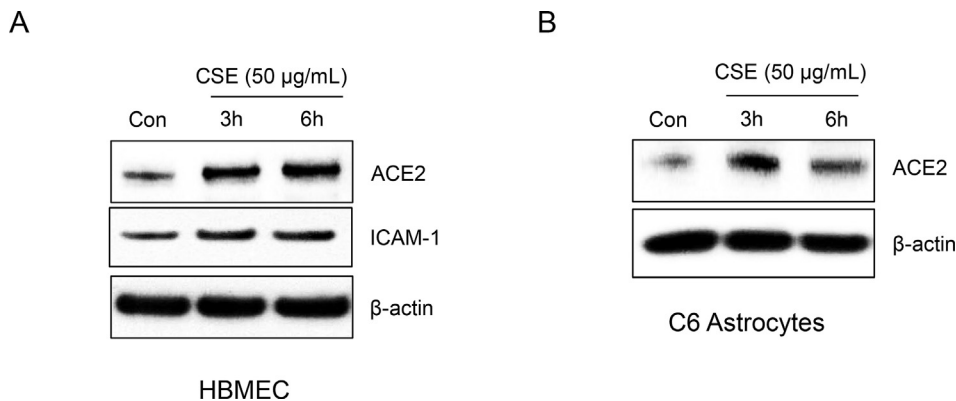


Fig. 3. Expression changes of *ACE2* in endothelial cells and astrocytes after CSE exposure. (A) HBMECs were exposed to 50 μ g/mL of CSE for the indicated time (3 and 6 h) and lysed in RIPA buffer. Lysates were probed with antibodies against *ACE2* and *ICAM-1*. β -actin was used as a loading control for the lysates. (B) C6 cells were exposed to 50 μ g/mL of CSE, lysed in RIPA buffer, and the lysates were probed with antibodies against *ACE2*. β -actin was used as a loading control for the lysates.

enough evidence to know how COVID-19 evokes CVD, including strokes. Recent reports suggest a possibility that COVID-19 triggers inflammation involved in the pathological progression of CVD, such as clot formation in the blood [26]. Blood clotting is formed by

cellular components such as histones [27], HMGB1 [28], micro-particles [2], and secreted granule proteins [29], which can be degraded by pro-fibrinolytic agents released from the endothelial cells [30]. Thus, in the surface of endothelial dysfunctions, cellular

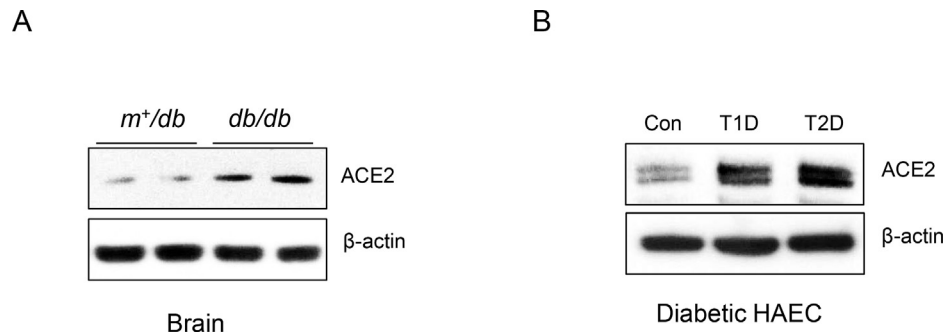


Fig. 4. The alteration of ACE2 expression in diabetes.

(A) Immunoblot analysis of ACE2 expression in brain samples of *db/db* and *m⁺/db* mice cortices. The lysates were probed against ACE2. β-actin was used as a loading control for the lysates. (B) Comparison of ACE2 expression in control and type I and II diabetic HAECs. Cells were lysed in RIPA buffer. The lysates were probed against ACE2. β-actin was used as a loading control for the lysates.

and protein materials congregate, and produce a blood clot as a consequence [30]. In particular, regarding the disease, an increase of blood clots and BBB disruption have been observed in patients with type II diabetes [31]. Our present data showed an increase in vascular ACE2 levels in T1D and T2D patients compared to healthy controls (Fig. 4). These results support the possibility that cellular entry of SARS-CoV-2 through ACE2 onto vessels makes the vessels weaker than they were before the onset of diabetes, and may disrupt the blood supply to the brain via clot blocking. However, the exact mechanism underlying COVID-19-induced CVD is required.

Like diabetes, smoking is known as a risk factor for CVD as well as lung diseases, including chronic obstructive pulmonary disease (COPD). The deleterious effects of CSE on the BBB are known to upregulate inflammation-related genes, including ICAM1 and VCAM1. In blood vessels, the increase of ICAM1 and VCAM1 in response to pro-inflammatory cytokines plays a crucial role in the adhesion of leukocytes, including neutrophils and macrophages, resulting in BBB disruption and brain inflammation. However, ACE2 expression with cigarette smoking is controversial. A recent clinical study showed that ACE2 expression is increased in the airways of smokers, which correlates with the expression of the $\alpha 7$ subtype of nicotine acetylcholine receptors [26]. In contrast, nicotine, a component of cigarette smoking, reduced ACE2 expression in primary neurons and glial cells of CVD [32]. Nonetheless, in this study, we clearly showed upregulation of ACE2 following CSE in brain endothelial cells and astrocytes (Fig. 3). Under the same conditions, CSE increased ICAM1 protein levels in primary human brain vessels (Fig. 3). Furthermore, our previous study provided evidence that CSE induces brain inflammation via astrocyte reactivation [15]. Taken together, these results suggest that a weaker vascular system among COVID-19 patients with a history of smoking makes them more vulnerable to severe symptoms.

In this study, we demonstrated that ACE2 expression was increased in the brain after a stroke. In the mouse brain or human blood vessels with diabetes, ACE2 expression was upregulated. We showed the *in vitro* effect of CSE on ACE2 expression in blood vessels. Finally, although the *in vivo* relevance of our data needs to be verified using a human population, caution may be warranted against COVID-19 among individuals with underlying diseases, such as strokes, diabetes, and smoking.

Declaration of competing 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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