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Short Communication

Hypoxia alters the expression of ACE2 and TMPRSS2 SARS-CoV-2 cell entry mediators in hCMEC/D3 brain endothelial cells

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ARTICLE INFO

Keywords:

Hypoxia
Human cerebral microvascular endothelial cells (hCMEC/D3)
Covid19
SARS-CoV-2
Angiotensin-converting enzyme 2 (ACE2)
Transmembrane protease serine 2 (TMPRSS2)
Blood-brain barrier (BBB)

ABSTRACT

The mechanisms by which the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) induces neurological complications remain to be elucidated. We aimed to identify possible effects of hypoxia on the expression of SARS-CoV-2 cell entry mediators, angiotensin-converting enzyme 2 (ACE2) receptor and transmembrane protease serine 2 (TMPRSS2) protein, in human brain endothelial cells, *in vitro*. hCMEC/D3 cells were exposed to different oxygen tensions: 20% (Control group), 8% or 2% O₂ (Hypoxia groups). Cells were harvested 6-, 24- and 48 h following hypoxic challenge for assessment of mRNA and protein, using qPCR and Western Blot. The response of the brain endothelial cells to hypoxia was replicated using modular incubator chambers. We observed an acute increase (6 h, $p < 0.05$), followed by a longer-term decrease (48 h, $p < 0.05$) in ACE2 mRNA and protein expression, accompanied by reduced expression of TMPRSS2 protein levels (48 h, $p < 0.05$) under the more severe hypoxic condition (2% O₂). No changes in levels of von Willebrand Factor (vWF – an endothelial cell damage marker) or interleukin 6 (IL-6 – a pro-inflammatory cytokine) mRNA were observed. We conclude that hypoxia regulates brain endothelial cell ACE2 and TMPRSS2 expression *in vitro*, which may indicate human brain endothelial susceptibility to SARS-CoV-2 infection and subsequent brain sequelae.

1. Introduction

The coronavirus disease 2019 (Covid-19), caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China, in December 2019 (Huang et al., 2020) as a primary respiratory disease. Since then, however, several reports have shown neurological manifestations associated with Covid-19, including meningoencephalitis, encephalopathy, and ischaemic stroke (Beyroufi et al., 2020; Chen et al., 2020; Mao et al., 2020; Moriguchi et al., 2020).

The routes of infection and the mechanisms underlying SARS-CoV-2-induced central nervous system (CNS) damage are not fully understood. Viral infections can impact the brain both directly (by infecting neurons) or indirectly (by inducing a hyper-inflammatory state/cytokine storm), both of which can induce brain disease and lead to relatively long-lasting sequelae, as observed in Covid-19 patients (Paterson et al., 2020). Emerging evidence linking SARS-CoV-2 infection to coagulopathies and vascular damage has directed interest towards the brain

endothelium (Paterson et al., 2020; Tang et al., 2020).

The angiotensin-converting enzyme 2 (ACE2) receptor and transmembrane protease serine 2 (TMPRSS2) are postulated to be the two main facilitators of SARS-CoV-2 penetration within host cells: ACE2 binds to the coronavirus spike (S) glycoproteins, while TMPRSS2 induces the proteolytic cleavage of both ACE2 and S, resulting in viral uptake (Heurich et al., 2014; Hoffmann et al., 2020). ACE2 and TMPRSS2 are expressed by brain endothelial cells (BECs) (Baig et al., 2020; Desforgues et al., 2019; Hamming et al., 2004). Considering that systemic hypoxia is common in cases of Covid-19 clinical reports and that oxygen tension is an established regulator of ACE2 and other components of the renin-angiotensin-aldosterone system in some cells and somatic tissues (Ito et al., 2002; Joshi et al., 2019; Li et al., 2017; Mao et al., 2010), we sought to elucidate the *in vitro* effects of moderate (8% O₂) and severe (2% O₂) hypoxic conditions on ACE2 and TMPRSS2 in the brain endothelium. This information will contribute to our understanding of how SARS-CoV-2 might penetrate the brain, as well as, a

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Received 22 October 2020; Received in revised form 30 May 2021; Accepted 1 August 2021

Available online 18 August 2021

0026-2862/© 2021 Published by Elsevier Inc.

number of the endothelial pathologies that have recently been associated with Covid-19.

2. Material and methods

2.1. Cell culture and reagents

The human cerebral microvascular endothelial cell line (hCMEC/D3, Cedarlane Labs #CLU512, Burlington, Canada) was cultured at 37 °C, in 5% CO₂ and 20% O₂ with EndoGRO™-MV Complete Culture Media Kit® (Millipore, Canada, #SCME004). Human basic fibroblast growth factor (1 ng/mL; Sigma, #F0291) and 1% penicillin-streptomycin (10,000 units-10,000 µg/mL, Life Technologies, #15140-122) were supplemented to the media. All procedures, treatments, and analyses were performed with the cells at passage 30.

2.2. Experimental design

hCMEC/D3 cells were plated in clear flat bottom 6-well TC-treated polystyrene culture plates (Costar, Kennebunk, USA, #3516) pre-coated with type 1 collagen (Gibco, #A1048301) at a density of 25,000 cells/cm². 24 h after seeding, cells were challenged with different oxygen tensions: 20% (normoxia, the control group), 8%, or 2% O₂ (hypoxia groups). The plates and wells were randomly assigned to the different groups. Cells were collected for protein or mRNA analysis after 6-, 24- and 48-h of challenge. In order to confirm the brain endothelial cell response to hypoxia, we replicated the experiment in independent cultures, exposing hCMEC/D3 cells to 2% and 1% O₂ using modular incubator chambers (Billups-Rothenberg, Del Mar, CA, USA) for 6, 24 and 48 h. The different oxygen tensions did not affect cell viability (ranged from 100% to 86.49%) at any time point during the study (Supplementary Fig. 3).

2.3. mRNA analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen #74104, Toronto, Canada), following the manufacturer's instructions. Nano-Drop1000 Spectrophotometer (Thermo Scientific, Wilmington, USA) was used to determine the RNA purity/concentration, and total RNA (1 µg) was reverse transcribed to cDNA using iScript™ Reverse Transcription Supermix (Bio-Rad #1708840, Mississauga, Canada). SYBR Green (Sigma #S9194) was used to run the quantitative polymerase chain reaction (qPCR) using the CFX 380 Real-Time system C1000™ Thermal Cycler (Bio-Rad), with the following parameters: 1 cycle of 95 °C for 2 mins, and 40 cycles of 95 °C for 5 s and 60 °C for 20s. 40 ng or 10 ng of RNA was used per reaction depending on the gene of interest, and samples were run in triplicate. In some cases, not all replicates amplified, generating some variation in the number of samples per group. The specific number of samples used in each group per target is displayed under each column in the graphs. Geometric mean of beta actin (*ACTB*) and DNA topoisomerase type 1 (*TOP1*) was used to normalize the expression of the target genes. Table 1 shows the sequences and origin of the primers used in this study. The 2^{-ΔΔCT} Method

Table 1
Sequence of primers used to perform qPCR analysis in this study.

| Target | Primer | | Reference |
|----------------|--------------------------|---------------------------|---|
| | Forward | Reverse | |
| <i>ACE2</i> | GGAGTGATAGTGGTTGGCATTGTC | GCTAATATCGATGGAGGCATAAGGA | Bloise et al., 2021 Lye et al., 2021 |
| <i>ACTB</i> | CTGGAACGGTGAAGGTGACA | AAGGGACTTCTCTAACAATGCA | Weber et al., 2014 |
| <i>IL-6</i> | TCAATGAGGAGACTTGCCTG | TGGCTTGTTCCTCACTACTCT | Imperio et al., 2021 |
| <i>VEGF</i> | CGGGCCTCCGAAACCATGAACCT | CCCTCTCTCTTCTGCCATGGGT | Lye et al., 2013 |
| <i>vWF</i> | GCAGTGAGAAACAGTGGTG | GTGGCAGCGGGCAAAC | Starke et al., 2011 |
| <i>TMPRSS2</i> | AGCTGCAGAAGCCTCTGACTTTC | AGCGTTCCAGCACTTCTGAGGTC | Bloise et al., 2021 |
| <i>TOP1</i> | GATGAACCTGAAGATGATGGC | TCAGCATCATCTCATCTCG | Lye et al., 2013 |

(Livak and Schmittgen, 2001) was used to calculate the relative mRNA expression, and the control group was normalized to 1.

2.4. Protein analysis

Cells were collected in lysis buffer (1 mol/L Tris-HCL pH 6.8, 10% SDS, and 10% glycerol) with protease and phosphatase inhibitor (Thermo Scientific, Mississauga, Canada, #78440), and the protein was extracted by sonication. Protein concentration was determined using the Pierce BCA Protein assay kit (Thermo Scientific). Total protein (20 µg) was boiled (5 min) and loaded on 8% SDS polyacrylamide gels for electrophoretic separation (100 V, 1 h). Proteins were transferred from gels to polyvinylidene difluoride membranes (Bio-Rad, #1704157) using the Bio-Rad Trans-Blot® Turbo™ Transfer System. Membranes were blocked for 1 h with 5% skim milk in Tris-Buffered Saline containing 0.1% Tween (TBS-T), followed by overnight incubation at 4 °C with primary antibodies anti-ACE2 (Abcam #ab15348, 1:1000), anti-TMPRSS2 (Abcam #ab92323, 1:1000) or anti-beta actin (Sigma, #2066, 1:20000) as the loading control. Membranes were then washed (3×) with TBS-T and incubated (1 h) with HRP-linked anti-rabbit secondary antibody (1:10000; GE Healthcare Bio-Science, Baie d'Urfe, Canada). Chemiluminescence was assessed using the SuperSignal™ West Femto (Thermo Scientific, #34095, for ACE2 and TMPRSS2) or the Luminata™ Crescendo Western HRP Substrate (Millipore, #WBLUR0100, for beta actin) for 5 min, and detected under UV using a ChemiDoc™ MP Imaging system (Bio-Rad). ACE2 and TMPRSS2 protein bands were quantified by densitometric analysis using the Image Lab™ software and normalized against beta-actin signal for total protein assessment.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism (Inc., San Diego, USA) software version 7. Normal distribution was assessed using the Shapiro-Wilk test. Grubbs' test was used to exclude outliers. Gene and protein expression were analyzed using Two-way ANOVA, followed by Tukey's multiple comparisons test comparing between different oxygen tensions at each time point. The number of replicates (n) varied between 3 and 6 per group. Specific numbers are provided in the figures. Data are presented as mean ± standard error of the mean (S.E.M.). Differences were significant when p < 0.05.

3. Results

3.1. VEGF is upregulated in the human brain endothelium hypoxic model

In this study, we used the vascular endothelial growth factor (VEGF) to confirm the response of hCMEC/D3 cells to different levels of hypoxia (Javam et al., 2014; Lye et al., 2013). VEGF mRNA levels were upregulated under 8% hypoxia at 6 h (p < 0.05), and under 2% hypoxia at 6- (p < 0.001), 24- (p < 0.05) and 48 h (p < 0.001) (Fig. 1A). A subsequent study utilizing modular incubator chambers demonstrated a significant increase in VEGF mRNA expression in the 2% O₂ group (at 6, 24, and 48

h, $p < 0.01$ and $p < 0.05$) and 1% O₂ group (at 24 h, $p < 0.05$) compared to the control (C, 20% O₂) group (Supplementary Fig. 1).

3.2. Hypoxia induces time-dependent modulation of ACE2 in hCMEC/D3 cells

ACE2 mRNA expression was upregulated under 2% hypoxia at 6 h ($p < 0.05$), unchanged at 24 h, and downregulated under 2% hypoxia at 48 h ($p < 0.05$, Fig. 1B). However, TMPRSS2 mRNA levels did not exhibit changes under 8% or 2% hypoxia at any time point investigated (Fig. 1C). Using the modular incubator chambers, we found a similar response of hCMEC/D3 cells to hypoxia at 6 h, with increased ACE2 mRNA expression under 2% O₂ compared to control ($p < 0.01$, Supplementary Fig. 1). TMPRSS2 mRNA expression was unaltered under hypoxic conditions.

3.3. Hypoxia did not induce changes in von Willebrand Factor (vWF) or interleukin (IL)-6 mRNA expression in hCMEC/D3 cells

vWF and IL-6 were investigated as markers of endothelial dysfunction (Balta, 2020). No alterations at the mRNA level were found in vWF or IL-6 expression in hypoxic BECs in this study (Fig. 1D and E).

3.4. Hypoxia acutely enhances ACE2, and chronically reduces ACE2 and TMPRSS2 protein expression in hCMEC/D3 cells

ACE2 relative protein expression was increased under 2% hypoxia at 6 h ($p < 0.05$), and decreased under 2% hypoxia at 48 h ($p < 0.001$) (Fig. 2A and B). In contrast, TMPRSS2 expression was decreased under 2% hypoxia after 48 h ($p < 0.05$) of exposure (Fig. 2C and D). Using the modular incubator chambers, we found a trend towards an increased ACE2 expression in the 1% O₂ group ($p < 0.06$) at 6 h, and reduced expression of ACE2 in the 1% O₂ group ($p < 0.05$) at 48 h, compared to the control groups (Supplementary Fig. 2A and B). Interestingly, TMPRSS2 protein expression was also elevated acutely (6 h) after exposure to 2% ($p < 0.01$) and 1% ($p < 0.05$) O₂, compared to control.

No changes were observed in the 2% O₂ group, but decreased TMPRSS2 expression was observed in the 1% ($p < 0.001$) O₂ group at 48 h (Supplementary Fig. 2C and D).

4. Discussion

This study demonstrates, for the first time, that hypoxic conditions modulate SARS-Cov-2 cell entry-mediators ACE2 and TMPRSS2 in human BECs. An acute increase of ACE2 protein and mRNA expression following 6 h of severe hypoxic exposure (2% O₂) may indicate an increased BEC susceptibility to SARS-CoV-2 infection acutely, with this susceptibility decreasing over time. This indicates a time-dependent regulatory mechanism and is consistent with previous findings in lung epithelial cells (37).

TMPRSS2 mRNA expression was not changed in BECs under acute or longer-term (48 h) hypoxic conditions, contrasting with two reports of decreased TMPRSS2 mRNA expression under hypoxia in human prostate and breast cancer cell lines (Fernandez et al., 2015; Gkogkou et al., 2020). We observed high variability between same-sample replicates, which is characteristic of low expressed genes of interest. Therefore, cell-specific characteristics and response of TMPRSS2 under hypoxia may explain the divergent observations found in mRNA expression. At the protein level, however, we found TMPRSS2 at the expected molecular weight using a well-established antibody. *In vitro*, TMPRSS2 appears to be higher and more consistently expressed when the cells reach confluence (48 h). To our knowledge, this is the first demonstration of the effect of hypoxia on TMPRSS2 protein expression in any cell-type. We found decreased TMPRSS2 protein expression after 48 h of severe hypoxia in BECs. Little is known about the physiological function of TMPRSS2 in BECs, and our results highlight the need for further studies on TMPRSS2 participation in the cellular adaptations to hypoxic conditions, and its possible relevance for altered susceptibility to SARS-CoV-2 infection in hypoxic brain endothelium.

In addition to the numerous cases of stroke associated with SARS-CoV-2 infection (Avula et al., 2020; Hess et al., 2020), emerging evidence is suggesting that Covid-19 is predominantly a vascular

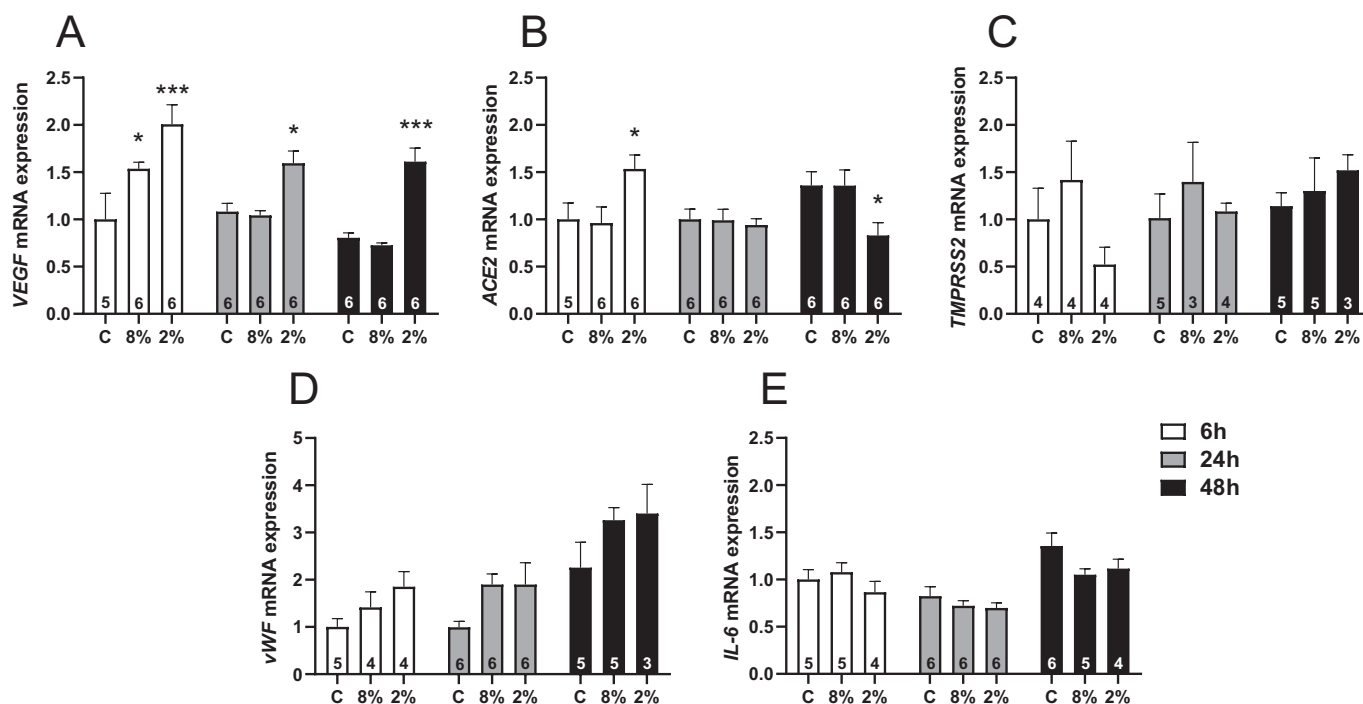


Fig. 1. VEGF (A), ACE2 (B), TMPRSS2 (C), vWF (D) and IL-6 (E) mRNA levels in hCMEC/D3 cells 6-, 24- or 48 h after exposure to 20% (Control), 8% or 2% oxygen, measured by qPCR. N = 3–6/group, specific n is presented under each column. Statistical analysis: two-way ANOVA followed by Tukey's multiple comparisons test. Mean \pm SEM. * $p < 0.05$ and *** $p < 0.001$.

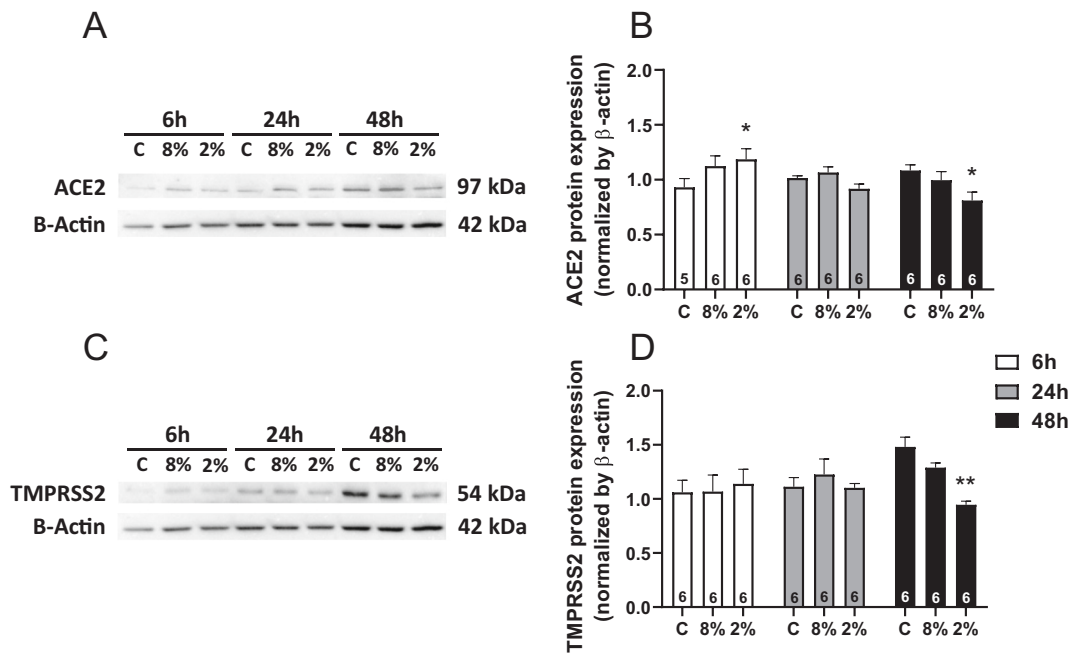


Fig. 2. ACE2 (A and B) and TMPRSS2 (C and D) protein levels in hCMEC/D3 cells 6-, 24- or 48 h after exposure to 20% (Control), 8% or 2% oxygen, measured by Western Blot. A and C show representative blots of each group, C and D present the results of densitometry. Beta actin was used as the loading control. N = 6/group. Statistical analysis: two-way ANOVA followed by Tukey's multiple comparisons test. Mean \pm SEM. * p < 0.05 and ** p < 0.01.

pathology/disease. This includes the occurrence of cerebral microbleeds (Paterson et al., 2020), the correlation between brain MRI hemorrhagic findings with clinical indicators of Covid-19 severity (Kremer et al., 2020), as well as the association of abnormal coagulation parameters combined with a prothrombotic state with poor outcome of Covid-19 patients (Tang et al., 2020). Thus, it is plausible to assume an involvement of endothelial dysfunction and vascular damage with Covid-19 infections. This suggestion has been further endorsed by a report identifying direct SARS-CoV-2 infection of endothelial cells accompanied by endothelial inflammation in the small intestine and lung (Varga et al., 2020).

It is imperative to understand how the brain could be affected in the context of altered BEC function. In this study, we investigated the expression of *vWF* and *IL-6* as indicators of potential endothelial dysfunction induced by hypoxia. *vWF* is produced and secreted by endothelial cells following disturbance or damage (Lip and Blann, 1997). It has been implicated in the development of coagulopathies and systemic inflammatory response (Plautz et al., 2020), and was found increased in severe Covid-19 patients (Escher et al., 2020). *IL-6*, a pro-inflammatory cytokine, was found to contribute to Covid-19-related hyperinflammatory syndrome (Hlh Across Speciality Collaboration UK et al., 2020) and to correlate with Covid-19 severity (Han et al., 2020; Herold et al., 2020). Further, some Covid-19 patients responded positively to *IL-6* inhibition (Atal and Fatima, 2020; Price et al., 2020). In other model systems, both *vWF* and *IL-6* have been shown to respond to hypoxic conditions (Michiels et al., 2000; Mojiri et al., 2013). However, in the current study, we found no statistical changes in *vWF* or *IL-6* mRNA levels after acute or sustained hypoxia. Although not significant, a trend towards an increase in *vWF* mRNA expression was observed in cells between 24 and 48 h of culture. Since *vWF* is an endothelial cell marker, it is possible that this effect may result from increased cell numbers across the course of the experiment. Taken together, our data suggest that altered ACE2 and TMPRSS2 expression induced by hypoxia occurs independently of compromised endothelial function and/or enhanced inflammatory state, at least over the time frame that we investigated.

Although modest, the effect hypoxia on ACE2 mRNA and TMPRSS2

mRNA is consistent and it is reproducible using different hypoxic systems. We recognize that using only one brain endothelial cell line is a limitation of the study, as it may introduce a bias towards this specific cell line. Nonetheless, we believe this study is extremely relevant because it is the first to describe the effects of hypoxia on SARS-CoV-2 receptors in brain endothelial cells. Other studies using primary cells or other cell lines will be important to confirm these effects and elucidate the mechanisms of hypoxia leading to ACE2 and TMPRSS2 regulation in brain endothelial cells.

In conclusion, this study has clearly demonstrated that SARS-CoV-2 cell entry mediators ACE2 and TMPRSS2 are modified under hypoxic conditions in human brain endothelium *in vitro*. Since hypoxia can result from Covid-19, this may have profound clinical ramifications, including increased vulnerability of the endothelium to infection. In the context of BECs, this may in turn increase the risk for Covid-19-related brain sequelae. Further studies are necessary to evaluate the impact of multiple variables, combining direct viral injury, hypoxia and hyperimmune stimulation. It will also be important to consider how astrocyte and microglia activation may contribute to the hyperinflammatory state evoked by SARS-CoV-2 in the human brain.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mvr.2021.104232>.

Abbreviations

| | |
|------------|---|
| ACE2 | angiotensin-converting enzyme 2 |
| ACTB | beta actin |
| BBB | blood-brain barrier |
| BECs | brain endothelial cells |
| CNS | central nervous system CNS |
| Covid-19 | Novel coronavirus disease 2019 |
| hCMEC/D3 | human cerebral microvascular endothelial cells |
| IL | interleukin |
| VEGF | vascular endothelial growth factor |
| <i>vWF</i> | von Willebrand Factor |
| SARS-CoV-2 | Severe Acute Respiratory Syndrome coronavirus 2 |
| TBS-T | Tris-Buffered Saline containing 0.1% Tween |

TMPRSS2 transmembrane protease serine 2

Funding

EB is supported by Coordenação de Aperfeiçoamento Pessoal de Nível Superior (CAPES), finance Code 001, Capes-Print fellowship and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, 310578/2020-5); SJL is supported by Canadian Institutes of Health Research (CIHR) grant (FDN-143262); SGM is funded by the Canadian Institutes of Health Research (SGM; FDN-148368).

CRediT authorship contribution statement

Guinever E Imperio: Methodology, Investigation, Validation, Data analysis, Writing - Original Draft; Phetcharawan Lye: Methodology, Validation; Hafsa Mughis: Investigation, Validation, Data analysis, Writing - Review & Editing; Hirotaka Hamada: Methodology; Enrico Bloise: Writing - Review & Editing, Resources, Supervision; Stephen J Lye: Conceptualization, Resources, Supervision; Stephen G Matthews: Conceptualization, Writing - Review & Editing, Resources, Supervision.

Declaration of competing interest

The authors declare no conflict of interest.

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