Hypoxia modifies levels of the SARS-CoV-2 cell entry proteins, angiotensin-converting enzyme 2, and furin in fetal human brain endothelial cells

Hafsah Mughis, MSc; Phetcharawan Lye, MEd; Stephen G. Matthews, PhD; Enrrico Bloise, PhD

BACKGROUND: It is not known whether human fetal brain endothelial cells that form the blood-brain barrier express angiotensin-converting enzyme 2, transmembrane serine protease 2, and furin, which are SARS-CoV-2 cell entry proteins. Moreover, it is unclear whether hypoxia, commonly observed during severe maternal COVID-19, can modify their level of expression. We hypothesized that human fetal brain endothelial cells isolated from early- and midpregnancy brain microvessels express angiotensin-converting enzyme 2, transmembrane serine protease 2, and furin. Furthermore, we hypothesized that hypoxia modifies their expression levels in a gestational age– and time-of-exposure–dependent manner.

OBJECTIVE: This study aimed to investigate whether early- and midpregnancy human fetal brain endothelial cells express angiotensin-converting enzyme 2, transmembrane serine protease 2, and furin SARS-CoV-2–associated cell entry proteins and to determine the effects of hypoxia on angiotensin-converting enzyme 2, transmembrane serine protease 2, and furin expression levels in human fetal brain endothelial cells.

STUDY DESIGN: This was a prospective study where human fetal brain endothelial cells isolated from early-pregnancy (12.4±0.7 weeks of gestation) and midpregnancy (17.9±0.5 weeks of gestation) fetal brain microvessels (6 per group) were exposed to different oxygen tensions (20%, 5%, and 1% oxygen) for 6, 24, and 48 hours. Angiotensin-converting enzyme 2, transmembrane serine protease 2, and furin messenger RNA and protein levels and localization were assessed using quantitative polymerase chain reaction, Western blot testing, and immunofluorescence.

RESULTS: Angiotensin-converting enzyme 2, transmembrane serine protease 2, and furin co-localize with the endothelial cell marker von Willebrand factor in human fetal brain endothelial cells isolated from early pregnancy and midpregnancy. In early pregnancy, *TMPRSS2* messenger RNA expression was decreased by 5% oxygen compared with 20% oxygen after 6 hours of exposure ($P<.05$). In midpregnancy, 5% oxygen down-regulated *ACE2* messenger RNA compared with 20% oxygen after 24 hours ($P<.05$). *Furin* messenger RNA expression was decreased under 5% and 1% oxygen compared with 20% oxygen ($P<.05$) after 24 hours. In midpregnancy, angiotensin-converting enzyme 2 protein levels were decreased under 5% and 1% oxygen ($P<.001$) after 24 hours. In contrast, furin protein levels were increased under 1% oxygen compared with 20% oxygen after 24 hours ($P<.05$). At 48 hours, 1% oxygen increased angiotensin-converting enzyme 2 protein levels compared with 20% oxygen ($P<.01$).

CONCLUSION: Hypoxia modifies the expression of selected SARS-CoV-2 cell entry proteins in human fetal brain endothelial cells in a gestational age– and time-of-exposure–dependent manner. As severe COVID-19 may lead to maternal hypoxia, an altered expression of these proteins in the developing human blood-brain barrier could potentially lead to altered SARS-CoV-2 brain invasion and neurologic sequelae in neonates born to pregnancies complicated by SARS-CoV-2 infection.

Key words: SARS-CoV2, Developing Blood-Brain Barrier, Hypoxia, angiotensin-converting enzyme 2 (ACE2), transmembrane serine protease 2 (TMPRSS2), furin

Introduction

Pregnant people are at an increased risk of complications related to COVID-19.[1] SARS-CoV-2 infection during pregnancy may induce maternal hypoxia[2] and lead to fetal hypoxic-ischemic injury.[3] Neonates born to pregnancies with SARS-CoV-2 infection present a greater likelihood of exhibiting signs of fetal distress, being born preterm, having low birthweight, and being admitted to the neonatal intensive care unit.[4,5]

The best-described mechanisms of SARS-CoV-2 cell entry consist of binding of the N-terminal portion of the outer spike (S) protein to its host cell membrane receptor, the angiotensin-converting enzyme 2 (ACE2) ectoenzyme.[6] This process is followed by cellular furin cleavage at the multibasic site Arg-Arg-Ala-Arg, located at the spike S1-S2 junction and transmembrane serine protease 2 (TMPRSS2) cleavage of the viral spike S2′ region.[7] These specific proteolytic cleavages induce extensive conformational changes in the S protein, which promote fusion of SARS-CoV-2 and target cell membranes, releasing viral RNA into the host cell cytoplasm.[6–8]

The human placenta co-expresses ACE2, TMPRSS2, and furin at the messenger RNA (mRNA) and protein levels[9,10] at the syncytiotrophoblast, whereas ACE2 has also been localized at fetal blood vessels of the placental villi.[9,11,12] Thus, SARS-CoV-2 may potentially infect the placenta and be vertically transmitted. SARS-CoV-2 has been detected in the syncytiotrophoblast[13,14] and has been shown to induce placental pathologies associated with intrauterine hypoxia and asphyxia, such as placental malperfusion, massive perivillous fibrin deposits, fetal vasculopathy, and villous trophoblast necrosis.[3,15–17] Transplacental SARS-CoV-2 transmission is rare but can occur followed by neonatal viremia[18,19] and is assumed to be the likely primary cause of neurologic

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AJOG MFM at a Glance

Why was this study conducted?

This study aimed to investigate whether human fetal brain endothelial cells (hfBECs) isolated from brain microvessels express angiotensin-converting enzyme 2 (ACE2), transmembrane serine protease 2 (TMPRSS2), and furin proteins associated with SARS-CoV-2 cell entry and probe how hypoxia modifies their level of expression.

Key findings

hfBECs expressed ACE2, TMPRSS2, and furin, which are modified by hypoxia in a gestational age– and time-of-exposure–dependent manner.

What does this add to what is known?

ACE2, TMPRSS2, and furin are expressed in hfBECs of the developing human blood-brain barrier (BBB). ACE2 and furin are modified under hypoxic conditions in vitro in midpregnancy. Given that complications of pregnancy often involve hypoxia, this study suggests an increased vulnerability of the developing BBB to SARS-CoV-2 infection and COVID-19, brain sequelae during hypoxia in pregnancy.

alterations in neonates born to pregnancies with SARS-CoV-2 infections[18,20–23]; however, this remains somewhat controversial.[13,14]

SARS-CoV-2 has been detected in low levels in the postmortem human adult brain,[24–27] indicating that it may invade the adult brain in specific clinical circumstances. COVID-19 may elicit important acute and long-term neurologic consequences, such as anosmia (loss of smell), stroke, encephalitis, encephalopathy, primary psychiatric syndromes,[26,28] and brain multifocal microvascular injury.[25]

Previously, we demonstrated that adult brain endothelial cells (BECs) exposed to different oxygen tensions (20%, 8%, and 2% oxygen) exhibited altered mRNA and protein levels of ACE2 and TMPRSS2,[29] showing that hypoxia may modulate adult BEC susceptibility to SARS-CoV-2 infection and subsequent viral neuroinvasion. As severe COVID-19 may promote maternal hypoxia and fetal hypoxic-ischemic injury,[2,3] we hypothesized that human fetal BECs (hfBECs), isolated from early- and midpregnancy brain microvessels, express ACE2, TMPRSS2, and furin mRNA and protein and that expression of these SARS-CoV-2 cell entry proteins may be modulated by hypoxia. Modification of SARS-CoV-2 cell entry proteins in the fetal blood-brain barrier (BBB) during hypoxia could represent a potential path to neurologic sequelae in some neonates born to pregnancies with SARS-CoV-2 infection.[30]

Material and Methods

Donor fetal brain collection

Human fetal brains were collected after elective termination of pregnancies, by the Research Centre for Women's and Infants' Health BioBank program at Sinai Health System, in adherence to institutional research ethics boards (REBs). Written informed consent (protocol number: 18-0057-E) was obtained. Fetal brains (with no gross morphologic abnormality) were collected in early pregnancies (12.4±0.7 weeks of gestation; n=6) and midpregnancies (17.9± 0.5 weeks of gestation; n=6). The institutional REB does not allow for the retrieval of any clinical information of the donors. Therefore, we were not able to provide further clinical information on the donors in this manuscript.

Oxygen treatments

Immediately after the elective termination of pregnancies, hfBECs were isolated from fetal brain specimens as previously described[31] and grown at 20% oxygen (5% CO_2, 37°C) until they reached confluence and stored in liquid nitrogen. hfBECs were plated in 6-well tissue-culture–treated polystyrene culture plates (number: 3516; Costar, Kennebunk, ME) precoated with type 1 collagen (number: A1048301; Thermo Fisher Scientific, Waltham, MA) at a density of 25,000 cells/cm^2. Of note, 24 hours after seeding, cells were challenged with different oxygen tensions: 20%, 5%, or 1%[32] using modular incubator chambers (Billups-Rothenberg, Del Mar, CA), each flushed with 1% and 5% oxygen for 5 minutes before culture. Cell-free media was infused with 1% and 5% oxygen for 24 hours before each experiment. Once flushing was complete, clamps on the inlet and outlet ports were closed, and the chamber was placed in an incubator at 37°C. Cells in the 20% oxygen group were placed in a chamber as well, open to the air for 5 minutes, and then placed inside the incubator with the clamps open, at 37°C.[33] An oxygen sensor (OXYLogger, PreSens, Regensburg, Germany) was placed in the 1% oxygen chamber, and oxygen measurements were logged throughout the experiment.[33] Cells were counted, and all plates were seeded at the same time. Each 6-well plate was seeded with cells derived from 6 individual fetuses per gestational age (ie, 1 well per treatment/subject/time; a total of 9 plates per gestational age). After confirming cell attachment, the plates were assigned to different oxygen chambers (20%, 5%, and 1%; 3 plates per chamber). As such, all plates contained cells from the 6 individual fetuses allowing direct comparison. Cells were collected for protein or mRNA analysis after 6, 24, and 48 hours of treatment. The different oxygen tensions did not affect cell viability at any time point during the study as previously described.[33]

Immunofluorescence

Immunofluorescence experiments were performed as described previously[31,34] with adaptations. In brief, Phosphate-buffered saline (PBS)-rinsed hfBECs (3 subjects in duplicates per group) were fixed with 4% paraformaldehyde for 15 minutes and then permeabilized with Triton X-100 (X100-1L, laboratory grade; Sigma-Aldrich, St. Louis, MO)

(0.2%, 5 minutes, room temperature [RT]). Autofluorescence was reduced using 0.1% Sudan Black (12806DE; Sigma-Aldrich, St. Louis, MO) in 70% ethanol (1 minute) and blocked using 1% bovine serum albumin (BSA) (BioShop Canada Inc, Burlington, Ontario) for 1 hour. hfBECs slides were incubated with the primary antibodies ACE2 (ab15348, 1:100; Abcam, Toronto, Ontario), TMPRSS2 (ab109131, 1:100; Abcam), furin (ab3467, 1:100; Abcam), and von Willebrand factor (vWF; ab11713, 1:500; Abcam) overnight at 4°C. Subsequently, cells were washed 3 times and incubated with fluorescent secondary antibodies, the antirabbit Alexa 488 (A21206, 1:1000; Thermo Fisher Scientific) or the antisheep Alexa 555 (A21436, 1:000; Thermo Fisher Scientific) and counterstained with 4′,6-diamidino-2-phenylindole (1 μg/mL, 1 hour). Fluorescent microscopy was performed using a spinning disk confocal microscope at various magnifications (DMI6000 B; Leica, Concord, Ontario).

Messenger RNA analysis

Total RNA was extracted using the RNeasy Mini Kit (number 74104; Qiagen, Toronto, Ontario), as previously described.[31] NanoDrop1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA) was used to determine the RNA purity and concentration, and total RNA (1 μg) was reverse transcribed to cDNA using iScript Reverse Transcription Supermix (number: 1708840; Bio-Rad Laboratories, Mississauga, Ontario). SYBR Green (number: S9194; Sigma-Aldrich, St. Louis, MO) was used to run the quantitative polymerase chain reaction using the CFX 380 Real-Time System C1000 TM Thermal Cycler (Bio-Rad Laboratories, Mississauga, Ontario), with the following parameters: 1 cycle of 95°C for 2 minutes and 40 cycles of 95°C for 5 seconds and 60°C for 20 seconds. Of note, 50, 10, or 5 ng of RNA was used per reaction depending on the gene of interest, and samples were set up in technical triplicates, where the cycle threshold (CT) value of each replicate per sample was recorded (6 per gestational age). The geometric mean of reference genes, *SDHA*, and *CYC1* (early pregnancy) and *ACTB*, *SDHA*, and *CYC1* (midpregnancy) was used to normalize the expression of target genes. The $2^{-\Delta\Delta CT}$ method[35] was used to calculate the relative mRNA expression. The Table shows the sequences and origin of the primers used in this study.

Table List of primers used in this study

Primer name	Sequence	Reference
ACE2	F: 5'- GGAGTGATAGTGGTTGGCATTGTC -3' R: 5'- GCTAATATCGATGGAGGCATAAGGA -3'	10,11,29
TMPRSS2	F: 5′- AGCTGCAGAAGCCTCTGACTTTC -3′ R: 5′- AGCGTTCAGCACTTCTGAGGTC -3′	10,29
Furin	F: 5′- CCTGGTTGCTATGGGTGGTAG -3' R: 5′- AAGTGGTAATAGTCCCCGAAGA -3′	54
SDHA	F: 5′- TGGGAACAAGAGGGCATCTG -3′ R: 5′- CCACCACTGCATCAAATTCATG -3′	55
ACTB	F: 5′- CTGGAACGGTGAAGGTGACA -3′ R: 5′- AAGGGACTTCCTGTAACAATGCA -3′	29
CYC1	F: 5′- CAGATAGCCAAGGATGTGTG -3′ R: 5′- CATCATCAACATCTTGACCC -3′	56

ACE2, angiotensin-converting enzyme 2; *ACTB*, actin beta; *CYC1*, cytochrome c1; *SDHA*, succinate dehydrogenase complex flavoprotein subunit A; *TMPRSS2*, transmembrane serine protease 2.

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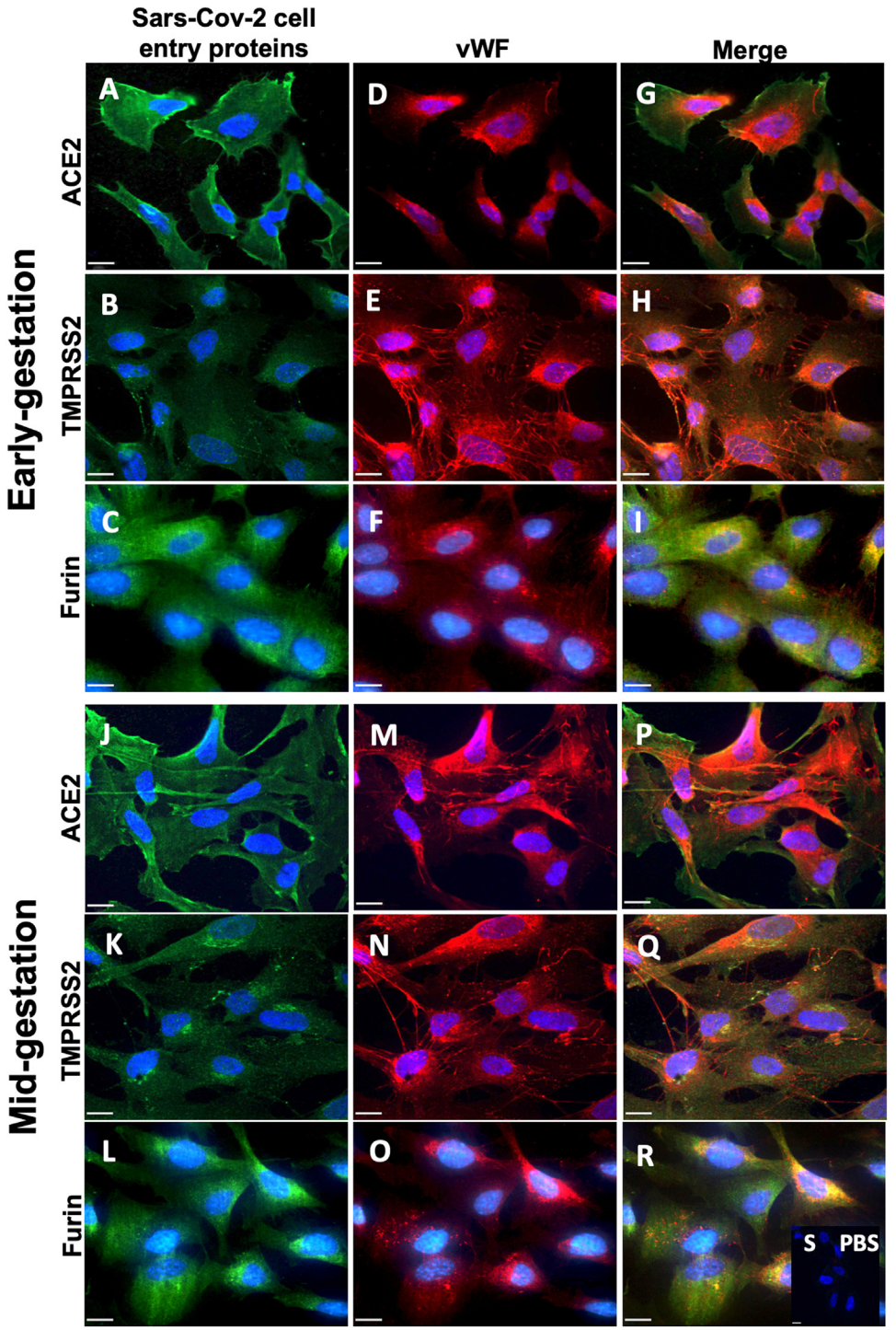
Protein analysis

Western blot testing was performed as previously described with adaptations.[11,29,31] Protein isolated from cultured cells was extracted by sonication using lysis buffer (1 mol/L Tris hydrochloride, pH 6.8, 2% Sodium Dodecyl Sulfate (SDS), and 10% glycerol) with protease or phosphatase inhibitor cocktail (number: 78420; Thermo Fisher Scientific, Waltham, MA). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Total protein (30 μg) was boiled (5 minutes) and loaded on 8% SDS polyacrylamide gels for electrophoretic separation (100 V, 1 hour). Proteins were transferred (10 minutes) from gels to polyvinylidene difluoride (PVDF) membranes using Trans-Blot Turbo (Bio-Rad Laboratories, Mississauga, Ontario). Membranes were blocked for 1 hour with 5% skim milk in Tris-buffered saline containing 0.1% Tween (TBS-T), followed by incubation at 4°C with primary antibodies anti-TMPRSS2 (number: ab242384, 1:5000; Abcam) for 2 nights and anti−ß-actin (number: 2066, 1:1000, loading control; Sigma-Aldrich) for 1 night. For the primary antibody anti-ACE2 (number: ab108252, 1:1000; Abcam), membranes were blocked for 1 hour with 5% BSA in TBS-T, followed by incubation at 4°C for 2 nights, and for the anti-furin antibody (number: ab3467, 1:1000; Abcam), membranes were blocked for 1 hour with 3% BSA in TBS-T, followed by incubation at 4°C for 1 night. All membranes were subsequently washed (3 times) with TBS-T and incubated (1 hour) with Horseradish peroxidase (HRP)-linked antirabbit secondary antibody (1:10000; GE Healthcare, Baie-D'urfe, Québec, Canada). Chemiluminescence was assessed using the SuperSignal West Femto (number: 34095; ThermoFisher Scientific) for ACE2 and TMPRSS2 or the Luminata Crescendo Western HRP Substrate (number: WBLUR0100; MilliporeSigma, Burlington, MA) for furin and ß-actin for 5 minutes and detected under ultraviolet illumination using a ChemiDoc (Bio-Rad Laboratories, Mississauga, Ontario). Protein bands were quantified by densitometric analysis and normalized against ß-actin signal for total protein assessment.

Statistical analysis

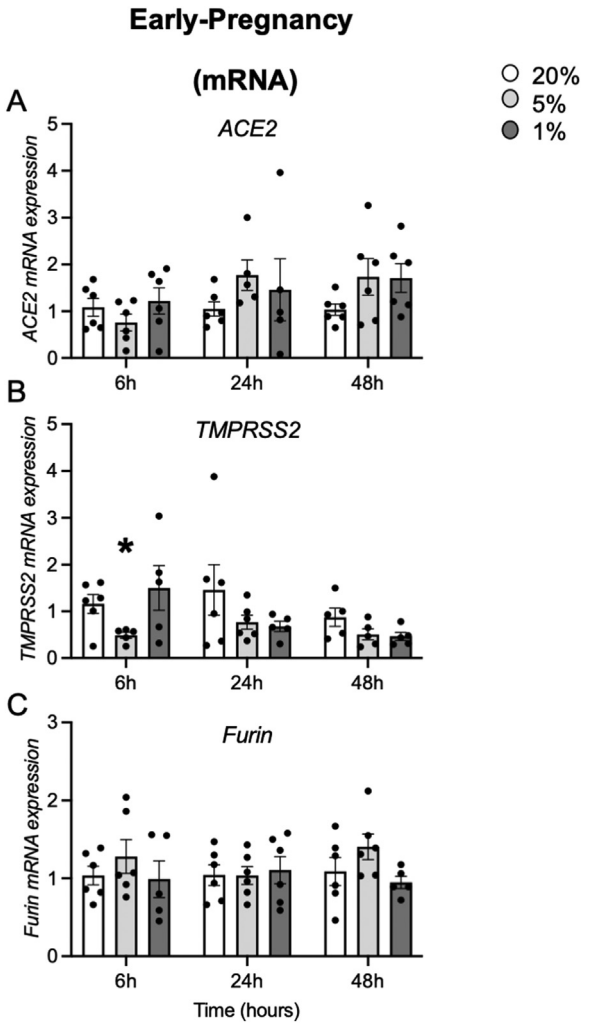
Statistical analyses were performed using GraphPad Prism software (version 9.2; GraphPad Software, Inc, San Diego, CA). The Grubbs method

FIGURE 1
ACE2, TMPRSS2, and furin are localized in early- and midpregnancy hfBECs



Representative immunofluorescence (*green*) of ACE2 (**A** and **J**), TMPRSS2 (**B** and **K**), and furin (**C** and **L**) detected in early- and midpregnancy hfBECs, respectively. ACE2 was localized in the plasma membrane and cytoplasm of early-pregnancy (**A**) and midpregnancy (**J**) hfBECs, TMPRSS2 exhibited a uniform cytoplasmic staining and granular staining in early pregnancy (**B**) and midpregnancy (**K**), whereas furin was stained in the cytoplasm but was also localized in the plasma membrane in early-pregnancy (**C**) and midpregnancy (**L**) hfBECs. The endothelial cell marker vWF (*red*) was detected in the cytoplasm and the cell surface of early-pregnancy (**D**–**F**) and midpregnancy (**M**–**O**) hfBECs. Co-localization of ACE2, TMPRSS2, furin, and vWF staining confirmed ACE2 (**G** and **P**), TMPRSS2 (**H** and **Q**), and furin (**I** and **R**) localization in early- and midpregnancy hfBECs, respectively. **A**–**R,** DAPI (*blue*; a nuclear marker). **S,** PBS (negative control). There are 3 per gestational age (representative images presented). The *scale bar* represents 12 μm.

FIGURE 2
***TMPRSS2* mRNA expression is decreased under 5% oxygen in early-pregnancy hfBECs**



ACE2 (**A**), *TMPRSS2* (**B**), and *Furin* (**C**) mRNA levels in early-gestation hfBECs exposed to 20%, 5%, or 1% oxygen for 6, 24, or 48 hours, respectively, measured using quantitative polymerase chain reaction (5−6 per group). The *open white bars* indicate 20% oxygen, the *light gray bars* indicate 5% oxygen, and the *dark gray bars* indicate 1% oxygen. Statistical analysis: 2-way analysis of variance followed by the Dunnett multiple comparisons tests. Mean±standard error of the mean. *Asterisk* denotes $P<.05$.

ACE2, angiotensin-converting enzyme 2; *hfBEC*, human fetal brain endothelial cell; *mRNA*, messenger RNA; *TMPRSS2*, transmembrane serine protease 2.

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identified outliers, and normality was tested using the Shapiro-Wilk test. Gene and protein expressions were analyzed using the 2-way analysis of variance, followed by the Dunnett multiple comparisons tests, comparing different oxygen tensions at each time point. Data are presented as mean±standard error of the mean. Differences were significant when $P<.05$.

Results

Angiotensin-converting enzyme 2, transmembrane serine protease 2, and furin are expressed by early- and midgestation human fetal brain endothelial cells

ACE2 was localized in the plasma membrane and cytoplasm in early pregnancy (Figure 1, A) and midpregnancy (Figure 1, J). TMPRSS2 exhibited a uniform cytoplasmic or plasma membrane staining, which was also present in intracellular vesicles in early pregnancy (Figure 1, B) and midpregnancy (Figure 1, K). Furin staining was present at the cytoplasm or plasma membrane in early- (Figure 1, C) and midpregnancy (Figure 1, L) hfBECs. In addition, we investigated whether these proteins would co-localize with the endothelial cell marker vWF. As previously demonstrated,[31] early- (Figure 1, D−F), and midpregnancy (Figure 1, M−O) hfBECs exhibited prominent vWF staining in the plasma membrane and cytoplasm. vWF co-localized with the 3 proteins studied in early pregnancy (Figure 1, G−I) and midpregnancy (Figure 1, P−R).

Hypoxia has limited effect on angiotensin-converting enzyme 2, transmembrane serine protease 2, and furin messenger RNA and protein expression in human fetal brain endothelial cells derived in early pregnancy

In early pregnancy, different oxygen tensions did not alter the mRNA expression of *ACE2* and *Furin* in the hfBECs (Figure 2, A and C) at any time point investigated (6, 24, and 48 hours). However, *TMPRSS2* mRNA expression was decreased by 5% compared to 20% oxygen after 6 hours of exposure ($P<.05$) but was not significantly different at other time points (Figure 2, B). Protein levels of all 3 SARS-CoV-2 cell entry proteins were not significantly affected by oxygen tension at any time point investigated (Figure 3, A−D).

ACE2, angiotensin-converting enzyme 2; *DAPI*, diamidino-2-phenylindole; *hfBEC*, human fetal brain endothelial cell; *TMPRSS2*, transmembrane serine protease 2; *vWF*, von Willebrand factor.

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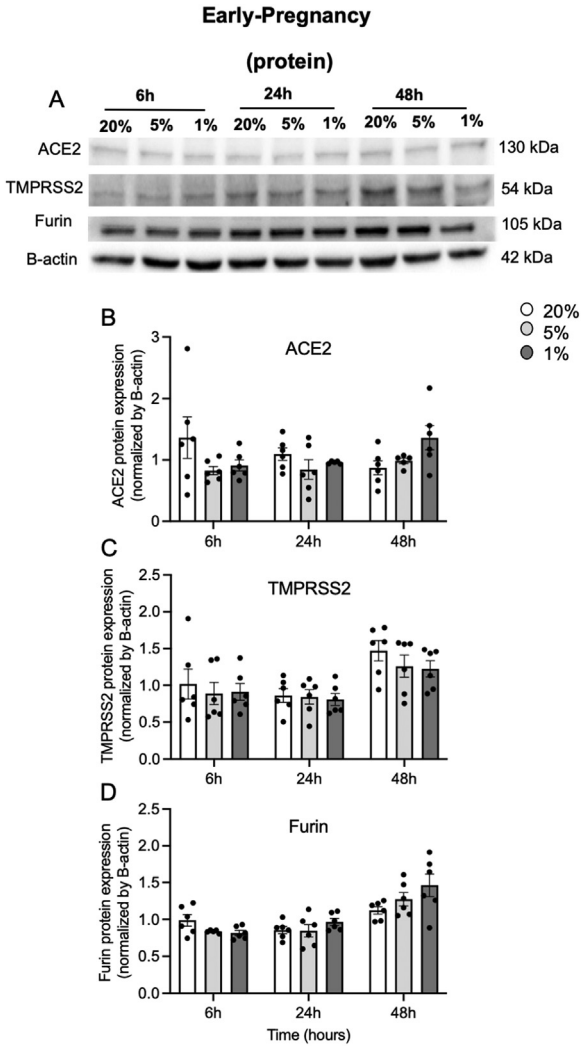
FIGURE 3
Hypoxia does not alter ACE2, TMPRSS2, and furin levels in early-pregnancy hfBECs



Representative images of Western blots (**A**). Densitometric analysis of total ACE2 (**B**), TMPRSS2 (**C**), and furin **(D)** protein in early-pregnancy hfBECs exposed to 20%, 5%, or 1% oxygen for 6, 24, or 48 hours, respectively, measured using Western blot testing. Beta-actin was used as the loading control (5−6 per group). The *open white bars* indicate 20% oxygen, the *light gray bars* indicate 5% oxygen, and the *dark gray bars* indicate 1% oxygen. Statistical analysis: 2-way analysis of variance followed by the Dunnett multiple comparisons tests. Mean±standard error of the mean.

ACE2, angiotensin-converting enzyme 2; *hfBEC*, human fetal brain endothelial cell; *TMPRSS2*, transmembrane serine protease 2.

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Hypoxia modifies angiotensin-converting enzyme 2, transmembrane serine protease 2, and furin in midgestation human fetal brain endothelial cells

In midpregnancy hfBECs, mRNA expression of *ACE2* was down-regulated by 5% oxygen compared with 20% oxygen at the 24-hour time point ($P<.05$) but was not significantly different at any other time points (6 and 48 hours) (Figure 4, A). Similarly, *Furin* mRNA was significantly decreased under 5% and 1% oxygen compared with 20% oxygen at 24 hours ($P<.05$), with no difference observed at other time points (Figure 4, C). We did not observe any significant changes in *TMPRSS2* mRNA expression by oxygen (Figure 4, B).

At the level of protein, we observed an oxygen and time-dependent pattern of ACE2 modulation in hfBECs. A time-dependent increase in protein expression of ACE2 under 20% oxygen after 24 hours was detected, consistent across all samples. Accordingly, at the 24-hour time point, ACE2 expression was significantly decreased under 5% and 1% oxygen compared with 20% oxygen ($P<.001$), whereas after 48 hours, ACE2 was increased by 1% oxygen compared with 20% oxygen ($P<.01$) (Figure 5, A and B). At midpregnancy, furin protein levels were increased under 1% oxygen compared with 20% oxygen after 24 hours ($P<.05$) (Figure 5, A and D). TMPRSS2 protein levels were not modulated by oxygen at any time point investigated (Figure 5, C). Overall, most significant changes were observed after 24 hours for furin and ACE2, in both mRNA and protein in midpregnancy hfBECs.

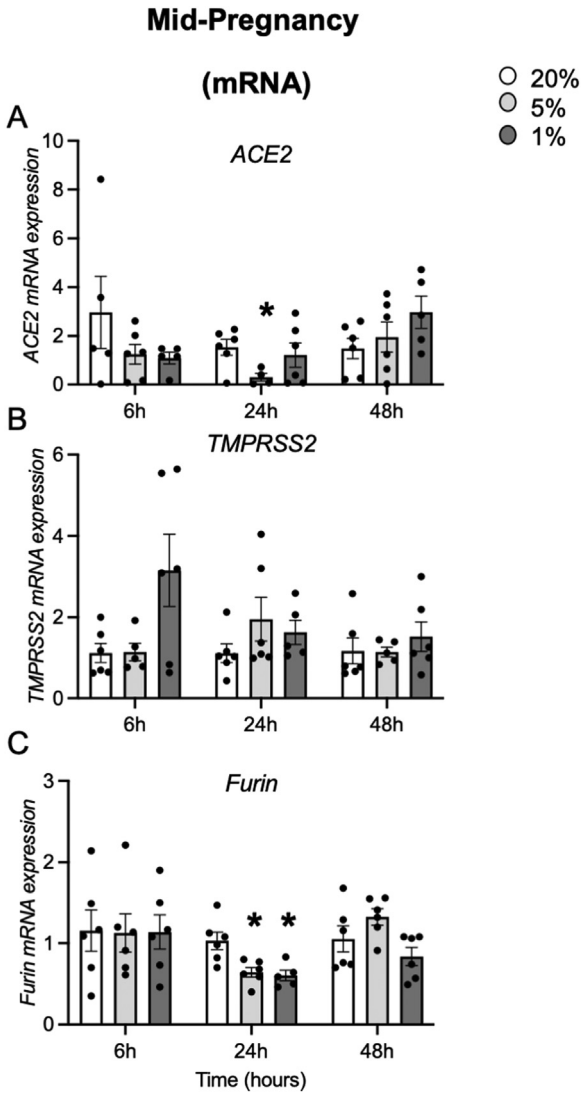
Comment

Principal findings

We demonstrated that hfBECs express the SARS-CoV-2 cell entry mediators ACE2, TMPRSS2, and furin. In addition, we demonstrated that hypoxia modulates their mRNA and or protein expression in an oxygen tension−, gestational age−, and time-of-exposure −dependent manner.

Previously, we reported that hfBECs express the endothelial cell marker vWF, the BBB tight junctions, zonula occludens-1, and claudin-5 and are capable of forming tubelike structures when cultured in Matrigel, confirming their BEC phenotype.[31] Importantly, compared with early- and midpregnancy hfBECs exposed to 20% oxygen, early- and midpregnancy hfBECs exposed to 1% oxygen exhibited an up-regulation of vascular endothelial growth factor mRNA levels,[33] a known hypoxia marker in endothelial cells,[36] indicating that hfBECs mount robust biological responses to a hypoxic environment in

FIGURE 4
Oxygen modulates *ACE2* and *Furin* mRNA expression in midpregnancy hfBECs



ACE2 (**A**), *TMPRSS2* (**B**), and *Furin* (**C**) mRNA levels in midpregnancy hfBECs exposed to 20%, 5%, or 1% oxygen for 6, 24, or 48 hours, respectively, measured using quantitative polymerase chain reaction (5–6 per group). The *open white bars* indicate 20% oxygen, the *light gray bars* indicate 5% oxygen, and the *dark gray bars* indicate 1% oxygen. Statistical analysis: 2-way analysis of variance followed by the Dunnett multiple comparisons tests. Mean±standard error of the mean. *Asterisk* denotes $P<.05$.

ACE2, angiotensin-converting enzyme 2; *hfBEC*, human fetal brain endothelial cell; *mRNA*, messenger RNA; *TMPRSS2*, transmembrane serine protease 2.

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vitro. Furthermore, we have previously shown that isolated hfBECs express the toll-like receptors 3, 4, and 8 and are capable of sensing and mounting specific proinflammatory responses to pathogens, such as SARS-CoV-2.[37]

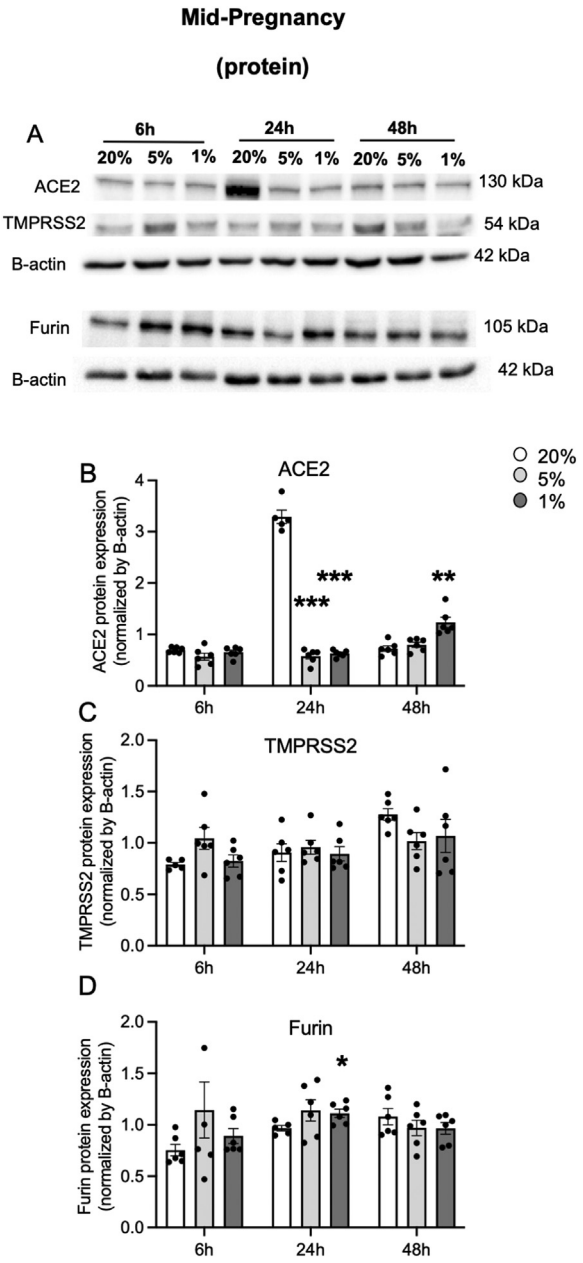
Clinical implications

There are limited reports demonstrating the presence of SARS-CoV-2 proteins or RNA in the fetal brain and or the possible routes of fetal brain infection. A single report has identified the SARS-CoV-2 localization in brain cells of a deceased infant brain in a pregnancy complicated by SARS-CoV-2 infection.[38] In addition, a large follow-up study, including 222 pregnancies with positive SARS-CoV-2 infection, revealed that prenatal SARS-CoV-2 may induce neurodevelopmental sequelae in some offspring.[39,40] We detected ACE2, TMPRSS2, and furin immunofluorescence localized at the plasma membrane, the cytoplasm, and at some intracellular vesicles of early- and midpregnancy hfBECs. Accordingly, they co-localized with the endothelial cell marker, vWF, suggesting that SARS-CoV-2, if vertically transmitted, has the potential to infect BECs of the developing BBB as early as 12 weeks of gestation and throughout the second trimester of pregnancy.

SARS-CoV-2 brain invasive routes are controversial but likely involve breaching the BBB.[27] Many viruses replicate in BECs and release infectious viral particles into the brain parenchyma.[41] Adult BECs express ACE2, TMPRSS2, and furin at the mRNA and protein levels,[29,42] and SARS-CoV-2 has been shown to infect human and mice BECs and transfer to the brain parenchyma via BEC transcellular transport,[43,44] indicating a possible route of SARS-CoV-2 entry into the BECs and potentially the brain. BEC entry of SARS-CoV-2 was impaired after viral co-incubation with antispike- and anti-ACE2–specific antibodies or the TMPRSS2 inhibitor, nafamostat.[43]

These findings are consistent with studies showing that there can be a connection between SARS-CoV-2 infection during pregnancy and greater rates of offspring neurodevelopmental sequelae.[38–40] This is especially important because, apart from the respiratory distress pregnant women may suffer during more severe forms of COVID-19, hypoxic stress during pregnancy can occur at high altitudes, because of maternal smoking, heart failure, anemia, placental insufficiency, placenta accrete, placenta previa, and preeclampsia.[49] Oxygen levels are physiologically lower during early pregnancy and continue to be low throughout pregnancy.

FIGURE 5
Oxygen modulates ACE2 and furin protein expression in midpregnancy hfBECs



Representative images of Western blots (**A**). Densitometric analysis of total ACE2 (**B**), TMPRSS2 (**C**), and furin (**D**) protein in midpegnancy hfBECs exposed to 20%, 5%, or 1% oxygen for 6, 24, or 48 hours, respectively, measured using Western blot testing. Beta-actin was used as the loading control (5—6 per group). Beta-actin Western blot tests were run separately for ACE2 and TMPRSS2 and furin. The *open white bars* indicate 20% oxygen, the *light gray bars* indicate 5% oxygen, and the *dark gray bars* indicate 1% oxygen. Statistical analysis: 2-way analysis of variance followed by the Dunnett multiple comparisons tests. Mean±standard error of the mean. *Asterisk* denotes $P<.05$; *double asterisks* denote $P<.01$; *triple asterisks* denote $P<.001$.

ACE2, angiotensin-converting enzyme 2; *hfBEC*, human fetal brain endothelial cell; *TMPRSS2*, transmembrane serine protease 2.

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Mean oxygen concentrations at the end of the third trimester of pregnancy are approximately 3.7% in the umbilical vein and 2.5% in the umbilical artery.[32] Further deprivation of oxygen can cause severe injury to vital fetal organs, including the developing brain.[45,46] In addition, hypoxia can lead to an increase in BBB permeability resulting in greater fetal brain toxicity.[47] Given these factors, it is imperative to understand the effects of hypoxia on the susceptibility of the developing brain to SARS-CoV-2 infection. Hypoxic situations can make the developing brain more vulnerable to the transfer of SARS-CoV-2 from the mother (because of increased BBB vulnerability), therefore making the BBB an important target for drugs developed to prevent or facilitate recovery from neurologic damage that may be caused by SARS-CoV-2.[30]

Research implications

Hypoxia did not change ACE2 and furin expressions during early pregnancy. An acute decrease in *TMPRSS2* mRNA expression was observed under 5% oxygen compared with 20% oxygen after 6 hours only, indicating a somewhat transient degree of protection to SARS-CoV-2 infection acutely, which decreased over time. Studies examining the effects of hypoxia on TMPRSS2 are limited but indicate decreased expression in lung epithelial cells under hypoxia in vitro and in vivo (in mice).[48]

The decrease in Furin mRNA expression under 5% and 1% oxygen (compared to 20%) after 24h in midgestation contrasts the increase in protein expression under 5% and 1% oxygen after 24h. This indicates a time-dependent effect of hypoxia on Furin. The addition of a furin cleavage site on SARS-CoV-2 is one of the factors making the virus more infectious than its ancestor viruses. Cases of increased furin levels overlap with greater vulnerability to more severe forms of COVID-19.[49] The results of the disconnect between furin mRNA and protein have been reported previously, and there is

increasing evidence that alterations in furin expression and dysregulation in the cleavage of its substrates can contribute toward the pathophysiology of neurogenerative diseases,[50] which can be further exacerbated by hypoxia.

The time-dependent steep increase in ACE2 expression after 24 hours at 20% oxygen at midpregnancy was perplexing but consistent in all our samples. This resulted in a significant decrease in expression under 5% and 1% oxygen. The experiment was independently replicated, and the full analysis ran twice, but the same phenomenon was observed consistently. None of the other markers, including the loading control (actin), displayed a similar result at any time point or in any replicate, ensuring it is a biological effect. At 24 hours, a decreased ACE2 expression by physiological and severe hypoxia suggests that fetal hypoxia would transiently decrease the likelihood of BEC infection by SARS-CoV-2 via ACE2 down-regulation. However, at 48 hours, severe hypoxia (1% oxygen) increased its expression, suggesting that fetal hypoxia might lead to a greater chance of SARS-CoV-2 fetal neuroinvasion, but this requires further investigation.

There are reports of ACE2 playing a protective role in the brain. ACE2 deficiency in the cerebral arteries contributed to BEC dysfunction, increased inflammation, and oxidative stress in mice.[51] This would suggest a protective role of the hypoxia-increased ACE2 observed in our midpregnancy samples, although only at the 48-hour time point.

Strengths and limitations

We have shown that hypoxia exposure and gestational age can regulate gene and protein expression of specific SARS-CoV-2 cell entry proteins in hfBECs, suggesting the existence of windows of vulnerability in which SARS-CoV-2 has a greater likelihood of infecting and crossing the developing BBB. However, we did not assess whether SARS-CoV-2 is capable of infecting and traversing these endothelial cells at the developing BBB. Furthermore, astrocytes are important modulators of BEC function at the BBB.[52] Future studies investigating whether SARS-CoV-2 infects hfBECs and how SARS-CoV-2 cell entry proteins in hfBECs cocultured with fetal astrocytes respond to hypoxia will aid in our understanding of the mechanisms of fetal brain SARS-CoV-2 neuroinvasion. We recognize that the sample size and the lack of clinical information on the donors may limit the interpretation of our findings. In this connection, as mandated by our REB, we were unable to karyotype our isolated hfBECs to assess whether they are genetically normal. Chromosomal abnormalities can cause cells to respond differently to hypoxia[53] and thus potentially affect the expression response of SARS-CoV-2–associated cell entry proteins to hypoxia in the developing BBB.

Conclusions

Our study determined that ACE2, TMPRSS2, and furin are present and are modified under hypoxic conditions in hfBECs derived from early pregnancy and midpregnancy. Given that complications of pregnancy usually involve hypoxia, this study determined some of the effects of hypoxia on SARS-CoV-2 BBB infective markers, indicating an increased vulnerability of the BECs to infection and a higher risk of COVID-19–related brain sequelae during development. Further studies are imperative to determine the role of hypoxia and COVID-19 in affecting fetal brain development. ■

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