SARS-CoV-2 triggers pericyte-mediated cerebral capillary constriction

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The SARS-CoV-2 receptor, ACE2, is found on pericytes, contractile cells enwrapping capillaries that regulate brain, heart and kidney blood flow. ACE2 converts vasoconstricting angiotensin II into vasodilating angiotensin-(1-7). In brain slices from hamster, which has an ACE2 sequence similar to human ACE2, angiotensin II evoked a small pericyte-mediated capillary constriction via AT1 receptors, but evoked a large constriction when the SARS-CoV-2 receptor binding domain (RBD, original Wuhan variant) was present. A mutated non-binding RBD did not potentiate constriction. A similar RBD-potentiated capillary constriction occurred in human cortical slices, and was evoked in hamster brain slices by pseudotyped virions expressing SARS-CoV-2 spike protein. This constriction reflects an RBD-induced decrease in the conversion of angiotensin II to angiotensin-(1-7) mediated by removal of ACE2 from the cell surface membrane and was mimicked by blocking ACE2. The clinically used drug losartan inhibited the RBD-potentiated constriction. Thus, AT1 receptor blockers could be protective in COVID-19 by preventing pericyte-mediated blood flow reductions in the brain, and perhaps the heart and kidney.

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Introduction

Despite the primary site of infection by SARS-CoV-2 being the respiratory tract, the virus evokes dysfunction of many other organs, including the brain, heart and kidney: 36% of hospitalized patients show neurological symptoms, 20% develop myocardial injury and 41% experience acute kidney injury. This could reflect either a spread of virus via the blood or the effects of inflammatory mediators released from the lungs. These effects may contribute to ‘long Covid’, in which clouding of thought and physical exhaustion extend for months after the initial infection.

The receptor1,2 for SARS-CoV-2 is the enzyme angiotensin converting enzyme 2 (ACE2, part of the renin–angiotensin system that regulates blood pressure), which converts3 vasoconstricting angiotensin II into vasodilating angiotensin-(1-7). The Spike protein of SARS-CoV-2 binds to ACE2 to trigger its endocytosis.4 For the closely related SARS virus, binding of only the receptor binding domain (RBD) is sufficient5 to evoke internalization of ACE2.

In the heart and brain,6,10,11 the main cells expressing ACE2 are pericytes enwrapping capillaries (with some expression in endothelial cells), and pancreas and lung pericytes also express ACE2.12,13 Pericytes express contractile proteins and in pathological conditions have been shown to constrict capillaries and decrease blood flow in the brain,14,15 heart16 and kidney.17 Interestingly, a decrease of blood flow has been reported for SARS-CoV-2 infection in the brain18–20 and kidney.21 One brain study21 was a single case report that used arterial spin label (ASL) and dynamic susceptibility contrast MRI techniques to show an asymmetric marked reduction of cerebral blood flow in the bilateral frontoparietal regions. A further perfusion imaging study of 11 patients19 found bilateral fronto-temporal hypoperfusion in all of them. Another ASL MRI study20 of 51 patients who had recovered from coronavirus disease 2019 (COVID-19) showed that patients who had severe disease suffered from a prolonged and widespread decrease of cerebral blood flow.

Because pericytes have been reported to be infected by SARS-CoV-2 in COVID-19,11,19 these blood flow reductions could be due to pericyte dysfunction caused by SARS-CoV-2 reducing the activity of ACE2, either by occluding its binding site for angiotensin II (although this is thought not to occur either for the related SARS virus22 or for SARS-CoV-2)23,24 or by promoting removal of the enzyme from the membrane.6,25 In the presence of angiotensin II (either renally derived and reaching the brain parenchyma via a compromised blood–brain barrier or generated by the brain’s own renin–angiotensin system),26 a reduction of ACE2 activity would increase the concentration of vasoconstricting angiotensin II and decrease the concentration of vasodilating angiotensin-(1-7). We therefore investigated the effect of the SARS-CoV-2 RBD and Spike protein on the control of capillary diameter by pericytes.

Imaging of pericyte-mediated constriction

Pericytes on cortical capillaries were identified visually as previously described (Fig. S1 in Nortley et al.25 and see below) and imaged with a charge-coupled device (CCD) camera as described.26 Diameter was measured in Metamorph software (Molecular Devices) by drawing a line across the vessel between the inner walls of the endothelial cells.

RBD and mutant RBD synthesis

Codon-optimized Genblocks (IDT Technology) for the receptor binding domain (RBD amino acids 330–532) of SARS-CoV-2 (original Wuhan variant; Genbank MN908947) and human Angiotensin Converting Enzyme 2 (ACE2, amino acids 19–615) were inserted into the vector pOPINTTGneo (PMID: 25447866) incorporating a C-terminal BirA-His6 tag and pOPINTTGneo-3C-Fc to make C-terminal fusions to Human IgG Fc. The (non-binding) RBD-Y489R mutant was generated by first amplifying the RBD-WT gene using oligos TTGneo_RBD_F and RBD-Y489R_R, as well as RBD-Y489R_F and TTGneo_RBD_R; followed by joining the two resultant fragments with TTGneo_RBD_R and TTGneo_RBD_F. The constructs TTGneo_RBD_F, TTGneo_RBD_R, RBD-Y489R_F and RBD-Y489R_R are, respectively: 5′-ctgatgtaagcggccggcataactctgttg-3′; 5′-TGATGATGATGATACTTTTTCGTGC CGCACAC-3′; 5′-GGCGTCTCAGGTTTATTAAGTGTGCCTCCACTCTCA GTCATACGGG-3′; and 5′-CCGATTGACTAAGTGAAAGCCTGACATT AAAACCTCCGACGCC-3′.

The gene carrying the Y489R mutation was then inserted into the vector pOPINTTGneo incorporating a C-terminal His6 tag by Infusion® cloning. The plasmid was sequenced to confirm that the mutation had been introduced successfully. Recombinant protein was transiently expressed in Expi293™ (ThermoFisher Scientific) and purified from culture supernatants by immobilized metal affinity chromatography using an automated protocol implemented on an Äktamap GE Healthcare) followed by a Superdex 200 10/300 GL column, using PBS pH 7.4 buffer. Recombinant RBD-WT and ACE2-Fc were produced as described.27 The sequence of the RBD was: ETGPNPTLCFPPFTVPNNATRFASYYAWNNKRSNCVADYSVLNYSA SFSTFKCYGVCFTKLNLDCLFTNYAVDPSFIRGEVRQAPGQFGSKDYN YKLKDPFTGCVIAWNSNLKDGKVNLYVRJFKNKLFPERFDIRTEI YQAAGSTPCNGEVCNFCYFPQLSYFQGTNGVQFYFRVVLFSFLLHAP AVTCGPK垦TNKHHPH, where the residues in italics are derived from the expression vector. Glycosylated residues are shown in bold (N) and the tyrosine that is responsible to arginine (Y489R) in the mutant RBD is shown underlined.

Surface plasmon resonance

Experiments were performed using a Biacore T200 system (GE Healthcare). All assays were performed using a Sensor Chip Protein A (GE Healthcare), with a running buffer of PBS pH 7.4, supplemented with 0.005% vol/vol surfactant P20 (GE Healthcare), at 25°C. ACE2-Fc was immobilized onto the sample flow cell of the sensor chip; the reference flow cell was left blank. RBD-WT or RBD-Y489R (0.1 μM) was injected over the two flow cells, at a flow rate of 30 μl/min with an association time of 60 s.

Solutions

Brain slices were superfused at 33–36°C with solution containing (mM): 124 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2, 1 NaH2PO4, 26 NaHCO3, 10 d-glucose and 0.1 ascorbate, bubbled with 20% O2/70% N2/5%
CO₂ to ensure a physiological [O₂] was achieved in the slice. The high molecular weight of the RBD (~31 kDa) implies it will not diffuse rapidly from the superfusion solution into brain slices so, to apply the RBD, we pre-incubated each slice in solution containing RBD (at 35°C, to allow time for diffusion) prior to placing the slice in the imaging chamber, where it was superfused with the same solution containing the RBD. This 30 min pre-incubation time was mimicked for slices that RBD was not applied to. The same procedure was followed for the mutant RBD and for pseudovirus application.

Immunohistochemistry

Hamster brain slices were fixed in 4% paraformaldehyde (PFA) while shaking at room temperature for 20 min (except for experiments with pseudotyped virions; 1 h) and washed 3 times in PBS. For detection of ACE2, antigen retrieval using sodium citrate buffer (consisting of 10 mM sodium citrate, 0.05% Tween 20 and HCl to adjust the pH to 6.0) for 20 min was performed and the slices were left to cool down for 20 min before being washed in PBS for 5 min. Brain slices with or without antigen retrieval were transferred to blocking solution containing 10% horse serum, 0.2% saponin (Sigma-Aldrich, S7900 for ACE2 detection) or 0.3% Triton X-100, 200 mM glycine and 150 μM bovine serum albumin in PBS at 4°C, and shaken for 4 h at room temperature or overnight at 4°C for ACE2 detection. Slices were incubated in the blocking solution with primary antibodies for 24 h (72 h for ACE2 detection) at 4°C with agitation, washed with PBS 4 times, incubated with the secondary antibody overnight at 4°C with agitation and washed again 4 times with PBS. For nucleus counterstaining, slices were incubated in PBS containing DAPI (100 ng/ml) for 1 h, washing once with PBS. Imaging of Z stacks (approximately 320 μm × 320 μm × 20 μm) was performed on a confocal microscope. The first 20 μm from the surface were discarded to exclude cells killed by the slicing procedure.

Pericyte death assessment

Brain slices (300-μm thick) were incubated for 3 h at 35°C in extracellular solution (bubbled with 20% O₂, 5% CO₂ and 75% N₂) containing 7.5 μM propidium iodide (PI; Sigma-Aldrich, 81845) and IB4 conjugated to Alexa Fluor 647, with and without RBD (0.7 mg/l) and/or angiotensin II (50 nM). The slices were fixed with 4% PFA for 1 h and washed three times with PBS, for 10 min each time. Nucleus counterstaining was achieved by incubating the slices in PBS containing DAPI (100 ng/ml) for 1 h, washing once with PBS. Imaging of Z stacks (approximately 320 μm × 320 μm × 20 μm) was performed on a confocal microscope. The first 20 μm from the surface were discarded to exclude cells killed by the slicing procedure.

Quantification of ACE2 expression on the pericyte surface

Brain slices (200-μm thick) were incubated in extracellular solution gassed with 20% O₂, 5% CO₂ and 75% N₂, with and without RBD (0.7 mg/l) for 3 h. Immunohistochemistry was performed to detect ACE2 and PDGFβR expression. Images of randomly selected pericytes (~39 μm × 39 μm) were taken using a confocal microscope. For each image, a mask of PDGFβR, which is expressed on the cell membrane, was created and the mean fluorescence intensity representing ACE2 expression within that membrane area was measured (isolated puncta in the PDGFβR image, located away from pericytes, were digitally removed). For the soma the ACE2 intensity in the intracellular space bounded by the membrane (defined as described) was also measured.

Pericyte identification

Pericytes were identified morphologically (pericytes are located on the outside of capillaries with their nuclei showing a ‘bump on a log’ morphology, and at the intersection of capillary branches), when visualized through staining either the basement membrane with isoelectin B4 (IB4) or the pericyte cell membrane using anti-PDGFRβ or anti-NG2 antibodies (Supplementary Fig. 1). Pericytes are completely embedded in (encircled by) the basement membrane, which in hamsters can be labelled with IB4 conjugated to Alexa Fluor 647 (Invitrogen, I32450, 3.3 μg/ml, applied for 30 min before fixation and subsequent immunohistochemistry (in rats and mice IB4 also works when applied with the secondary antibodies during immunohistochemistry)). In contrast, a smaller population of perivascular cells that expresses PDGFβR like pericytes are fibroblasts, which have a flatter soma and are outside the basement membrane and so show IB4 labelling only on the capillary side of the cell. We found that out of 30 PDGFβR expressing peri-capillary cells, 93.3% were completely surrounded by IB4 and hence were pericytes. We have also previously shown that identifying pericytes morphologically gives excellent agreement with identification based on IB4 labelling (see Fig. S1 in Noritley et al. [32]).
by lysing cells with the Bright-Glo luciferase kit (Promega), using a Glomax plate reader (Promega).

**Pseudotyped virion application**

Brain slices (300-μm thick) were incubated in extracellular solution containing either pseudotyped virions (8375 TCID50/ml final concentration when applied at a 1:10 dilution from the harvested viral supernatant) or DMEM (1:10; as a control), oxygenated with 20% O2, 5% CO2 and 75% N2 for 30 min. Angiotensin II (50 nM) was added to the solution and then slices were incubated for another 30 min. The slices were fixed with 4% PFA for 1 h to inactivate the virions. Immunohistochemistry for CD31 and PDGFRβ with nuclear counterstaining was performed and the slices were imaged as Z stacks (~320 μm × 320 μm × 16 μm) with a confocal microscope. Pericyte somata were identified by expression of PDGFRβ and DAPI. The capillary diameter was measured at each pericyte soma and 5, 10 and 15 μm away from the soma,15 by drawing a line across the vessel between the outer walls of the endothelial cells (as defined by the CD31 signal).

**Statistics**

Data are presented as mean ± SEM averaged over pericytes (the responses of which show more variance than that between animals) or image stacks; number of animals from which the data were taken are given in the figure legends. Experiments using drugs were interleaved randomly with control experiments lacking drugs. For bar graphs individual data-points are superimposed on the mean data. Data normality was assessed with Shapiro–Wilk or D’Agostino–Pearson omnibus tests. Comparisons of normally distributed data were made using two-tailed Student’s t-tests. Equality of variance was assessed with an F test, and heteroscedastic t-tests were used if needed. Data that were not normally distributed were analysed with Mann–Whitney tests. P-values were corrected for multiple comparisons using a procedure equivalent to the Holm–Bonferroni method (for n comparisons, the most significant P-value is multiplied by n, the second most significant by n–1, the third most significant by n–2, etc.; corrected P-values are significant if they are less than 0.05).

**Data and code availability**

Data plotted in the figures are available in the Supplementary material. No custom code was used in this study.

**Results**

**Angiotensin II evokes pericyte-mediated capillary constriction via AT1 receptors**

To study the effect of the SARS-CoV-2 RBD on cerebral capillary pericyte function, we employed live imaging26 of brain slices from Syrian golden hamsters. Hamsters have an ACE2 sequence, in the part of the protein that binds the SARS-CoV-2 Spike protein, which is more similar to that in humans than is the rat and mouse ACE2 sequence.34 In particular amino acid 353 in hamsters and humans is a lysine (K) rather than a histidine (H), and this is a key determinant40 of how well coronaviruses bind to ACE2, making hamsters a good model for studying SARS-CoV-2 effects.38

We assessed the location of ACE2 and contractile properties of pericytes in the cerebral microvasculature of the hamster, which have not been studied previously (Fig. 1). Immunohistochemistry (IHC) revealed ACE2 to be predominantly expressed in capillary pericytes expressing NG2 and PDGFRβ (Fig. 1A and B). Quantification of overlap with the pericyte marker PDGFRβ revealed ~75% co-localization (Fig. 1C) and comparison of expression in capillaries and penetrating arterioles showed that capillaries exhibited ~75% of the ACE2 expression (Fig. 1D). These results are consistent with transcriptome and IHC data from mouse and human brain10,11 and human heart.9 As for brain pericytes in rats,35 the thromboxane A2 analogue U46619 (200 nM) evoked a pericyte-mediated capillary constriction and superimposed glutamate evoked a dilation (Fig. 1E).

Applying angiotensin II (150 nM) evoked a transient constriction, which was inhibited by the AT1 receptor blocker losartan (20 μM; Fig. 1F). Immunohistochemistry revealed the presence of AT1 receptors on capillary pericytes, as well as on other cortical cells (Supplementary Fig. 1A and B). Similar angiotensin-evoked pericyte-mediated capillary constriction has been reported in the kidney36 (where the angiotensin receptors were shown to be on the pericytes themselves37) and retina38 (where angiotensin evokes a rise in [Ca2+], in pericytes),39 and cultured human brain pericytes have been shown to express AT1 receptors40 (transcriptome data13 also show AT1R expression at the mRNA level in brain pericytes). The transience of the constriction might reflect receptor desensitization41 at this relatively high angiotensin II concentration, or a delayed activation of Mas receptors after the angiotensin II is converted to angiotensin-(1-7). Blocking either AT2 receptors (with 1 μM PD123319) or Mas receptors (with 10 μM A779) increased the angiotensin II-evoked constriction (approximately 4.5-fold for MasR block, P < 10–4; Fig. 1G and H), consistent with the AT1R-mediated constriction being opposed by angiotensin II activating AT2 receptors, by angiotensin-(1-7) activating Mas receptors, or by activation of AT2/Mas heteromeric42 receptors.

**SARS-CoV-2 binding potentiates angiotensin II-evoked capillary constriction**

Acute application of the RBD of COVID-19 (at 0.7 mg/l or ~22.5 nM, which is approximately five times the EC50 for binding43 for up to 40 min evoked a small and statistically insignificant reduction of capillary diameter at pericytes (Fig. 2A). On applying a very high level of angiotensin II (2 μM) in the absence of RBD, a transient constriction of capillaries at pericytes was observed (6.3 ± 3.6% in six capillaries, not significantly different from the 7.5 ± 1.6% observed using 150 nM angiotensin II in nine capillaries in Fig. 1F, P = 0.73). However, if brain slices were exposed for 30 min to RBD (0.7 mg/l) before the same concentration of angiotensin II was applied together with the RBD, then the angiotensin II evoked a 5-fold larger constriction of 31.5 ± 9.3% in four capillaries (significantly different to that seen in the absence of RBD, P = 0.019, Fig. 2B). The 30-min pre-exposure period was used in order to allow time for the large RBD molecule to diffuse into the slice, and was mimicked for the experiments without the RBD. This large constriction-potentiating effect of the RBD was not a non-specific effect on the pericytes’ contractile apparatus, because the contractile response to U46619 (200 nM) was unaffected by the RBD (Fig. 2C), and is consistent with the RBD reducing ACE2 activity and decreasing generation of the MasR-activating vasodilator angiotensin-(1-7).

The high concentration of angiotensin II used in Fig. 2B is probably not physiological and evokes a transient response for reasons that are discussed above. We therefore switched to a lower angiotensin II concentration (50 nM; Fig. 2D), which is more similar to
Figure 1 Cerebral pericytes express ACE2 and constrict capillaries in response to Ang II. (A) Labelling of hamster cortical slice with antibodies to the SARS-CoV-2 receptor ACE2, the pericyte markers NG2 and PDGFRβ and with DAPI to label nuclei. (B) Lower magnification (note different scale bar) maximum intensity projection of ACE2 and PDGFRβ labelling, showing capillaries and penetrating arteriole. (C) Integrated ACE2 labelling overlapping with a binarized mask of PDGFRβ labelling and with the inverse of this mask. (D) Integrated ACE2 labelling over capillaries versus penetrating arterioles (PA). In (C) and (D) the number of image stacks is on the bars; data in (C) and (D) were each from two animals. (E) Average normalized diameter changes (mean ± SEM) at seven pericytes (in different brain slices from four animals) exposed to the thromboxane A₂ analogue U46619 (200 nM) and then with the neurotransmitter glutamate (500 µM) superimposed. (F) Average normalized diameter changes at nine pericytes (in different slices from seven animals) exposed to 150 nM angiotensin II alone (i.e. in artificial CSF, aCSF) and 10 pericytes (from three animals) exposed to angiotensin II in the presence of the AT1R blocker losartan (20 µM). (G) As in F (aCSF plot is the same) but showing angiotensin II response in the presence of the AT2R blocker PD123319 (1 µM, nine pericytes from four animals) or the Mas receptor blocker A779 (10 µM, five pericytes from two animals). (H) Peak constriction evoked by angiotensin II in different conditions (number of pericytes studied shown on bars). Points superimposed on bar graphs here and in subsequent figures are individual data-points (pericytes or image stacks) contributing to the mean.
levels that have been found physiologically within the kidney and heart. In the presence of the RBD, the constricting response to angiotensin II was increased from an insignificant dilation of 4.5 ± 3.0% to a constriction of 7.8 ± 3.6% (9 capillaries each, \( P = 0.02 \)), i.e. effectively a constriction of ∼12% (from 100 × [1 − (92.2% / 104.5%)]).

Using surface plasmon resonance to assess binding of RBD mutants to immobilized ACE2, we identified the Y489R mutation as reducing binding by ∼94% (Fig. 2E). Applying this mutated RBD (for which glycosylation of the protein is expected to be the same as for the normal RBD) had essentially no effect on the response to angiotensin II (Fig. 2F). Thus, the potentiation of the angiotensin II response by the RBD is a result of it binding to ACE2.

The RBD effect is mimicked by blocking ACE2 and blocked by losartan

We hypothesized that the potentiating effect of the RBD on the response to angiotensin II reflects a decrease in the conversion by ACE2 of vasoconstricting angiotensin II into vasodilating angiotensin-(1-7). Such a decrease is expected if RBD binding promotes ACE2 internalization or if it occludes the angiotensin II binding site. We therefore tested the effect of an ACE2 inhibitor (MLN-4760, 1 µM) on the response to 50 nM angiotensin II. This closely mimicked the potentiating effect of the RBD, confirming that the RBD reduces effective ACE2 activity (Fig. 3A and B). Furthermore, applying the ACE2 inhibitor after inducing constriction with angiotensin II in the presence of the RBD evoked no further constriction (Supplementary Fig. 2A and B). This occlusion of the potentiation of the constrictions evoked by the RBD and by the ACE2 inhibitor is consistent with the effect of the RBD being to effectively decrease ACE2 activity.

Activating the Mas receptor that angiotensin-(1-7) acts on [using the stable angiotensin-(1-7) analogue AVE0991], after the capillaries had been constricted by applying the RBD and angiotensin II, led to a large dilation, resulting in a small net constriction similar to that produced by angiotensin II in the absence of the RBD (Supplementary Fig. 2C and D). This is consistent with the large constriction seen in the presence of RBD and angiotensin II being the result of the RBD blocking production of angiotensin-(1-7) by ACE2. Applying the MasR blocker A779 (10 µM) during the constriction evoked by angiotensin in the presence of the RBD evoked no further constriction (Supplementary Fig. 2C and D), which is also
SARS-CoV-2 constricts capillaries

Figure 3 The effect of RBD is mimicked by blocking ACE2 and reduced by losartan. (A) Capillary constriction at pericytes in response to 50 nM angiotensin II in the absence (n = 9) and presence (n = 9) of the RBD (mean ± SEM, replotted from Fig. 2D) or the presence of the ACE2 inhibitor MLN4760 (1 μM, nine pericytes from three animals, with no RBD). (B) Constriction in A between t = 29.67 and 30.00 min. (C) Response to 50 nM angiotensin II after 30-min incubation in (and continued perfusion with) artificial CSF (aCSF) containing RBD (0.7 mg/l, replotted from Fig. 2D) or additionally losartan (20 μM, 10 pericytes from three animals). (D) Constriction in C between t = 29.67 and 30.00 min.

consistent with the RBD inhibiting the generation of angiotensin-(1-7) by ACE2.

The reduction by the RBD of ACE2 activity may reflect ACE2 removal from the surface membrane, either by internalization or (as seen for the related SARS virus) by cleavage and release into the extracellular solution. To assess this, after 3 h of brain slices to solution containing or lacking the RBD (0.7 mg/l), we used IHC to quantify the amount of ACE2 that remained in the cell membrane (defined by overlap in location with PDGFRβ: Supplementary Fig. 3A and D). Incubation with the RBD reduced the surface membrane ACE2 level defined in this way (but not the PDGFRβ level) by 32% (P < 0.0001; Supplementary Fig. 3G and H). This figure is an underestimate because of the limited ability of immunohistochemistry to spatially distinguish ACE2 in the membrane from ACE2 internalized to an intracellular position that may be just under the cell membrane, especially in the processes of the pericytes which are too thin for antibody labelling and light microscopy to resolve any intracellular space with no PDGFRβ labelling (see PDGFRβ labelling of processes in Supplementary Fig. 3); indeed, the fact that the RBD produces a potentiation of the angiotensin II-evoked constriction which is similar to that produced by blocking ACE2 (Fig. 3A and B) implies that essentially all of the ACE2 is removed from the surface membrane Measuring the mean intensity of intracellular ACE2 labelling within pericyte somata (which was not feasible for the fine processes of pericytes) showed that the RBD evoked a reduction of level of 25% (P = 0.002; Supplementary Fig. 3E, F and I). This could reflect an RBD-evoked decrease of ACE2 synthesis and targeting of internalized ACE2 for degradation, or an overall loss of ACE2 from the cell as a result of RBD-evoked cleavage and ectodomain release. Crucially, however, the pharmacological data presented in this paper (Figs 1G, 2D, 3A and C and Supplementary Fig. 2A and C) imply a loss of functional ACE2 from the outer surface of pericytes and a resulting loss of Mas receptor-evoked dilation (opposing AT1R-mediated constriction) in response to angiotensin II.

With a view to reducing SARS-CoV-2-evoked capillary constriction and any associated reduction of microvascular blood flow, we tested whether the AT1 receptor blocker losartan prevented the constriction-potentiating effect of the RBD. Losartan completely blocked the angiotensin II-evoked constriction seen in the presence of the RBD (Fig. 3C and D).

In human SARS-CoV-2 infection it has been suggested that one pathological mechanism is a loss of pericytes caused by viral infection reducing their viability or their interactions with endothelial cells. In a transgenic model of pericyte loss (decreasing PDGFRβ signalling) it was found that endothelial cells upregulated von
Willebrand Factor (vWF) production, and thus produced a pro-thrombotic state, which could explain the coagulopathy seen in SARS-CoV-2 patients. However, exposing hamster brain slices to RBD (0.7 mg/l) for 3 h, in the absence or presence of 50 nM angiotensin II, had no significant effect on pericyte death as assessed by propidium labelling (Fig. 4A). Nevertheless, infection with the actual virus might have more profound effects on pericyte function or viability than does exposure to the RBD.

**Capillary constriction is potentiated by SARS-CoV-2 RBD in human capillaries**

To assess whether the potentiation of capillary constriction, characterized above in hamsters, also occurs in human capillaries, we employed brain slices made from live human brain tissue that was removed in the course of tumour removal surgery. Consistent with the similar binding of the SARS-CoV-2 RBD to human and hamster ACE2, we found that the RBD greatly potentiated the pericyte-mediated constriction evoked in human capillaries by 50 nM angiotensin II (Fig. 4B and C). SARS-CoV-2 binding would therefore be expected to decrease human cerebral blood flow assuming that, as in rodents, the largest resistance to flow within the brain parenchyma is provided by capillaries.

**Pseudovirus expressing SARS-CoV-2 spike protein evokes capillary constriction**

To check whether a viral stimulus more realistic than the RBD alone would also evoke pericyte-mediated capillary constriction, we constructed SARS-CoV-2 spike protein pseudotyped non-replicating HIV-1 virions (as previously described, see ‘Materials and methods’ section). After pre-incubating hamster brain slices with these virions, applying 50 nM angiotensin II evoked a constriction of capillaries at pericyte somata of ~11%, compared to the diameter seen in the absence of the virions (Fig. 4D). This is remarkably similar to the potentiated constriction seen when applying the RBD in Figs 2D and 4B. Plotting the capillary diameter as a function of distance from the pericyte soma (Fig. 4E) showed that the diameter at the soma was larger than that at a distance 10–15 μm from the soma in the absence of the virions, but was smaller than that at a distance of 10–15 μm in the presence of the virions. A similar variation of diameter with distance in the presence of a constricting agent has previously been shown to be consistent with the distribution of circumferential processes as a function of distance from the pericyte soma.

**Discussion**

The data presented above are consistent with the scheme shown in Fig. 4F and G. ACE2 expression in the brain appears to be largely on pericytes in both rodents and humans (some papers that did not use pericyte or vascular markers have also reported it on endothelial cells, neurons and astrocytes, however, endothelial and astrocyte labelling at the RNA level could reflect contamination with fragments of pericytes or smooth muscle cells). Binding of the SARS-CoV-2 RBD to ACE2 in pericytes leads to a decrease in effective surface membrane ACE2 activity, which could occur either as a result of ACE2 removal from the membrane (via internalization or cleavage and release into the extracellular solution) or due to occlusion of the angiotensin II binding site (we favour removal as the mechanism, because we detect a decrease in the amount of ACE2 in the surface membrane (Supplementary Fig. 3G) and because it is known that, for both the related SARS virus and for SARS-CoV-2, binding to ACE2 does not occlude the binding site for angiotensin II). This loss of ACE2 function leads to an increase in the local concentration of vasoconstricting angiotensin II and a decrease in the concentration of vasodilating angiotensin-(1-7) (note, however, that this postulated mechanism is based on the pharmacological experiments reported in Figs 1G, 2D, 3A and C and Supplementary Fig. 2A and C, and not on direct measurements of peptide concentrations, the local values of which at pericytes may not be reflected in the bulk concentrations in the solution perfusing the slice). The resulting activation of contraction via AT1 receptors in capillary pericytes reduces capillary diameter locally by ~12% when 50 nM angiotensin II is present. As most of the vascular resistance within the brain parenchyma is located in capillaries, this could significantly reduce cerebral blood flow (as occurs following pericyte-mediated constriction after stroke and in Alzheimer’s disease). In addition, constriction of some capillaries but not others can lead to tissue hypoxia even without a large reduction of blood flow. Presumably the same mechanisms could evoke a similar reduction of blood flow and oxygen delivery in other organs where pericytes (or other nearby cells) express ACE2 and AT1 receptors.

We have assumed in this discussion that the AT1 receptors that mediate SARS-CoV-2-evoked constriction are both located on pericytes. However, AT1 receptors are also expressed on other cell types (Supplementary Fig. 1A and B) and, although a direct pericyte contractility-regulating effect of angiotensin seems likely, we cannot rule out an indirect effect mediated by AT1Rs on another cell type. Furthermore, even if the AT1Rs are located on pericytes, it may not even be necessary for the ACE2 which is effectively inactivated by SARS-CoV-2 to be located on the same pericytes: depending on how far the angiotensin-(1-7) made by ACE2 can diffuse (i.e. how local its actions are), it is conceivable that removal of ACE2 from the membrane of other cells close to pericytes could also promote the vasoconstricting action of angiotensin II on the pericytes.

Constriction of capillaries by pericytes decreases cerebral blood flow in three ways. First, the reduction of capillary diameter increases the local flow resistance because, by Poiseuille’s law, resistance to the flow of a liquid is inversely proportional to the fourth power of diameter (e.g. if the 12% diameter reduction mentioned above occurred uniformly in the vasculature then the blood flow would be reduced by 40% [from (1–0.12)4 = 0.6]; however, pericytes occur only every 30–100 μm (depending on age) along capillaries, implying a less profound effect on resistance). Second, the presence of red blood cells results in the blood viscosity increasing dramatically at small diameters, so that even small pericyte-mediated constrictions can have a large effect. Third, complete stalling of blood flow in capillaries can occur as a result of neutrophils (which are less distensible than red blood cells) becoming stuck at narrow parts of the vessel, for example near constricted pericytes, and this also increases the reduction of blood flow produced by a small constriction. In the Supplementary Information we estimate that the first two of these factors would reduce overall flow by ~16%, to which neutrophil block may add another 5%. A combined reduction of cerebral blood flow by ~20% is expected to lead to cognitive impairment such as an inability to maintain attention and white matter damage. How long this reduction of blood flow lasts may depend on the time needed for the surface membrane ACE2 level to recover after SARS-CoV-2 infection, which may in turn depend on whether long-term damage is evoked in pericytes.
In order for SARS-CoV-2 to evoke pericyte-mediated capillary constriction (or to cause pericyte dysfunction that upregulates vWF production) the virus would need to bind to the ACE2 that is located in pericytes located on the opposite side of the endothelial cell barrier from the blood. Infection of brain pericytes by SARS-CoV-2 has been reported, raising the question of how the virus can access the pericytes. This might occur via initial infection of the nasal mucosa and movement from there up the olfactory nerve into the brain. Alternatively, movement of the S1 part of the spike protein across the blood–brain barrier by transcytosis has been reported.
crossing the endothelial cell layer may also occur via infection of monocytes (which express ACE2 highly and can cross endothelial cells) or via breakdown of the blood–brain barrier as a result of cytokines released as a result of lung inflammation.69 The reduction of blood flow produced by pericyte-mediated capillary constriction, together with any upregulation of vWF that may occur,49 will tend to promote clotting in the microvasculature. SARS-CoV-2 infection is associated with thrombus formation in large vessels that can be imaged, but it seems possible that thrombi of microvascular origin may add to this, and could perhaps even seed these larger clots. Together, capillary constriction and thrombus formation will reduce the energy supply to the brain and other organs, initiating deleterious changes that probably contribute to the long duration symptoms of ‘long Covid’. Indeed, the decrease of cerebral blood flow occurring during SARS-CoV-2 infection18,19 outlasts the acute symptoms.20

Our data suggest an obvious therapeutic approach, i.e. that the reduction of cerebral and renal blood flow that is observed in SARS-CoV-2 infection18–21 might be blockable using an AT1 receptor blocker such as losartan. A small clinical trial of the possible beneficial effects of losartan in SARS-CoV-2 infection reported no effect on hospitalization rate,73 but did not assess effects on organ blood flow or long-term outcome such as the incidence of ‘long Covid’. In contrast, a retrospective study48 concluded that angiotensin receptor blockers had beneficial effects on clinical outcome in COVID-19.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at Brain online.

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