In a mouse model of transient middle cerebral artery (MCA) ischemia, authors from the United States investigated the possible mechanisms through which severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike (S) protein influences acute ischemic stroke. Since the SARS-CoV-2 S protein binds to human angiotensin-converting enzyme-2 (ACE-2) receptors, researchers chose humanized ACE-2 knock-in (hACE2 KI) mice, expressing humanized ACE2 receptors. In their previous publication, the same research group showed downregulation of brain expression of ACE2, increased brain inflammation, and disruption of the renin-angiotensin-aldosterone system (RAAS) balance in hACE2 KI mice after injection of SARS-CoV-2 S protein. They, therefore, hypothesized that SARS-CoV-2 S protein could exacerbate stroke and cerebrovascular complications by disrupting the RAAS balance, increasing coagulation, and reducing fibrinolysis.

The authors pointed out the crucial role of the ACE-2 enzyme in the RAAS balance. ACE-2 degrades the bioactive form angiotensin II (Ang II), which can bind to the angiotensin type receptor 1 (AT1R), which is more abundant, or the angiotensin type receptor 2 (AT2R). Activation of the Ang II/AT1R axis pathway leads to vasoconstriction, thrombosis, and generation of reactive oxygen species. The Ang II/AT2R axis opposes the effects of AT1R activation through vasodilation, anti-inflammatory responses, and maintenance of hemostasis. SARS-CoV-2 S protein binds to ACE-2 and downregulates the ACE-2 receptor. This decreases Ang II degradation and increases activation of the AT1R, which is more abundant than AT2R and activates the RAAS destructive arm.

About the study

The authors investigated the effect of SARS-CoV-2 S protein in humanized ACE-2 knock-in (hACE2 KI) mice, which express humanized ACE2 receptors to replace mouse ACE2. Transient focal ischemia was induced by chemical thromboembolic occlusion of the middle cerebral artery (MCA) with ferric chloride (MCA/FeCl3model), where a clot forms and spontaneously recanalizes within a few hours. The recombinant SARS-CoV-2 S protein was injected intravenously seven days before induction of the stroke. Losartan, an AT1R blocker, was administered immediately after the injection of the recombinant S protein (10 mg/kg body weight). The relatively higher lipophilicity of losartan increases its ability to cross the blood-brain barrier.

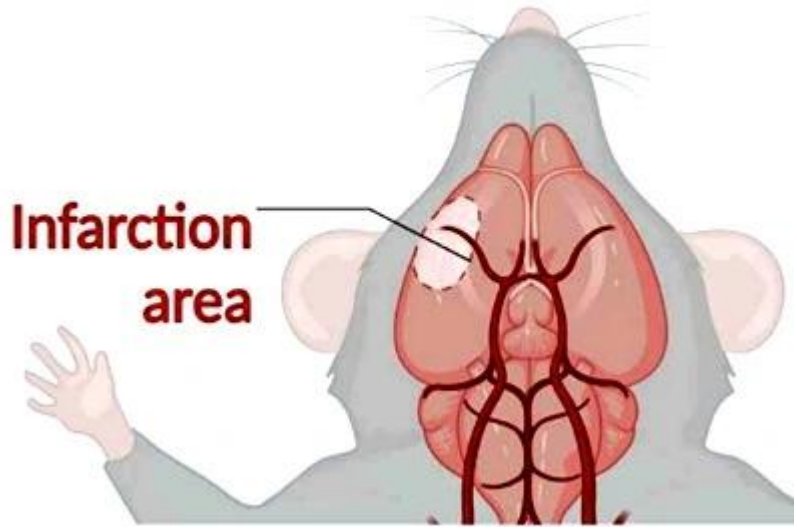
hACE2 KI mice were randomly assigned to four groups: sham, stroke, stroke + SARS-CoV-2 S protein injection, and stroke + SARS-CoV-2 S protein injection + losartan. Sham animals were exposed to the MCA thromboembolic surgery without FeCl3 treatment.

24 hours after transient focal ischemia, brain infarct volume was measured. Before SARS-CoV-2 S protein injection and 24 hours after MCA occlusion, animal recognition memory and learning functions were assessed by Novel Object Recognition testing. Cerebral blood flow was measured with the Laser Speckle Imaging System at 1, 2, 3, 6, and 24 hours after MCA occlusion.

Tissue factor III (TF-III) and plasminogen activator inhibitor-1 (PAI-1) were measured by immunoblotting. TF-III is expressed and activated by endothelial cells, vascular smooth

muscle cells, and monocytes. It is commonly released after the disruption of the endothelial matrix and activates the coagulation cascade, leading to thrombin activation. PAI-1 is located in the extracellular matrix and in the extracellular exosome. It inhibits the activation of the endogenous tissue-type plasminogen activator and the conversion of plasminogen to plasmin, thus hindering fibrin degradation.

The effects of SARS-CoV-2 S protein were also studied in cultures of primary human brain microvascular endothelial cells (HBMECs) treated with recombinant SARS-CoV-2 S protein (100 µM) with or without losartan (100 µM) for 24 hours. After 24 hours, HBMECs were either maintained under normoxic conditions or placed in a hypoxia chamber for 6 hours.



Results

Cognitive impairment

The Novel Object Recognition Test, which assesses the memory and learning ability of hACE2 KI mice, showed no differences between the groups in terms of total distance traveled. However, the mice with transient focal ischemia showed a significant decrease in the number of entries into the zone containing the novel object and the time spent interacting with the novel object.

Pretreatment with SARS-CoV-2 S protein further decreased the time spent interacting with the novel object. In contrast, the AT1R blocker losartan, administered immediately after injection of recombinant S protein, significantly improved cognitive function in hACE2 KI mice, as evidenced by increases in the time spent with the novel object and in the number of

entries into the zone containing the novel object.

RAAS Imbalance

The main finding of this study is that the SARS-CoV-2 S protein disrupted the renin-angiotensin-aldosterone system (RAAS) balance in the brain vasculature.

Ischemic insult increased the AT1R expression in the brain by 2-fold. SARS-CoV-2 S protein injection given to hACE2 KI mice seven days before transient focal brain ischemia increased AT1R expression by 4-fold. It also downregulated the ACE2 gene expression and the RAAS protective arm AT2R. AT1R blocker losartan, administered immediately after S protein injection, restored ACE2 gene expression downregulated by the recombinant S protein.

These results were confirmed in cultures of primary human brain microvascular endothelial cells treated with recombinant SARS-CoV-2 S protein and exposed to hypoxia. Even under normoxic conditions, the S protein increased AT1R expression and decreased AT2R expression. These effects were further enhanced under hypoxic conditions. According to these results, the S protein increased AT1R expression in the brain's endothelial cells at the expense of the protective AT2R. Losartan reduced the effects induced by the SARS-CoV-2 S protein, but insignificantly.

Brain inflammation, infarct volume, and cerebral blood flow

The transient focal ischemia increased inflammation in both brain hemispheres (the affected and the contralateral). Importantly, administration of SARS-CoV-2 S protein seven days before induction of the stroke significantly increased infarct volume and inflammation, as evidenced by increased nuclear factor kappa B (NF-kB), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 gene expression in the brain. The AT1R blocker losartan, administered immediately after the recombinant S protein injection, significantly reduced infarct volume.

In cultures of primary human brain microvascular endothelial cells, treatment with recombinant SARS-CoV-2 S protein significantly increased the expression of TNF-α under normoxic conditions and further increased inflammation under hypoxic conditions.

Cerebral blood flow was decreased in the brain hemisphere affected by transient focal ischemia compared to the contralateral unaffected hemisphere. However, it was restored within six hours after a transient thromboembolic occlusion of the MCA. Treatment with SARS-CoV-2 S protein seven days before stroke induction significantly intensified a

decrease in cerebral blood flow and delayed recanalization to over 6 hours. The AT1R blocker losartan significantly decreased the effects of recombinant SARS-CoV-2 S protein by reducing recanalization time.

Coagulation

Transient focal ischemia upregulated TF−III and PAI-1 expression in the brain homogenate of hACE2 KI mice. Treatment with recombinant SARS-CoV-2 S protein before stroke further increased TF−III and PAI-1 expression.

These results were confirmed in cultures of primary human brain microvascular endothelial cells treated with recombinant SARS-CoV-2 S protein, which favored clot formation by increasing coagulation and decreasing fibrinolysis in hypoxic conditions. According to these results, the S protein increased coagulation by increasing TTF-III and PAI-1 expression. Losartan decreased hypercoagulation induced by the S protein.

Conclusion

This study provides new evidence that SARS-CoV-2 S protein injection given to hACE2 KI mice seven days before transient focal brain ischemia disrupted the RAAS balance by increasing Ang II/AT1R signaling in the brain's cells at the expense of the Ang II/AT2R protective arm.

Recombinant SARS-CoV-2 S protein increased coagulation and decreased fibrinolysis, which worsened ischemic stroke outcomes, as evidenced by increased infarct size, reduced cerebral blood flow, and increased cognitive impairment. Losartan, an AT1R blocker, restored RAAS balance and reduced recombinant SARS-CoV-2 S protein-induced effects.

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