Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Spike-mediated ACE2 down-regulation was involved in the pathogenesis of SARS-CoV-2 infection

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SUMMARY

The ongoing global pandemic of Coronavirus disease 2019 (COVID-19) poses a serious threat to human health, with patients reportedly suffering from thrombus, vascular injury and coagulation in addition to acute and diffuse lung injury and respiratory diseases. Angiotensin converting enzyme 2 (ACE2) as the receptor for SARS-CoV-2 entry, is also an important regulator of renin-angiotensin system (RAS) homeostasis, which plays an unsettled role in the pathogenesis of COVID-19. Here, we demonstrated that SARS-CoV-2 Spike protein activated intracellular signals to degrade *ACE2* mRNA. The decrease of ACE2 and higher level of angiotensin (Ang) II were verified in COVID-19 patients. High dose of Ang II induced pulmonary artery endothelial cell death *in vitro*, which was also observed in the lung of COVID-19 patients. Our finding indicates that the downregulation of ACE2 potentially links COVID-19 to the imbalance of RAS.

Introduction

Coronavirus disease 2019 (COVID-19) outbreak caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is still a global pandemic [1]. Angiotensin converting enzyme 2 (ACE2) is the major recognized cell-surface receptor enabling cellular entry of SARS-CoV-2 and SARS-CoV [2,3], widely expressed in lung, cardiovascular system, gut, kidney, central nervous system and adipose tissue [4]. As a zinc metalloproteinase, ACE2 plays an important role in maintaining the homeostasis of renin-angiotensin system (RAS) [5,6]. Angiotensin converting enzyme (ACE) cleaves angiotensin (Ang) I to generate Ang II [7], while ACE2 cleaves Ang I to generate Ang 1–9 and Ang II to generate Ang 1–7. The ACE-Ang II-AT1R axis leads to vasoconstriction, water and sodium retention, inflammatory response, oxidative stress and cardiovascular remodeling [8]. In contrast, ACE2-Ang1–7-MAS axis antagonizes the ACE-AngII-AT1R axis, thus exerting the functions of vasodilation, natriuretic, diuretic, anti-inflammatory, anti-oxidative stress and anti-cardiovascular remodeling [9].

SARS-CoV-2 infection has been reported to activate RAS system and destroy endothelial cells, inducing cardiovascular dysfunction, oxidative stress and thrombosis [10]. Several clinical cohort studies have also found that cardiovascular disease like myocardial damage were more common in COVID-19 patients [11–13]. Increased circulating Ang II was also found in the serum of COVID-19 patients than that in healthy controls [14]. These findings suggest that SARS-CoV-2 infection more usually generates the cardiovascular diseases in COVID-19 patients, while the underlying mechanisms remain unclear.

Abbreviations: COVID-19, Coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; RAS, renin-angiotensin system; Ang I, angiotensin I; Ang II, angiotensin II; AT1R, Angiotensin II Type 1 Receptor; AT2R, Angiotensin II Type 2 Receptor; HPAEC, Human pulmonary artery endothelial cells; c-casp3, caspase-3; VWF, Von Willebrand Factor; TCID50, 50% tissue culture infectious dose; WB, western blotting; IHC, Immunohistochemistry; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin; rhACE2, recombinant soluble form of human ACE2; SPP, Spike protein-pseudotyped; DAB, 3,3′-diaminobenzidine; ACE, Angiotensin converting enzyme; ACE2, Angiotensin converting enzyme 2.

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Given the versatility of ACE2 in SARS-CoV-2 infections, we investigate the crucial roles of ACE2 in the cardiovascular pathology of COVID-19. Here, we show that SARS-CoV-2 Spike protein downregulates ACE2 expression, thus causing cardiovascular stress by failure to cleave Ang II and subsequently leading to lung injury and cardiovascular complication-associated mortality.

Results

SARS-CoV-2 downregulated ACE2 expression through the Spike protein

ACE2 is the receptor of SARS-CoV-2 entering host cells [15], and its expression is greatly reduced after viral infection. This was confirmed in both Vero-E6 and hACE2-HeLa cells (Fig. 1a). To dissect which proteins from SARS-CoV-2 were responsible for the decrease in ACE2, 26 viral proteins were transfected in hACE2-HeLa cells and ACE2 protein level was detected by WB. Intriguingly, significant ACE2 reduction was detected only in Spike-expressed hACE2-HeLa cells rather than other viral protein-expressed cells (Fig. 1b). In addition, Spike-induced ACE2 reduction is dose-dependent in hACE2-HeLa cells (Fig. 1c). hACE2-HeLa cells were constructed by inserting LTR from retroviral vector to initiate endogenous ACE2 expression [16]. Spike proteins can also reduce exogenous ACE2 expression form plasmid transfection in hACE2–293T cells (Fig. 1c). The expression of ACE2 was also lower in the bronchial epithelial cells in COVID-19 patient than that in health control (Fig. 1d). Histopathological analysis also showed that ACE2 expression was significantly reduced in the bronchial epithelial cells of SARS-CoV-2 infected mice (Fig. 1e). These results clearly indicated that SARS-CoV-2 infection can reduce the ACE2 expression at the protein level *in vivo*.

SARS-CoV-2 Spike consists S1 and S2 subunits. S1 subunit was responsible for binding with ACE2, and S2 mediated virus fusion with host cell [17]. To explore which subunit of Spike reduced ACE2 expression, Spike 1/ Spike 2 and ACE2 plasmids were co-transfected in HEK-293T cells, and ACE2 expression was detected by WB. Significant ACE2 reduction was found in both Spike and Spike 1 expressed HEK-293T cells rather than Spike 2 expressed HEK-293T cells (Fig. 1f, g). These results suggest that SARS-CoV-2 infection can decrease the protein level of ACE2 through Spike S1 subunit.

Spike mediated ACE2 protein reduction is independent of endocytosis

S1 subunit rather than S2 subunit mediated ACE2 decline suggests that ACE2 may have been degraded during endocytosis after binding with Spike. Virus receptors will be internalized into the endosome and degraded during virus entry process [18]. To verify whether Spike-mediated ACE2 reduction is dependent on virus endocytosis, SARS-CoV-2 Spike pseudovirus that only mimics the virus entry process was used to infect hACE2-HeLa cells. Unexpectedly, SARS-CoV-2 Spike pseudovirus infection does not downregulate ACE2 levels as the live virus infection does (Fig. 2a, b). We further verify this observation using hACE2-HeLa cells treated with 1 μg/ml Spike protein for 24 h *in vitro*, and no significant changes of ACE2 protein expression were observed in this assay (Fig. 2c). Furthermore, we detected the protein levels of ACE2 at 2 and 24 h post infection of live SARS-CoV-2 virus. Consistently, at the entry phase without detected viral proteins, the protein levels of ACE2 were not affected. When Spike proteins were intracellularly accumulated at 24 h, the ACE2 proteins were decreased significantly (Fig. 2d). These results suggested that while the extracellular signals stimulated by Spike did not affect the expression of ACE2, intracellular SARS-CoV-2 Spike protein accumulation potentially downregulated ACE2 expression.

ACE2 reduction is not achieved through protein degradation pathways

Proteasome and lysosome are two important organelles that degrade the protein in mammalian cells [19]. We tested whether Spike-mediated reduction of ACE2 is achieved through protein degradation. HEK-293T cells were treated with proteasome inhibitor MG132, lysosomal inhibitor E64D and bafilomycin A1, or DMSO as control after co-transfected with Spike and ACE2. Interestingly, the ACE2 protein was reduced in the presence of these inhibitors in Spike-expressed cells (Fig. 3a). Similar results can also be observed on SARS-CoV-2 infected hACE2-HeLa cells. Although MG132 and E64D inhibited the infection of SARS-CoV-2, they failed to rescue the expression of ACE2 (Fig. 3b). Thus, Spike mediated ACE2 protein reduction was independent of the protein degradation.

Spike activates intracellular signals to degrade ACE2 RNA

Next, we wondered whether Spike protein can reduce *ACE2* at RNA levels. Vero-E6 cells were infected with 0.3 MOI SARS-CoV-2 and total RNA was extracted for RT-qPCR. Unexpectedly, the RNA levels of *ACE2* were significantly reduced in SARS-CoV-2 infected cells (Fig. 4a). This result was further confirmed in hACE2-HeLa cells (Fig. 4b). Furthermore, RNA-seq analysis also revealed the reduction of *ACE2* RNA in SARS-CoV-2 infected cells compared with mock cells (Fig. 4c). Overexpression of Spike can also reduce the level of *ACE2* mRNA (Fig. 4d). Thus, Spike mediated both endogenous and exogenous ACE2 reduction, indicating that the reduction of *ACE2* RNA was not regulated at transcriptional levels.

High Ang II induces pathological damage

Given the critical regulatory role of ACE2 in RAS, we went on to test whether the RAS is dysregulated in COVID-19 patients. Serum samples from 30 SARS-CoV-2-infected patients and 11 healthy donors as control were collected. We found that the levels of Ang II were significantly increased in COVID-19 patients compared with healthy controls (Fig. 5a). Consistent with previous report [20], the treatment with high level of Ang II induced the death of pulmonary artery endothelial cells HPAEC, but did not damage lung epithelial cells Calu3 (Fig. 5b & Supplementary Fig. S1a). Indeed, treatment with Ang II induced AT1R but not AT2R expression in HPAEC cells (Fig. 5c). Combination of Ang-II and AT1R caused vasoconstriction, cell proliferation, inflammatory responses, blood coagulation, and extracellular matrix remodeling[21]. To confirm the involvement of endothelial injury, we examined lung tissues from a COVID-19 patient and an age-matched control for evidence of apoptosis using cleaved caspase-3 (c-casp3). C-casp3 positive endothelial cells (VWF^{+}) was only found in the lung of COVID-19 patient but not the control (Fig. 5d). The increased VWF expression in the lung of COVID-19 patient also suggested vascular damage. HE staining revealed a large number of inflammatory cells infiltrated around the blood vessels, destroying the integrity of the blood vessel structure and forming vasculitis (Supplementary Fig. S1b). Thus, COVID-19 disease is accompanied by endothelial cell damage, which affects normal cardiovascular function.

Discussion

Clinical studies have found that cardiovascular disease is more common in COVID-19 patients [22], while hypertension and cardiovascular patients are more likely to develop severe disease after contracting the SARS-CoV-2 [23]. The pivotal mechanisms mediating the detrimental effects of SARS-CoV-2 on cardiovascular disease remain largely unknown. Our study shows that SARS-CoV-2 Spike not only binds to ACE2 to mediate virus entry but also

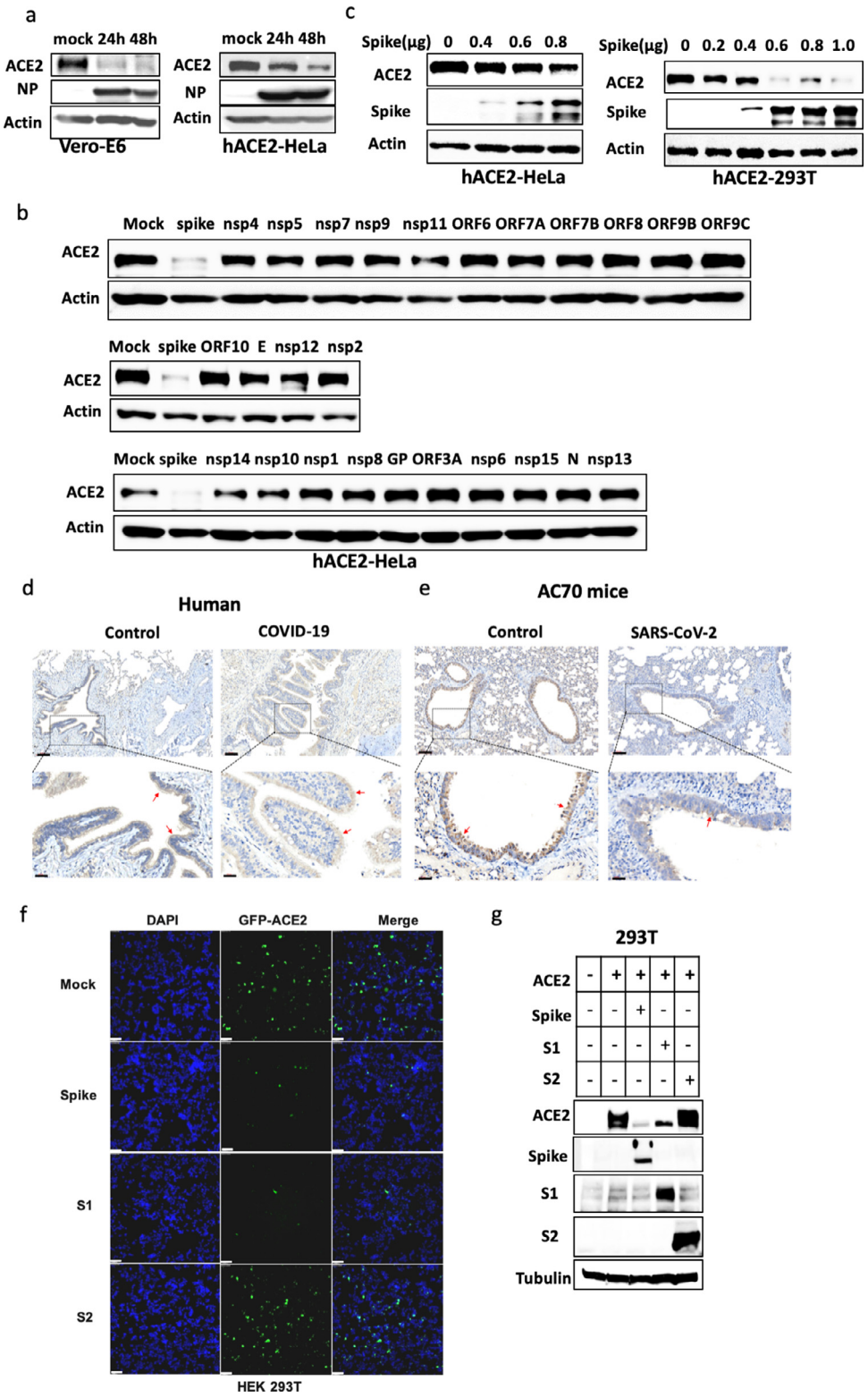

Fig. 1. SARS-CoV-2 Spike downregulated the expression of ACE2. a. ACE2 was downregulated during SARS-CoV-2 infection. Vero-E6 and hACE2-HeLa cells were infected with 0.3 MOI SARS-CoV-2 for 1h, washed with PBS, and replaced with fresh media for 24h and 48h. The cell lysates were analyzed by western blotting using indicated antibodies. b. SARS-CoV-2 Spike was responsible for ACE2 reduction. hACE2-HeLa cells were transfected with expression vectors of NSP4, NSP5, NSP7, NSP9, NSP11, ORF6, ORF7A, ORF8, ORF9B, ORF9C, ORF10, E, NSP12, NSP2, NSP14, NSP10, NSP1, NSP8, GP, ORF3A, NSP6, NSP15, N, NSP13. The cell lysates were analyzed by western blotting using ACE2 antibody and Actin was used as the loading control. c. hACE2-HeLa and hACE2-293T cells were transfected with different amounts of SARS-CoV-2 Spike expression vector. The cell lysates were analyzed by western blotting using indicated antibodies. d. Immunohistochemistry staining of ACE2 in the lungs of healthy control or severe COVID-19 patient. Scale bars: 90 μm. The lower row shows the enlarged images of the bronchi areas above. Scale bars, 20 μm. The red arrow indicates ACE2 staining on bronchial epithelial cells. e. Immunohistochemistry staining of ACE2 in the lungs of control or AC70 transgenic mice infected with SARS-CoV-2. Scale bars: 90 μm. The lower row shows the enlarged images of the bronchi areas above. Scale bars, 20 μm. The red arrow indicates ACE2 staining on bronchial epithelial cells. f. HEK-293T cells were co-transfected with 1 μg of GFP-ACE2 expression vector and 1 μg different Spike, Spike 1 or Spike 2 plasmid for 24h. Then the cells were subjected to microscopy. The green signal shows the expression level of ACE2, and DAPI was used to stain nuclei. Scale bars: 20 μm. g. HEK-293T cells were co-transfected with 1 μg of ACE2 expression vector and 1 μg different Spike, Spike 1 or Spike 2 plasmid for 24h. The cell lysates were analyzed by western blotting using indicated antibodies.

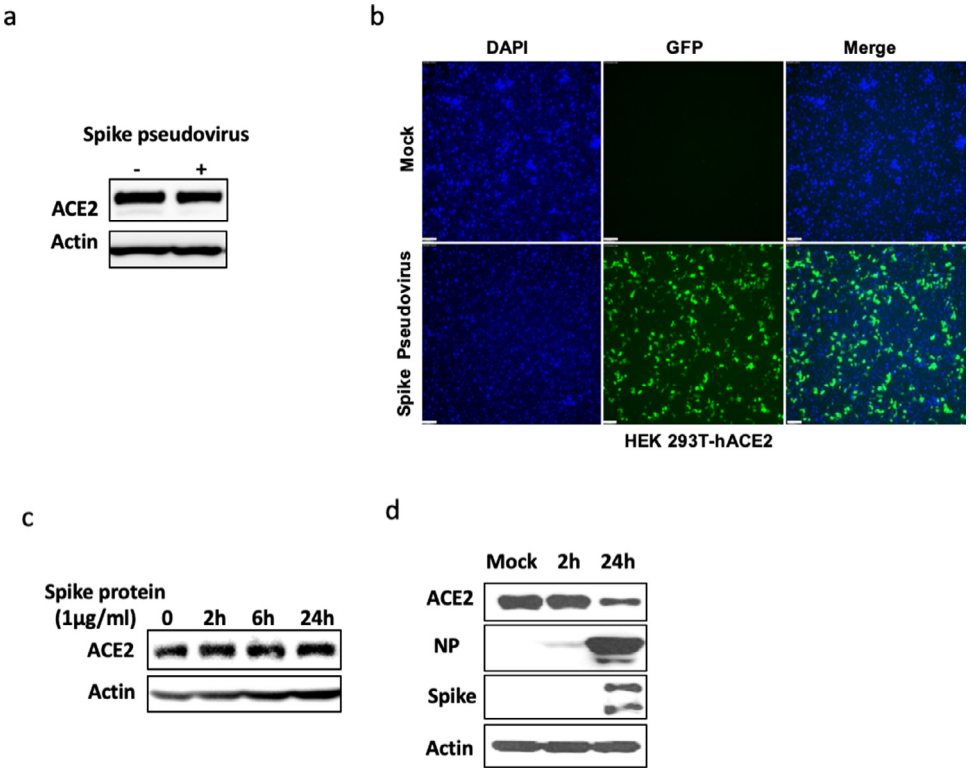

Fig. 2. Spike mediated ACE2 protein reduction is independent of endocytosis. a. hACE2-HeLa cells were infected with SARS-CoV-2 Spike pseudovirus for 48 h. The cell lysates were analyzed by western blotting using indicated antibodies. b. hACE2-HeLa cells were infected with GFP-bearing SARS-CoV-2 Spike pseudovirus for 48 h. Then the stimulated cells were subjected to microscopy. The green signal shows pseudovirus-infected cells, DAPI was used to stain nuclei. Scale bars: 20 μm. c. hACE2-HeLa cells were stimulated with SARS-CoV-2 spike protein for different amount of time as indicated. The cell lysates were analyzed by western blotting using indicated antibodies. d. hACE2-HeLa cells were infected with 0.3 MOI SARS-CoV-2 for 1 h, washed with PBS, and replaced with fresh media for 2 and 24 h. The cell lysates were analyzed by western blotting using indicated antibodies.

down-regulated the expression of ACE2, indicating that the down-regulation of ACE2 potentially links COVID-19 to its imbalance of RAS.ACE2 downregulation has also been observed in some other viral infections, such as influenza and SARS-CoV [24,25]. A recent study also found that SARS-CoV-2 infection down-regulated ACE2 expression, and found that ACE2 links COVID-19 to its metabolic complications, while ACE2 activators Imatinib and methazolamide improve metabolic disorders under SARS-CoV-2 infection [26]. However, these studies do not explain the molecular mechanism of ACE2 downregulation. A recent study found that Spike pseudovirus infected hamsters and HPAEC cells reduced ACE2 expression [27]. But we were unable to replicate that the expression of ACE2 can be down-regulated after Spike pseudovirus infection in HeLa-ACE2 cells. No significant changes of ACE2 protein expression were also observed in Spike protein treated HeLa-ACE2 cells. This may be because the cell lines we used are different. Our study found that SARS-CoV-2 Spike protein activates intracellular signals to reduce *ACE2* RNA, possibly altering *ACE2* RNA modification to affect its stability. More detailed mechanisms require future studies. Moreover, the decrease of ACE2 was verified in SARS-CoV-2 infected patient and mice. Finally, the decrease of ACE2 would lead to an increase of Ang II secretion, which affected the normal endothelial cell function and aggravated cardiovascular disease (Fig. 6).

Our previous study found that SARS-COV-2 infected lung epithelial cells induced high expression of inflammatory cytokines, and cytokines such as IFN and IL-6 can up-regulate ACE2 expression [28]. In contrast, the expression of ACE2 was down-regulated in SARS-COV-2 infected hypoinflammatory cells such as Vero-E6 and Hela-ACE2, suggesting that ACE2 was down-regulated in the truly infected cells, while the expression of ACE2 was up-regulated in bystander cells. Thus, the bystander cells expressing high levels of ACE2 were more susceptible to SARS-CoV-2. While SARS-CoV-2 hijacks ACE2 to facilitate its entry into host cells and then destroys host RAS by down-regulating ACE2 in the infected target cells.

As the main receptor mediating SARS-CoV-2 entry, targeting ACE2 has become one of the therapeutic strategies for COVID-19 [29]. ACE2-targeting monoclonal antibody 3E8 has been shown to broadly block the invasion of multiple coronaviruses, including SARS-CoV, SARS-CoV-2 mutant variants, without markedly affecting the physiological activities of ACE2 or causing severe toxicity in ACE2 transgenic mice [30]. APN01, a recombinant soluble form of human ACE2 (rhACE2), was reported to be effective in blocking viral infection and protecting the lung from pathological damage [31]. This peptide drug was slated for a multicenter trial in Europe (NCT04335136). A similar rhACE2 drug was applied for clinical trials in China, but it was subsequently withdrawn (NCT04287686) [32]. Overall, ACE2 based drugs represent a double-edged sword in coronavirus infection [33]. Low expression of ACE2 promote the progression of Systemic hypotension, hypokalemia and lung injury, aggravating the severity of the disease [34–38]. These data suggested that possible dysregulation of patient's RAS should be noted when targeting ACE2 for COVID-19 therapy.

Materials and methods

Ethics statement

This study was reviewed and approved by the Medical Ethical Committee of Shenzhen Third People's Hospital (2021–030).

One COVID-19 patient in this study was from the Shenzhen Third People's Hospital. Control lung tissues were from normal tissues far from the tumor of lung adenocarcinoma patients. Series

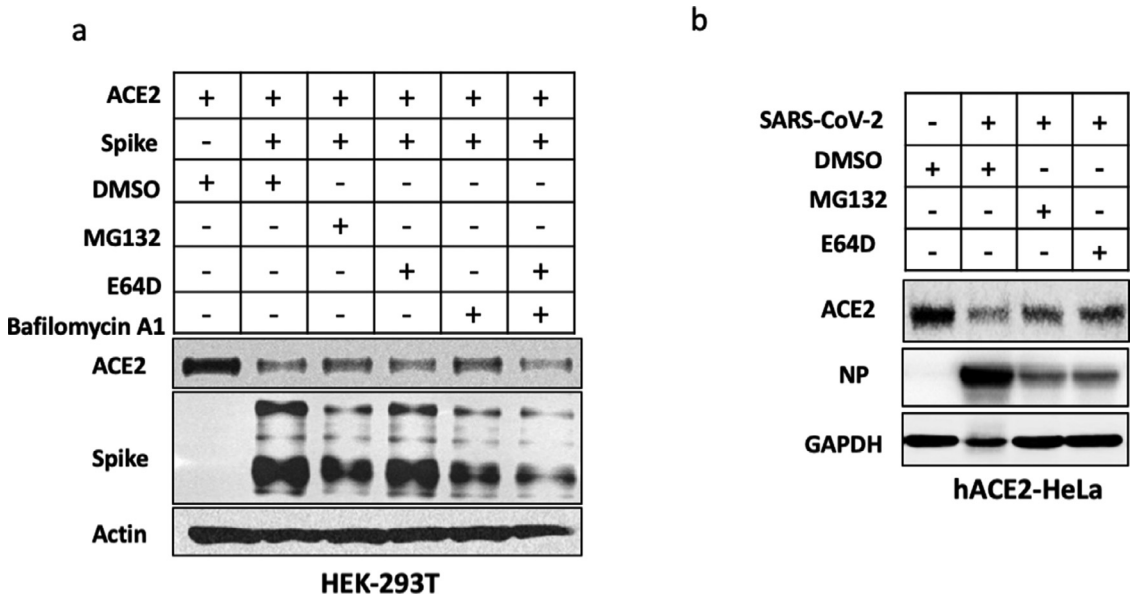

Fig. 3. Spike-mediated ACE2 reduction does not go through the protein degradation pathway. a. HEK-293T cells were co-transfected with expression vectors of Spike and ACE2 for 24 h, then treated with MG132(5 μM), E64D (10 μM) or Bafilomycin A1(200 nM) for 2 h before samples collection. The cell lysates were analyzed by western blotting using indicated antibodies. b. hACE2-HeLa cells were infected with SARS-CoV-2 for 24 h, then incubated with MG132(5 μM) / E64D (10 μM) or DMSO for 4 h. The cell lysates were analyzed by western blotting using indicated antibodies.

plasma were obtained from COVID-19 patients who had positive RT-PCR test results during hospitalization.

Cells, plasmids and reagents

Vero-E6 cells, hACE2-HeLa, hACE2–293T and HEK-293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% FBS (Gibco), 100 U/mL Penicillin-Streptomycin. Calu3 cells were cultured in Modified Eagle Medium (MEM, Gibco) supplemented with 20% FBS (Gibco), 100 U/mL Penicillin-Streptomycin (Gibco). HPAEC were purchased from ATCC and cultured in Medium 200 supplemented with Low Serum Growth Supplement (LSGS) containing FBS.

SARS-CoV-2 plasmids (NSP4, NSP5, NSP7, NSP9, NSP11, ORF6, ORF7A, ORF8, ORF9B, ORF9C, ORF10, E, NSP12, NSP2, NSP14, NSP10, NSP1, NSP8, GP, ORF3A, NSP6, NSP15, N, NSP13) were kindly provided by professor Nevan J. Krogan (University of California San Francisco). SARS-CoV-2 Spike (VG40589-NF), Spike 1 (VG40591-CH), Spike 2 (VG40590-CY) were purchased from Sino Biological. MG132 (S2619) and E64D (S7393) were obtained from Selleck Chemicals. Angiotensin II (ALX-151–039-M005) was purchased from Cayman.

SARS-CoV-2 preparation and infection

All experiments involving SARS-CoV-2 infection were carried out in the biosafety level-3 (BLS-3) laboratory of Shenzhen Third People's Hospital. SARS-CoV-2 isolate SZTH-003 was sourced from a COVID-19 patient. The genomic sequence of SZTH-003 has been deposited in the Global Initiative of Sharing All Influenza Data (GISAID, EPI_ISL_406,594). SARS-CoV-2 was amplified once in Vero-E6 cells and viral stocks were contained at −80 °C. For Vero-E6 cells and hACE2-HeLa cells infection, virus (MOI=0.3) was poured on cells directly and incubated for 1 hour for viral adsorption at 37 °C 5% CO2. Subsequently, infection media was removed and cells were incubated in 2% FBS media in 37 °C 5% CO2 condition and harvested for analysis at indicated time points.

For titration of the 50% tissue culture infectious dose (TCID50) of SARS-CoV-2, Vero-E6 cells were plated in 96-well plates at 20, 000 cells per well in DMEM containing 5% FBS, Penicillin/Streptomycin, L-Glutamine, and 15 mM HEPES (Life Technologies). The cells were incubated overnight in a 5% CO2 environment at 37 °C, washed once with PBS and then cultured in serum-free DMEM containing Penicillin/Streptomycin, L-Glutamine, 15 mM HEPES and 1 μg/mL TPCK-treated trypsin. A 10-fold initial dilution of samples with one freeze-thaw cycle was made in octuplicate wells of the 96-well plates followed by 6 serials 10-fold dilutions. The last row served as negative control without addition of any sample. After a 4-day incubation, the plates were observed for the presence of cytopathogenic effect (CPE) using an inverted optical microscope. Any sign of CPE was categorized as a positive result. The endpoint titers were calculated by means of a simplified Reed & Muench method.

Mice

The animal experiments were conducted in the BLS-3 animal facility approved for the studies of SARS-CoV-2. The experiment protocol has been approved by the Animal Ethics Committee of the Second Military Medical University. AC70 CAG-hACE2 transgenic mice (Shanghai Model Organisms, NM-TG-200,002) (6–8 weeks of age, male) were intranasal infected with 20 μl of SARS-CoV-2 (1×10^4 PFU). On the 4th day of infection, mice in each group were sacrificed and their lungs were removed and fixed with formalin for 48 h and prepared for paraffin embedding and subsequent IHC staining.

Generation of pseudotyped lentivirus

SARS-CoV-2 spike (S) glycoprotein plasmid were kindly provided by professor Ping Zhao (Second Military Medical University, Shanghai, China). HEK-293T cells were grown in DMEM containing 10% FBS and co-transfected with HIV Gag/Pol, HIV rev, plenti-EGFP and the SARS-CoV-2 spike expression plasmids using Lipofectamine 2000 reagent (Thermo Fisher). The supernatant with produced virus (Spike protein-pseudotyped (SPP) or (VSV-G lentivirus)) was harvested at 48 h post transfection, clarified by

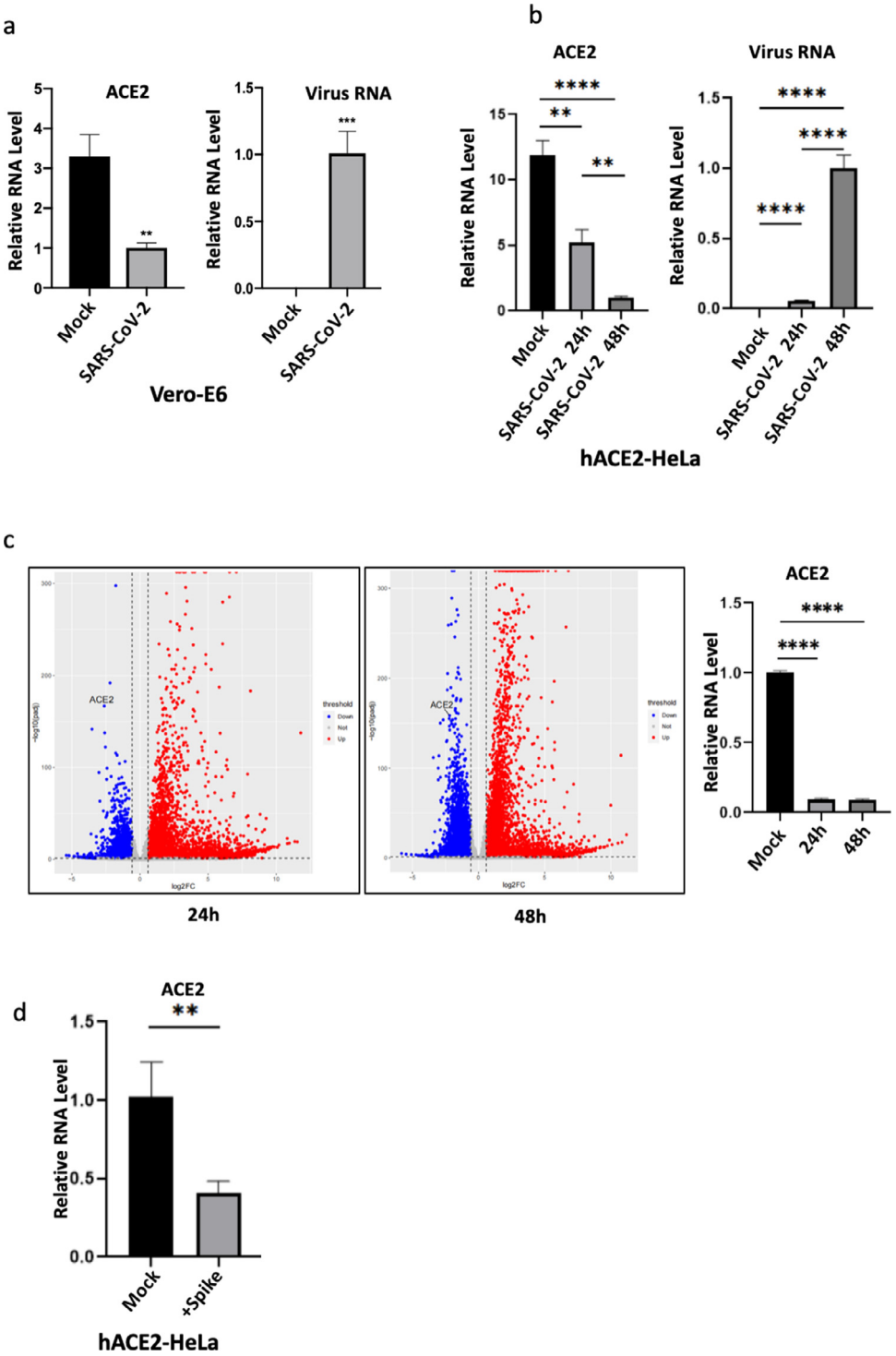

Fig. 4. Spike activates intracellular signals to degrade ACE2 RNA. a. Vero-E6 cells were infected with 0.3 MOI SARS-CoV-2 for 2 h, washed with PBS and replaced with fresh media for 24 h. Total RNA was extracted for RNA-seq. Paired *t*-test was used in RT-qPCR analysis ($^{***}P < 0.001$). b. hACE2-HeLa cells were infected with 0.3 MOI SARS-CoV-2 for 1 h, washed with PBS, and replaced with fresh media for 24 h and 48 h. The levels of ACE2 and viral RNA in hACE2-HeLa cells were determined by qPCR. ($^{**}P < 0.01$, $^{****}P < 0.0001$). c. hACE2-HeLa cells were infected with SARS-CoV-2 for 24 h and 48 h. Total RNA was extracted for RNA-seq. The volcano shows the levels of *ACE2* RNA were significantly reduced (left:24 h and right: 48 h). d. hACE2-HeLa cells were transfected with 1 μg SARS-CoV-2 Spike expression vector. The levels of ACE2 were measured by qPCR. ($^{**}P < 0.01$).

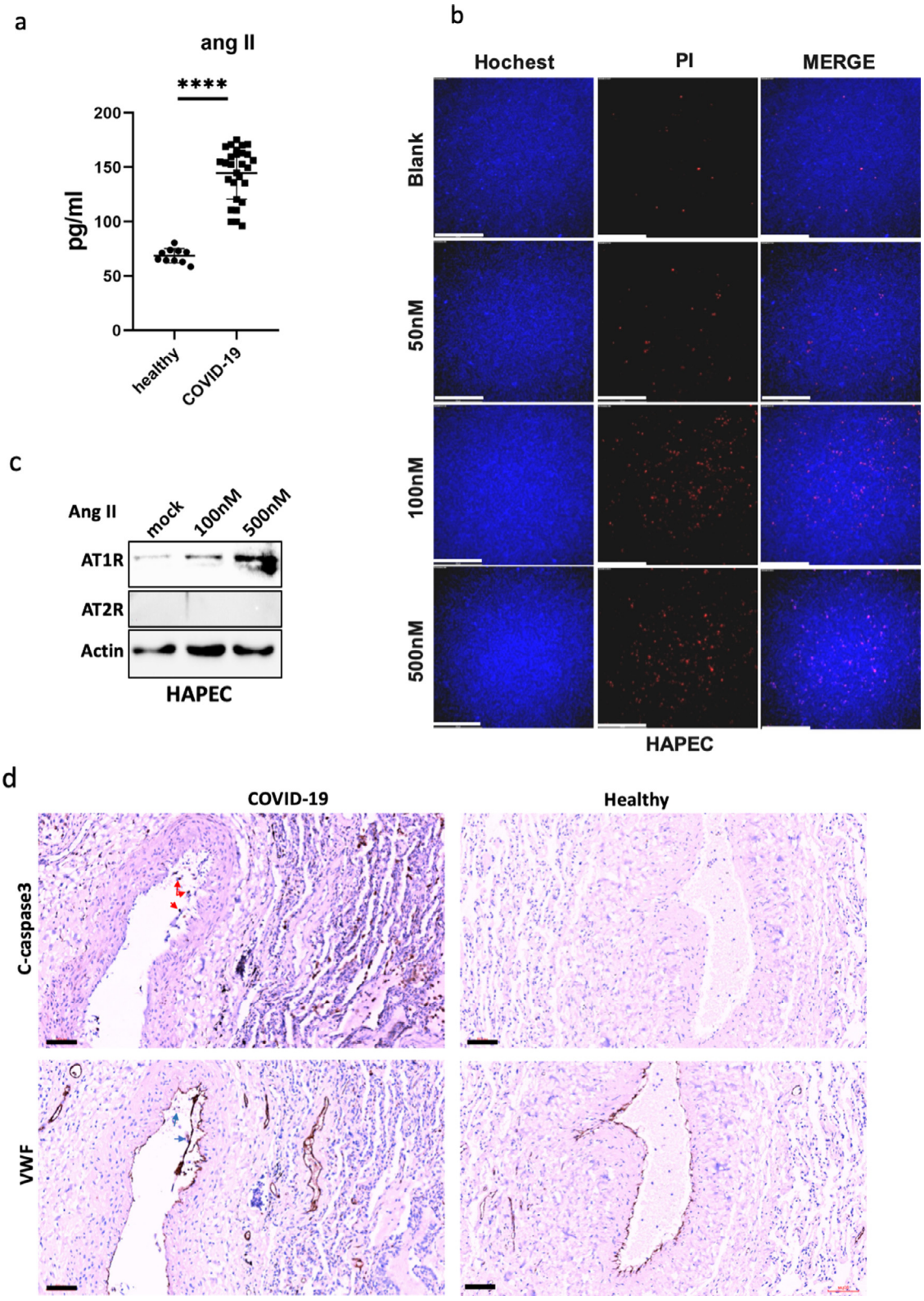

Fig. 5. High Ang II induces pathological damage. a. Higher level of Angiotensin II in severe COVID-19 patients. Angiotensin II levels in plasma of healthy controls ($n = 11$) and COVID-19 patients ($n = 30$). Un-paired t-test was used in analysis (***, $P < 0.001$). b. Ang II induced Lung endothelial cell injury through activating Angiotensin II receptor I. HPAEC cells were stimulated with different concentrations (50 nM, 100 nM and 500 nM) of Ang II for 48 h. Then the stimulated cells were stained with PI dye and Hochest, and then subjected to microscopy. Scale bars: 100 μm. c. HPAEC cells were stimulated with different concentration of Ang II as indicated for 48 h. The cell lysates were analyzed by western blotting using indicated antibodies. d. Immunohistochemistry staining of cleaved caspase-3 (c-casp3) and VWF in lung of severe COVID-19 and age-matched control. Scale bars, 20 μm. The apoptosis marker c-casp3 and Vascular Endothelial Cells marker VWF were analyzed by immunohistochemistry on serial sections of severe COVID-19 and controls. The red arrows indicate the cells in the blood vessels that have undergone apoptosis, the blue arrow shows the VWF positive cells at that location.

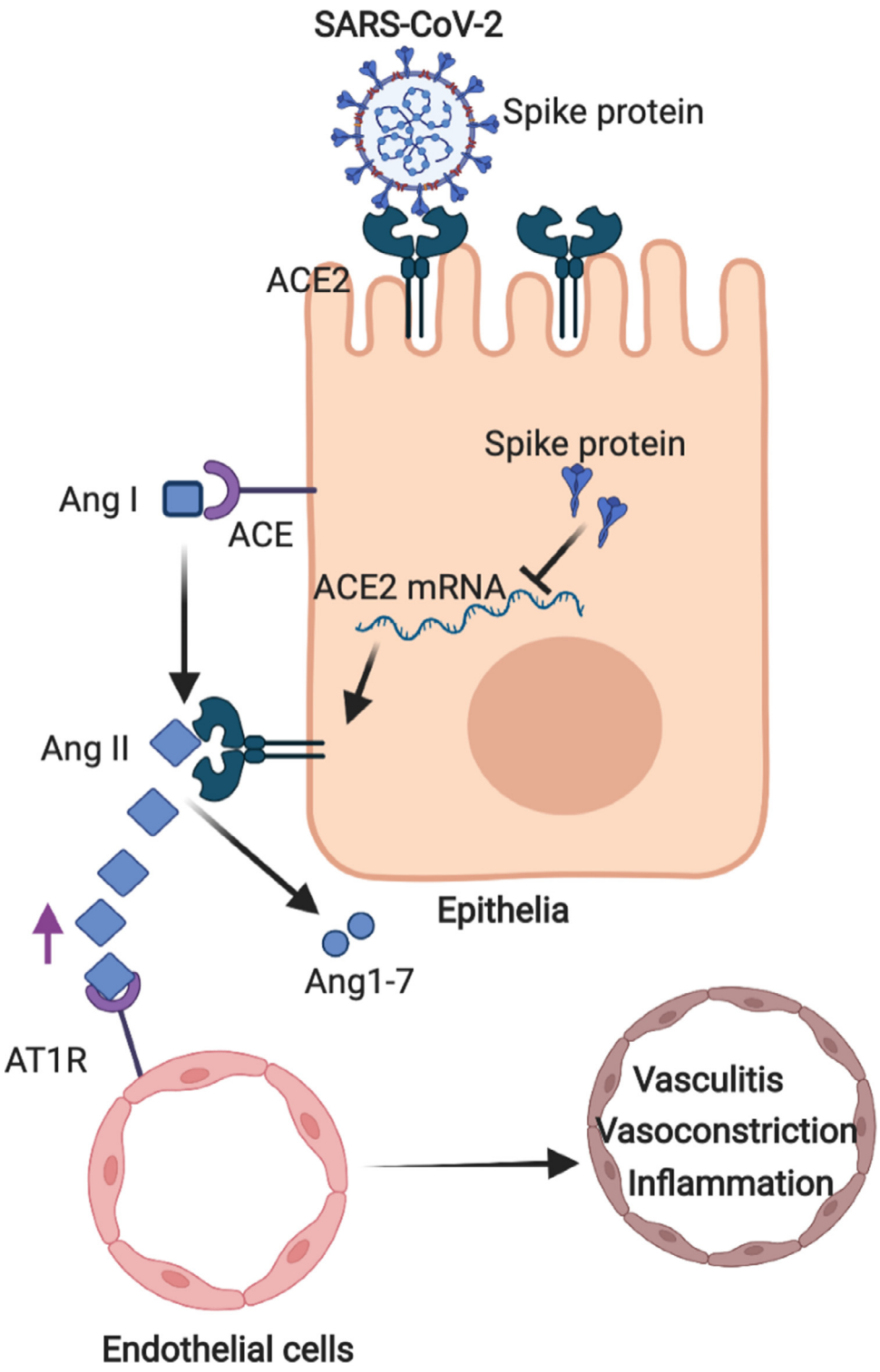

Fig. 6. The abstract overview of this study. SARS-CoV-2 Spike destroys the renin-angiotensin system in the lungs by down-regulating the expression of ACE2.

centrifuging at 1, 200 rpm for 5 min at 4 °C and then filtered with 0.22 μm membrane. The virus was stored at −80 °C.

RT-qPCR

Total RNA was extracted with TRIzol™ Reagent in accordance with the manufacturer's instructions. Reverse transcription into cDNA was performed with a High-Capacity cDNA Reverse Transcription Kit (Takara, RR036A). The expression levels of indicated RNA were determined by qPCR analysis using Power SYBR Green PCR Master Mix (Vazyme, Q311–02). The primers used RT-qPCR were human ACE2-F:5′-GGAGTTGTGATGGGAGTGAT-3′; ACE2-R: 5′-GATGGAGGCATAAGGATTTT-3′; human GAPDH-F:5′-GGAGCGAGATCCCTCCAAAAT-3′; GAPDH-R: 5′-GGCTGTTGTCATAC TTCTCATG-3′; SARS-CoV-2(Spike)-F: 5′-TGCAGGTATATGCGCTAGTTA TCAG-3′; SARS-CoV-2(Spike)-R: 5′-CACCAAGTGACATAGTGTAGGCAAT-3′.

Western blotting

Cells were harvested and boiled in 2X lammili sample buffer containing 10% βME (Sigma, M3148). Cell lysates were separated by 9% SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, IPFL10100). After being blocked with 5% BSA in TBS buffer containing 0.05% Tween 20, the blot was probed with indicated first antibodies and the horseradish peroxidase (HRP)-conjugated secondary antibody sequentially. Protein bands were detected by SuperSignal West Pico Chemiluminescent substrate (Bio-Rad). The following antibodies were used: ACE2 (Abcam, ab108209), SARS-CoV-2 Nucleoprotein (Sino Biological, 40,588-T62), SARS-CoV-2 Spike (Sino Biological, 40,591-MM42), Actin (TransGen Biotech, HC201), GAPDH (TransGen Biotech, HC301), AT-1R (Abcam, ab18801), AT-2R (Abcam, ab92445), anti-mouse IgG (TransGen Biotech, HS201–01) and anti-rabbit IgG (TransGen Biotech, HS101–01).

Immunohistochemistry

Formalin-fixed paraffin-embedded Lung tissue was cut into 4 μm sections and mounted on frosted glass slides. After deparaffinization and rehydration, slides were submerged into pH 8.0 EDTA buffer and boiled for 2 min with high-pressure for antigenic retrieval. Slides were washed in PBS, incubated in 3% hydrogen peroxide for 10 min and then blocked with 1% bovine serum albumin (BSA) for 1 h. The following primary antibodies were used: cleaved-Caspase3 (Cell Signaling Technology, 9661), ACE2 (Abcam, ab108209) and VWF (Cell Signaling Technology, 65707S). Slides were then washed in PBST (PBS plus 0.1% Tween 20), incubated with the biotinylated secondary antibody (Zymed, San Francisco, CA) and visualized with 3,3′-diaminobenzidine (DAB) under the microscopy.

Plasma angiotensin II measurement

The plasma samples from COVID-19 infected patients were separated from blood samples in the BSL-3 laboratory. The concentrations of plasma angiotensin II were detected by ELISA assay according to the manufacturer's instructions (Sigma, RAB0010).

RNA sequencing (RNA-seq) analysis

Differentially expressed genes (DEGs) are defined as the ratio of the infected group to the uninfected group (Mock group) by the rank-sum test. The P values were adjusted using the FDR method. Corrected *P* values of 0.05, and the value of FC greater than 1.5 or less than 1/1.5, were set as the threshold for significant differential expression. The differential expression results were displayed with the volcano map through the ggplot2 package of the R project.

Statistics analysis

All statistical analyses are performed using GraphPad Prism version 8.0.1. Paired *t*-test was used in qPCR analysis. Data were derived from the average of three biological replicate experiments, and calculated as the mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.001$.

Author contributions

This project was designed and directed by Z.Z. and G.X. S.Z. and X.G. conducted the SARS-CoV-2 infection experiments. J.G. and G.Z. conducted pathological section experiments. Y.W., L.F. and J.Z. contributed some experimental data. The manuscript was written by Z.Z., G.X. and X.G.

Declaration of Competing Interest

The authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2022.06.030.

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