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Full Length Article

RhoA-Rho associated kinase signaling leads to renin-angiotensin system imbalance and angiotensin converting enzyme 2 has a protective role in acute pulmonary embolism

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ABSTRACT

Introduction: Acute pulmonary embolism (APE) is a cardiovascular disease with high morbidity and mortality. Although the anatomical obstruction of the pulmonary vascular bed initiates APE, recent studies have suggested that vasoconstrictors in the renin-angiotensin system (RAS) play a role in the severity of APE.

Materials and methods: We performed a 5-year retrospective clinical study to analyze the key RAS components in APE patients, including angiotensin converting enzyme (ACE), ACE2, angiotensin II (Ang II) and angiotensin 1-7(Ang(1-7)). The role of RhoA-Rho associated kinase (ROCK) signaling in regulating RAS vasoconstrictors was detected in rat pulmonary artery endothelial cells and in an APE rat model.

Results: In clinical study, we found that the levels of RAS vasoconstrictors were correlated with the clinical classification of APE patients, ACE and Ang II were unregulated, whereas ACE2 and Ang(1-7) were down-regulated in the high-risk group compared to the healthy volunteers. In animal study, we found that activated RhoA-ROCK signaling was responsible for the imbalance in RAS vasoconstrictors both *in vitro* and *in vivo*, and further evidence indicated that ROCK inhibitors (Y27632 or HA1077) and an ACE2 activator (Resorcinol naphthalein) restored the dysregulated RAS vasoconstrictors significantly and had a protective role in an APE rat model.

Conclusions: Our study revealed that RhoA-ROCK signaling leads to RAS imbalance in APE patients, and ACE2 activation might be a novel therapeutic target in APE treatment.

1. Introduction

Acute pulmonary embolism (APE) is a leading cause of vascular death worldwide after myocardial infarction and stroke [1,2]. The clinical classification of the severity of APE is based on the estimated APE-related early mortality risk, and the high-risk group has a higher 30-day mortality than other groups [2]. The major reason for APE is the anatomical obstruction of the pulmonary vascular bed, which leads to an increase in pulmonary vascular resistance (PVR) and interferes with blood gas exchange [3]. APE induced vasoconstriction also contributes to the increased PVR [4,5].

Previous studies have suggested that the renin-angiotensin system (RAS), which regulates blood pressure and fluid balance, is involved in the pathogenesis of various pulmonary diseases, including pulmonary hypertension (PH) [6,7], lung fibrosis [8], and lung injury [9]. Vasoconstrictive factors in RAS are suggested to be involved in the pathogenesis of pulmonary embolism [10,11]. Angiotensin converting enzyme (ACE) and its homologue ACE2 are key regulators in the RAS.

Abbreviations: A-aDO_2, alveolar-arterial difference in oxygen partial pressure; ACE, Angiotensin converting enzyme; ACE2, Angiotensin converting enzyme 2; AngII, angiotensin II; Ang(1-7), angiotensin 1-7; APE, Acute pulmonary embolism; ESC, European Society of Cardiology; HIF-1α, hypoxia-inducible factor 1α; I-high, intermediate-high; I-low, intermediate-low; LPA, lysophosphatidic acid; PaO_2, arterial oxygen tension; PCR, polymerase chain reaction; PESI, pulmonary embolism severity index; PH, pulmonary hypertension; PVR, pulmonary vascular resistance; RAS, renin-angiotensin system; ROCK, Rho-associated kinase; rPAECs, rat pulmonary artery endothelial cells; RV, right ventricle; RVSP, right ventricular systolic pressure

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ACE cleaves angiotensin I (AngI) to generate AngII, which is one of the major vasoconstrictors in the RAS [12]. ACE2 counter-regulates the RAS by converting AngII into the vasodilator Ang(1-7) [13,14]. Decreased expression of ACE2 is associated with pulmonary vascular diseases [15]. Likewise, pharmaceutically activated ACE2 can restore overactivated RAS in the endothelium [16–18].

RhoA is a member of the Rho family, a small GTP-binding protein, and its downstream effector Rho-associated kinase (ROCK) plays an important role in the pathogenesis of pulmonary vasoconstriction and vascular remodeling [19]. RhoA-ROCK signaling is activated by vasoconstrictors such as AngII [20] and endothelin-1 [21]. Specific ROCK inhibitors such as Y-27362 [22] and HA-1077 (Fasudil) [23] can reverse sustained vasoconstriction induced by many kinds of agonists [24,25]. Little is known about the characteristics of RhoA-ROCK signaling in APE.

Previously, we observed that ACE was a direct target of hypoxia-inducible factor 1α (HIF-1α), and the accumulated AngII was a key mediator in the downregulation of ACE2 by HIF-1α [26]. Furthermore, dysregulation of the ACE/ACE2 ratio enhanced the proliferation and migration of pulmonary artery smooth muscle cells and contributed to the pathogenesis of hypoxic PH [27,28]. Overexpression of ACE2 attenuated the elevation in right ventricular systolic pressure (RVSP) and pulmonary vascular remodeling and protected animals from cardiovascular and pulmonary disorder in PH [29,30]. However, whether ACE or ACE2 contributes to the severity of APE and the therapeutic potential for APE is still unknown. We hypothesized that activated RhoA-ROCK signaling in APE was responsible for dysregulation of the ACE/ACE2 ratio.

In this study, we evaluated ACE and ACE2 levels in APE patients with different clinical classifications through a retrospective clinical study. We also investigated the role of RhoA-ROCK signaling in the regulation of ACE and ACE2 in rat pulmonary artery endothelial cells and in an APE rat model.

2. Materials and methods

2.1. Study population and risk assessment

One hundred and fourteen patients with an initial diagnosis of APE were enrolled into this study from January 2009 and December 2014 at Sir Run Run Shaw Hospital, Medical School of Zhejiang University. The diagnosis of APE followed the 2014 European Society of Cardiology (ESC) guidelines [2] and was mainly based on clinical features, blood examination and imaging. Exclusion criteria included a history of malignancy, cor pulmonale, interstitial lung disease, severe COPD, tuberculosis, hypertension, coronary heart disease, advanced heart failure with left ventricular ejection fraction < 50%, severe liver disease, or end stage renal failure. In addition, children (below 18 years), elderly persons (over 80 years), and pregnant women were also excluded.

After screening, 32 patients were excluded due to malignancy (n = 6), thyroid cancer (n = 1), intracranial germ cell tumors (n = 1), cor pulmonale (n = 1), severe COPD (n = 3), tuberculosis (n = 1), hypertension (n = 4), coronary heart disease (n = 4), left ventricular dysfunction (n = 1), severe liver disease (n = 1), end stage renal failure (n = 1), coronary heart disease (n = 3), aged over 80 years old(n = 4), and pregnancy (n = 1).

Therefore, the remaining 82 patients constituted the study population and were assigned to the low risk group (n = 32), the intermediate-low risk group (n = 17), the intermediate-high risk group (n = 23), or the high risk group (n = 10) according to the 2014 ESC guidelines [2] using PE-related risk and the patient's clinical status and comorbidities (Fig. 1). Briefly: shock or hypotensive patients were identified as high-risk PE. Normotensive patients with a pulmonary embolism severity index (PESI) Class ≥ III or a simplified PESI ≥1 constituted the intermediate-risk group. Within the intermediate-risk group, patients who displayed evidence of both right ventricle (RV) dysfunction and elevated cardiac biomarkers were classified into the intermediate-high (I-high) risk category, and patients in whom RV was normal and/or cardiac biomarkers were normal were in the intermediate-low (I-low) risk group. Normotensive patients with PESI Class ≤ II or a simplified PESI = 0 constituted the low-risk group. The plasma samples form 82 patients were collected within 24 h when APE was identified. We recruited 30 age and sex matched healthy volunteers who had a general medical examination at the Health Promotion Center of Sir Run Run Shaw Hospital to constitute the control group. Plasma specimens were collected from APE patients (before anticoagulation or thrombolysis administration) and healthy volunteers.

2.2. Experimental animals and animal facilities

Male Sprague-Dawley rats (12 weeks old) were obtained from the Animal Experimental Center of Zhejiang University, China. All animals were housed in temperature-controlled conditions (25 ± 1 °C) and maintained on a 12:12-hour light: dark cycle with free access to water and food. The Institutional Animal Care and Use Committee of Zhejiang University approved all procedures involving experimental animals.

2.3. Isolation and culture of rat pulmonary artery endothelial cells

Primary rat pulmonary artery endothelial cells (rPAECs) were isolated as previously described [31]. Cells were characterized by morphology and activity and validated by immunostaining for von Willebrand factor, CD31 and CD34, but not for α-smooth muscle actin, smooth muscle myosin heavy chain or CD90/Thy-1. Cells were used at passages 4–6 and were subjected to serum starvation for 24 h before each experiment.

2.4. Reverse transcription-polymerase chain reaction

Total RNA extracted from rPAECs and rat lungs was used to carry out reverse transcription-polymerase chain reaction (RT-PCR) and fluorescence real-time quantitative RT-PCR according to a previous report [32]. The specific primers of genes were RhoA: F 5′-AAA CTG GTG ATT GTT GGT GAT G-3′, R 5′-TCA GGG CTG TCG ATG GAA A-3′; ACE: F 5′-GGT CCT ATT CCC GCT CAT CT-3′, R 5′-CCA GCC CTT CTG TAC CAT TG-3′; ACE2: F 5'-CGG AGC CAA TGA AGG GTT-3′, R 5′-CCA TCC ACC TCC ACT TCT CTA A-3′; ROCK 1: F 5′-ACA GAA GCA GTT AGA AGA AGC G-3′, R 5′-GGT CTC CAA TCA TCT CAG AAT C-3′; ROCK2: F 5′-TGC TAA CAG TCC GTG GGT G-3′, R 5′-TGT TAT CGG GCT TCA CAT CTC T-3′; GAPDH: F 5′-CCC CAA TGT ATC CGT TGT G-3′, R 5′-CTC AGT GTA GCC CAG GAT GC-3′(rat for all). GAPDH was used as an internal control and the relative changes were calculated as 2-ΔΔCT.

2.5. Western blot analysis

Total protein was extracted from rPAECs and rat lungs with RIPA lysis buffer (Beyotime, Haimen, China). Protein lysates were resolved by SDS-PAGE gel and then transferred to PVDF membranes (Millipore, Bedford, USA). Anti-RhoA (26C4), anti-ROCK1 (H-85), anti-ROCK2 (C-20), anti-ACE (H-170) and anti-ACE2 (H-175) antibodies (dilution 1:200 for all) were purchased from Santa Cruz Biotechnology. Anti-β-tubulin antibody (dilution 1:1000) was obtained from ComWin Biotechnology. The protein signals were detected by a Las-4000 Imaging System (Fujifilm, Tokyo, Japan) with an ECL chemiluminescent kit (BioInd, Israel). Relative densitometry was normalized to β-tubulin.

2.6. Enzyme-linked immune sorbent assay (ELISA)

Anticoagulant blood from human subjects was centrifuged at 2500 rpm for 20 min to obtain plasma samples, ACE (DACE00, R&D systems, Minneapolis, USA), ACE2 (E4528, BioVision, Milpitas, USA),

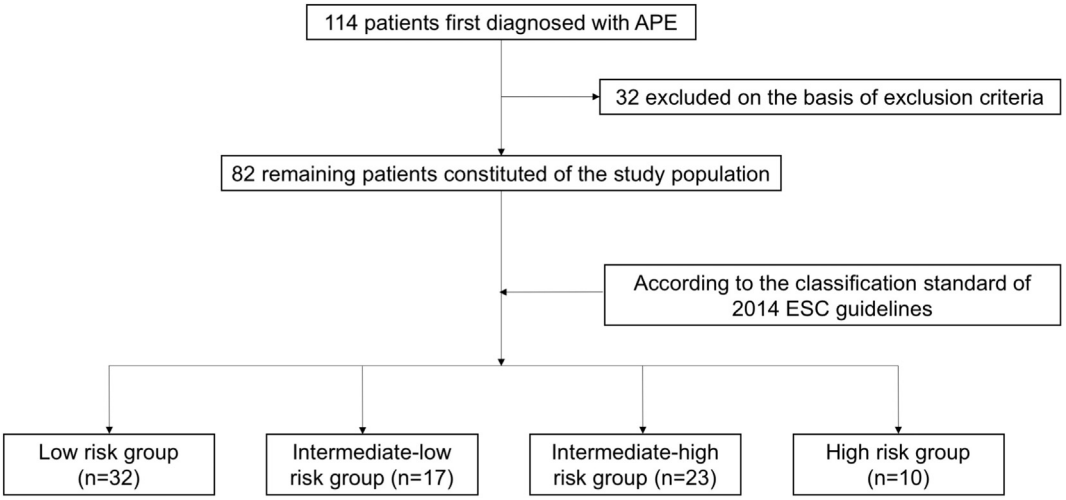

Fig. 1. Flow chart of patients enrolled and grouped into this study. One hundred and fourteen patients with an initial diagnosis of APE were enrolled into this study, 32 patients were excluded, and 82 enrolled APE patients were assigned to 4 groups based on PE-related risk and the patient's clinical status and comorbidities.

Table 1
Baseline characteristics.

Characteristics	Control group (n = 30)	Low risk group (n = 32)	I-low risk group (n = 17)	I-high risk group (n = 23)	High risk group (n = 10)	p value
Demographic characteristics						
Mean age, yr	54.3 ± 9.3	55.9 ± 9.7	58.6 ± 9.1	63.4 ± 9.6	58.4 ± 8.1	0.433
Male sex	11	17	8	9	6	0.569
Body mass index	23.3 ± 1.1	24.1 ± 1.3	23.6 ± 1.6	23.7 ± 1.2	23.5 ± 1.2	0.991
Past history, %						
Lung disease	0	2	1	1	1	0.655
Stroke	0	1	1	2	1	0.509
Hematologic Disease	0	1	0	0	0	0.641
Autoimmune Disease	0	0	0	1	1	0.239
Hormone Therapy	0	0	0	1	1	0.239
Smoking	7	11	6	5	3	0.764
Vital sign						
Systolic blood pressure, mmHg	116.8 ± 6.8	119.2 ± 7.5	121.8 ± 9.2	120.2 ± 10.1	99.5 ± 16.7	0.041
Heart rate, bpm	74.6 ± 9.5	82.4 ± 14.4	87.6 ± 13.1	84.4 ± 12.2	92.7 ± 10.9	0.13
Respiratory rate, /min	14.4 ± 1.8	16.9 ± 3.5	18.1 ± 3.8	20.1 ± 3.4	21.3 ± 4.1	0.02
SpO_2, %	98.4 ± 1.3	96.0 ± 2.8	93.6 ± 3.7	92.1 ± 4.7	89.5 ± 6.4	0.03
Laboratory test						
Troponin I, ng/ml	0.05 ± 0.03	0.07 ± 0.03	0.13 ± 0.08	0.23 ± 0.07	0.29 ± 0.05	0.002
CK-MB, IU/L	10.1 ± 4.0	9.9 ± 3.8	15.9 ± 5.0	25.2 ± 6.9	40.6 ± 8.8	0
D-dimer, ug/mL	0.11 ± 0.05	3.53 ± 1.40	3.82 ± 1.28	4.88 ± 1.45	5.65 ± 1.48	0
BNP, pg/mL	148.1 ± 49.0	161.8 ± 42.3	908.1 ± 129.3	1971.6 ± 417.2	2603.2 ± 463.8	0

SpO_2, oxygen saturation; CK-MB, creatine kinase MB fraction; BNP, brain natriuretic peptide; I-high: intermediate-high; I-low: intermediate-low.

Ang II (E4527, BioVision, Milpitas, USA), and Ang(1-7) (023394, United States Biological, Salem, USA) were measured using commercially available ELISA kits following the manufacturer's instructions. Anticoagulant blood from rats was centrifuged at 2500 rpm for 20 min to obtain plasma samples, culture supernatants were also collected from rPAECs, ACE (24743–1-AP, Proteintech, Rosemont, USA), ACE2 (MA5–31272, Invitrogen, NY, USA), Ang II (023412, United States Biological, Salem, USA), and Ang(1-7) (023394, United States Biological, Salem, USA) were measured using commercially available ELISA kits following the manufacturer's instructions.

2.7. Production of the rat RhoA overexpression vector

The rat RhoA coding region was amplified by PCR using the following pair of primers: F, 5′-TAT TC*G GCG CGC C*GC CAC CAT GGC TGC CAT CAG GAA GAA-3′, and R, 5′-AGT GC*G* TTT AAA *C*TC ACA AGA TGA GGC ACC CCG-3′. The resulting fragments were cloned into a pLenti6.3-IRES-EGFP vector (pLenti6.3-RhoA-IRES-EGFP; Invitrogen, USA). Positive clones were extracted and sequenced by Genepharma (Shanghai, China).

2.8. Primary rat pulmonary artery endothelial cells (rPAECs) treatment

rPAECs were treated with PBS (Blank group), Lenti6.3-EGFP (Control group), Lenti6.3-RhoA-IRES-EGFP (RhoA group), Lenti6.3-RhoA-IRES-EGFP + ROCK inhibitor Y27632 (1 μmol/L, Sigma Chemical, St. Louis, MO) (RhoA + Y27632 group), or Lenti6.3-RhoA-IRES-EGFP + ROCK inhibitor HA1077 (50 μmol/L, Asahi Kasei, Japan) (RhoA + HA1077 group). The multiplicity of infection was 30 for all groups. Ectopic expression of RhoA was confirmed using RT-PCR and Western blot. rPAECs were also treated with lysophosphatidic acid (LPA, Sigma, L7260) (40 μm) for 48 h.

2.9. APE rat model

An APE rat model was created using intrajugular injection of

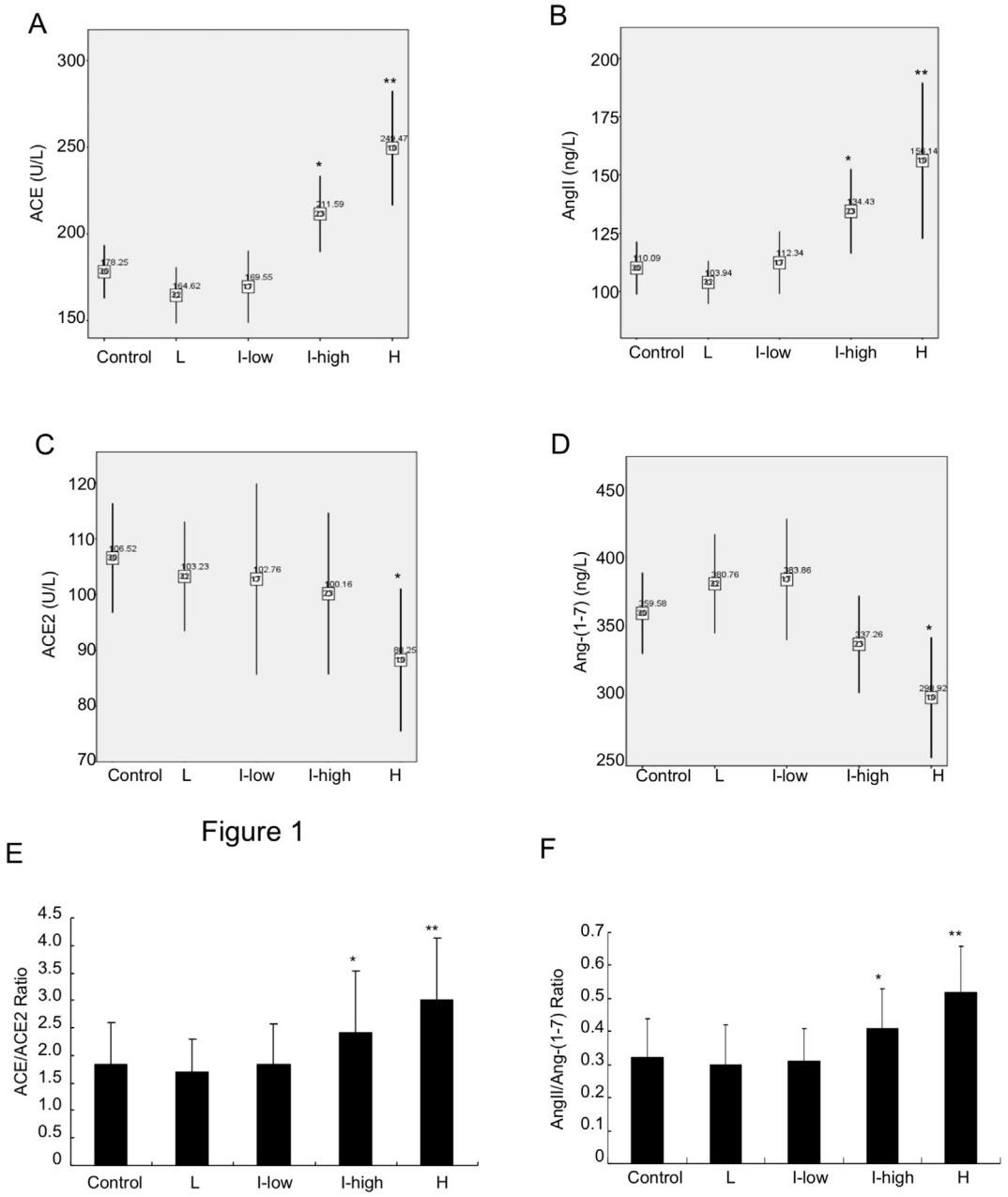

Figure 1

Fig. 2. Evaluation of key RAS components in APE patients. The protein levels of key RAS components ACE (A), AngII (B), ACE2 (C) and Ang(1-7) (D) were measured by ELISA in plasma samples from APE patients and healthy volunteers (Control) (n = 30). The ratio of ACE/ACE2 (E) and AngII/Ang(1-7) (F) were also calculated. APE patients were divided into four groups: low (L) risk group (n = 32), immediate-low (I-low) risk group (n = 17), immediate-high (I-high) risk group (n = 23) and high (H) risk group (n = 10). * $P < 0.05$ and ** $P < 0.01$ compared with healthy volunteers.

polystyrene microsphere as previously reported [33,34]. This model produces occlusion of the pulmonary artery with physiological alteration similar to that observed in human APE [35]. Male Sprague-Dawley rats (12 weeks old) were anesthetized with intraperitoneal ketamine (7 mg/100 g) and xylazine (1 mg/100 g) and then intubated through the trachea with an 18-gauge angiocatheter (IV Catheter, BD Insyte, USA). While breathing spontaneously, the left jugular vein was catheterized with PE-50 tubing (PORTEX, Smiths Medical, Ashford, UK) as previously described [33,36]. Rats were randomly assigned to the following five groups: control group, mock group, Y27632 group, HA1077 group, and Resorcinol naphthalein (Res) group. The control group was injected with vehicle (1.75 mL/kg saline), and 1 h later saline was injected again. For the remaining 4 groups, polystyrene microsphere beads (0.75 ml/kg with 1 ml/kg of saline flush; mean diameter 26 μm, 7525A, Duke Scientific) were administered. One hour later, saline was injected into the mock group, ROCK inhibitor Y27632 (3 mg/kg) was injected into the Y27632 group, ROCK inhibitor HA1077 (3 mg/kg) was injected into the HA1077 group, and ACE2 activator Res (Cayman Chemical, Ann Arbor, MI, USA) (2 mg/ml Res in 50% DMSO of saline) was implanted subcutaneously (6 μg/h) into Res group.

2.10. Hemodynamics and arterial blood gas analysis

Five hours after administration of saline, ROCK inhibitors, or an ACE2 activator, rats were anesthetized again and placed in a supine position. A PE-50 catheter (PORTEX, Smiths Medical, Ashford, UK) filled with heparin-saline solution (40 U/ml), was inserted into the right external jugular vein and forwarded to the right ventricle. RVSP was

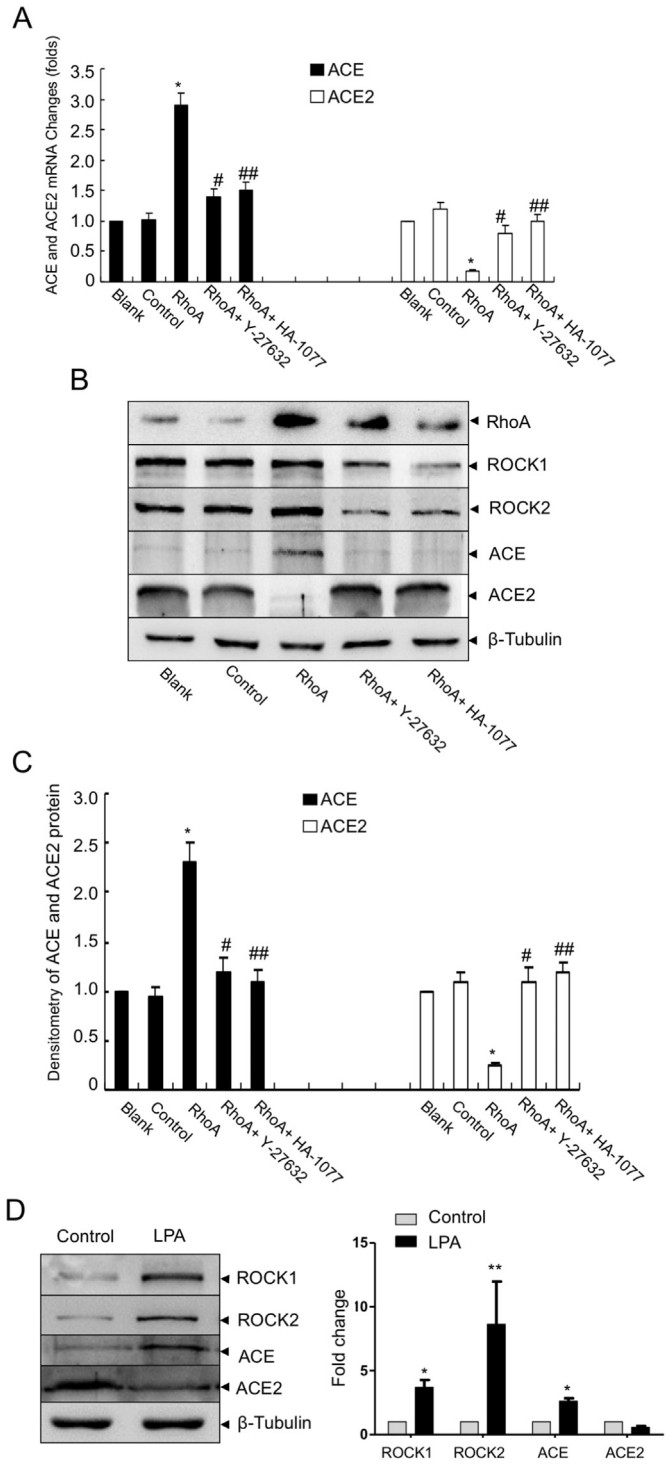

Fig. 3. RhoA-ROCK signaling regulated RAS components in rPAECs. Primary isolated rat pulmonary artery endothelial cells (rPAECs) were infected with RhoA lentivirus (RhoA group) or control virus (Control group), and further treated with ROCK inhibitors (RhoA + Y27632 group, RhoA + HA1077 group) as indicated. ACE and ACE2 mRNA levels were measured by qPCR (A). RhoA, ROCK1, ROCK2, ACE and ACE2 protein levels were measured by Western blot with β-tubulin as an internal control (B). The Western blot bands of ACE and ACE2 were scanned and quantified (C). rPAECs were treated with ROCK activator lysophosphatidic acid (LPA) (40 μm) for 48 h and proteins were detected and quantified with β-tubulin as an internal control (D). All results were presented as the mean ± SD of 3 independent tests. * $P < 0.05$ and ** $P < 0.01$ compared with the control group, # $P < 0.05$ and ## $P < 0.01$ compared with the RhoA group.

detected through a liquid pressure transducer (YP200, Xinhang Machine and Equipment, Gaobeidian, China) and recorded by the MedLab-U/501H® biological signal collecting-processing system (Medease Science and Technology, Nanjing, China) as previously described [37].The position of the catheter was estimated by the waveform of the pressure tracing and validated by postmortem examination. The femoral artery was cannulated for the measurement of systemic arterial blood pressure as previously described [38]. Arterial blood samples were collected from the femoral artery and analyzed by blood gas analyzer (ABL90FLEX, Radiometer, Denmark) within 30 min. Once the hemodynamics and arterial blood samples were obtained, the animals were sacrificed. The lungs were removed for gene expression analysis.

2.11. Statistical analysis

Statistical analysis was performed using SPSS for Windows (Version 16.0, SPSS Inc., Chicago, IL, USA). The data were expressed as the mean ± SD of three independent experiments. One-way analysis of variance (ANOVA) was applied for statistical comparisons between different groups with Graphpad Prisms 5.0 (Graphpad Software Inc., San Diego, CA, USA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Dysregulation of the RAS in APE patients

To investigate the correlations among RAS components in different clinical classifications for APE patients, we collected plasma samples from 82 APE patients and 30 healthy volunteers for a retrospective study. APE patients were categorized into four groups: a low risk group ($n = 32$), an I-low risk group ($n = 17$), an I-high risk group ($n = 23$) and a high risk group ($n = 10$), according to their PE-related risk and clinical status and comorbidities on admission as recommended by the 2014 ESC guidelines [2]. Baseline characteristics and diagnostic information for each group are summarized in Table 1. We measured the protein levels of ACE, ACE2, Ang II and Ang(1-7), which are four key components in the RAS, in plasma from APE patients, with healthy volunteers as controls. Interestingly, we found that levels of ACE and AngII were positively correlated with the clinical classification of APE. ACE protein was 18% higher in the I-high risk group ($P = 0.012$) and 40% in the high-risk group ($P = 0.001$) than in the healthy volunteers (Fig. 2A). Consistently, AngII protein increased by 22% in the I-high risk group ($P = 0.014$) and by 42% in the high risk group compared with controls ($P = 0.001$) (Fig. 2B). Conversely, ACE2 and Ang(1-7) were negatively correlated with the severity of APE, with a 14.4% decrease ($P = 0.037$) in ACE2 (Fig. 2C) and a 16.8% decrease ($P = 0.035$) in Ang(1-7) (Fig. 2D) in the high-risk group compared with healthy volunteers. We calculated the ratios of the RAS components and found a significantly increased ACE/ACE2 ratio and AngII/Ang(1-7) ratio in the I-high risk group ($P = 0.018$ and $P = 0.013$, respectively) and the high risk group ($P = 0.0001$ and $P = 0.0001$, respectively) (Fig. 2E, F), which indicated involvement of the RAS in the severity of APE. Overall, our clinical retrospective study demonstrated that RAS activation was significantly correlated with the clinical severity of APE.

3.2. RhoA-ROCK signaling regulated the RAS components in rPAECs

We found that overexpression of RhoA increased ACE mRNA expression (~3-fold) but decreased ACE2 mRNA expression in primary rPAECs compared to untreated control cells (Fig. 3A). Furthermore, the dysregulation of ACE and ACE2 in RhoA overexpressed rPAECs was significantly blocked by two ROCK inhibitors, Y27632 and HA1077 (Fig. 3A). ACE protein was upregulated over the control expression by 2.5- fold in RhoA-overexpressed cells, but ACE2 protein was

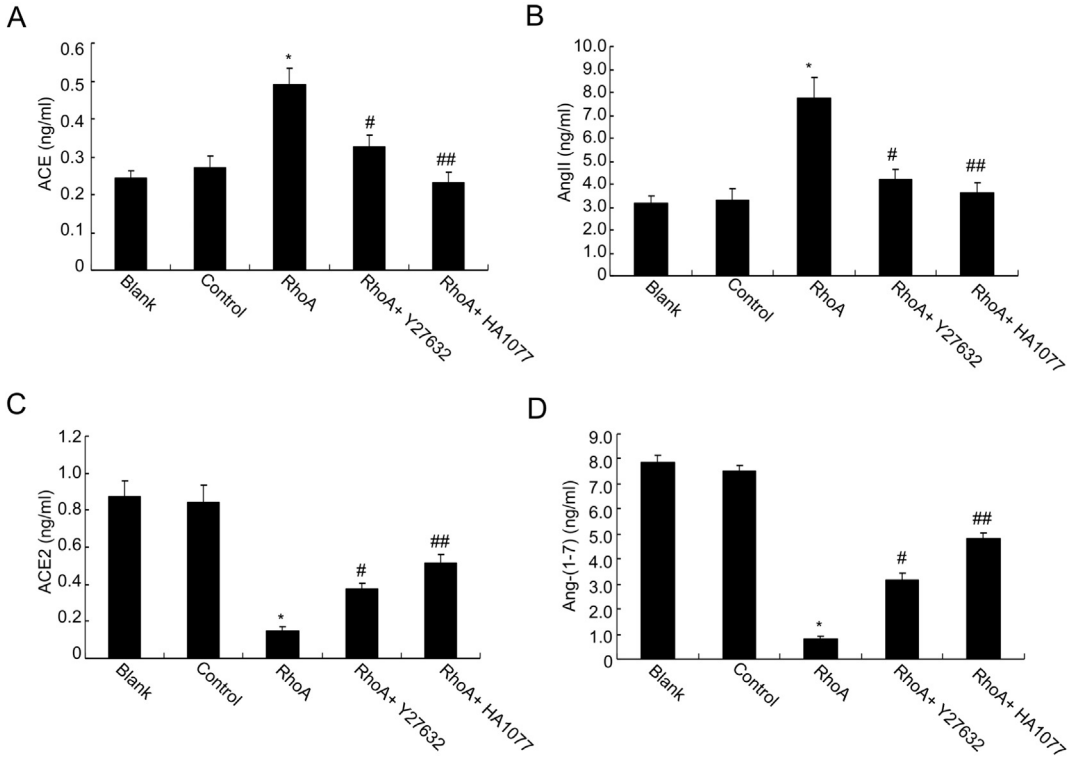

Fig. 4. Blockade of RhoA-ROCK signaling ameliorated dysregulation of RAS components in RhoA overexpressing rPAECs. Primary isolated rat pulmonary artery endothelial cells (rPAECs) were infected with RhoA lentivirus (RhoA group) or control virus (Control group), and further treated with ROCK inhibitors (RhoA + Y27632 group, RhoA + HA1077 group) as indicated. Cell culture media were harvested and measured by ELISA for ACE (A), AngII (B), ACE2 (C) and Ang (1-7) (D). All results were presented as the mean ± SD of 3 independent tests. * $P < 0.05$ and ** $P < 0.01$ compared with the control group, # $P < 0.05$ and ## $P < 0.01$ compared with the RhoA group.

downregulated by 65% (Fig. 3B, C). Consistently, the dysregulation of the ACE and ACE2 proteins were completely blocked by the two ROCK inhibitors as shown in Fig. 3B, C. We used ROCK activator LPA [39] and found that LPA activated ROCK1 3.7-fold and ROCK2 8.6-fold and increased ACE expression (2.63-fold), but it reduced ACE2 expression (44% reduction) in rPAECs compared to controls (Fig. 3D). Collectively, our results demonstrated that ROCK signaling was responsible for regulation of ACE and ACE2 expression in the RAS.

We further measured levels of ACE, ACE2, AngII and Ang-(1-7) in rPAECs culture supernatants by ELISA after RhoA activation. Overexpression of RhoA increased ACE and its target AngII (2.0-fold and 2.5-fold, respectively) in the cell culture supernatants over control levels (Fig. 4A, B). The upregulation of ACE and AngII were significantly attenuated by ROCK inhibitors, Y27632 and HA1077 (Fig. 4A, B). Conversely, RhoA activation reduced ACE2 and Ang(1-7) expression (~80% and ~90%, respectively) in the cell culture supernatants compared to control cell supernatants (Fig. 4C, D). Consistently, the downregulation of ACE2 and Ang(1-7) was significantly attenuated by ROCK inhibitors, Y27632 and HA1077 (Fig. 4C, D).

Overall, our data indicated that dysregulation of the RAS in APE was mediated by RhoA-ROCK signaling *in vitro*.

3.3. ROCK inhibitors and the ACE2 activator ameliorated dysregulation of the RAS in APE rats

We adopted an APE rat model to explore dysregulation of the RAS by RhoA-ROCK signaling *in vivo*. After injecting polystyrene microsphere beads into the left jugular vein, rats developed APE in 5 h. RhoA was upregulated in APE rat lung tissue (2.2-fold increase over control tissue, $P = 0.017$) (Fig. 5A). We further investigated whether RhoA contributed to ACE and ACE2 dysregulation in APE rats. We found that ROCK inhibitors, Y27632 and HA1077, significantly ameliorated the increase in ACE and the decrease in ACE2 in APE rat lung tissue at both mRNA (Fig. 5B) and protein (Fig. 5C, D) levels. ACE2 activator, Res, rescued ACE/ACE2 dysregulation in APE rats (Fig. 5B–D).

To further verify this observation, we measured the protein levels of AngII and Ang(1-7) in plasma samples from APE rats after ROCK inhibitors or ACE2 activator treatment. The variation in AngII and Ang(1-7) was consistent with ACE and ACE2 after ROCK inhibitor or ACE2 activator treatment (Fig. 5E, F). We found that the ACE2 activator significantly restored the dysregulation in the ACE/ACE2 ratio ($P = 0.007$) and the AngII/Ang(1-7) ratio ($P = 0.006$) (Fig. 5G, H). Overall, our data suggested that both ROCK inhibitors and the ACE2 activator ameliorated dysregulation of the RAS in APE rats.

3.4. ROCK inhibitors and the ACE2 activator improved hemodynamics and arterial blood gas exchange in APE rats

We performed an *in vivo* treatment of ROCK inhibitors and an ACE2 activator in an APE rat model. We found that administration of ROCK inhibitors or an ACE2 activator in APE rats resulted in significant attenuation of RVSP ($P = 0.04$) (Fig. 6A). Systolic blood pressure (SBP) was decreased in APE rat compared to controls ($P = 0.001$) and was rescued by ROCK inhibitors or the ACE2 activator (Fig. 6B). The clinical improvements were also confirmed by the decreased in alveolar-arterial difference in oxygen partial pressure (A-aDO_2) ($P = 0.035$) (Fig. 6C) and increased in arterial oxygen tension (PaO_2) compared to controls ($P = 0.03$) (Fig. 6D). Overall, our data demonstrated that both ROCK inhibitors and the ACE2 activator have potential clinical value for APE treatment.

4. Discussion

In this study, we investigated that high-risk APE patients had

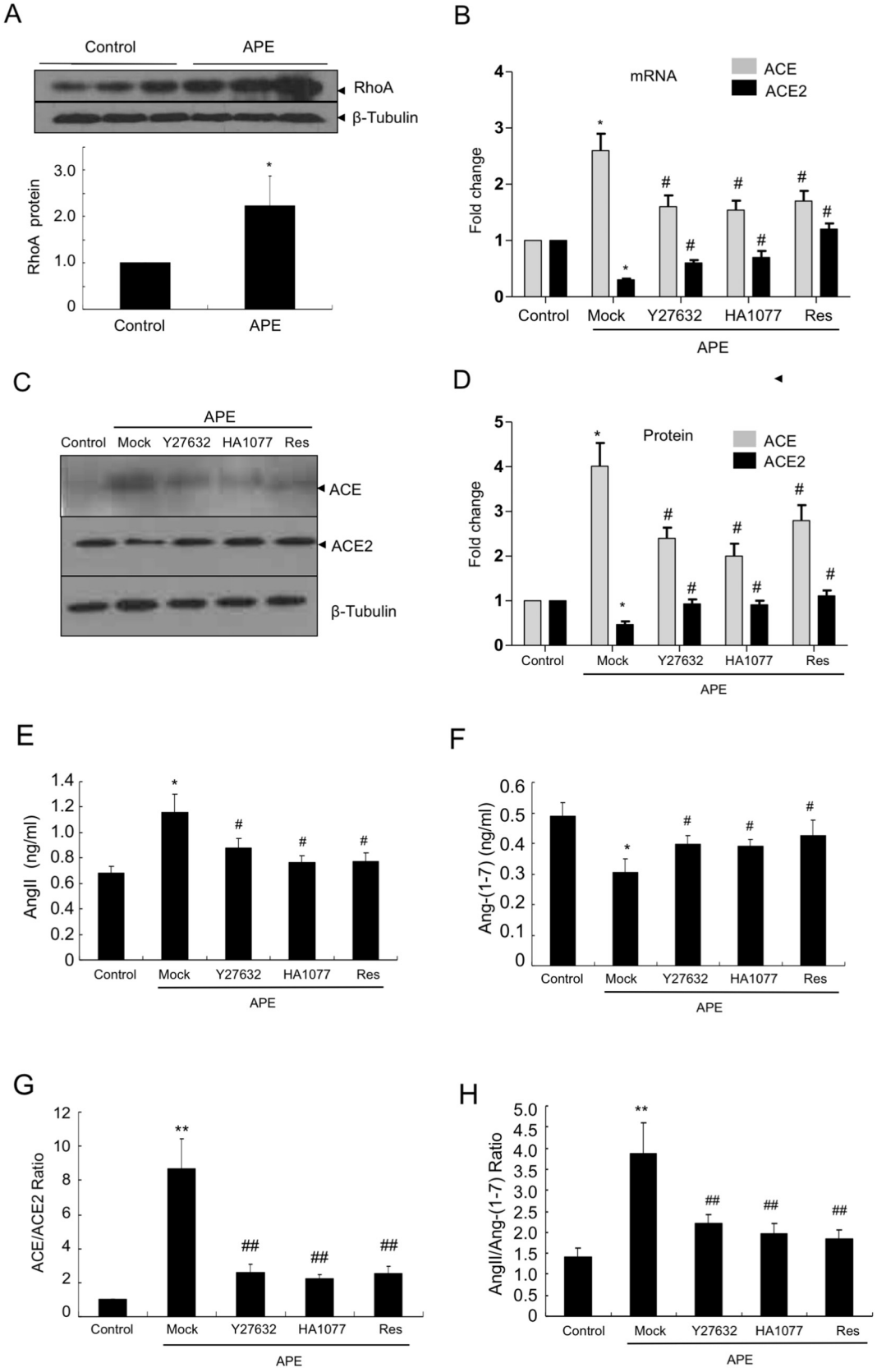

Fig. 5. ROCK inhibitors and ACE2 activator regulate RAS in APE rats. RhoA protein levels were measured in lungs from control and APE rats with β-tubulin as an internal control (A). Lung tissue samples were collected from control and APE rats with ROCK inhibitor (Y27632 or HA1077) or ACE2 activator (Res) administration. ACE and ACE2 mRNA (B) and protein (C-D) in rat lung tissues were measured by qPCR and Western blot, respectively. Blood plasma samples from control and APE rat were harvested as indicated. AngII (E) and Ang-(1-7) (F) were measured by ELISA assays. The ratio of ACE/ACE2 (G) and AngII/Ang-(1-7) (H) were quantified. Values were represented as the mean ± SD ($n = 6$ in each groups). * $P < 0.05$ and ** $P < 0.01$ compared with the control group, # $P < 0.05$ and ## $P < 0.01$ compared with the mock APE group.

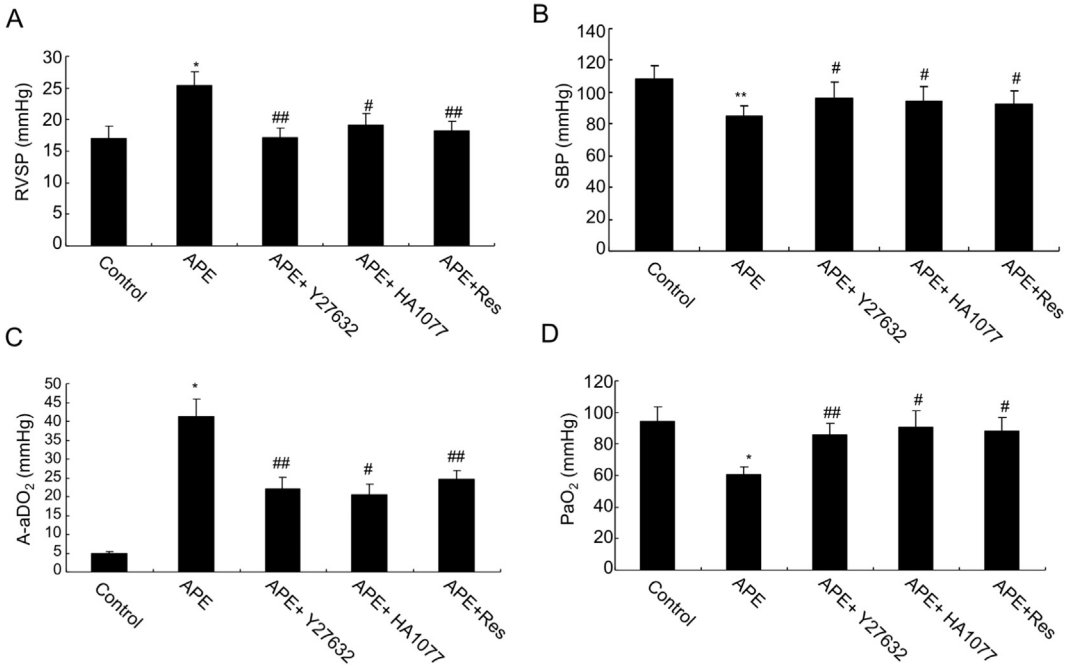

Fig. 6. ROCK inhibitors and ACE2 activator improved hemodynamics and arterial blood gas exchange in APE rat. APE rats were treated with ROCK inhibitor or ACE2 activator as indicated. Right ventricular systolic pressure (RVSP) (A), systolic blood pressure (SBP) (B), alveolar-arterial difference in oxygen partial pressure (A-aDO_2) (C) and arterial oxygen tension (PaO_2) (D) were measured. Values were represented as the mean ± SD ($n = 6$ in each groups). * $P < 0.05$ and ** $P < 0.01$ compared with control group, # $P < 0.05$ and ## $P < 0.01$ compared with APE group.

increased ACE and AngII but decreased ACE2 and Ang-(1-7) levels in their circulating system in a retrospective clinical study. In an animal study, we revealed that the activation of RhoA-ROCK signaling was related to the dysregulation of the RAS vasoconstrictive factors both *in vitro* and *in vivo*. ROCK inhibitors or an ACE2 activator restored the dysregulation in the RAS and had a protective role in an APE rat model.

The principle factors causing death in APE are PH, right ventricular failure and hypoxemia. PH is characterized by a progressive elevation in pulmonary arterial pressure, which is caused by mechanical obstruction of the pulmonary vascular bed and subsequent vasoconstriction [40]. Hypoxia occurs when perfusion and ventilation of the pulmonary vascular bed becomes mismatched. RhoA-ROCK signaling plays an important role in the pathogenesis of various experimental models of PH [19]. Y-27632 is a specific ROCK inhibitor that competes with ATP for binding to the kinase [22], and pharmacological inhibition of ROCK with Y-27632 has been shown to attenuate acute pulmonary vasoconstriction in PH animal models [24,41]. HA-1077 (Fasudil) is a selective ROCK inhibitor, and is the first approved ROCK for clinical use in the treatment of ischemia-induced brain damage [23,25]. However, little is known about whether RhoA-ROCK signaling participates in the regulation of the RAS in APE. To investigate the dysregulation of the RAS in APE, we studied RhoA-ROCK signaling and found that RAS vasoconstrictive factors were dysregulated in primary isolated rPAECs and the APE rat model. Our results showed ACE and ACE2 were regulated by RhoA-ROCK. However, the detailed mechanism was unclear. Kataoka and his colleagues found that the Rho/Rho-kinase pathway induced inflammatory factor expression *via* angiotensin II type 1 receptors in a coronary arteriosclerosis rat model [42]. Whether RhoA-ROCK signaling regulates ACE and ACE2 expression by a similar mechanism needs to be confirmed. Furthermore, our study demonstrated that administration of ROCK inhibitors could attenuated RVSP and ameliorated hypoxemia in APE rats. These results indicated that ROCK inhibitors could represent a potential method for APE treatment.

ACE2 is predominantly expressed in endothelial cells and smooth muscle cells of the pulmonary vascular wall where it suppresses the RAS through destroying AngII [43,44]. Our *in vivo* data provided evidence that the ACE2 activator, Res, attenuated RVSP and ameliorated hypoxemia in APE rats. This finding was consistent with a previous report that ACE2 overexpression had protective effects in PH [7]. In another previous study, AngII receptor antagonist, Losartan, failed to protect against APE [45], which suggested that directly targeting ACE2 would be a better clinical approach. AngII generated by ACE had deleterious effects [46], such as vasoconstriction, vascular inflammation, platelet aggregation, oxidative stress, and endothelial dysfunction through the angiotensin II type 1 receptor [4,47–49]. ACE2 hydrolyzes AngII to generate the negative regulatory heptapeptide Ang(1-7), which exerts vasodilation, anti-inflammation and anti-oxidative stress actions by antagonizing AngII [50–54]. This indicated that the protective role of ACE2 in APE is possibly due to increased Ang(1-7) production and reduced AngII. However, an ACE2 activator also improved endothelium dysfunction [16], inhibited neointimal formation [29], and exerted anti-inflammatory actions [55,56]. These studies indicated that ACE2 might benefit APE through other unknown mechanisms. Furthermore, the role of other vasoconstrictors such as endothelin-1 and thromboxane A2, in APE progress also need intensive study.

In the future, comparing the effects of ACE2 activators, ACE inhibitors or angiotensin-receptor blockers in APE treatment will be important to understand the complex mechanisms of the RAS in APE pathogenesis and their therapeutic potentials. Several limitations should be mentioned for the present study. One limitation is that a microsphere embolism model could mimics anatomical obstruction of the pulmonary vascular bed, but whether this model could mimic the biochemical or physiologic effects of thromboembolism was not explored. Furthermore, other than detecting RVSP, echocardiography may be better for dynamically monitoring right ventricle function or measuring of cardiac output in the APE rat model.

5. Conclusions

In summary, our study evaluated RAS dysregulation in APE patients with different clinical severities. We found that RhoA-ROCK signaling led to an ACE/ACE2 imbalance in APE rast, and revealed the potential

value of ROCK inhibitors and ACE2 activator for APE treatment.

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Ethics approval and consent to participate

The study was approved by the ethics committee of Sir Run Run Shaw Hospital, Medical school of Zhejiang University, Zhejiang, China. All subjects have given written informed consent. All methods were performed according to the approved documents. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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