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Original Article

VE-cadherin involved in the pulmonary microvascular endothelial cell barrier injury induced by angiotensin II through modulating the cellular apoptosis and skeletal rearrangement

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Abstract: Objective: Angiotensin II (AngII) involved in the pathogenesis of pulmonary injury through impairing the integrity of pulmonary microvascular endothelial barrier, but the mechanism is still not clear. We aim to determine the roles of VE-cadherin, playing crucial roles in the adhesion of the vascular endothelial barrier and the barrier function, in the pulmonary microvascular endothelial cell (PMVEC) barrier injury mediated by AngII. Methods: Mice acute lung injury (ALI) model was induced through pumping of AngII. The infiltration of macrophages and neutrophils as well as the PMVEC permeability were determined in order to determine the barrier injury in vivo and in vitro. Knockdown of VE-cadherin was established using siRNA technique, and its roles in the apoptosis and skeletal rearrangement in the PMVECs were evaluated. Results: After AngII interference, the expression of VE-cadherin in the PMVECs and pulmonary tissues in mice was down-regulated. Upon VE-cadherin knockdown through siRNA technique, AngII induced susceptibility of PMVECs to apoptosis. Knockdown of VE-cadherin contributed to the skeletal rearrangement in the endothelial cells, together with increase of permeability. Conclusions: VE-cadherin expression is closely related to the apoptosis and skeletal rearrangement of PMVECs induced by AngII.

Keywords: VE-cadherin, acute lung injury, endothelial cell barrier, angiotensin II, apoptosis, skeletal rearrangement

Introduction

Many aortic dissection (AD) patients may present acute lung injury (ALI) which hampers the prognosis of AD severely [1]. Our previous study revealed inflammatory cell infiltration and inflammatory factors were the major causes for the pathogenesis of acute aortic dissection (AAD) complicated with ALI. In this process, the cellular apoptosis and injury of mechanical barrier of pulmonary microvascular endothelial cells (PMVECs) mediated by angiotensin II (AngII) played crucial roles. PMVECs, localized at the internal side of the blood-air barrier, constituted endothelial barrier together with the adjacent endothelial cells [2]. Besides, the endothelial adherens junctions play pivotal roles in the physiological and pathological processes, including cellular differentiation, maintenance of entire monolayer integrity, and the remodeling [3].

Vascular endothelial cadherin (VE-cadherin), the major component for the adherens junctions between endothelial cells [4], has been reported to be closely involved in various disorders as its expression and phosphorylation could induce vascular endothelial dysfunction and increase of microvascular permeability [5, 6]. In structure, the VE-cadherin was consisted of 780 amino acids, which was divided into three domains including extra- and intra-cellular region, as well as the transmembrane domain. It has been reported that VE-cadherin could adhere to each other through the extracellular region [7]. For the intra-cellular region, the amino acid sequences were considered to be highly conserved, among which the tyr685, tyr658 and tyr731 could be phosphorylated by the tyrosine kinase [8, 9]. Afterwards, the phosphorylated VE-cadherin could recruit the molecular proteins containing SH2/SH3 domains, including src, Nck, PI3K and Csk, which finally

Table 1. Clinical features of the patients

Variables	Control (n=12)	Non-ruptured aneurysm (n=12)	AAD	
			Complicated with lung injury AAD (n=21)	Non lung injury AAD (n=37)
Age, yr	40±9	58±11	47±6	51±6
Male sex, n %	8 (66.7)	8 (66.7)	17 (81.0)	30 (81.1)
Average duration from onset, h	N/A	N/A	9.2	10.7
Smoking, n (%)	4 (33.3)	5 (41.7)	11 (52.4)	19 (51.4)
Circulating AngII (pg/mL)	424±122	583±113	1163.7±312*,#	900±246*

AngII, angiotensin II; AAD, acute aortic dissection; ALI, acute lung injury. *P < 0.05 versus control, #P < 0.05 versus AAD without ALI.

activated the signaling molecules downstream [10-13]. All these indicated that VE-cadherin was closely participated in the structural function and signaling transduction pathways, and its expression and phosphorylation may be related to the dysfunction of PMVECs and the mechanical barrier.

In this study, RNA interference (RNAi) was used to knock down the expression of VE-cadherin. On this basis, we investigated the roles of VE-cadherin in the PMVECs apoptosis and skeletal rearrangement mediated by AngII. Our results indicated that down-regulation of VE-cadherin was noticed in PMVECs after AngII treatment. Besides, VE-cadherin could modulate the sensitivity of apoptosis and skeletal rearrangement in PMVECs.

Materials and methods

Patients

Fifty-eight AAD patients admitted in the ICU of our hospital from September 2014 to July 2015 were included in this study. Meanwhile, 12 matched individuals and 12 non-ruptured aortic aneurysm patients were registered. The diagnosis of AAD and aortic aneurysm was performed according to the CT findings and ultrasonic examination. ALI was defined as PaO_2/FiO_2 of ≤ 300 mmHg in the first 24 hour after diagnosis [14]. The exclusion criteria were as follows: patient admitted to our hospital 7 days after the onset; those with cancer, chest trauma and pulmonary infection within one month. Blood sample was collected and PaO_2/FiO_2 was determined 1 h after admission. Twenty-one AAD patients showed combined hyoxemia before the surgery. The patient characteristics were listed in **Table 1**. Each patient signed the informed consent based on their wills. The study protocols were approved by the Ethical Committee of the Wuhan University Renmin Hospital.

Induction of ALI by AngII

The ALI model was induced according to our previous description [15]. Male mice (8-week-old) were provided by the HFK Bioscience Co., Ltd (C57BL/6J, Beijing, China). The mice were fed by normal diet, combined without (control group) or with osmotic mini pumps (Alzet, Cupertino, CA) filled with AngII (1 μg/kg per minute, Sigma-Aldrich) for 1 week (AngII group). The animal handling was consistent with the Wuhan Directive for Animal Research and the current Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. Mice were sacrificed after anesthesia using 2% phenobarbital (40 mg/Kg), and extensive measures were taken to decrease the sufferings of the animals. The animal study protocols were approved by the Ethical Committee of the Wuhan University Renmin Hospital.

The mice were randomly divided into three groups, including blank control (n=8), sham control (n=8) subject to 0.9% normal saline (1 μl/kg) per minute for 1 week, and AngII group (n=8) subject to ALI induction followed by treating with AngII for 1 week.

Cell culture and RNAi

Rat PMVECs of passages 2~8 were purchased from BeNa Culture Collection Co., Ltd. (category No. BNCC338210; Beijing, China). Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10%

fetal bovine serum (FBS), 0.5% fungizone (Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin. The mixtures were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. Subsequently, the cellular growth was arrested by replacing RPMI 1640 containing 10% FBS with FBS-free RPMI 1640 for 24 h. The cells were divided into four groups, including (i) control group; (ii) AngII group, which was subject to 1 μM AngII; (iii) AngII+VE-cadherin knockdown group, which was subject to RNAi followed by AngII; and (iv) VE-cadherin knockdown group, subject to RNAi as follows.

RNAi was used to knock down the VE-cadherin expression in rat PMVECs as previously described [16]. Briefly, SmartPool siRNA for VE-cadherin was obtained from Dharmacon (Lafayette, CO). Transient transfection of PMVECs with sequence specific siRNA or scrambled control, was conducted through oligofectamine (Invitrogen, CA, USA), 200 nM siRNA and 1.5×10^6 cells per reaction. About 72 h after RNAi, the cells were harvested to determine the cellular apoptosis, skeletal rearrangement and the permeability of cell monolayer. The knockdown was validated using Western blot analysis.

ELISA

Serum AngII in patients and matched individuals was measured using commercial ELISA kits (category No. EK0459, Biofavor Biotech Service Co., Ltd. Wuhan, China) according to the manufacture's instructions. All tests were conducted at least in triplicate.

Histopathological examination

Upon collection of lung tissues, the samples were fixed and embeded. Afterwards, H&E staining and immunohistostaining were performed to determine the expression of CD68 (category No. 333801, Biolegend Inc.), MPO (category No. 66177-1-Ig, Proteintech Co, Ltd.) and VE-cadherin (category No. PA1-84328, Thermo) according to the previous description [6, 17, 18]. VE-cadherin was labeled with Cy3 (category No. BA1032, Boster Co., Ltd. Wuhan, China) with strict adhesion to the manufacturer's instructions. Afterwards, the images were observed under a BX51 light microscope (Olympus, Tokyo, Japan) and the Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan), respectively.

Western blotting

The VE-cadherin expression was determined using Western blot according to the previous study [19]. VE-cadherin was extracted from the lung tissues and cultured PMVECs, respectively. Then the membrane was blocked with 10% skimmed milk at room temperature for 1 h, followed by incubating with the primary antibody against VE-cadherin (1:1000; category No. PA1-84328, Thermo) and β-actin (1:700; Santa Cruz Biotechnology) overnight at 4°C. Afterwards, the mixture was incubated with the horseradish peroxidase-conjugated secondary antibody (1:5000; Zhongshan-Golden Bridge Biological Technology Co., Ltd., Beijing, China) for 1 h at room temperature. The same membrane probed with and β-actin served as loading control.

Flow cytometry

Flow cytometry was performed to analyze the cell apoptosis. All cells were immunostained using the Annexin V/PI apoptosis kit, according to the manufacturer's instructions. The apoptosis ratio was analyzed according to the previous study [20].

Immunofluorometric assay for the cellular skeleton

The endothelial cells were fixed using 4% paraformaldehyde, followed by addition of Actin-Tracker Green (1:100; category No. C1033, Beyotime Biotech). The mixture was incubated at room temperature for 30 min. Subsequently, counterstaining was performed after washing with PBS containing 0.1% Triton X-100 (category No. ST795, Beyotime Biotech). Fluoromount-G (Southern Biotech, category No. 0100-01, Birmingham, USA) was used to block the coverslip [21]. Finally, the images were observed under the BX53 fluorescence microscope (Olympus, Tokyo, Japan).

Permeability assay

Transendothelial electrical resistance, a term used to define the endothelial cell barrier function, was determined by using an electric cell substrate impedance sensing system (Applied BioPhysics, CA, USA) as described previously [22]. In brief, the cells were plated on sterile eight-chambered goldplated electrode arrays (8W10E plus) precoated with fibronectin and

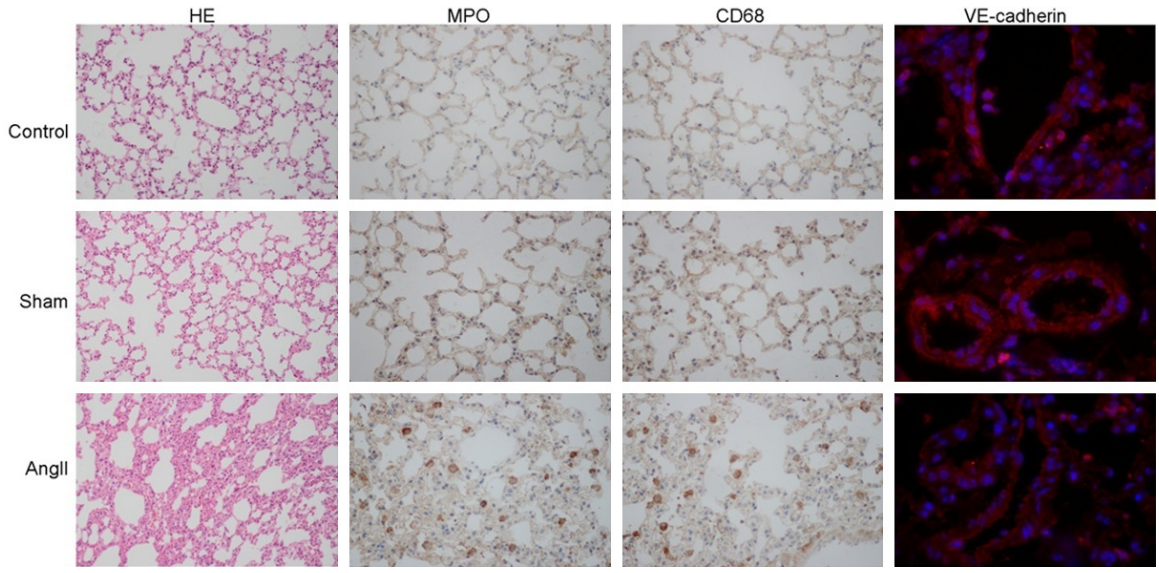

Figure 1. Obvious pulmonary edema was noticed in mice model of ALI after administration of AngII. Besides, infiltration of neutrophils (MPO) and macrophages (CD68) was noticed, together with PMVEC injury (VE-cadherin). HE staining, immunohistochemistry results and immunofluorescent images were observed under a magnification of 200×, 400×, and 1000×, respectively.

Table 2. Determination of MDA, SOD and W/D in each group

Group	MDA (nmol/mgprot)	SOD (U/mgprot)	W/D
Normal control	1.29±0.21	49.26±5.73	3.19±0.48
AngII group	4.45±0.38*	23.46±4.36*	4.72±0.48*
Sham group	1.31±0.29	48.97±6.52	3.22±0.50

*P < 0.05, compared with normal control.

grown to full confluence. Afterwards, the electrode arrays were mounted on the electric cell substrate impedance sensing system within an incubator at 37°C in 5% CO_2. Finally, the monolayer resistance was recorded at 15 kHz for 3 h in 5-min intervals.

Additional methods

The expression of malondialdehyde (MDA) content and the wet- to -dry weight ratios (W/D ratios) and superoxide dismutase (SOD) in lung were determined in this study. All the tests were carried out independently at least in triplicate.

Statistical analysis

The statistical analysis was performed with SPSS 20.0 software. Quantitative data were presented as mean ± standard error of mean (SEM). Student's t-test was used for the intergroup comparison, while the one-way ANOVA using Dunnett's test was conducted in multiple comparisons. *P* values of less than 0.05 were considered statistically significant.

Results

Elevation of serum AngII in AAD complicated with ALI patients

The concentration of AngII in the patients with AAD complicated with ALI was remarkably elevated compared to the normal individuals, the non-ruptured aneurysm patients, and the AAD patients without ALI (**Table 1**), which implied AngII may be associated with the onset of AAD complicated with ALI.

AngII induced ALI combined with impairment of endothelial barrier

Based on the remarkable elevation of AngII in AAD complicated with ALI patients, the mice model was established through infusion of AngII. Procedures were performed to verify whether the mice model was successfully induced. For the HE staining and immunohistostaining, obvious pulmonary interstitial edema was noticed in the mice model. Meanwhile, massive infiltration of inflammatory cells was noticed in the lung tissues in the AngII group, together with injury of PMVECs (**Figure 1**). Further, the MDA content and the W/D ratios in lung was remarkably elevated together with obvious decrease of SOD activities (**Table 2**).

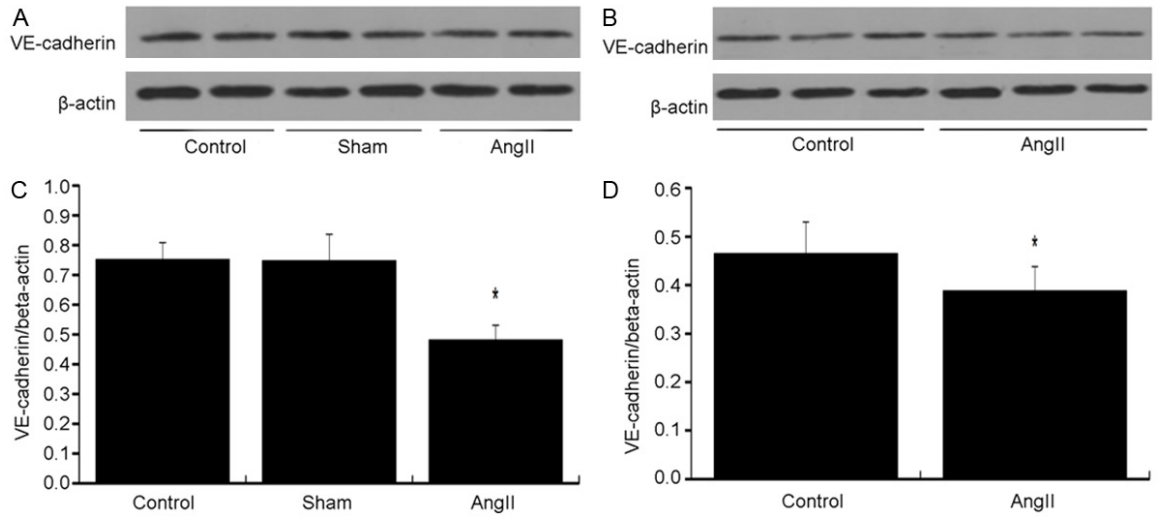

Figure 2. AngII induced obvious down-regulation of VE-cadherin expression in lung and cultured PMVECs. A, C. In the ALI model, the expression of VE-cadherin was remarkably down-regulated compared with the control group and Sham group. B, D. AngII induced down-regulation of VE-cadherin expression in cultured PMVECs. *P < 0.05 versus control group.

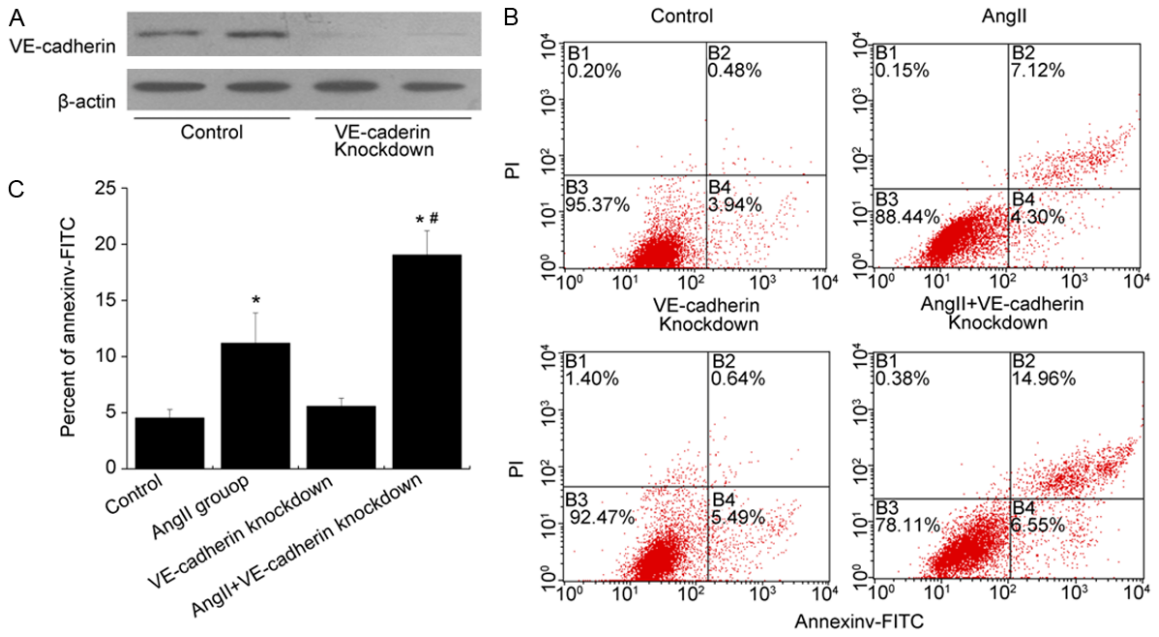

Figure 3. Down-regulation of VE-cadherin contributed to the sensitivity of PMVECs to apoptosis induced by AngII. A. Knockdown of VE-cadherin by RNAi was validated using Western blot analysis. B, C. Cell apoptosis determined by flow cytometry. *P < 0.05 versus control group; #P < 0.05 versus AngII group.

VE-cadherin down-regulation mediated by AngII in lung and PMVECs

To evaluate the effects of AngII on VE-cadherin expression in lung tissues, AngII-infused mice were sacrificed and lung sections were immunolabeled for VE-cadherin, and changes of lung VE-cadherin protein level were examined. As shown in **Figure 2A**, the expression of VE-cadherin in the AngII-infused mice was decreased compared with the normal group and Sham group.

To confirm the effects of AngII on VE-cadherin expression in vitro, we next examined the changes of protein level in cultured PMVECs. As

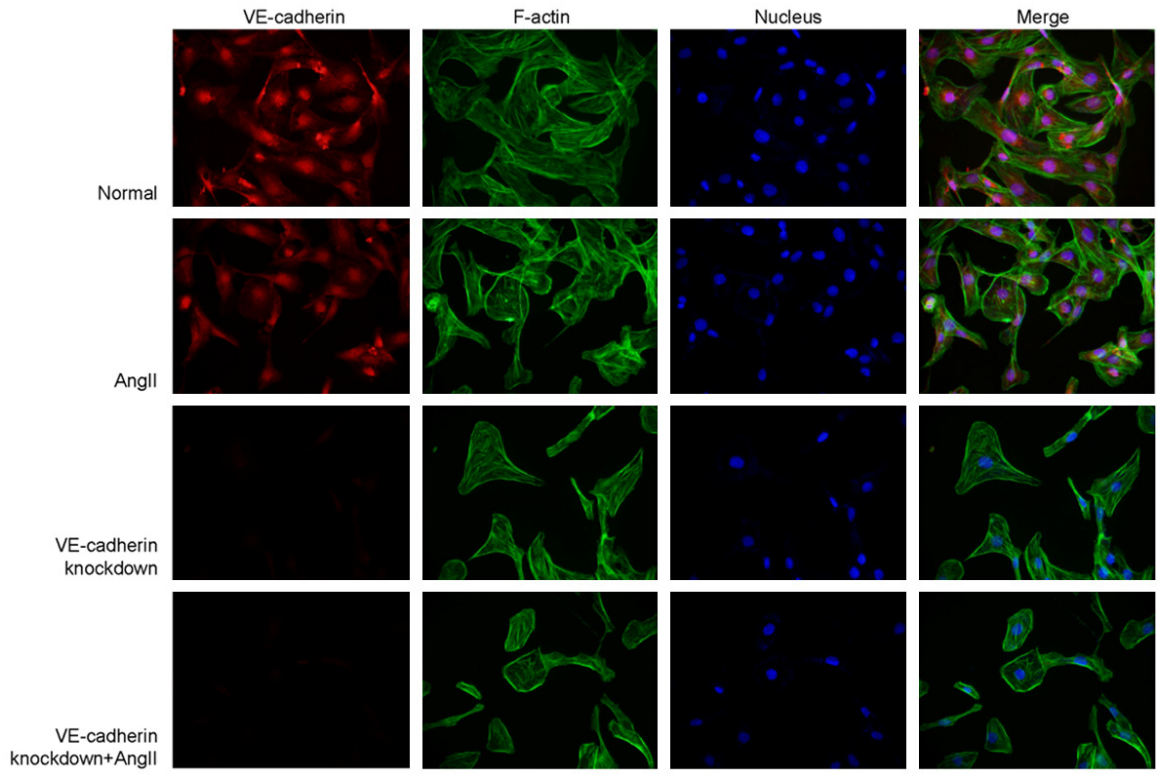

Figure 4. Down-regulation of VE-cadherin hampered the skeletal stability of PMVECs. AngII induced the down-regulation of VE-cadherin in PMVECs, together with obvious changes in the morphology and distribution of F-actin featured by stress fiber, filipodium and lamellipodia. After VE-cadherin knockdown, condensed stress fiber, endothelial cell shrinkage, and widening of intercellular space was observed in PMVECs. The images were observed under a magnification of 400×.

shown in **Figure 2B**, AngII treatment induced down-regulation of VE-cadherin protein expression in PMVECs.

Down-regulation of VE-cadherin contributed to the sensitivity of PMVEC to apoptosis

Our previous study indicated that AngII could induce apoptosis of PMVECs [23]. As shown in **Figure 3**, inhibition of VE-cadherin expression could not induce the apoptosis of PMVECs. Nevertheless, after the interference of AngII, the apoptosis of PMVECs was remarkably elevated. This indicated that means inhibited the expression of VE-cadherin could increase the sensitivity of apoptosis in PMVECs.

Down-regulation of VE-cadherin induced skeletal rearrangement in PMVECs

Actin cytoskeletal rearrangements and perturbations were reported to be related to the other model systems as they were involved in the PMVEC barrier disruption [24, 25]. Meanwhile, the VE-cadherin was closely related to the cellular rearrangement of the skeleton [26, 27]. Thus, the actin in fixed cells was determined through phalloidin staining and fluorescence microscopy. As shown in **Figure 4**, the F-actin in the rat PMVECs was mainly distributed along the cell membrane, and the microfilament was regularly arranged in the cytoplasm. After treating with AngII, the expression of VE-cadherin was decreased. Meanwhile, obvious changes were noticed in the morphology and distribution of the F-actin, together with anomalies in the stress fiber, filipodium and lamellipodia. Nevertheless, after knock down of VE-cadherin, accumulation of stress fiver, shrinkage of endothelial cells and widening of intercellular space were noticed. This indicated that the stable expression of VE-cadherin played a pivotal role in the stability of PMVEC skeleton.

Down-regulation of VE-cadherin promoted the permeability of PMVECs

The endothelial permeability of pulmonary microvascular vessels is crucial for the pulmonary injury [28]. Therefore, to investigate the

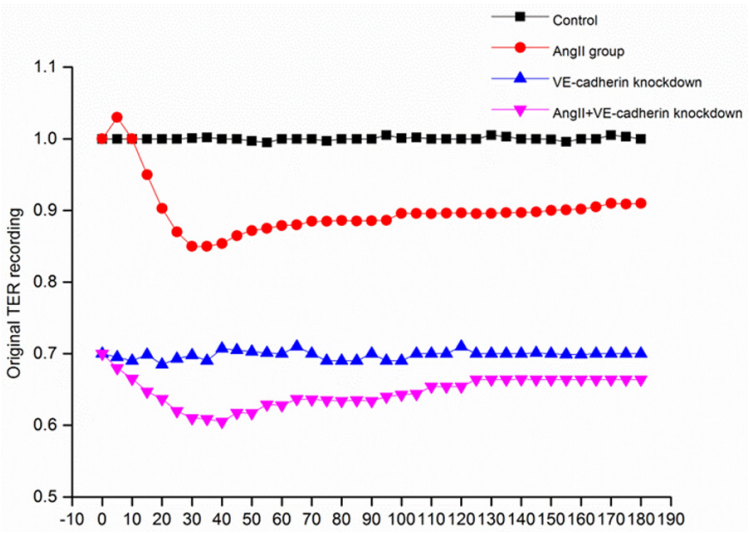

Figure 5. PMVECs were grown to confluence and quiescent monolayer, and TEER was recorded. AngII induced decrease of resistance in PMVECs, together with increased permeability. After knockdown of VE-cadherin, the monolayer resistance in PMVECs showed further decrease, together with obvious increase in the permeability.

endothelial barrier function, we measured monolayer PMVECs permeability using the electrical cell substrate impedance sensing system. As shown in **Figure 5**, the stimulation of AngII could induce decrease of resistance in the PMVECs, together with elevated permeability. Meanwhile, knock down of VE-cadherin expression by RNAi could decrease the resistance in the PMVECs obviously together with remarkable elevation in the permeability.

Discussion

AngII has been reported to be closely related to the pathogenesis of AAD complicated with ALI. Vascular endothelial barrier dysfunction including endothelial apoptosis and elevated permeability was the pathological basis for the AAD complicated with ALI [23]. Therefore, we aim to investigate the correlation between the AngII and endothelial barrier injury in the pulmonary microvascular vessels. In the presence of vascular endothelial barrier dysfunction, accumulation of macrophages and neutrophils in the pulmonary stroma may be induced together with onset of pulmonary edema. Therefore, it is reasonable to speculate that the dysfunction of vascular endothelial barrier may be closely related to the AngII.

Endothelial barrier plays a crucial role in the permeability of large molecules and the circulating cells from the blood to the specific tissues. The adherens junctions between the endothelial cells was reported to be closely involved in the cellular junction of the endothelial cells. In structure, the junction was composed of the VE-cadherin complex formed by VE-cadherin, catenin and the actin microfilament [29]. VE-cadherin was reported to play crucial roles in maintaining the integrity of endothelial barrier. This leads us to investigate the association between VE-cadherin and the vascular endothelial barrier. Currently, most of the VE-cadherin related studies have been focusing on the effects of tyrosine residue phosphorylation in the VE-cadherin complex and endothelial adhesion stability. For example, Potter et al reported that ICAM-1 stimulation resulted in phosphorylation of VE-cadherin, and this process was a prerequisite for adherens junction disassembly. In HUVECs, the kinases Src and Pyk2 phosphorylated VE-cadherin on the p120 and β-catenin binding sites tyrosine residues 658 and 731, respectively. This process inhibited the binding between VE-cadherin and p120 or β-catenin [9]. Besides, Gavard et al revealed Rac1 activation was associated with the phosphorylation of VE-cadherin on serine 665, which signaled its clathrin-dependent internalization [30]. Furthermore, VE-cadherin was reported to involve in various biological processes, including cellular survival and signaling transduction [31, 32]. In this study, we confirmed that VE-cadherin played pivotal roles in the vascular endothelial barrier dysfunction mediated by AngII. Meanwhile, two major mechanisms including endothelial apoptosis and skeletal recombination were closely related to the onset of vascular endothelial barrier dysfunction. On this basis, we investigated the correlation between VE-cadherin and the endothelial apoptosis and skeletal recombination in such process.

In our previous study, AngII was reported to induce apoptosis of PMVECs, while the roles of VE-cadherin in such process were not well defined despite the VE-cadherin complexes have been confirmed to be central force transducers in endothelial barrier. Up to now, most of the studies on VE-cadherin have been focused on its roles in the cell-to-cell adhere, and few studies investigated its roles in the apoptosis. According to the in vivo results in mice, AngII interference induced down-regulation of VE-cadherin in the PMVECs. On this basis, we established knock-down of VE-cadherin through RNAi, and the apoptosis of PMVEC induced by AngII was remarkably increased. However, VE-cadherin knock-down was not correlated to the apoptosis directly. This indicated that VE-cadherin may play a protective role in the PMVECs, while its down-regulation may increase the sensitivity of endothelial cells to the apoptosis. Furthermore, Liu et al indicated that VE-cadherin was associated with the cellular apoptosis in the vascular endothelial cells. To be exact, VE-cadherin exhibited an anti-apoptosis effect through enhanced PKC signaling and an enhanced cell proliferation pathway[33].

It has been well acknowledged that EC barrier integrity is critically depending on the cytoskeletal structure [34], while VE-cadherin is crucial for the skeletal stability [35-37]. Our results indicated the expression of VE-cadherin was down-regulated after AngII interference, and obvious changes were noticed in the morphology and distribution of F-actin in PMVECs. Nevertheless, after VE-cadherin knockdown, the VE-cadherin complex was interrupted, which was featured by accumulation of stress fibers in the cytoplasm and obvious decrease of cellular adhesion. Taken together, it is reasonable to speculate that VE-cadherin expression is pivotal for the skeletal stability in PMVCEs and the integrity of cell-cell junction. In ALI, the endothelial cell barrier is interrupted, which subsequently leads to increase of vascular permeability.

In this study, we investigated the roles of VE-cadherin in the pulmonary microvascular endothelial cell barrier injury mediated by AngII through determination of trans-endothelial electrical resistance (TEER) of monolayer PMVECs. The results indicated that the expression of VE-cadherin was down-regulated in the PMVECs after AngII interference, which in turns promoted the susceptibility of PMVECs to apoptosis. Meanwhile, the down-regulation of VE-cadherin contributed to the skeletal rearrangement. All these are essential for the elevation of pulmonary microvascular endothelial permeability. Our study provides new strategies for the management of AAD complicated with ALI.

Disclosure of conflict of interest

None.

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