Roles of VE-Cadherin in Hypoxia Induced Injury of Pulmonary Microvascular Endothelial Barrier

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ABSTRACT

Background: Hypoxia induced injury of pulmonary microvascular endothelial barrier is closely related to the pathogenesis of acute lung injury after lung transplantation. VE-cadherin is an important structural molecule for pulmonary microvascular endothelial barrier. In this study, we aim to investigate the roles of VE-cadherin in hypoxia induced injury of pulmonary microvascular endothelial barrier.

Methods: Rat model of hypoxia and cultured pulmonary microvascular endothelial cells (PMVECs) were utilized. Determination of PMVECs apoptosis, skeleton combination was conducted to verify the effects of hypoxia on injury of pulmonary microvascular endothelial barrier. In addition, VE-cadherin expression was modulated by administration of siRNA in order to investigate the roles of VE-cadherin in hypoxia induced PMVECs apoptosis and skeleton recombination.

Results: Our data indicated that expression of VE-cadherin was down-regulated in hypoxia-exposed PMVECs. Whereas, in the cells treated using siRNA, down-regulation of VE-cadherin did not trigger PMVECs apoptosis, but it increased the sensitivity of PMVECs to the hypoxia induced apoptosis. In cases of hypoxia, the expression of VE-cadherin was significantly down-regulated, together with endothelial skeleton recombination and increase of permeability, which then triggered endothelial barrier dysfunction.

Conclusions: These data verify that VE-cadherin expression played an important role in hypoxia induced apoptosis and cellular skeletal recombination.

INTRODUCTION

Most of the studies on lung transplantation have been focused on the immunological rejection. Little is known about the molecular mechanism and prevention of vascular endothelial injury after pulmonary ischemia and hypoxia. To date, no effective treatment options and prevention methods are available for the ischemia-reperfusion injury of lung.

Hypoxia induced injury of pulmonary microvascular endothelial cells (PMVECs), such as cellular apoptosis and barrier function injury, which then triggered infiltration of inflammatory cells and release of inflammatory factors that were considered as the major causes for acute lung injury after pulmonary transplantation. PMVECs, localized in the internal side of the blood air barrier [Stevens 2011], formed the endothelial barrier together with the adjacent endothelial cells. The adherens junction formed by adjacent PMVECs was the most complex structure in the endothelial junction, with its function serving as an important type of junction [Taveau 2008].

VE-cadherin is the major structural molecule for the adherens junction of vascular endothelial cells [Abu Taha 2014]. Its expression could trigger injury of vascular endothelial barrier function and increase of microvascular permeability, which played important roles in the pathogenesis of various diseases [Kandasamy 2015; Huang 2015]. In this study, RNAi technique was utilized to modulate the expression of VE-cadherin, and then the roles of VE-cadherin in hypoxia induced PMVECs apoptosis and skeletal recombination was investigated. Our findings indicated that hypoxia induced down-regulation of VE-cadherin and increase of sensitivity of PMVECs to the hypoxia induced apoptosis. Besides, VE-cadherin down-regulation resulted in recombination of PMVECs skeleton. These indicated that VE-cadherin played crucial roles in hypoxia induced endothelial barrier dysfunction.

MATERIALS AND METHODS

Establishing hypoxia induced pulmonary injury model in rats: Male Sprague-Dawley rats (7 weeks old) were anesthetized after trachea cannula connecting the respirator
with a respiratory frequency of 80-90/min. The respiratory exchange ratio was set at 1.2-1.3 with a working pressure of 2-4 kPA as previously described [Wu 2016].

Rats were divided into four groups: (i) sham control group (N = 7), which were subjected to sham operation; (ii) ischemia for 30 min (N = 7); (iii) ischemia for 3h group (N = 7); and (iv) ischemia for 12h group (N = 7). The chest was entered by the 4th intercostal space at the left body side, followed by heparinization and ligation of left inferior lung lobe for 30 min, 3h, and 12h, respectively. The animals were sacrificed at these time points to obtain the samples from left inferior lung and serum samples. The animal study protocols were approved by the Ethical Committee of the Beihua University Hospital and all animal handling was performed in accordance with the Jilin Directive for Animal Research and the current Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health. Rats were sacrificed by decapitation after being successfully anesthetized with 2% Phenobarbital (40 mg/Kg). Unnecessary suffering was avoided as far as possible.

Cell culture: Rat PMVECs purchased from Bena Culture Collection (category No. BNCc38210, Suzhou, China) were cultured using the standard method as previously described [Wu 2016]. Briefly, cells were cultured in endothelial culture medium (No. 1001, Sciencell) supplemented with 5% fetal bovine serum (FBS, No. 0025, Sciencell), 1% endothelial cell growth supplement (No. 1052, Sciencell) and 1% penicillin/streptomycin solution (No. 0503, Sciencell) in 21% oxygen and 5% carbon dioxide at 37°C. PMVECs (P2-4) were expanded in monolayers in cell culture bottle. The culture medium was changed to a serum-free solution for 24 h prior to usage.

To investigate the roles of VE-cadherin in hypoxia induced PMVECs apoptosis in vitro, cells were divided into four groups, including the control group; hypoxia interference group treated by 5% O₂; VE-cadherin knockdown group subject to knockdown of VE-cadherin by RNAi technique; and hypoxia interference +VE-cadherin knockdown group subject to 5% O₂; and knockdown of VE-cadherin by RNAi.

Knockdown of VE-cadherin expression by RNAi: Smart Pool siRNA for VE-cadherin was obtained from Dharmacon (Lafayette, CO). Transient transfection of PMVECs with sequence specific siRNA or scrambled control was completed using oligofectamine (Invitrogen, Carlsbad, CA), 200 nm siRNA and 1.5x10⁵ cells per reaction as previously described [O’Leary 2010]. Cells were then treated with DNR, and collected at 72 h for evaluation of apoptosis and cytoskeleton.

Histopathological examination: After lung tissue sample collection, HE staining was performed to determine the pulmonary injury extent. The images were obtained from a BX51 light microscope (Olympus Corporation, Tokyo, Japan). Meanwhile, an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan) was utilized for the imaging collection and analysis.

Western blotting analysis: VE-cadherin was detected using a standard Western blot protocol to determine the expression of VE-cadherin protein [Orsenigo 2012]. The transferred membrane was blocked with 10% skimmed milk for 1 h at room temperature, and then was incubated with the primary antibody against VE-cadherin (1:1000) and β-actin (1:1000) overnight at 4°C. After incubation with the horse-radish peroxidase-conjugated secondary antibody (1:5000) for 1 h at room temperature, the immunoblotting signals were visualized using a Luminescent Detection kit (Vigorous Bio-technology, Beijing, China).

TUNEL assay: Cellular apoptosis was determined using TUNEL assay as previously described [Wu 2018; Takeda 2015]. Sections were incubated with TUNEL reaction mixture (11684817910, Roche, Germany), according to the manufacturer’s instructions. Sections were then observed using an Eclipse 80i fluorescence microscope (Nikon, Tokyo, Japan). Subsequently, 10 visual fields randomly were selected and observed under a magnification of 200×.

Immunofluorometric assay: Immunofluorometric assay was carried out to determine the cellular skeleton. The coverslip wrapped by the PMVECs was fixed using paraformaldehyde, and then Actin-Tracker Green (1:100, C10330, Beyotime) was added and incubated at room temperature for 30 min after washing with PBS containing 0.1% Triton X-100, the cells were counterstained. The mixture was then incubated at room temperature for 30 min. Fluoromount-G (category No. 0100-01, Birmingham, USA) was used to block the coverslip [Auda 2013]. Finally, the images were observed using a BX53 fluorescence microscope (Olympus, Tokyo, Japan). A comet assay was performed to determine the total number of DNA strands.

Determination of pulmonary microvascular permeability: Pulmonary microvascular permeability was evaluated through administration of azovan coeruleus via caudal vein injection. The content of azovan coeruleus was determined under a wavelength of 625 nm utilizing to evaluate the extent of pulmonary microvascular permeability.

Measurement of laboratory indices: Indices including expression of malondialdehyde (MDA), superoxide dismutase (SOD) activity, and the weight-to-dry weight (W/D) ratio in pulmonary tissues were detected. The mean value was obtained after at least three triplicates.

Statistical analysis: SPSS 20.0 software was utilized for the statistical analysis. Quantitative data was presented as the mean ± standard error of mean (SEM). Statistically significant differences in mean values were tested by Student’s t-test or by one-way ANOVA using Dunnett’s test in multiple comparisons. P < 0.05 was considered to be statistically significant.
Hypoxia is a type of inevitable injury factor for organ transplantation. It affects the functional recovery of implanted organs and is closely related to acute rejection and chronic implanted organ hypofunction [Requiao-Moura 2011]. Recently, the standardization of donation after cardiac death (DCD) contributed to the organ transplantation, but there still are some challenges. This extends the organ source for the patients with end-stage diseases, however, hypoxia induced injury during storage, transmission and transplantation may hamper the efficiency of organ utilization [Elgharably 2015].

Hypoxia may trigger pulmonary microvascular endothelial barrier function, including endothelial apoptosis and increase of permeability, which then resulted in acute lung injury. Therefore, we aimed to investigate the correlation between hypoxia and pulmonary microvascular endothelial barrier injury. To our best knowledge, pulmonary microvascular endothelial barrier can limit the large molecules generated in endothelial cells and circulating cells in blood other than penetrating into the tissues. Meanwhile, the permeability of endothelial cells mainly was modulated by the endothelial adherens junction.

The endothelial adherens junction is the most important type of cellular junction. It is formed by VE-cadherin catenin complex consisting of VE-cadherin, catenin, and actin microfilament [Padmanabhan 2015]. VE-cadherin molecule consisted of 780 amino acids, which was divided into extracellular region, intracellular region, and transmembrane domain. Specifically, the amino terminal (extracellular region) showed isoform junction [Baumgartner 2000], which could form adherens junction with the VE-cadherin related molecules in the adherens junction [Guo 2015]. As an important structural molecule in endothelial adherens junction, VE-cadherin played important roles in apoptosis of endothelial cells. On this basis, we utilized RNAi to modulate the expression of VE-cadherin in order to evaluate its roles in hypoxia induced PMVEC apoptosis and permeability. As shown in Figure 3, inhibition of VE-cadherin could not trigger PMVEC apoptosis. (Figure 3) However, in the presence of hypoxia, it could enhance apoptosis of PMVECs. This indicated that inhibition of VE-cadherin expression promoted the apoptosis of PMVECs.

Roles of VE-cadherin in PMVEC skeletal recombination induced by hypoxia: There was accumulation of F-actin fiber in PMVECs in normal rats in the peripheral tissues of adjacent cell membrane. (Figure 4) In addition, the microfibril in cytoplasm was arranged in a regular manner. In cases of hypoxia, the expression of VE-cadherin was down-regulated, and obvious changes were noticed in the profile and distribution of F-actin fibers, which were featured by stress fiber, filipodium, and lamellipodia. There were massive stress fibers in the cytoplasm after knockdown of VE-cadherin, together with endothelial cell shrinkage and formation of intracellular fissures. On this basis, we speculated that the expression of VE-cadherin played crucial roles in the stability of PMVECs skeleton.

**Table 1. Determination of pulmonary microvascular permeability and W/D in each group**

<table>
<thead>
<tr>
<th>Group</th>
<th>Pulmonary microvascular permeability (μg/mg)</th>
<th>W/D</th>
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<tbody>
<tr>
<td>Normal control</td>
<td>0.049±0.003</td>
<td>3.28±0.61</td>
</tr>
<tr>
<td>Hypoxia 30 min group</td>
<td>0.050±0.006</td>
<td>3.31±0.52</td>
</tr>
<tr>
<td>Hypoxia 3h group</td>
<td>0.107±0.02*</td>
<td>4.93±0.70*</td>
</tr>
<tr>
<td>Hypoxia 12h group</td>
<td>0.171±0.04*</td>
<td>5.53±0.85*</td>
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*P < 0.05, compared with normal control

**Table 2. Determination of MDA, and SOD in each group**

<table>
<thead>
<tr>
<th>Group</th>
<th>MDA (nmol/mgprot)</th>
<th>SOD (U/mgprot)</th>
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<tbody>
<tr>
<td>Normal control</td>
<td>1.3±0.41</td>
<td>51.49±7.53</td>
</tr>
<tr>
<td>Hypoxia 30 min</td>
<td>1.34±0.39</td>
<td>49.77±8.25</td>
</tr>
<tr>
<td>Hypoxia 3h group</td>
<td>2.94±0.58*</td>
<td>34.63±6.03*</td>
</tr>
<tr>
<td>Hypoxia 12h group</td>
<td>4.41±0.78*</td>
<td>28.35±7.11*</td>
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*P < 0.05, compared with normal control

Figure 1. Hypoxia induced animal model based on ligation of left inferior lung lobe. Obvious edema and massive cellular infiltration was noticed in the lung tissues in the model group after HE staining. (A) sham control group; (B) ischemia for 30 min; (C) ischemia for 3h group; (D) ischemia for 12h group.

DISCUSSION

Hypoxia may trigger pulmonary microvascular endothelial barrier function, including endothelial apoptosis and increase of permeability, which then resulted in acute lung injury. Therefore, we aimed to investigate the correlation between hypoxia and pulmonary microvascular endothelial barrier injury. To our best knowledge, pulmonary microvascular endothelial barrier can limit the large molecules generated in endothelial cells and circulating cells in blood other than penetrating into the tissues. Meanwhile, the permeability of endothelial cells mainly was modulated by the endothelial adherens junction.

The endothelial adherens junction is the most important type of cellular junction. It is formed by VE-cadherin catenin complex consisting of VE-cadherin, catenin, and actin microfilament [Padmanabhan 2015]. VE-cadherin molecule consisted of 780 amino acids, which was divided into extracellular region, intracellular region, and transmembrane domain. Specifically, the amino terminal (extracellular region) showed isophil junction [Baumgartner 2000], which could form adherens junction with the VE-cadherin related molecules in the adjacent vascular endothelial cells. These demonstrated...
that VE-cadherin contributed to intact of endothelial barrier. In recent studies, VE-cadherin could modulate the cellular viability and involve in signaling transmission [Birdsey 2008; Carmeliet 1999]. Therefore, it has been considered as an important target for endothelial barrier function. VE-cadherin expression alternation may be associated with biological behaviors of PMVECs and barrier function.

In a previous study, hypoxia could induce apoptosis of PMVECs [Guo 2015], whereas little is known about the VE-cadherin serving an important molecule role in the adherens junction. In this study, the expression of VE-cadherin was down-regulated in the PMVECs under hypoxia conditions. Our study further indicated that after knockdown of VE-cadherin using RNAi, the PMVECs viability showed no statistical difference, but it could contribute to the hypoxia induced PMVECs apoptosis accordingly. These findings implied that VE-cadherin played protective roles on PMVECs, and its down-regulation could increase the sensitivity of endothelial cells to apoptosis.

Endothelial barrier intact has been reported to be associated with the endothelial skeleton [Kasa 2015], while VE-cadherin is crucial for the stability of cellular skeleton [Orsenigo 2012; Weidert 2014; Wallez 2007]. Our findings indicated that the F-actin in the PMVECs of normal rats was mainly accumulated in the peripheral tissues of the adjacent membrane, and the arrangement of the microfilament in the cytoplasm was regular. In cases of hypoxia, the expression of VE-cadherin was down-regulated, and the profile and distribution

Figure 2. Hypoxia induced significant down-regulation of VE-cadherin in lung tissues and cultured PMVECs. (A) In the model group, the expression of VE-cadherin in lung tissues showed significant decrease compared with control. (B) In the presence of hypoxia, the VE-cadherin expression in cultured PMVECs showed significant decrease. *P < 0.05 versus control group

Figure 3. Down-regulation of VE-cadherin increased the sensitivity of PMVECs to the hypoxia-induced apoptosis. TUNEL revealed apoptosis of PMVECs. *P < 0.05 versus control group; # P < 0.05 versus hypoxia group

Figure 4. VE-cadherin down-regulation hampered the skeleton of PMVECs. In the presence of hypoxia, the VE-cadherin was down-regulated in PMVECs. Additionally, there were significant changes in the distribution and profile of F-actin in PMVECs, which were featured by presence of stress fiber, filipodium and lamellipodia. After VE-cadherin knockdown, massive stress fiber was noticed in PMVECs together with endothelial shrinkage and formation of inter-cellular fissure.
of F-actin showed obvious changes, which led to formation of stress fiber, filipodium and lamellipodia. Whereas, in the presence of VE-cadherin knockdown, massive stress fiber was noticed in the cytoplasm together with shrinkage of endothelial cells and formation of intracellular space. This indicated that stable expression of VE-cadherin was crucial for the stability of PMVEC skeleton.

There were some limitations to this study. First of all, the sample size was not large for the in vivo experiments. To solve this problem, in our subsequent study, we may expand the sample size. Secondly, we cannot identify the exact mechanism of VE-cadherin down-regulation in PMVECs. In the future, we will focus on it.

In summary, hypoxia could induce pulmonary injury through hampering the endothelial barrier function. For the mechanism, it may be related to the down-regulation of VE-cadherin in PMVECs in presence of hypoxia, which in turn contributed to the sensitivity of PMVECs to the hypoxia induced apoptosis. Besides, VE-cadherin down-regulation promoted skeletal recombination of PMVECs.

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REFERENCES


