Enhanced Expression of Robo4 Ameliorates LPS Induced Acute Lung Injury in Mice through Binding to Rhoa

Xueling Wu1, Xiaoyuan Sun1, Bin Liu1 and Yunfeng Zhao2* 

1Department of Respiratory Medicine, Renji Hospital, School of medicine, Shanghai Jiao Tong University, Shanghai, PR China 
2Department of Respiratory Medicine, Punan Hospital, Pudong New District, Shanghai, PR China 

Abstract

Robo4 maintains the barrier function of the mature vascular network by inhibiting endothelial permeability induced by pro-angiogenic factor and the function of Robo4 was tissue specific. The aim of this study was to investigate the role and signaling pathways of Robo4 in pulmonary endothelial permeability in Lipopolysaccharides (LPS) induced acute lung injury (ALI) in mice. Mice were challenged by intra-peritoneal (IP) injection of LPS (15 mg/kg) 48 h after gene transfer of Retro-hRobo4. Protein in bronchoalveolar lavage fluid (BALF), lung wet/dry ratio, lung injury score, MPO activity, secondary cytokine and survival rate was recorded. Co-immunoprecipitation (Co-ip) was used to detect the binding of Robo4 and RhoA. Robo4 mRNA and protein concentration in mice lungs were significantly increased after retrovirus-mediated gene delivery. Up-regulation of Robo4 significantly decreased protein in BALF, lung wet/dry ratio, lung injury score and MPO activity while intercellular tight junction protein ZO-1 was significantly increased. However, up-regulation of Robo4 didn’t change the concentration of TNF-α, vWF and VEGF in serum of mice. In addition, administration of Retro-hRobo4 significantly improved the survival of mice. Robo4 was bind to RhoA confirmed by Co-ip. These findings suggest that up-regulation of Robo4 enhanced pulmonary micro-vascular integrity and reduced lung edema in LPS induced ALI partially through binding to Rhoa.

Keywords: Roundabout 4 (Robo4); Acute lung injury; Endothelial permeability; Tight junction (TJ); Rhoa

Abbreviations: ALI: Acute Lung Injury; Robo4: Roundabout 4; TJ: Tight Junction; BALF: Broncho Alveolar Lavage Fluid; IL: Interleukin; TNF-a: Tumor Necrosis Factor a; Co-ip: Co-Immunoprecipitation; TLR4: Toll-Like Receptor 4; LPS: Lipopolysaccharide; qRT-PCR: Quantitative Real Time PCR; ELISA: Enzyme Linked Immunosorbert Assay

Introduction

Lung edema induced by increased endothelial permeability is a life-threatening complication accompanying acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) [1]. Bacterial can significantly increase pulmonary endothelial permeability through inflammatory responses in the infected lungs [2]. Unfortunately, except small tidal volume ventilation strategies, no other standard treatment exists for high permeability induced lung edema, thus there is a strong demand to have novel targets that could reduce endothelial permeability [3].

Roundabouts (Robos) are a class of neural guidance receptors that bind the slit family of guidance cues and primarily mediate axon repulsion signals [4]. Slit-Robo signaling has also been implicated in inhibition of leukocyte migration [5]. Three Robo receptor family members have been identified, in which robo4 is the predominant is expressed in embryonic vasculature during development [6]. Various studies have shown Robo4 is essential for angiogenesis [7]. Robo4 maintains the barrier function of the mature vascular network by inhibiting endothelial permeability induced by pro-angiogenic factors. Robo4 siRNA increased the permeability of human retinal vascular endothelial cells (HRVECs) mono-layers and that LIM-kinase 1 (LIMK1)/cofilin signal transduction system may be involved in the modulating process [8]. In another study, pretreatment with an active N-terminus fragment of Slit2, a Robo4 agonist, protected lymphatic endothelial cells from HIV-1 gp120-induced hyper-permeability by inhibiting c-Src kinase activation [9] Because the function of Robo4 was tissue specific [10], we aimed to study whether up-regulation of Robo4 could reduce lung endothelium permeability in acute lung injury. Furthermore, we explored whether Robo4 could enhance the lung vascular integrity through binding to Rhoa.

Procedure

Generation of retroviral vectors

hRobo4 was generated by PCR and sub-cloned into the pR-RES vector. cDNA coding for hRobo4 was excised from pR-RES-hRobo4 and ligated into the retroviral plasmid vectors pMSCV to generate the plasmid pMSCV-hRobo4. The retroviral plasmid vectors pMSCV- hRobo4, pVSV, and pGAG-POL were cotransfected into the packaging cell line 293 EBNA under mediation of lipofectamine. A high-titer retrovirus was obtained as a result and the titer of viruses was determined in 293T cells by real time RT-PCR (Takara). Meanwhile, a control vector, free of any transgenes, was constructed using the same method. The recombinant and control virus were named as Retro-hRobo4 and Retro-V respectively.

Establishment of LPS-induced ALI mouse model and experimental design

The experimental protocol was performed with the approval of the Animal Care Committee of Renji Hospital, Shanghai Jiaotong University followed the ARRIVE and Directive 2010/63/EU guidelines

*Corresponding author: Yunfeng Zhao, Department of Respiratory Medicine, Punan Hospital, Pudong New District, Shanghai, PR China, Tel: +86 18617197928; Fax: 86-21-20302592; E-mail: yzfzh71@126.com

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[11]. Balb/c mice (18-22 g) were intra-nasally inoculated with 1.2 × 10^6 TU/ml of Retro-hRobo4 or Retro.V. LPS (Escherichia coli 0127: B8, Sigma, St Louis, Mo) at 15 mg/kg was intra-peritoneally (IP) injected to establish ALI mouse model 48 h after administration of Retro-hRobo4. The mice were killed at 0 h, 6 h, 12 h after LPS challenge (n=5 per time point in each group).

To evaluate the effect of Robo4 on the outcome of LPS-induced ALI, another 30 mice were divided into two equal groups: (1) Retro. V+ LPS, and (2) Retro-hRobo4+ LPS mouse group and given the same treatments as above. For survival experiments, the survival time was recorded daily for 7 days.

**Quantitative Real-Time PCR**

Total RNA from lungs were extracted with Trizol reagent (Invitrogen, Carlsbad, CA). 2 μg RNA was reversely transcribed to complementary deoxyribonucleic acid and 40 ng of complementary deoxyribonucleic acid was used as a template for real-time reverse transcription polymerase chain reaction in ABI RISM 7900. Gene-specific oligonucleotides were designed using Primer Express 2.0 software (Applied Biosystems, Carlsbad, CA) as previously described (24). The primer sequences of Robo4 and ZO-1 were shown in Table 1. The amplification cycling reactions (40 cycles) were performed as follows: 15 second at 95 °C and 1 min at 60 °C. Relative quantification values of the target genes were standardized according to the comparative threshold cycle (2-ΔΔCt).

**Western blotting assay**

The proteins were loaded in SDS-PAGE and the proteins were transferred to PVDF membranes using a steady flow model (200mA) over 60 min after electrophoresis. Then being blocked with non-fat milk in a TBST buffer containing 0.05% Tween-20, and the membranes were incubated with primary antibody at 4 °C overnight. The next day, the primary antibody was detected by chemo-luminescence using an appropriate peroxidase conjugated secondary antibodies. The intensity of each band was analyzed by the Image J software (NIH, USA).

**Lung injury score**

Lung tissue slides were stained with hematoxylin and eosin (HE) for histologic evaluation. Lung injury was scored by a blinded observer according to the following three criteria: (a) alveolar and interstitial edema, (b) alveolar hemorrhage, and (c) infiltration or aggregation of neutrophils. Each criterion was graded according to a four-point scale: 0=normal, 1=mild change, 2=moderate change and 3=severe change. The scores for criteria 1 through 3 were summed to represent the lung injury score in mice (total score, 0-9).

**Determination of lung myeloperoxidase activity**

To measure tissue myeloperoxidase (MPO) activity, frozen lungs were thawed and extracted for MPO, following the homogenization and sonication procedure as described previously. MPO activity in the supernatant was measured and calculated from the absorbance (at 460 nm) changes resulting from decomposition of H₂O₂ in the presence of o-dianisidine.

**Secondary cytokine release**

TNF-α, vWF and VEGF levels in serum of mice were measured by commercially available enzyme-linked immunosorbent assay (ELISA) kits for mouse according to the protocols provided by the manufacture.

**Analysis of the BALF protein**

Bronchoalveolar lavage fluid (BALF) was done by intra-tracheal instillation of 1.0 ml of PBS into the lungs in situ with gentle and repeated flushing three times. The BALF was then centrifuged at 306 g for 10 min, and the supernatant was frozen at -80 °C for subsequent analysis. The concentration of protein in the BALF was measured using the Micro BCA Protein Assay Kit (Pierce, MA).

**Lung wet/dry ratio**

The mouse chest cavity was opened by a median sternotomy after euthanasia, and the whole lungs were excised. Exsanguinated entire lung wet weights were measured on an electronic scale to obtain the wet weight, and then dried in oven at 55 °C for 72 h before recording the dry weight.

**Co-immunoprecipitation**

Lung tissues from mice were cut off and lysed with cold protein lysis buffer with 1 mM PMSF for 30 min and, were centrifuged at 12000 rpm for 10 min at 4°C. Supernatant of cell lysates were transferred into new tubes and mixed with primary antibodies and incubated at 4°C with gentle agitation overnight. Then protein A/G beads were added to capture antigen antibody complex, which subsequently proceeded heat denaturing and immunoblotting.

**Statistical analysis**

Data in text and figures are expressed as the mean ± SEM. ANOVA was used to compare experimental groups to control values. Comparisons between multiple groups were done using Student-Newman-Keuls test. Kaplan-Meier curves were analyzed using the log-rank test. Statistical significance was determined as P<0.05.

**Results**

**Retrovirus vector mediated the up-regulation of Robo4 in mice lungs**

To evaluate the effects of vector expression of Robo4, quantitative real-time RT-PCR and western blot were performed. The results showed that Retro-hRobo4 significantly increased the expression of Robo4 in mice lungs (Figure 1). Furthermore, Robo4 mRNA and protein was constitutively expressed in mouse lungs after LPS stimulation. Compared with those in Retro-V+LPS group, Robo4 mRNA and protein expression were still significantly increased in Retro-hRobo4+LPS group at each time point.

**Over-expression of Robo4 in the lungs decreased lung injury score in mice**

No significant histological changes were observed either in Retro-V or Retro-hRobo4 pretreated mice before LPS challenge (Figures 2A and 2B),

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (Forward)</th>
<th>Primer Sequence (Reverse)</th>
</tr>
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<tbody>
<tr>
<td>mGAPDH</td>
<td>TGTGCAGTCCAGCATCCTGCT</td>
<td>AGACCGCAGCTTCACCCCAT</td>
</tr>
<tr>
<td>homo&amp;mus Robo4</td>
<td>CAGACCGACGTGGGATGATGC</td>
<td>GGCCTCGCTGGTTAGGTAG</td>
</tr>
<tr>
<td>homo&amp;mus ZO-1</td>
<td>AGCCTCCAGGTGTGGGAGATG</td>
<td>GCCCTCAGCTGTTAGGGCAT</td>
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Table 1: Primer sets used for quantitative reverse transcriptase-polymerase chain reaction.
demonstrating that there was no effect of retrovirus on lung injury. After LPS challenge, typical histological features of ALI were observed in group Retro-V, inclusive of diffuse alveolar damage, infiltration of numerous neutrophils, alveolar hemorrhage, and interstitium edema (Figures 2C and 2D). However, in mice given Retro-hRobo4, the pathological changes in lung tissue were attenuated (Figures 2E and 2F). Compared with those in group Retro-V+LPS the lung injury scores were significantly reduced in group Retro-hRobo4+LPS at each time point (Figure 3A).

Robo4 suppressed lung tissue MPO activity

Before LPS challenge, lung tissue MPO activity were very low. The MPO activity in the lung tissue increased sharply after LPS injection (P<0.05 vs. 0-h time point). Compared with that in group Retro-V+LPS, the increased MPO activity in group Retro-hRobo4+LPS was significantly reduced at each time point (P<0.05) (Figure 3B).

Secondary cytokine release in vivo

Over-expression of Robo4 didn't change the concentration of...
TNF-α in the serum of the Retro-hRobo4+LPS group mice compared with that of the Retro-V+LPS group, but still higher than its own 0 h point (Figure 4A). Furthermore, the levels of vWF and VEGF in the serum of the Retro-hRobo4+LPS group mice were not different from that of the Retro-V+LPS group, but higher than its own 0 h point (Figures 4B and 4C).

**Over-expression of Robo4 decreased the vascular leak and mortality in mice**

To study the effects of Robo4 on LPS-induced vascular leak, the lung wet/dry ratio was measure. As shown in Figure 5A, the wet/dry ratio was similar between Retro-V and Retro-hRobo4 group. LPS intra-peritoneal injection significantly increased lung wet/dry ratio. However, compared with group Retro-V+LPS, it was significantly decreased in group Retro-hRobo4+LPS.

The BALF protein concentration, another indicator of pulmonary vascular permeability, in the Retro-hRobo4 group at 6 h, 12 h were significantly decreased compared to group Retro-V+LPS, but still higher than before LPS stimulation (Figure 5B).

To determine the effect of enhanced expression of Robo4 on the outcome of LPS-induced ALI, the survival rate between two groups was compared using the log-rank test. As shown in Figure 5C, the survival rate in group Retro-hRobo4+LPS was 73.3% and significantly decreased compared to 33.3% in group Retro-V+LPS.

**Robo4 enhanced the expression of tight junction ZO-1**

To test whether Robo4 promotes vascular stability by directly enhancing the tight junction protein ZO-1, the ZO-1 expression in lung was evaluated by quantitative real-time PCR and western blot. LPS decreased the mRNA and protein expression of ZO-1 in lung. Enhanced expression of Robo4 significantly increased the expression of ZO-1 mRNA and protein at 6 h and 12 h after LPS stimulation (Figure 6).

**Robo4 directly interacts with RhoA**

To explore the possible mechanism of Robo4 in endothelial permeability, we observed the interaction of Robo4 and RhoA by co-immunoprecipitation. As shown in Figure 7, Robo4 protein was detected by Western blot analysis after co-precipitation with RhoA antibody and vice versa. However, in the negative control experiments using normal non-specific IgG, neither Robo4 nor RhoA was precipitated. The result suggested that RhoA and Robo4 were physically associated with each other.

**Discussion**

ALI is a disease that is associated with high mortality and morbidity [12]. The typical pathological changes of lung injury are heterogeneous lung edema due to increased endothelial permeability and uncontrolled inflammation cascade. Many approaches have been tested before, such as Prostaglandin E2 (PGE2), activated protein C (APC), corticosteroid, etc., [13-16]. Unfortunately, none of them showed confirmed benefit in
the expression of Robo4 hypothesis, we use the retrovirus as a gene delivery vector to enhance ligand slit2 could still reduce endothelial permeability. To confirm the tonic Robo4 signaling under basal conditions is anti-angiogenic. Robo4 virus showed a marked reduction in basal migration suggesting migration to angiogenic stimuli [20]. Additionally, cells infected with the endothelial barrier and blunts vascular leak [19]. Over-expression administration of the exogenous Slit2, a ligand for Robo4, strengthens in receptor-dependant manner [7,18]. Previous study showed that slit2/robo4 maintains the barrier function of the mature vascular network by inhibiting neovascular tuft formation and endothelial hyper-permeability induced by pro-angiogenic factors in receptor-dependant manner [7,18]. These findings strongly suggest that enhanced expression of Robo4 play a protective role in LPS-induced ALI.

We speculated that Robo4 may play important role in ligand-independent manner, in which over-expression of robo4 without ligand slit2 could still reduce endothelial permeability. To confirm the hypothesis, we use the retrovirus as a gene delivery vector to enhance the expression of Robo4 in vivo. We examined whether Robo4 over-expression in vivo had an effect on endothelial barrier function. Robo4 retrovirus successfully enhanced the expression of Robo4 by intranasal transfection. Robo4 expression may be decreased secondary to endothelial cell injury or to inflammatory cytokines. Pretreatment with Retro-hRobo4 increased the Robo4 expression in lung. Furthermore, Protein concentration in BALF, lung wet/dry ratio in group Retro-hRobo4 + LPS was decreased compared with that in group Retro-V+LPS, so pre-treatment with Retro-hRobo4 decreased lung vascular permeability and lung edema. The lung injury score, MPO activity and survival rate in group Retro-V+LPS was both significantly decreased. These findings strongly suggest that enhanced expression of Robo4 play a protective role in LPS-induced ALI.

However, over-expression of Robo4 didn’t change the secondary cytokine production in lung demonstrates that the therapeutic effect of Robo4 is not due to a reduction in inflammatory cytokine. In addition, in vivo studies showed that Retro-hRobo4 group mice had the same inflammatory cytokine expression compared with that in Retro-V group mice after LPS injection.

The endothelial cell monolayer provides a critical barrier between the blood and extra-vascular lung tissue that regulates the passage of nutrients, ion and fluid into the interstitial space [21]. The integrity of this barrier is determined by homophilic interactions between the cell surface adhesion junction protein and tight junction on adjacent endothelial cells. The decrease of ZO-1, a tight junction protein, in mice lung occurred in response to LPS stimulation. Previous reports have shown that Slit2 regulates vascular endothelial cadherin localization. Our data showed that the up-regulation of Robo4 also enhanced the expression of ZO-1. We concluded that Robo4 regulate endothelial permeability via alterations in both tight junction and adhesion junction pathways.

LPS induced activation of RhoA in mouse lungs in vivo, which resulted in the disruption of the endothelial barrier and subsequent lung edema formation [22]. RhoA regulates endothelial barrier function by disrupting intercellular junctions [23]. Our data demonstrated that Robo4 interacted with RhoA which was confirmed by Co-ip. Other study also implies Rho GTPases in Robo4-mediated vascular permeability [24]. In addition, inhibition of RhoA with Y-27632 inhibits 50% of TGF-induced permeability [25]. Robo4 cytoplasmic domain is dispensable for vascular permeability and neovascularization [26]. And Robo4 is also located in cytoplasma, so the role of Robo4 in endothelial barrier function may be in part due to RhoA pathway. We demonstrated that Robo4 physically directly interacts with RhoA by Co-ip.

Our results support the initial hypothesis that over-expression of Robo4 decreased endothelial permeability in LPS-induced ALI. Our data suggest that an alternative approach to enhance endothelial barrier is to up-regulate the expression of Robo4. This therapeutic approach may need to be used before the damage of vascular endothelium. We further showed that this strengthened barrier could protect mice from the lethal effects of ALI. Furthermore, enhancing the expression of Robo4 by retrovirus mediated gene delivery is more convenient than slit2 because the exogenous slit2 was short peptide and it need to be used frequently to maintain its activity. In addition, our results suggest that the slit2 is not required for the effect of Robo4 on endothelial barrier thus this protection is ligand-independent.

There are some limitations in this study. The model of LPS-induced lung injury may not represent full profile of live bacteria-induced lung injury. For exactly mechanisms how robo4 interact with RhoA to enhance tight junction expression is still unknown.

Conclusion
Over-expression of Robo4 results enhanced tight junction through RhoA thus improved endothelial barrier integrity, which reduces acute lung injury. This present study demonstrates that Robo4 plays a key role in regulating LPS-induced endothelial permeability and ALI in mice in ligand-independent manner, which suggested Robo4 might be a new target for ALI/ARDS treatment in the future.

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