International Immunopharmacology 72 (2019) 74–81

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

RhoA inhibitor suppresses the production of microvesicles and rescues high ventilation induced lung injury

Huijun Dai[1], Suisui Zhang[1], Xueke Du, Weikang Zhang, Ren Jing, Xiaoxia Wang, Linghui Pan*

Department of Anesthesiology, Guangxi Medical University Cancer Hospital, Nanning 530021, Guangxi Zhuang Autonomous Region, China.

ARTICLE INFO

Keywords:
RhoA/Rock signaling
Microvesicles
Ventilator
Lung injury

ABSTRACT

Microvesicles (MVs) have been extensively identified in various biological fluids including bronchoalveolar lavage fluid (BALF), peripheral blood and ascitic fluids. Our previous study showed that MVs are responsible for acute lung injury, but the exact mechanism underlying MVs formation remains poorly understood. In the present study, we investigate the potential role of RhoA/Rock signaling in MVs generation and the biological activity of MVs in ventilator-induced lung injury (VILI). Our results revealed that high tide ventilation induced super MVs releasing into the lung and subsequently caused lung inflammation. Strikingly, intratracheal instillation of MVs that isolated from highly ventilated mice triggered significant lung inflammation in naive mice. The MVs production is strongly correlated with lung inflammation and the upregulation of RhoA, Rock and phospho-Limk (phosphorylation of Limk is the activated form). RhoA inhibitor decreased the expression of Rock and the phosphorylation of Limk, decreased MVs production and alleviated lung inflammation. Rock inhibitor also decreased the phosphorylation of Limk, decreased MVs production and alleviated lung inflammation. Our data demonstrated that the production of MVs requires RhoA/Rock signaling, and VILI might be potentially prevented by targeting RhoA/Rock signaling pathway.

1. Introduction

Mechanical ventilation is an extensive used life-saving supportive approach for patients especially those critically ill ones. However, ventilation improperly will cause lung disorder, which has been identified even in previously healthy lungs. Mechanical ventilation could aggravate the lung effects of a 'first inflammatory hit', a concept known as ventilator-induced lung injury (VILI) [1,2]. VILI is a condition of total lung inflammation, and it usually comes from the sum of local lung inflammation with a heterogeneous distribution due to heterogeneous local lung susceptibility [1]. Currently, how the local inflammation become into the intensive is poorly understood.

MVs are characterized by the size of 0.1 to 1 μm [3], and are considered as a novel type of cell-cell communication, have attracted numerous researchers' attention these recent years. MVs shedding from the cells are distinct from the conventional cellular secretory, they carry things retained from their precursor cells, including signaling proteins, mRNA, miRNAs as well as cell receptors, over a distance to remote cells [4,5]. Given the potential of transporter capacity, MVs packing soluble mediators give an alternative novel way for intercellular communication and direct cell-to-cell contact and have been reported participating in diverse diseases development [6,7]. Previous studies have identified the appearance of MVs in bronchoalveolar lavage fluid (BALF) in ARDS patients [8,9]. However, physiological mechanisms underlying the role of MVs activities in mechanical ventilation remain a mystery.

Our previous works [10] have shown that inflammatory cytokines packed MVs are responsible for the lung inflammation during VILI. But the mechanism underlying the MVs production is still poorly understood. A previous study [11] indicated that MVs shedding from MDAMB231 breast cancer cells and HeLa cervical carcinoma cells lines depend on RhoA/Rock activation, suggesting that RhoA/Rock may have a role in regulating MVs formation in cancer cells in vitro. Given MVs are"actin-ring" structures characteristics [11], and it is a GTPase known for its capacity to reorganize the actin cytoskeleton. We hypothesized that RhoA may contribute to the MVs formation. Rho-kinase (Rock), originally identified as an effector of the small GTPase Rho, plays a crucial role in mediating rearrangements of the actomyosin cytoskeleton downstream of Rho [12,13]. Whether and how RhoA-Rock signaling affect MVs production during ventilation is not yet known.

* Corresponding author at Department of Anesthesiology, Guangxi Medical University Cancer Hospital, No. 71 Hedi Road, Qingxiu District, Nanning 530021, Guangxi Zhuang Autonomous Region, China.
E-mail address: plinghui@hotmail.com (L. Pan).
[1] These authors are co-first authors.

https://doi.org/10.1016/j.intimp.2019.03.059
Received 11 February 2019; Received in revised form 26 March 2019; Accepted 28 March 2019
Available online 05 April 2019

Here we report that high tide ventilation induced super MVs released into the lung and subsequently caused lung inflammation. The MVs production is strongly correlated with lung inflammation and the upregulation of RhoA, Rock and phospho-Limk (phosphorylation of Limk is the activated form). RhoA inhibitor decreased the expression of Rock and both RhoA inhibitor and Rock inhibitor decreased the phosphorylation of Limk, production of MVs and alleviated lung inflammation. It is our first to investigate the role of RhoA in MVs production which will aggravate lung inflammation in VILI development.

2. Materials and methods

2.1. Animals and VILI animal model

C57BL/6 mice 6 weeks old (25 ± 2 g), purchased from Guangxi Medical University. Animal protocols were approved by the Animal Committee of the Guangxi Medical University. Ketamine (Humanwell, Wuhan, China) 100 mg/kg and xylazine (Humanwell, Wuhan, China) 10 mg/kg were used to anesthetize mouse. Animals were orotracheally intubated with a 20-gauge catheter (Cusabio, Wuhan, China), then connected to an animal ventilator (Kent Scientific Corporation, CT, USA) at a respiratory rate of 80 breath/min. Mice with mechanical ventilation were ventilated either at 7 ml/kg (Low tide ventilation) or at 20 ml/kg (High tide ventilation) for 4 h, whereas control mice underwent orotracheal intubation but breathe spontaneously. During the 4 h, a quarter of the initial dose of ketamine was added to the mice via subcutaneous injection at 50-min intervals.

RhoA inhibitor Cethrin (1 mg/mg) (Cytoskeleton, Inc., Denver, CO), was administrated to mice through intratracheal instillation. The administration of Rock inhibitor Y-27632 (1 mg/kg) (Tocris Cookson Ltd., Bristol, UK,) as the Cethrin did and the equal volume of saline was added as sham control. After being treated with the Cethrin or Y-27632 for 1 h, animals were mechanically ventilated at 20 ml/kg for 4 h as described above. MouseOx (Life Science, PA, USA) were used to continuously monitor mouse oxygen saturation and heart rate. Finally, mice were euthanasia with an overdose of ketamine, and the samples were harvested as described below.

2.2. H&E staining for lung histopathology analysis

The right middle lung lobe was fixed in 4% formaldehyde (Sigma, MO, USA). Tissues were stained with hematoxylin and eosin (H&E) as previously describe [10], the results were assessed by an experienced pathologist. The extent of the lung pathological lesions was graded from 0 to 3, and the previous criteria [14] was performed to score the severity of VILI.

2.3. Wet/dry weight ratio of lung tissue

Lung wet/dry weight ratio was performed as a parameter of pulmonary edema formation. At the end of the experimental time point, mice were euthanasia and one of the left lung lobes were cut off and weighed immediately after removal (wet-weight) and then drying in an oven at 70 °C for 48 h (dry-weight).

2.4. Bronchoalveolar lavage harvest for MVs and cytokines analysis

In order to obtain high efficiency of BALF, 2.5 ml sterile saline was instilled into mouse trachea in five replicates as describe previously [10]. By this way, about 80% of the lavage fluid was recovered. Using a hemocytometer to count the cell number in BALF and BCA Protein Assay kit (Thermo Fisher, MA, USA) to detect the total protein concentration. And the rest of lavage fluid was restored at −80 °C for MVs isolation.

2.5. MVs isolation and instillation

First, BALF obtained from mice was centrifuged at a low speed (400 *g*, 5 min, 4 °C) to remove particles. Next, the supernatant was collected and centrifuged at a higher speed (2000 *g*, 10 min, 4 °C) to remove larger particles. Finally, centrifuged the supernatant at a super high speed (50,000 g, 120 min, 4 °C) to obtain MVs [3,15]. The MVs obtained from high ventilation groups were resuspended in 150 μl saline and 50 μl of which were intratracheally instilled into naive mice. After MVs instilling, animals kept breathing spontaneously for 4 h. The number of cells, levels of protein, IL-1β, IL-6 and TNF-α in BALF were measured as inflammatory parameter status. The other 100 μl of MVs were kept and stored at −80 °C for further study.

2.6. Transmission electron microscope (TEM) for MVs ultrastructural analysis

MVs isolated from mice were fixed with 4% paraformaldehyde at 4 °C for 8 h. 8 μl of MVs sample were placed on a formvar-carbon-coated grid. Rinsing the grids twice and then stained with 1% phosphotungstic acid for 2 min. The samples were finally assessed by an H-7650 transmission electron microscope (Hitachi, Japan) at 80 kV. The size of MVs was measured as previously described [10].

2.7. ELISA analysis for inflammation cytokine in BALF and MVs

MVs were disrupted using 1% Triton-100 in PBS to disrupt their membrane to release the content for analysis. IL-1β, IL-6 and TNF-α both in BALF and MVs were tested using ELISA kit (Cusabio, Wuhan, China) according to the manufacturer's instructions.

2.8. Western blot analysis

Lung samples were solubilized in RIPA buffer supplement with protein inhibitor for 30 min at 4 °C with shaking. BCA assay (Thermo Fisher, MA, USA) was used to detect the total protein concentration in BALF and MVs. Equal amounts of protein from the different groups were subject to electrophoresis on a 12% SDS polyacrylamide gel and subsequently transferred onto nitrocellulose membranes. Next, the membranes were blocked with 5% bovine serum albumin (BSA) for 2 h. Then membranes were incubated with primary antibodies at 4 °C overnight (diluted 1:1000; Cell Signaling Technology, MA, USA) against Flotillin-2, RhoA, Rock1, Rock2, phosphorylation of Limk, total Limk, or GAPDH. Blots were washed 3 times with 5 min each and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Pierce, Appleton, USA). Finally, the membranes were washed 3 times, and bands were visualized using enhanced chemiluminescence (Pierce, Appleton, USA). GAPDH served as a loading control.

3. Statistical analysis

The data were presented as mean ± SD ($n = 8$). Statistical analysis was performed by SPSS18.0 (IBM, USA), and $P < 0.05$ was defined as statistical significance. *t*-Tests were used between two groups comparisons. Differences between multiple experimental groups were compared using one-way analysis of variance followed by LSD method.

4. Result

4.1. High tide ventilation caused lung inflammation and induced super MVs releasing

It is well accepted that high ventilation cause lung damage [16]. We challenged mice with high tide ventilation (20 ml/kg, 4 h), and low ventilation (7 ml/kg, 4 h), and we use spontaneously breathing mice as

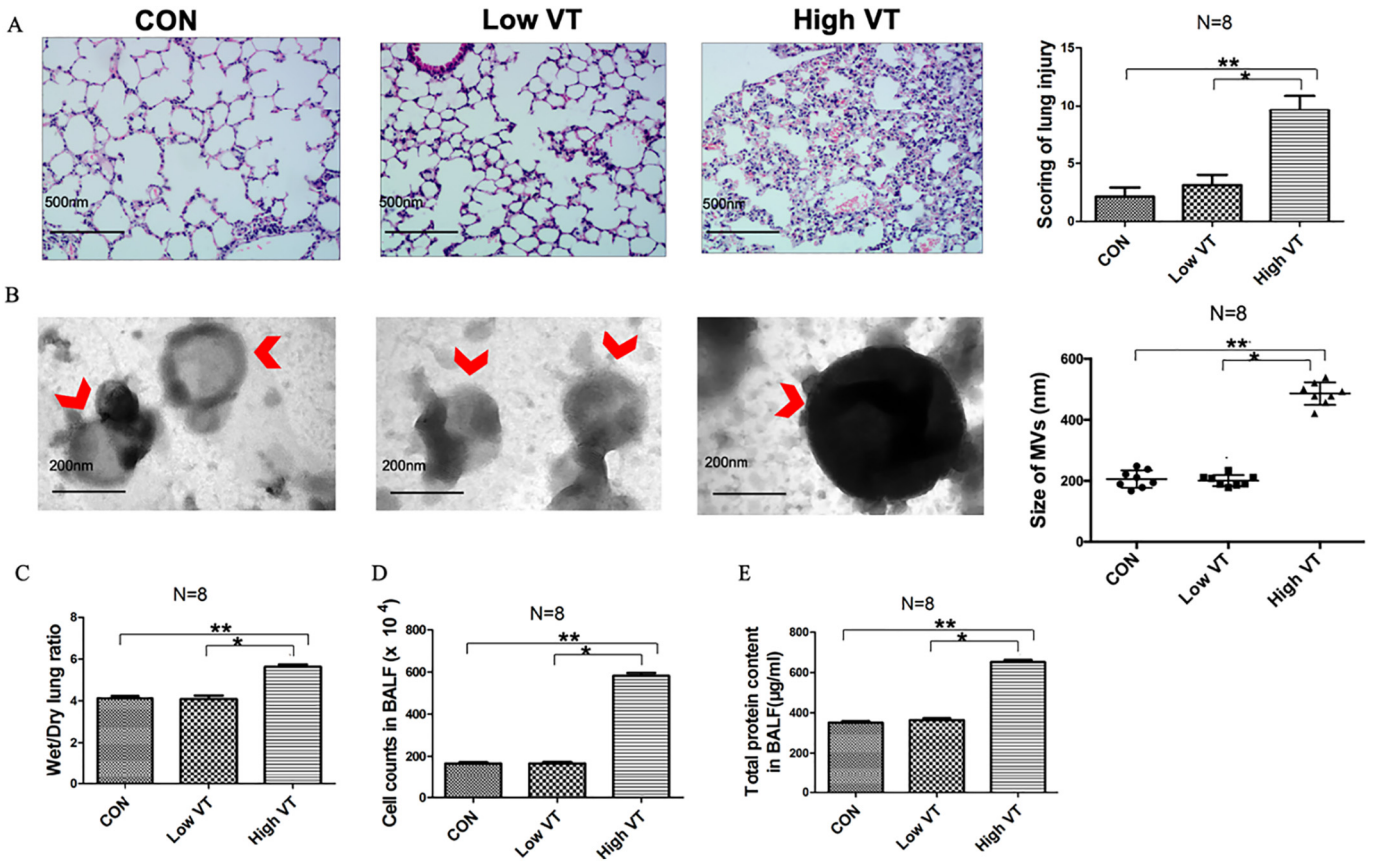

Fig. 1. High tide ventilation increases the level of the proinflammatory parameter and releases lager MVs. (A) Histopathologic examination for lung tissue. HE staining showed that high VT induced a marked increase in inflammatory cell infiltration, alveolar septal thickening, and pulmonary edema in the lung compared with control and low VT mice. (B) Transmission electron microscope for MVs (as arrow indicate) after ventilation or spontaneous breath. The size of MVs isolating from high VT mice is significantly bigger than the control and low VT mice. (C) Wet/dry weight ratios of lung tissues. (D) Total cell counts in BALF. (E) Levels of total proteins in BALF. All data are presented as means ± SD (n = 8 in each group). *$P < 0.05$ vs low VT; **$P < 0.05$ vs CON. CON: spontaneous breath; low VT: low tide ventilation; high VT: high tide ventilation.

control (under anesthesia as ventilation mice). As we expected, the lung from mice challenged with high VT showed significantly increased inflammatory cell infiltration, alveolar septal thickness, and pulmonary edema compared to the lungs from the mice with low VT or control mice There were no major histological differences in lungs observed between low VT mice and control group (Fig. 1A). Pulmonary permeability and edema were also evaluated using total protein concentration in BALF and wet/dry ratios of the lung. We found pulmonary severe edema (Fig. 1C, $P < 0.05$) and increased pulmonary permeability (Fig. 1E, $P < 0.05$) in high VT mice than those in low VT or the control mice. The number of inflammatory cells infiltrated into BALF was significantly increased in high VT mice comparing to the low VT or the control mice (Fig. 1D, $P < 0.05$), suggesting a severe inflammatory status in lungs from high VT mice. We then observed the MVs isolated from BALF using TEM, and found that the MVs from high VT mice were remarkable bigger size (486.0 ± 34.46 nm) than those from low VT mice (200.8 ± 17.16 nm) or from the control mice (205.75 ± 26.86 nm) (Fig. 1B, $P < 0.05$), suggesting that MVs in larger sizes may involve in VILI.

4.2. MVs enhance lung inflammation in high ventilation

MVs were considered to be critical communicator by transporting signaling proteins, mRNA, miRNAs as well as cell receptors, over a distance among cells. It is of great interest to investigate whether the large MVs found in the lungs of the high VT mice cause lung inflammation. We first instilled MVs obtained from high VT mice intratracheally to naive mice and found that the elevated level of cell counts, amount of total protein and concentration of IL-1β in BALF from MVs challenged naïve mice significantly higher compare that from sham mice (Fig. 2D–F, $P < 0.05$). These data showed a similar inflammatory parameter trend with the VILI model mice (Fig. 2A–C). These results indicate that MVs possess proinflammatory abilities in VILI.

4.3. MVs packing IL-1β, IL-6 and TNF-α inside during VILI

MVs shedding during high ventilation cause lung inflammation but the mechanisms underlying this event are unclear. Since MVs are believed to be a carrier for signaling proteins, mRNA, miRNAs as well as cell receptors, we set to measure whether there are any inflammation factors within their contents. Our result showed that, IL-1β, IL-6 and TNF-α within the contents of MVs from high VT mice significantly higher than those in control and low ventilation mice (Fig. 2G–I, $P < 0.05$).

4.4. High ventilation increased RhoA/Rock signaling expressing and RhoA blocking reduced MVs production then rescued lung inflammation

Though our data showed that MVs have proinflammatory capacity during the ventilation, but the upstream molecular mechanism mediates MVs production still unknown. RhoA triggers a specific signaling pathway essential for MV biogenesis in various human cancer cells [11]. We next evaluated the role of RhoA/Rock signaling in the

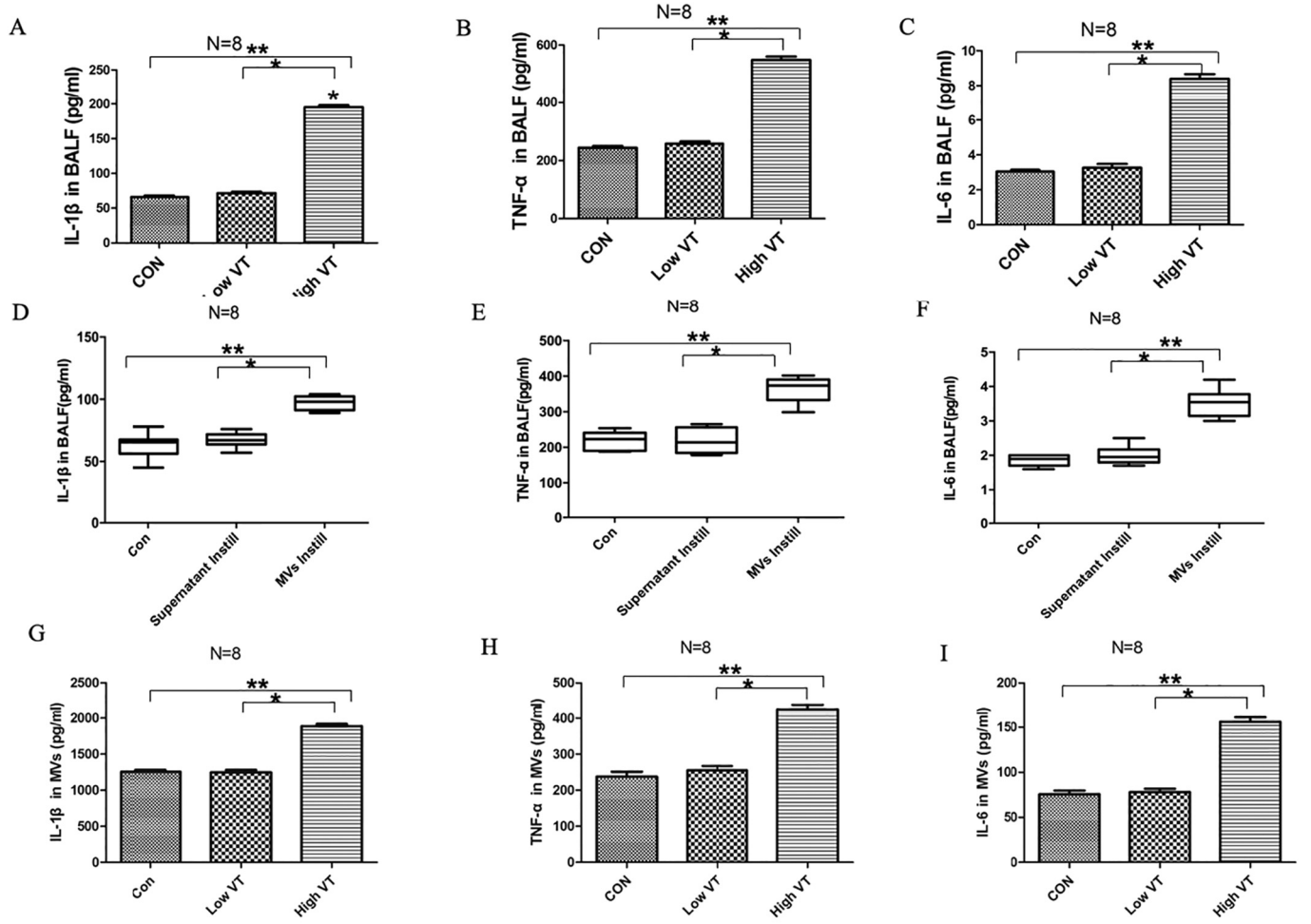

Fig. 2. MVs induce inflammation and packing IL-1β, IL-6 and TNF-α inside. A: Concentration of IL-1β in BALF, B: concentration of TNF-α in BALF, C: concentration of IL-6 in BALF in the different animal challenge with ventilation or spontaneous breath. D–F Illustrate the inflammatory parameter of naive mice challenged to the MVs (MVs were isolated from high ventilation mice). D: Concentration of IL-1β in BALF, E: concentration of TNF-α in BALF, F: concentration of IL-6 in BALF. G–I present the inflammatory factors levels packing by MVs. G: Concentration of IL-1β in MVs, H: concentration of TNF-α in MVs, I: concentration of IL-6 in MVs. All data are presented as means ± SD (n = 8 in each group). *$P < 0.05$ vs low VT or supernatant instill; **$P < 0.05$ vs CON. CON: naive mice as the control. MV: microvesicle.

production of MVs in high VT mice. Interestingly, we found that RhoA, Rock and phospho-Limk (phosphorylation of Limk is the activated form) were significantly upregulated accompanied with higher MVs (Flotillin-2 as the marker) production in the high ventilation mice than in the control and low ventilation animals (Fig. 3). This result implied that high ventilation may trigger RhoA/Rock signaling in the regulation of MVs releasing. When used RhoA inhibitor to block the pathway, we found that Rock1, Rock2 and phosphorylation of Limk were significantly decreased in lung from high VT mice (Fig. 4A), the production of MVs was also significantly decreased (Fig. 4A, Flotillin-2 as a marker of MVs). RhoA inhibitor can also greatly decrease the size of MVs (Fig. 4B). Blocking RhoA significantly attenuated inflammatory cell infiltration, alveolar septal thickness, and pulmonary edema in the lung tissue (Fig. 4B). And mice with RhoA inhibitor treatment prior to challenge with high ventilation, presented deceased level of IL-1β (Fig. 4C, $P < 0.05$), TNF-α (Fig. 4D, $P < 0.05$), and IL-6 (Fig. 4E, $P < 0.05$), and also lower level of total protein content (Fig. 4F, $P < 0.05$) in BALF. These data indicate that RhoA is the upstream signal of Rock1, Rock2 and phosphorylation of Limk, and govern the MVs formation during ventilation, and RhoA blocking can greatly improve lung inflammatory status.

4.5. Rock signal blocking decrease MVs formation and improve lung inflammatory status

It has been showed that Rock is a downstream signal of RhoA in cancer cells lines in vitro. It rouses us a question whether Rock delivers RhoA signal to downstream target to regulate MVs production in vivo during ventilation. We found that Rock inhibited by Y-27632 can significantly suppress phosphorylated LIMK and result in MVs reduction (Fig. 5A). What's more we can also observe that Rock blocking greatly decreased the size of MVs, reduced inflammatory cytokines and significantly attenuated lung inflammation status (Fig. 5B–F, $P < 0.05$). These results demonstrated that MVs production is also depended on Rock activation, and MVs may be a aggravating factor for spreading local inflammation to an extensive area of the lung.

5. Discussion

Extensive research focuses on ARDS in recent years and low tidal volume ventilation strategy is the ideal identified treatment modality to improve survival of ARDS patients [17,18]. However, more and more evidence demonstrated that mechanical ventilation, even at protective low tide ventilation, may trigger an inflammatory response in the lung and eventually led to VILI [19–21]. For example, Terragni [22] et.al had indicated that even at protective low tide ventilation may still

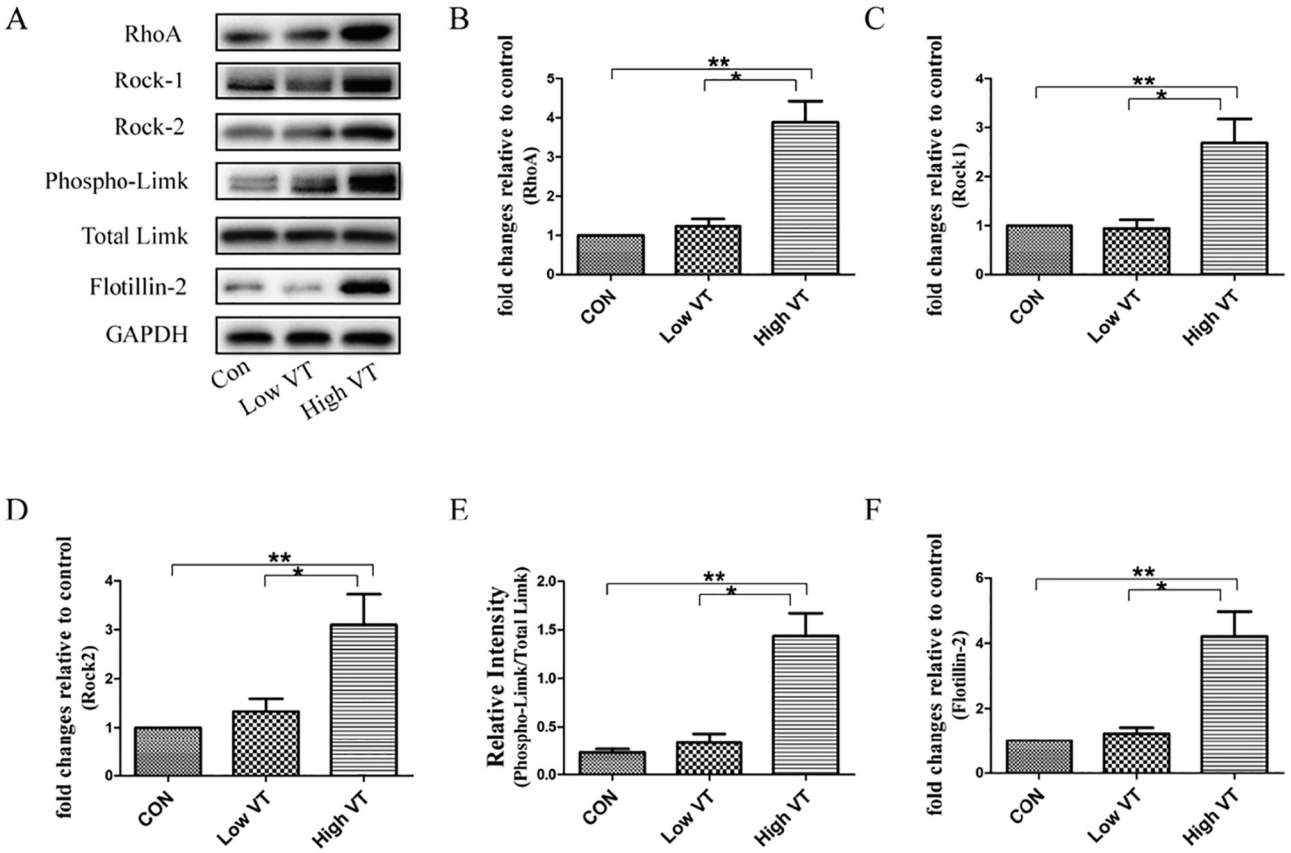

Fig. 3. RhoA/Rock signal overexpressing strongly correlates with high ventilation. (A) Expression of RhoA, Rock1, Rock2, Phospho-Limk and Flotillin-2 were accessed by Western blot. (B–F) Quantification of relative protein expression was performed by densitometric analysis and GAPDH was used as a loading control. B: Fold changes of RhoA. C: Fold changes of Rock1. D: Fold changes of Rock2. E: Fold changes of phospho-Limk. F: Fold changes of Flotillin-2. Flotillin-2 as a marker of MVs. ($n = 8$ in each group) $^{*}P < 0.01$ vs Low VT; $^{**}P < 0.01$ vs CON. CON: spontaneous breath; low VT: low tide ventilation; high VT: high tide ventilation.

cause tidal hyperinflation in ARDS patients due to its anatomical heterogeneity of damaged lung. So high tide mechanical ventilation is coming to be the most popular animal model for VILI and perhaps have a significant impact on clinical practice. And at our present study, we used 20 ml/kg as high tide volume ventilation to investigate the mechanism involving in the VILI.

Our previous studies [16,23] have clearly demonstrated that the physical forces of the high ventilation can activated TLR-MyD88 signaling pathway and caused the releasing of various intracellular mediators namely IL-1β, IL-6 and TNF-α, and led to lung inflammation. However, the mechanism of how local inflammation activated by over inflation alveolar and eventually became extensive in the whole lung and led to VILI is still unclear. MVs as a novel communicator between cells have received more and more attention. MVs are membrane-bound vesicles containing phospholipids shedding from diverse cell types, can transport proteins, miRNA and mRNA between cells, thus play an important role in intercellular communication [7,24–27]. They originate directly from the plasma membrane of the cell and reflect the antigenic content of the cells from which they originate [28,29]. Through these mechanisms, MVs releasing into the extracellular space can deliver bioactive molecules to specific target cells. In our previous work, we have shown that MVs packing IL-1β and TNF-α are involved in lung inflammation in VILI, but the mechanism underlying MVs production regulation remains poorly understood. Given the interesting capacities of MVs, we set out the following experiments to detect the role of MVs in VILI. MVs are “actin-ring” structures characteristics [11] prompt us to consider the potential roles of RhoA in the MVs formation, as the GTPase is known for their capacities to reorganize the actin cytoskeleton. And in our present study, we used inhibitors of RhoA and Rock to block the signal transmitting from RhoA/Rock to their downstream targets receivers to investigate the regulatory role involving in MVs production.

Having identified the present of MVs in alveolar BALF (Fig. 1), we further investigated the molecular mechanism of MVs proinflammatory activities involving in the VILI. The size of MVs in high ventilation mice shows larger than the control mice, suggesting that bigger MVs may be more activated (Fig. 1). Our data clearly showed that the contents of MVs namely IL-1β, IL-6 and TNF-α which isolated from high ventilation mice were much more than the control and low ventilation animals. To ensure the proinflammatory activities may be conducted by the contents of MVs. We extracted the MVs from high ventilation mice and instilled intratracheally to naive mice. And we found that MVs from high ventilation animals can significantly elevate the level of the inflammatory parameter (Fig. 2D–F). This suggested that IL-1β, IL-6 and TNF-α packed by MVs greatly enhanced the lung inflammation and played an important role in the VILI, given that IL-1β, IL-6 and TNF-α were identified inflammatory cytokines in lung inflammation. These results are consistent with Sanooj Soni et al. [3] who identified MVs role in infectious lung disease. Although Sanooj Soni et al. have shown the alveolar macrophage-derived MVs activities in the role of infectious lung inflammation model, the mechanism of non-infectious lung inflammation especially such kind of VILI is still unclear.

MVs are characterized by “actin-ring” structures [30]. And RhoA is a small GTPase protein in the Rho family, it is primarily associated with cytoskeleton regulation, mostly actin organization and actomyosin contractility [31]. It has been reported that RhoA activates Rock (RhoA kinase) which stimulates LIM kinase, which then inhibits cofilin, which effectively reorganizes the actin cytoskeleton of the cell [32]. These led

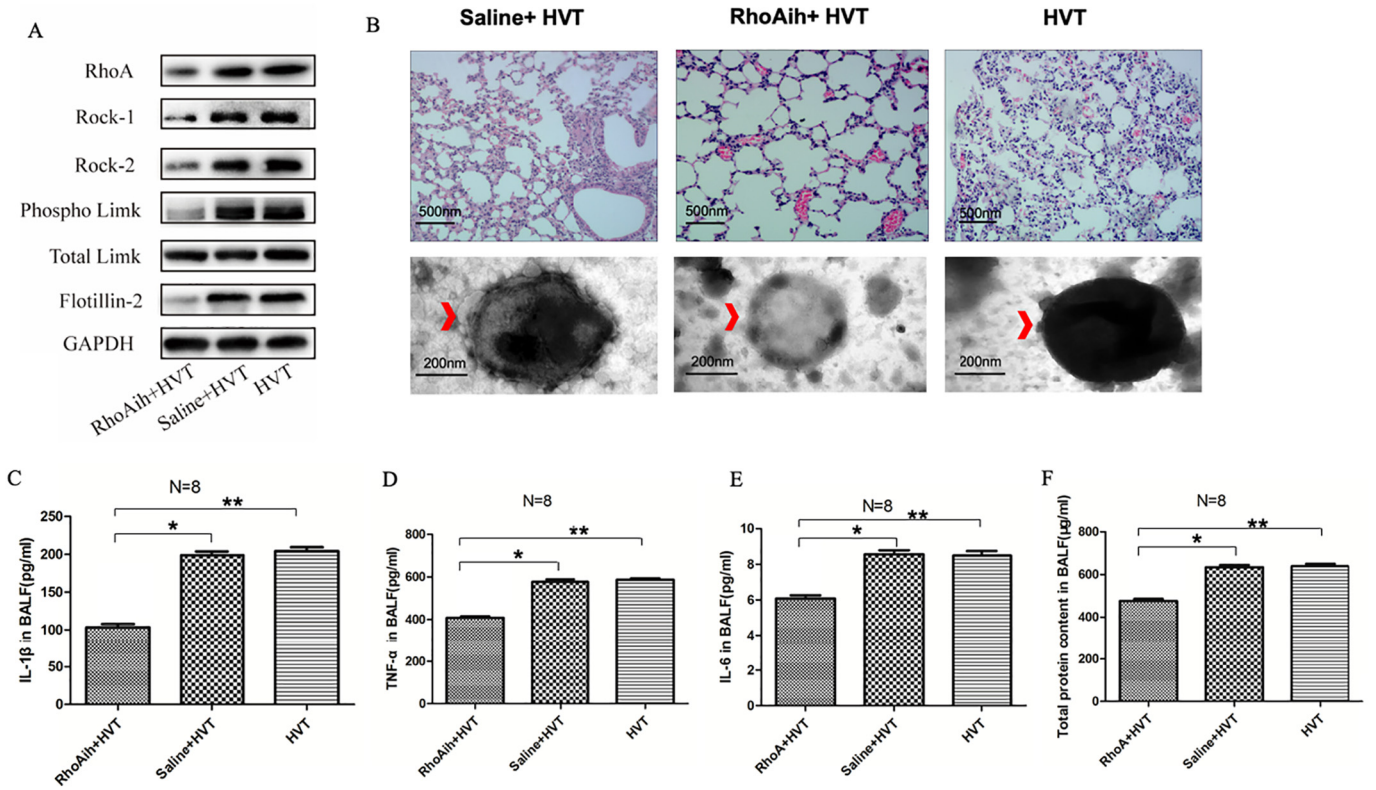

Fig. 4. RhoA inhibitor greatly downregulates RhoA/Rock signaling and MVs production then alleviate lung inflammation. (A) RhoA/Rock signaling level inhibit by RhoA inhibitor and analysis by western blot. (B) Histopathologic examination of lung tissue after mice challenged with RhoA inhibitor or saline plus high ventilation, and TEM for MVs. RhoA inhibitor not only remarkable decrees the size of MVs (as arrow indicate) compare with the mice from high VT and saline plus high VT, but also significantly attenuate inflammatory cell infiltration, alveolar septal thickening, and pulmonary edema in the lung. (C) Levels of IL-1β in BALF. (D) Levels of TNF-α in BALF. (E) Levels of IL-6 in BALF. (F) Levels of total proteins in BALF. All data are presented as means ± SD (n = 8 in each group). $^{*}P < 0.05$ vs saline + HVT; $^{**}P < 0.05$ vs HVT. RhoAih + HVT: mice subject to high tide ventilation plus with RhoA inhibitor, saline + HVT: mice subject to high tide ventilation plus with Saline as sham control. High VT: mice subject to high tide ventilation.

us to hypothesis that RhoA/Rock signaling pathway may play a role in MVs formation during VLLI. We found that level of lung inflammation is strongly correlated to RhoA, Rock1, Rock2 and Limk phosphorylation upregulation (Fig. 3). We further used RhoA inhibitor to test its role in MVs production. Our result showed that RhoA blocking can remarkably inhibit Rock1, Rock2 and Limk phosphorylation expressing, and significantly reduced MVs production was also observed (Fig. 4A). And the histopathologic changes in RhoA treatment mice were greatly improved compared to the control mice (Fig. 4B). What's more, inflammatory factors such as IL-1β, TNF-α and IL-6 in BALF in RhoA treatment mice were significantly decreased compare to the control mice (Fig. 4C–F). These data indicate that MVs production is regulated by RhoA, and RhoA blocking can greatly improve the lung inflammatory status. Next, we explore the role of Rock which is downstream of RhoA in VILI. After using Rock inhibitor, similar results of lung inflammatory status were observed as RhoA inhibitor treatment (Fig. 5). Taken our data together we concluded that during ventilation MVs production is RhoA/Rock signaling pathway dependent.

There is some limitation to our work. Our previous work had demonstrated that alveolar macrophages activation plays an important role in initiating lung inflammation during ventilation. And Sanooj Soni [3] showed that alveolar macrophage-derived MVs play an important role in infectious models. And whether RhoA-Rock signaling has an impact on the alveolar macrophage in MVs formation during ventilation (non-infectious model) is remain to be investigated in our following studies.

In conclusion, the present study showed that high ventilation can induce huge MVs releasing. And with the contents of IL-1β, IL-6 and TNF-α inside, MVs can cause lung inflammation in nude mice. The study for the first time demonstrates that RhoA/Rock signaling is required for the MVs production during VILI. The results revealed that MVs may be an important communicator in lung inflammation during ventilation.

Author contributions

LP designed the overall study. HD and SZ performed the experiments and drafted the manuscript. FL, XD, RJ, WZ, and XW performed the data analysis. All authors read and approved the final manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (81670079), the Basic Ability Improvement Project for Young and Middle-aged Teachers of Guangxi Zhuang Autonomous Region 2017 (2017KY0087), and the International Communication of Guangxi Medical University Graduate Education (2017).

Acknowledgments

We thank Hongwei Yang, Ph.D. (University of Massachusetts Medical School, MA, USA) and Dejun Yang, Ph.D. (University of Massachusetts Medical School, MA, USA) for reviewing the manuscript and for their invaluable suggestions.

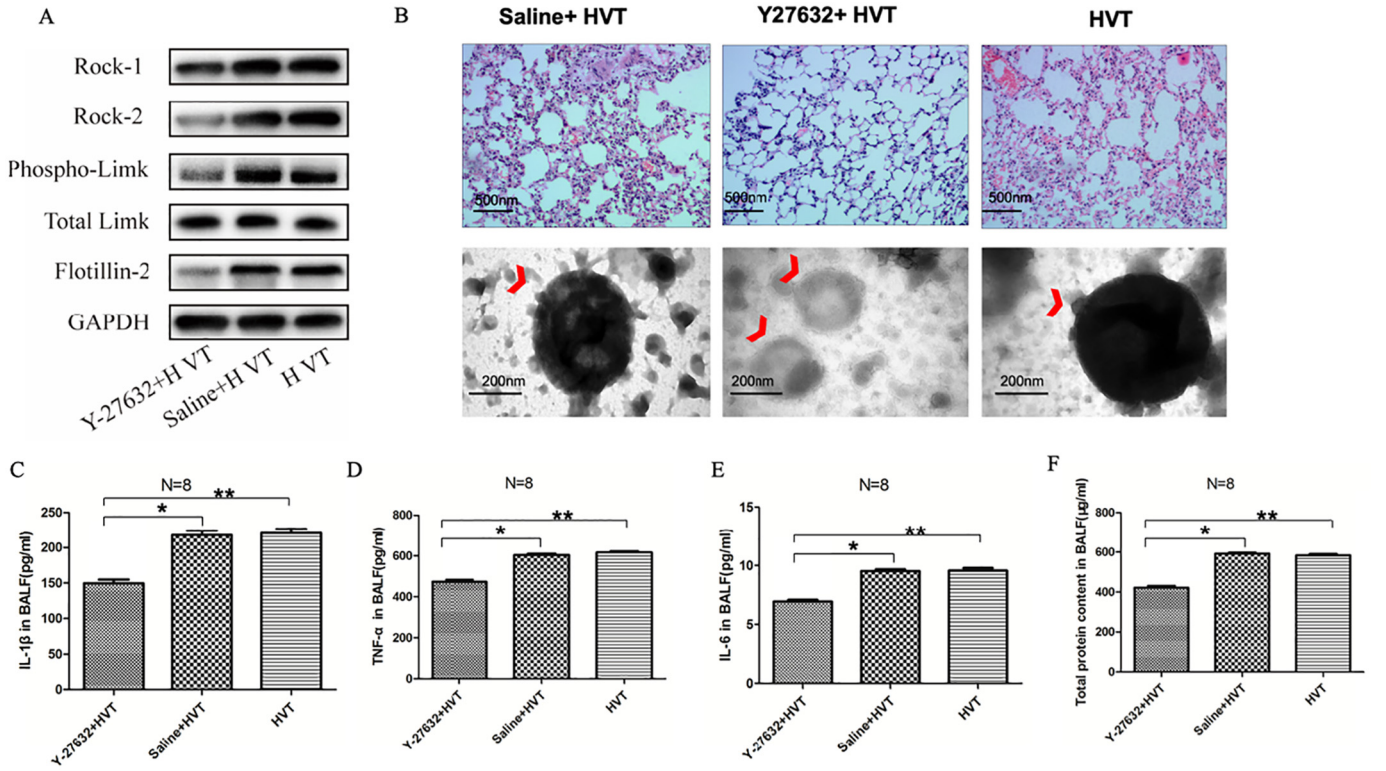

Fig. 5. Rock blocking reduces the production of MVs and attenuate lung inflammation. (A) Analysis of Rock downstream signal inhibition by Rock inhibitor (Y-27632) using western blot. (B) Histopathologic examination of lung tissue after mice challenged with Rock inhibitor or saline plus high ventilation, and TEM for MVs (as arrow indicates). Rock inhibitor Y27632 remarkably decrease the size of MVs compare with the mice from high VT and saline plus high VT, and also significantly reduced inflammatory cell infiltration, alveolar septal thickening, and pulmonary edema in the lung. (C) Levels of IL-1β in BALF. (D) Levels of TNF-α in BALF. (E) Levels of IL-6 in BALF. (F) Levels of total proteins in BALF. All data are presented as means ± SD (n = 8 in each group). $*P < 0.05$ vs saline + HVT; $**P < 0.05$ vs HVT. Y-27632 + HVT: mice subject to high tide ventilation with rock inhibitor, saline + HVT: mice subject to high tide ventilation with saline as control. High VT: mice subject to high tide ventilation.

Conflicts of interest

We declare that there are no interest conflicts.

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