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Original Article

Role of notch signaling pathway in mechanical ventilation induced lung injury

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Abstract: Objective: To investigate the expression of Notch signaling pathway after mechanical ventilation and its role in mechanical ventilation and lung injury. Methods: VILI model was established by mechanical ventilation at high tidal volume (VT = 20 ml/kg) for 4 hours. Lung injury and inflammatory response were evaluated. Hes-1 and Hes-5 mRNA levels were measured after mechanical ventilation, and the levels of NICD, Hes-1, and Hes-5 protein were measured. Furthermore, the Notch signaling pathway inhibitor (DAPT 100 mg/kg) was administered intraperitoneally before mechanical ventilation to assess the role of Notch signaling in VILI. Results: Compared with the control group, hypertonic mechanical ventilation induced VILI and increased the expression of inflammatory factors such as TNF-α, IL-6 and MIP-2 in the cell lavage fluid. The level of Hes-5 gene was significantly up-regulated in pulmonary macrophages with high tidal volume ventilation, and the protein levels of NICD and Hes-5 were up-regulated in pulmonary macrophages. The levels of TNF-α, IL-6, MIP-2 and other inflammatory cytokines in the alveolar lavage fluid decreased, and the levels of NICD and Hes-5 were also decreased, after DAPT pretreatment compared with the high tidal volume. The upregulation of p-IκBα and the degradation of IκBα protein in alveolar macrophages after mechanical ventilation was also observed. Conclusion: The expression of Notch signaling pathway protein in alveolar macrophages is upregulated after high tidal volume mechanical ventilation, and is involved in the regulation of mechanical ventilation and lung injury. Our results suggested that NF-κB pathway is likely involved in Notch-mediated mechanisms that underlie mechanical ventilation induced lung injury.

Keywords: Ventilator-induced lung injury, notch signaling pathway, macrophage, mechanical ventilation

Introduction

Mechanical ventilation is an indispensable part of intratracheal general anesthesia, as well as the major methods for the clinical treatment of ARDS and other causes of respiratory failure [1, 2]. Mechanical ventilation can lead to VILI (ventilator-induced lung injury), and the mechanism is complex. The current focus of the study on VILI is biotrauma, which suggests that the inflammatory response is not just a secondary response to lung cell injury, but is activated in the early mechanical ventilation, and is involved in the formation of VILI [3-5]. The innate immune system plays an important role in the development and progression of lung inflammatory reaction [6]. Alveolar macrophages are lung resident cells, which are the first natural immune barrier of lungs. When the lungs receive pathogen invasion or nociceptive stimulation, early activation of alveolar macrophages starts the immune and inflammatory response [7]. Mechanical stimulation can activate alveolar macrophages and play an important regulatory role in the mechanism of VILI production [8].

Notch signaling, thought to control the cell fate and regulate the organ development, can also respond to various cellular emergency events. Notch receptors are widely expressed on the surface of macrophages and Notch signaling pathway was up-regulated after macrophages were stimulated in vitro [9]. Additionally, Notch signaling pathway plays an important role in the regulation of lung inflammation induced by intraperitoneal injection of zymosan-induced lung injury [10] and cecal ligation

sepsis model [11]. Thus, it is reasonable to speculate that the Notch signaling pathway may also be activated in post-VILI alveolar macrophages. In the present study, we established a mice model of VILI model, and collected alveolar macrophages after VILI to assess the expression of Notch signaling pathway related molecules. We also speculated that the Notch signaling pathway is involved in the regulation of lung inflammatory response after mechanical ventilation in high tidal volume ventilation, and thus participates in the mechanical ventilation lung injury formation mechanism. Thus, we also explored the possible mechanisms of Notch signaling pathway in VILI.

Materials and methods

Mechanical ventilation lung injury model in mice

C57BL/6 mice were randomly divided into 3 groups: control group (Con), low tidal volume group (LVT, VT = 7 ml/kg), and high tidal volume group (HVT, VT = 20 ml/kg). Mice were preoperative fasted for 12 hours, and water was not limited. Before the experiment, atropine 10 μg/kg, and ketamine 100 mg/kg combined with 10 mg/kg xylazine were given intraperitoneally. After the onset of anesthesia, the neck skin received disinfection. Along the median incision, bending tweezers were used to blunt the anterior muscles, exposed trachea and tracheal band line. Sterile venous catheter was inserted along the direction of the trachea, and advanced about 1 cm, and was connected to small animal ventilator.

In the low-tidal volume group, tidal volume was 7 ml/kg, respiratory rate was 140 beats/min. In the high-tidal volume group, tidal volume was 20 ml/kg, respiratory rate was 100 beats/min. In all groups, the respiratory-inhalation ratio was 1:1, and mechanical ventilation was 4 hours. Both spontaneous ventilation and mechanical ventilation used 40% concentration of oxygen. The body temperature of the mice was monitored during ventilation. The temperature of the mice was maintained at 37°C using a heating pad. Additional anesthetics were given every 45 minutes to maintain anesthesia. After mechanical ventilation, lung tissue was collected for pathology examination; lung wet weight ratio, EBA index, and protein content in the alveolar lavage fluid. Lung inflammatory response was assessed by measuring the cell count of bronchoalveolar lavage fluid and inflammatory factors.

Role of notch signaling pathway in lung injury induced by mechanical ventilation in mice

To evaluate the effect of Notch signaling pathway on lung injury in mechanically ventilated mice, C57BL/6 mice were randomly divided into 3 groups (n = 6): control group (Con), high tidal volume group (HVT) and DAPT group. In the DAPT group, Notch signaling pathway inhibitor DAPT 100 mg/kg or vehicle was administered intraperitoneally 3 hours before mechanical ventilation. After the end of mechanical ventilation, lung injury was assessed and alveolar macrophages were collected.

Histology

Histological analysis was performed on formalin-fixed, paraffin-embedded lung tissue sections stained with H&E method. The sections were taken aftermechanical ventilation. The degree of fibrosis was quantified using the Ashcroft scoring method by observers who were blinded as to treatment groups.

Lung injury score

The histopathological features of the lung tissue were observed under light microscope and the acute lung injury was assessed from the following four aspects: alveolar cavity congestion; pulmonary hemorrhage; neutrophil aggregation or infiltration; alveolar or vascular wall thickness. Each aspect was scored according to a five-point scale: 0 point = the slightest injury; 1 point = minor injury; 2 = moderate injury; 3 = severe injury; 4 = the most serious injury. Therefore, the minimum possible score was 0, and the maximum was 16.

Lung EBA permeability index

One hour before the end of the experiment, preformulated EBA (20 mg/kg) was injected from the right jugular vein of the mice. At the end of the experiment, mice were sacrificed, blood was taken from the right ventricle, centrifuged and plasma was taken to measure the absorbance of EBA. The lungs were washed with 1 ml PBS and the left lungs were harvested for detection of EBA absorbance values in lung tis-

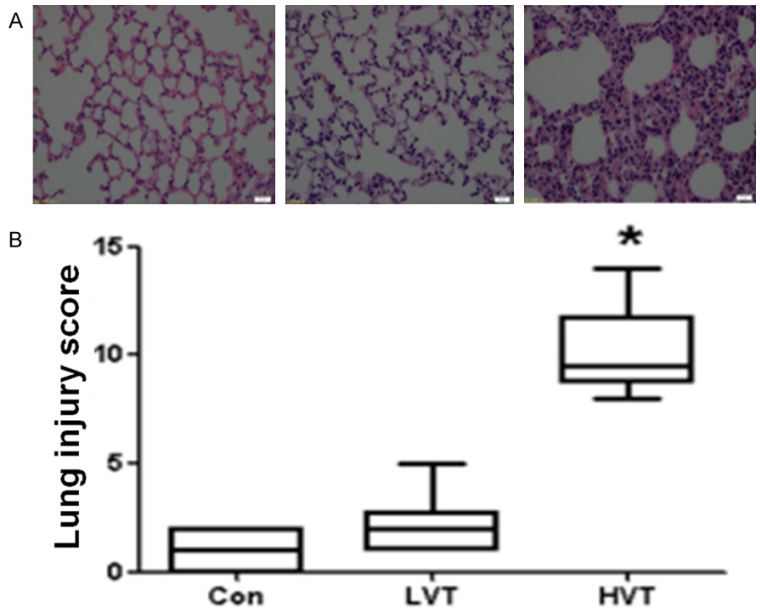

Figure 1. High tidal volume mechanical ventilation causes lung injury. A. Pathological examination of lung tissue using HE staining. HE staining images of the control group (CON), low tidal volume group (LVT), high tidal volume ventilation group (HVT) (× 200 Fold). High tidal volume ventilation caused lung inflammatory cell infiltration, alveolar structural damage and pulmonary congestion and bleeding. B. Urgent lung injury score. *P<0.05, Compared with the control group. Number of each group of experimental animals, N = 6.

sue. When the EBA absorbance value was measured, the lung tissue was homogenized in 2 ml of PBS and then 2 ml of formamide was added at 65°C for 18 hours. The solution was centrifuged at 15,000 g at 4°C for 30 minutes, and the supernatant was collected and the absorbance at 620 nm was measured with a spectrophotometer. The formula: EBA permeability index = (absorbance at 620 nm/per gram of lung tissue)/absorbance at 620 nm of EBA in plasma.

Cell count in alveolar lavage fluid

At the end of the experiment, the mice were sacrificed by intratracheal instillation of 4°C PBS (1 ml) slowly, and the fluid was slowly aspirated three times. The final extraction fluid was the bronchoalveolar lavage fluid (recovery rate was about 80%). The alveolar lavage fluid was centrifuged at 4°C at 2000 rpm for 10 minutes and the supernatant was cryoprotected at -80°C for protein concentration and inflammatory cytokines. The cell pellet was resuspended in PBS, the total number of cells was calculated under a microscope.

Lung wet/dry weight ratio

After sacrificing mice, the lungs were washed with PBS and quickly weighed (wet lung weight) after separation of the left lung. The wet lung was placed in a 65°C oven for 48 hours and then weighed again (dry lung weight). W/D was calculated as wet lung weight/dry lung weight.

ELISA

We used double antibody sandwich ELISA method. The anti-mouse monoclonal antibody was coated on the ELISA plate. OD value was measured at 450 nm, and the concentration was calculated based on the standard curve.

Isolation of murine pulmonary macrophages

After the end of ventilation, the mice were sacrificed, the thoracic cavity was opened, and the lungs were exposed. The lungs were injected slowly with 4°C PBS (1 ml). Then, the slow fluid was aspirated and the lungs were gently gavaged for 3-4 times. The perfusate was collected and centrifuged at 1000 rpm for 10 minutes to collect the cells. RPMI 1640 medium was used to resuspend the cells, and cells were counted under a microscope. The number of cells was then adjusted to $<10^6$/ml in RPMI 1640 medium containing 10% fetal bovine serum, 2×10^5 U/L penicillin, 2×10^5 U/L streptomycin, and inoculated into cell culture plates. Plates were placed in a 37°C, 5% CO_2 incubator for 1-2 hours. Cell culture medium was then removed, and cell surface was washed twice with warm PBS buffer at 37°C to remove the suspended cells. Giemsa staining was used to identify cell viability and purity.

Realtime quantitative RT-PCR

Total RNA was isolated from cultured pulmonary macrophages using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Five μg of the total

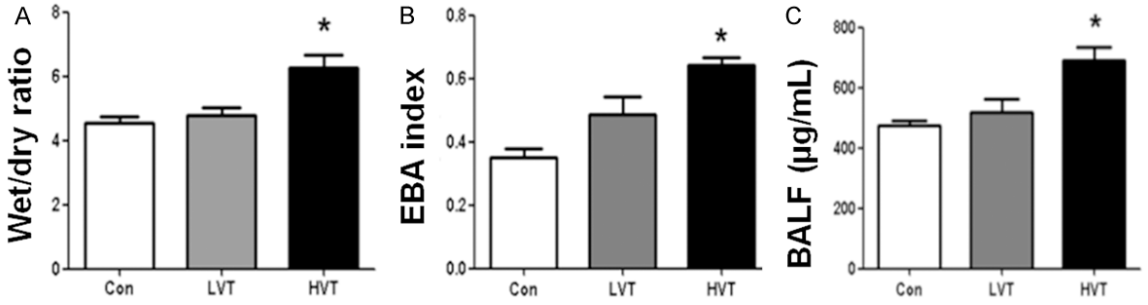

Figure 2. Effects of high tidal volume mechanical ventilation on lung tissue permeability. (A) Wet-dry weight ratio, (B) EBA Penetration index (C) Protein content in alveolar lavage fluid. All data are expressed as mean ± SD. *P<0.05, compared to the control group. Number of each group of animals, n = 6.

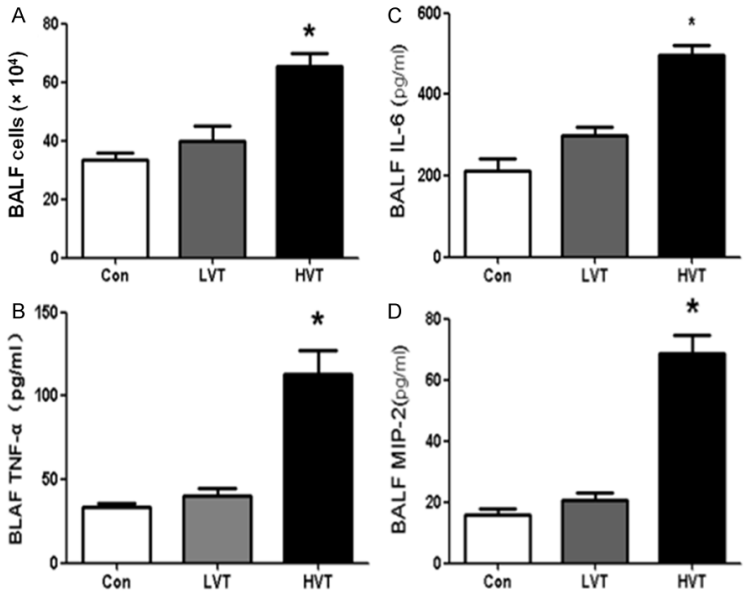

Figure 3. Effects of high tidal volume mechanical ventilation on the number of cells and inflammatory cytokines in alveolar lavage fluid. A. Number of cells. B. TNF-α level. C. IL-6 level. D. MIP-2 level. *P<0.05, Compared with the control group. Number of each group of experimental animals, N = 6.

RNA was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR assay was performed using gene specific double labelled fluorescent probes and sets of specific primers in an ABI PRISM 5700 sequence detection system (PE Applied Bio-Systems, Foster City, CA, USA). PCR was performed in a 50 μl volume with 5.0 μl of a cDNA sample, 1 μmol/l of both forward and reverse primers, 2 mM $MgCl_2$, 0.040 U/μl AmpliTaq Gold DNA polymerase, 200 nmol of probe, and 400 μM deoxyribonucleotide triphosphate mixture in 1× PCR-Gold buffer. Reaction mixtures were incubated at 50°C for 30 minutes. After inactivation of reverse transcriptase at 95°C for 12 minutes, 40 cycles of amplification were performed with denaturation at 95°C for 15 seconds and both annealing and extension at 60°C for 1 minute each. The primers are: Hes-1 F: CCAGCCAGTGTCAACACGA; R: AATGCCGGGAGCTATCTTTCT; Hes-5 F: AGTCCCAAGGAGAAAAACCGA; R: GCTGTGTTTCAGGTAGCTGAC; β-actin F: AGTGTGACGTTGACATCCGT; R: GCAGCTCAGTAACAGTCCGC.

To compensate for any differences in cell number and/or recovery of RNA, the copy number of each of the collagen mRNAs was determined relative to the β-actin mRNA determined simultaneously.

Western blot

Lung tissues were lysed in RIPA buffer and loaded onto SDS-PAGE gels, transferred to nitrocellulose membranes and blocked with 5% milk in TBST. Membranes were probed with primary antibodies NICD (Cell Signaling Technology, USA), Hes-1 (Santa Cruz, USA) Hes-5 (Santa Cruz, USA), p-IκBα (Cell Signaling Technology, USA), IκBα (Cell Signaling Technology, USA), and β-actin (Bioword, USA), followed by washing with TBST and incubation with horseradish peroxidase secondary antibody (Bioword, USA) for 1 h followed by development with an enhanced chemiluminescence (ECL) system

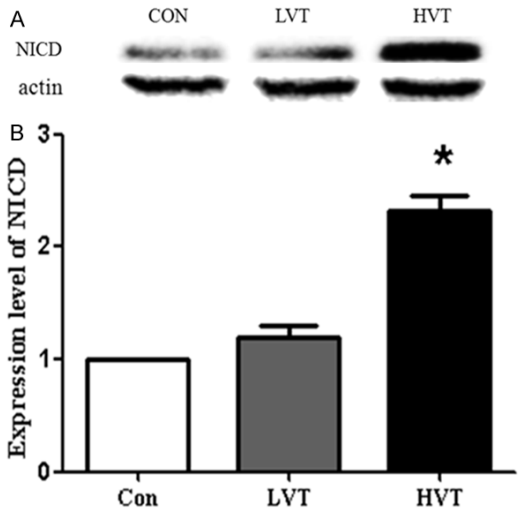

Figure 4. High tidal volume mechanical ventilation upregulates the expression of NICD in alveolar macrophages. (A) Representative Western Blot (B) Western Blot image analysis. *P<0.05: compared with the control group.

(Pierce Biotech, Rockford, IL, USA). Membranes were washed with stripping buffer (62.5 mM Tris-HCl at pH 6.7, 2% SDS, 100 mM beta-mercaptoethanol) to permit re-probing with antibodies to actin (i.e., loading control; 1:50,000, Santa Cruz Biotechnology, Santa Cruz, CA). Protein levels were quantified by densitometry, using NIH Image J software.

Cell immunofluorescence

The mouse lung macrophages were collected and plated in a 24-well plate pre-sterilized with 8 mm glass slides and incubated at 37°C for 2 hours in a CO_2 incubator. The medium was removed and washed three times with PBS. Cells were fixed at room temperature for 30 minutes and washed with PBS for 3 minutes and 3 times with a fixed solution (methanol: acetone = 1:1). Cell immunofluorescence blocking solution was then added at room temperature for 30 minutes. The primary antibody was diluted with antibody dilution at the recommended concentration and the cells were added and incubated overnight at 4°C in a wet box. Primary antibody was removed and the cells were incubated in PBS for 5 minutes × 3 times. Fluorescent secondary antibody (Biomart, China) was diluted with PBST containing 1% BSA, cells were added and incubated for 1 hour at room temperature in the dark, and washed for 5 minutes × 3 times with PBS. The nuclei were stained with DAPI at a concentration of 1 μg/ml for 15-30 minutes. The cells were washed with PBS for 1 minute × 3 times. Fluorescence was observed under fluorescence microscope, and the images were collected.

Statistical analysis

Data was shown as the mean ± standard deviation (SD). Discrete measurement was expressed as the median (range). One-way ANOVA analysis of variance was used to compare the differences between more than two groups, followed by Bonferroni when necessary. The statistical analysis of differences between discrete data was analyzed by Kruskkal-Wallis H test. P<0.05 was considered statistically significant. All statistical analysis were performed using the GraphPad Prism software (Version 5).

Results

The impacts of high tidal volume mechanical ventilation on the lung in mice

After HE staining, the pathological sections of the lung tissue were observed under light microscope. The infiltration of inflammatory cells in alveolar structure, alveolar septum and thickening of pulmonary alveolar septum were observed in the high tidal volume ventilation group. Control group had normal alveolus morphology and no infiltration of inflammatory cells. In low tidal volume group, the alveolus structure was normal but had a few inflammatory cells infiltrated (**Figure 1A**). The lung injury score of the control group was 1 (0-2), the low tidal volume group was 2 (1-5) and the high tidal volume group was 9.5 (8-12), which was significantly higher than that of the control group (P<0.05, **Figure 1B**).

As compared with the control group and low tidal volume group at 4 hours after ventilation, lung W/D (P<0.05, **Figure 2A**), EBA infiltration index (P<0.05, **Figure 2B**), and the content of protein in the lavage fluid alveolus (P<0.05, **Figure 2C**) were increased in high tidal volume group.

As compared with the control group and the low tidal volume group, the total number of cells in the alveolar lavage fluid and the levels of TNF-α

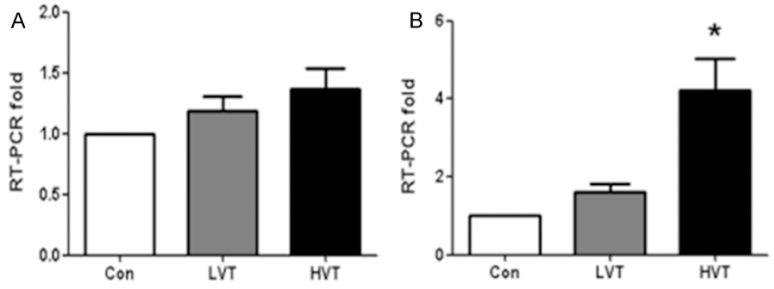

Figure 5. High tidal volume mechanical ventilation upregulates Hes-5 gene expression in alveolar macrophages. A. Real-time PCR was used to evaluate the effects of high tidal volume mechanical ventilation on Hes-1 gene expression. B. Real-time quantitative PCR was used to evaluate the effects of high tidal volume mechanical ventilation on Hes-5 gene expression. *P<0.05, compared with the control group.

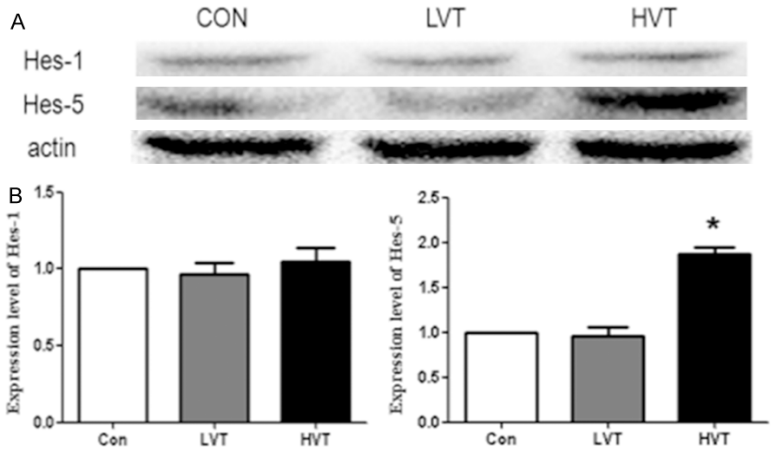

Figure 6. High tidal volume mechanical ventilation upregulates Hes-5 protein expression in alveolar macrophages. (A) Representative Western Blot (B) Western Blot image analysis. *P<0.05, compared with the control group.

(P<0.05, **Figure 3B**), IL-6 (P<0.05, **Figure 3C**), and MIP-2 (P<0.05, **Figure 3D**) in high tidal volume ventilation group were increased.

The changes of notch signaling pathway related molecules expression after mechanical ventilation

NICD protein is a marker for Notch signaling pathway activation. After mechanical ventilation, macrophages were isolated from the alveolar lavage fluid. Western Blot showed that there was no significant change in the NICD level in the low tidal volume group compared with the control group. However, the level of NICD in macrophages of the high tidal volume group was significantly increased (2.3 ± 0.3 fold) compared to the control group (**Figure 4**).

Hes-1 and Hes-5 genes are the target genes of Notch signaling pathway. Compared with the control group, the levels of Hes-1 and Hes-5 in the low tidal volume group had no significant changes. However, the Hes-5 level in the alveolar macrophages of high tidal volume group was increased 4.2 ± 1.9 times. In contrast, Hes-1 level did not change significantly (**Figure 5**).

The protein expression of Hes-5 in pulmonary macrophages was up-regulated after mechanical ventilation

After mechanical ventilation, macrophages were isolated from the alveolar lavage fluid of each group. Western Blot showed that the levels of Hes-1 and Hes-5 in the low tidal volume group had no significant change compared with the control group The expression of Hes-5 protein in macrophages was upregulated at 4 hours in high tidal volume group. However, Hes-1 protein level did not change significantly (**Figure 6**).

After macrophages were isolated from the alveolar lavage fluid of each group, the cells were immunofluorescence-stained. The results showed that the levels of Hes-5 in the low tidal volume group had no significant change, but the expression of Hes-5 protein in the high tidal volume group was upregulated in macrophages at 4 hours (**Figure 7**).

DAPT inhibited NICD after high tidal volume mechanical ventilation in mice

The expression of Notch signaling pathway protein NICD was significantly upregulated in macrophages at 4 hours in high tidal volume group, whereas DAPT significantly inhibited the upregulation of NICD (P<0.05, **Figure 8**).

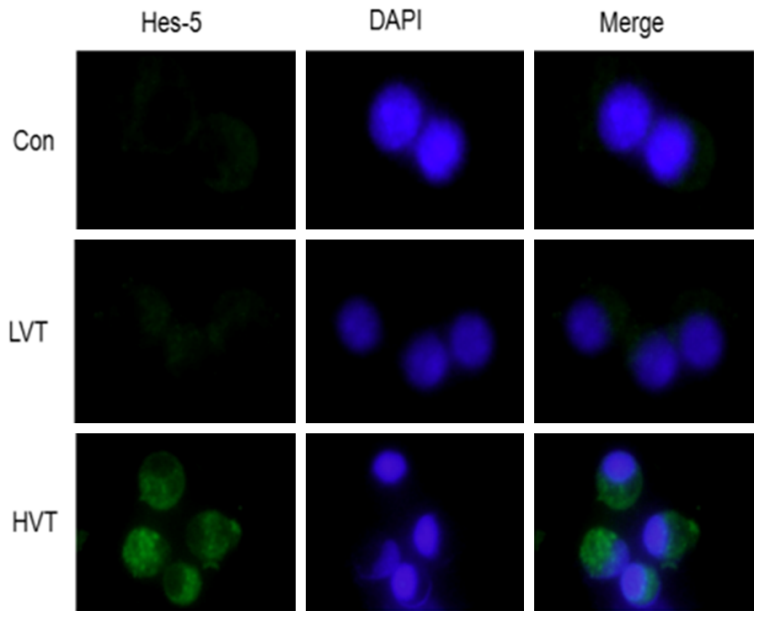

Figure 7. Immunofluorescence showed high tidal volume mechanical ventilation upregulated Hes-5 protein expression in alveolar macrophages. Green: Hes-5 expression; blue: DAPI staining.

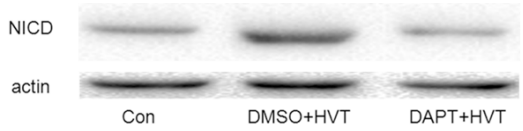

Figure 8. DAPT on high tidal volume mechanical ventilation induced Notch signal expression. Notch signaling pathway was inhibited by DAPT in alveolar macrophages after mechanical ventilation.

DAPT reduced lung injury after high tidal volume mechanical ventilation in mice

Lung histopathology results (**Figure 9A**) showed that the degree of lung injury in DAPT group was significantly lighter than that in high tidal volume ventilation group. Lung injury score was 6 (3-8), which is lower than that in high tidal volume group ($P<0.05$, **Figure 9B**). Lung W/D ratio ($P<0.05$, **Figure 9C**), EBA index ($P<0.05$, **Figure 9D**), protein content in lavage fluid ($P<0.05$, **Figure 9D**) in the DAPT group were all lower than those in the high tidal volume ventilation group.

DAPT attenuated lung inflammation after high tidal volume ventilation in mice

The number of cells ($P<0.05$, **Figure 10A**) and TNF-α ($P<0.05$, **Figure 10B**) and IL-6 ($P<0.05$ **Figure 10C**), and MIP-2 ($P<0.05$, **Figure 10D**) in the DAPT group were all lower than those of the high tidal volume ventilation group ($P< 0.05$).

DAPT attenuated the activation of the NF-κB pathway

After high tidal volume mechanical ventilation for 4 hours, Notch signaling was activated, and NICD and Hes-5 expression levels in pulmonary macrophages were upregulated. The upregulation of phospho-IκBα and downregulation of IκBα protein levels were also shown (**Figure 11A** and **11B**). These suggested that Notch signaling and NF-κB pathway were activated during mechanical ventilation lung injury in mice. DAPT, a Notch signaling inhibitor, was able to decrease Notch signaling activation. The levels of NICD and Hes-5, as well as IκBα activation were also decreased in DAPT group after high tidal volume mechanical ventilation.

Discussion

Our study successfully established the lung injury model of mechanical ventilation in mice. We found that the expression of NICD was increased, the mRNA level of Hes-5 was increased, and the protein levels of Hes-5 was upregulated in macrophages, after 4 hours of high tidal volume mechanical ventilation, suggesting the activation of Notch signaling in macrophage in VILI. Clinical mechanical ventilation lung injury mainly refers to the need for treatment of patients with ARDS. Mechanical ventilation itself can aggravate the original lung injury, which is the equivalent of giving the lung a “Second strike” [12]. However, study on the mechanisms of VILI in the healthy lungs can rule out the cellular signaling activations induced by original lung injury, thus making it easier to explore the mechanisms of mechanical ventilation related lung injury, especially the mechanisms of biological injury.

Mechanical ventilation-induced healthy lung injury model has been successfully replicated in a variety of animals [13-15]. Our study selected mice as the study object to establish mechani-

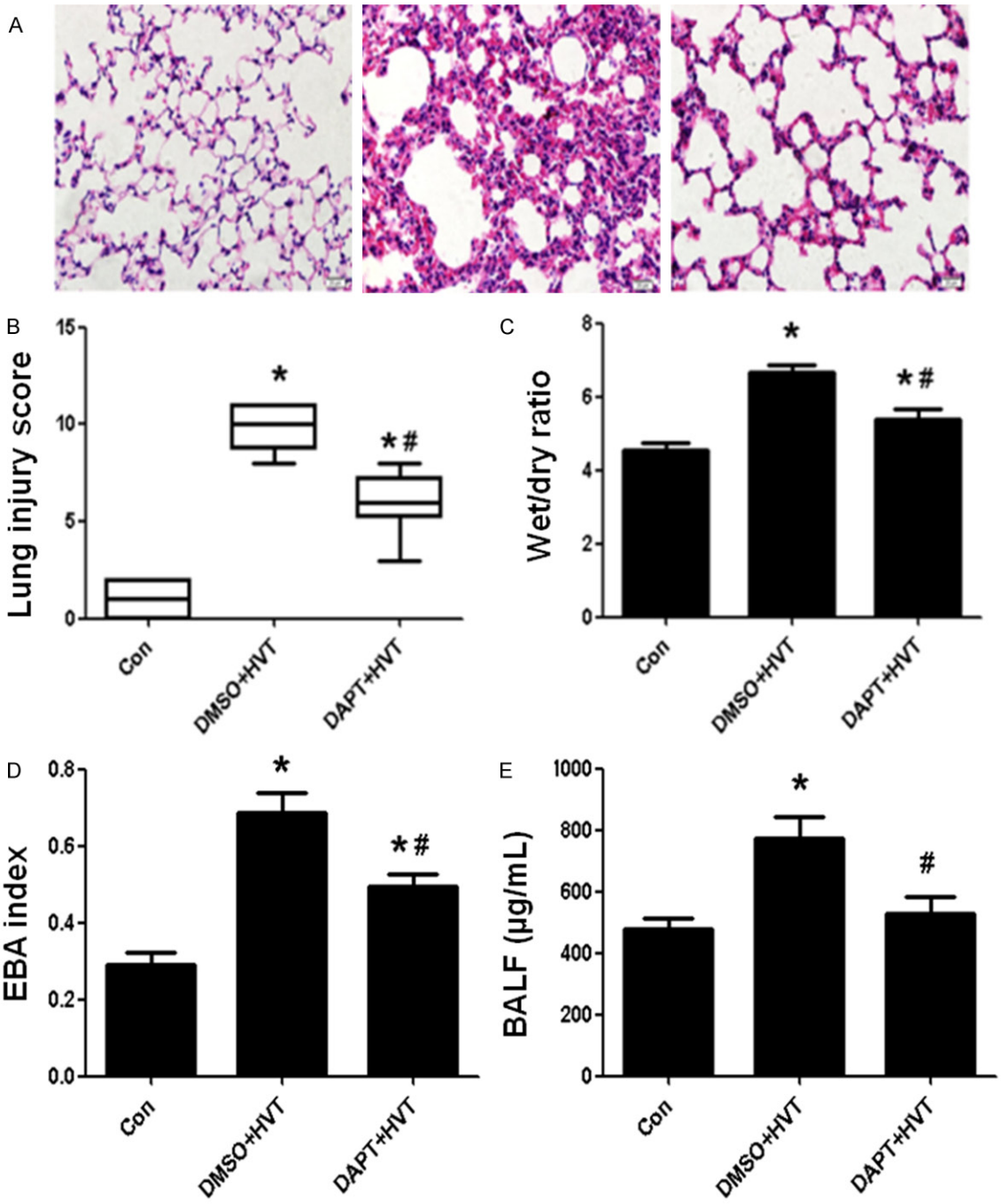

Figure 9. DAPT reduced lung injury after mechanical ventilation in mice. A. The paraffin section of lung tissue was stained by HE staining. B. Acute lung injury score; C. Wet weight ratio of lung tissue; D. EBA permeability index; E. Protein content of alveolar lavage fluid protein in the lung tissue paraffin section; *P<0.05, compared with the control group, #P<0.05, compared with the simple high tidal volume group.

cal ventilation lung injury model, based on the previous study [16, 17], which used a larger tidal volume to induce mechanical ventilation lung injury at 20 ml/kg of tidal volume for 4 hours. In animal experiments, the degree of lung injury is usually assessed by combining the pathological pulmonary HE staining, pulmonary edema, and vascular permeability indicators. In this study, after high tidal volume for 4 hours, HE staining showed a large number of inflammatory cell infiltrations, alveolar structural disorder, alveolar interval increase, visible

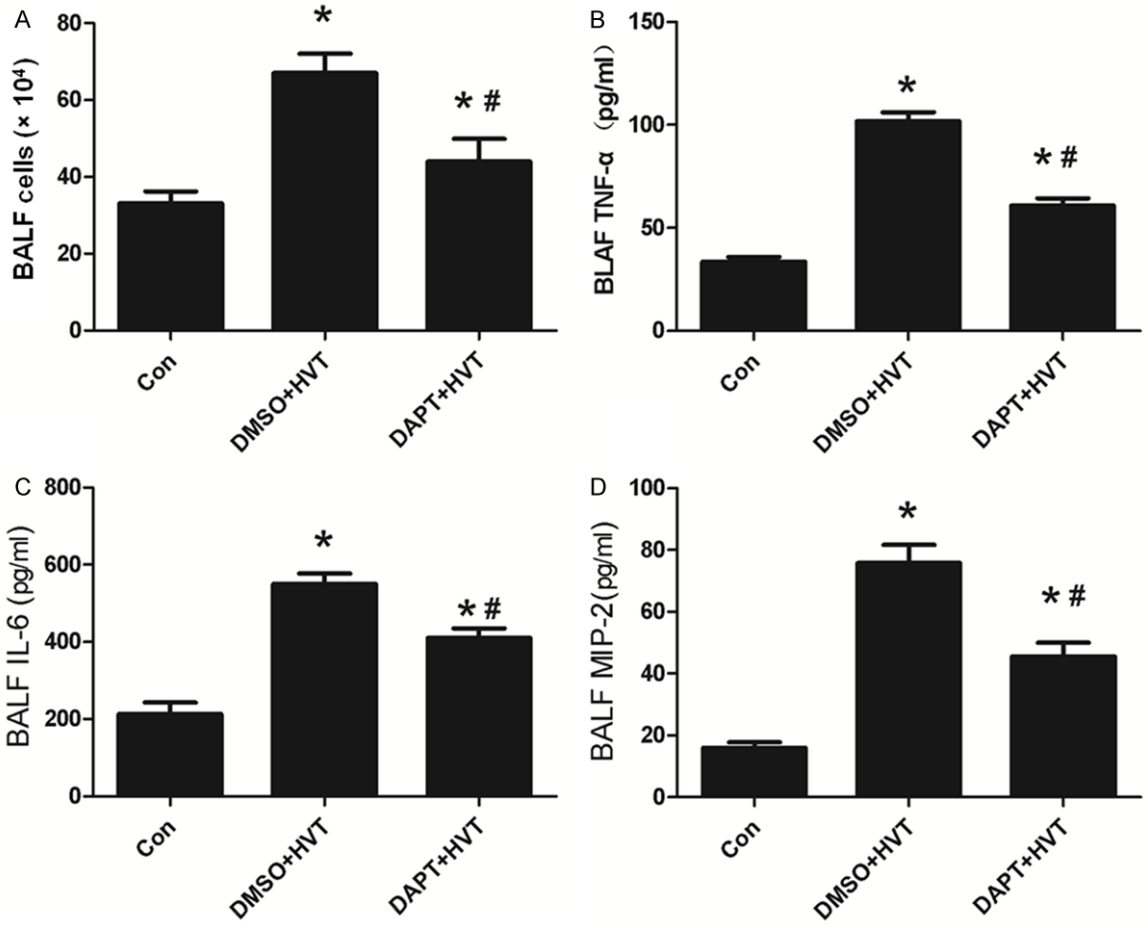

Figure 10. DAPT reduced lung inflammation after mechanical ventilation in mice. A. The total number of cells in BALF; B. TNF-alpha levels in BALF; C. IL-6 levels in BALF; D. MIP-2 levels in BALF. *P<0.05, compared with the control group, #P<0.05, compared with the simple high tidal volume group.

congestion, which are consistent with previous studies on mechanical ventilation lung injury [18, 19]. EBA index is a common indicator of pulmonary vascular permeability. The greater the EBA is index, the higher the pulmonary vascular permeability is [20, 21]. In this experiment, EBA index increased significantly after high tidal volume. The protein content in the alveolar lavage fluid was also increased significantly, reflecting vascular permeability. Wet/dry weight ratio also showed pulmonary edema. Both the EBA index and Wet/dry weight ratio both indicated the occurrence of pulmonary edema after high tidal volume mechanical ventilation.

The biological injury theory of mechanical ventilation lung injury claims that inflammation is not limited to secondary mechanical ventilation lung injury, but the mechanical ventilation can induce the inflammatory factors to further recruit the inflammatory cells to the lungs and amplify chemotactic factors leading to injury [22]. Studies have shown that macrophages were the major cells in the normal alveolar lavage fluid and neutrophils increased after mechanical ventilation [23]. In this study, after high tidal volume mechanical ventilation, the total number of cells in alveolar lavage fluid increased. This may be due to pulmonary neutrophil infiltration. Because of the need to further collect macrophages in the lavage fluid, our experiment did not count cells. The increased levels of TNF-α, IL-6 and MIP-2 in the alveolar lavage fluid are mainly associated with macrophages activation and neutrality aggregation. Neutrophil was recruited from peripheral blood vessels to the lung after activation in the inflammatory response. Neutrophil plays an important role in the biological injury mecha-

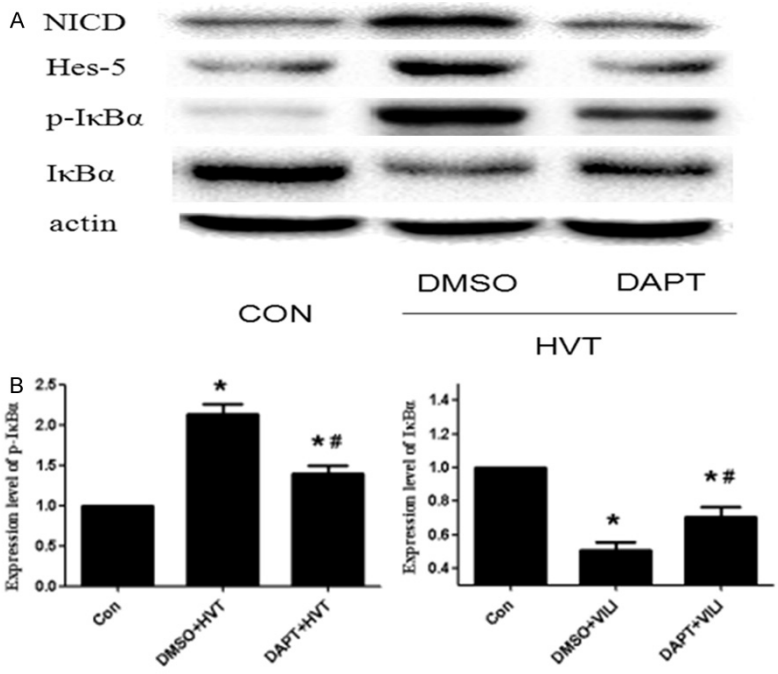

Figure 11. DAPT attenuated NF-κB pathway activation. High tidal volume mechanical ventilation after alveolar enhanced the expression of p-IκBα protein and decreased κBα in macrophages, indicating that NF-κB activation was increased. Notch signaling pathway blockers may attenuate activation of the NF-κB pathway. (A) Representative Western Blot (B) Western blot analysis of p-IκBα, IκBα. *P<0.05, compared with control group, #P<0.05, compared with the simple high tidal volume ventilation group.

nisms of VILI [24, 25]. Macrophages are the primary source of inflammatory mediators such as MIP-2 in the lungs when stimulated by mechanical ventilation [26], which has strong recruitingand chemotactic effects on neutrophils [27]. Alveolar macrophages play an important regulatory role in the pathogenesis of VILI [23, 25], so the cytological basis of Notch signaling activation in this study is likely dependent on macrophages.

Recent studies have shown that Notch signaling pathway is not only the developmental regulatory signaling pathway, but also plays a regulatory role in the immune response of macrophages. Notch receptors are mainly distributed on the surface of stem cells or primitive cells, and are also widely expressed on macrophage cell surface [28]. After Notch receptor binding to ligands, Notch receptor releases part of the extracellular fragment, and the intracellular part adhere in the cell membrane and release soluble Notch intracellular segment (i.e., NICD) after being digested by γ-secretase enzyme. NICD is the marker for Notch signaling pathway activation. In vitro studies showed that macrophage activation results in NICD up-regulation, which can in turn regulate macrophage function [29, 30]. As showed in many researches [31-33], the activation of Notch signaling was assessed by the expression levels of the NICD and the downstream molecules Hes-1, Hes-5 and protein molecules in this study. The results showed that the expression of NICD protein in macrophage was up-regulated at 4 hours after high tidal volume ventilation. The protein expression level of Hes-5 gene was also up-regulated simultaneously in the model of lung injury induced by mechanical ventilation. Cell immunofluorescence results also confirmed these findings. These results suggested that activation of Notch signaling pathway is likely to be involved in regulation of macrophage activation in mechanical ventilation induced lung injury.

Tsao et al. [11] found that DAPT, a Notch signaling pathway inhibitor administered 3 h before the sepsis model was established, could attenuate the expression of inflammatory cytokines such as TNF-α, IL-1β, IL-6 and decreased mortality, suggesting that 3 hours of time interval can effectively inhibit Notch signal activity. Studies have also confirmed [34] that DAPT given 3 hours after VILI can significantly inhibit the formation of NICD, and the effects continued up to 24 h. Therefore, DAPT was administered intraperitoneally 3 hours before VILI in our study. Our results demonstrated that DAPT could inhibit the activation of Notch signaling pathway in macrophages after mechanical ventilation. At the same time, the inhibition of Notch signaling pathway can reduce lung pathological changes and pulmonary permeability (wet dry weight ratio, protein content of alveolar lavage fluid, and EBA infiltration index), suggesting that Notch signaling pathway plays a

regulatory role in mechanical ventilation. Thus, DAPT pretreatment is beneficial to mechanical ventilation lung injury.

Our results suggested that DAPT attenuates lung injury and may be associated with reduced expression of TNF-α, IL-6, and MIP-2 in bronchoalveolar lavage fluid, which are consistent with other lung injury models [11, 35]. In vitro studies have shown that activation of Notch signaling pathway can regulate macrophage function through the NF-κB pathway [11, 36]. Our study also investigated whether it is also possible Notch signaling pathway can regulate inflammation response through NF-κB pathway after VILI. Under normal circumstances, NF-κB and its inhibitory protein IκBα are in the inactivated state, IκBα phosphorylation promotes its proteolytic [37]. After IκBα degradation, its inhibitory effects on NF-κB disappears, NF-κB is then transferred to the nucleus to regulate gene transcription. Because IκBα phosphorylation and degradation and NF-κB are closely linked, our study assessed the activation of NF-κB by detecting the expression of p-IκBα and IκBα. Our results demonstrated that high volume ventilation increased p-IκBα levels, promoted IκBα proteolysis, and resulted in decreased levels of IκB. However, inhibition of Notch signaling may partially reverses the proteolysis of IκBα, thereby inhibiting NF-κB activation. The upregulation of IκBα phosphorylation level depends on IKK-β phosphorylation [38], which may be regulated by Hes protein, because studies have shown that Hes can increase the phosphorylation of other molecules [32]. However, the specific mechanismhas not been fully elucidated.

This study confirmed that Notch signaling pathway can regulate the function of macrophages through NF-κB pathway and can participate in regulation of inflammatory response in mechanical lung injury.

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Disclosure of conflict of interest

None.

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