Annexin A1 peptide Ac2-26 mitigates ventilator-induced lung injury in acute respiratory distress syndrome rats by enhancing endothelial nitric oxide synthase activity

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Abstract

Background

Mechanical ventilation is an important therapeutic strategy for supporting patients with acute respiratory distress syndrome (ARDS). However, ventilation can exacerbate lung injury and lead to ventilator-induced lung injury (VILI). This study aimed to evaluate the effect and mechanism of Annexin A1 (AnexA1) peptide Ac2-26 on VILI in ARDS rats.

Methods

To evaluate the effect and mechanism of Annexin A1 (AnexA1) peptide Ac2-26 on VILI in ARDS rats, Male SD rats were anesthetized, and intubated, followed by injected with vehicle as the control S group or with lipopolysaccharides (LPS) to induce ARDS. The ARD rats were ventilated, randomized and injected intravenously with vehicle, Ac2-26 or Ac2-26 and L-NIO (an inhibitor of nitric oxide synthase) as the V, VA and VA/L groups, respectively. Their arterial blood gases were analyzed longitudinally for PaO2/FiO2 ratio, and the wet-to-dry lung weights and total protein concentrations in bronchoalveolar lavage fluid (BALF) were measured. The lung injury and inflammatory factors were quantified. The oxidative stress response was measured. The impact of Ac2-26 on the LPS-related cytotoxicity against A549 cells was analyzed.

Results

Compared with the S group, ARDS and ventilation impaired the lung function and caused lung injury in rats. Compared with V group, Ac2-26 treatment significantly ameliorated the lung function, reduced the VILI-related alveolocapillary permeability, lung injury and endothelial/epithelial cell apoptosis, oxidative stress and inflammation in VILI rats, but promoted AKT1 expression and eNOS phosphorylation, which were partially inhibited by co-treatment with L-NIO. Moreover, Ac2-26 protected against LPS-induced apoptosis of human A549 cells in vitro.

Conclusions

Ac2-26 effectively mitigated the severity of VILI partially by enhancing endothelial nitric oxide synthase expression in the lung of rats.

Introduction
Acute respiratory distress syndrome (ARDS) is a sudden and serious lung disease in the intensive care unit (ICU), and characterized by deterioration of lung function due to increased alveolar-vascular permeability, severe pulmonary interstitial edema, pulmonary focal lesions, hypoxemia and systemic inflammation [1]. A recent epidemiological study indicates that the prevalence of mild, moderate and severe ARDS is 30.0%, 46.6%, or 23.4%, respectively [2]. Moreover, approximately 39% of patients in the ICU receive mechanical ventilation (MV) to support their lung function [3]. Although MV can maintain oxygenation, it can cause lung injury, named as ventilator-induced lung injury (VILI) [4]. Evidence indicates that the imbalance of hypoxemia-elated inflammation and compensative anti-inflammatory response contributes to the pathogenesis of in ARDS and VILI [1, 5]. Actually, ventilation in pre-inflammatory lungs of ARDS can overstretch the alveoli [6], activate inflammatory cells and release pro-inflammatory factors to deteriorate the pre-injured lung damages [7, 8] and lead to severe hypoxemia and lung edema [9, 10]. Therefore, effective control of inflammation is a potential strategy to reduce the severity of VILI in an ARDS condition.

Annexin A1 (AnexA1) is an endogenous glucocorticoid-regulated anti-inflammatory protein and can suppress inflammation [11]. The N-terminal peptide Ac-ANX-A1 (Ac2-26) can reduce lung injury by activating the formyl peptide receptor and AKT/eNOS pathway [12]. Given that eNOS is an important regulator of acute lung injury and VILI [13, 14], and Ac2-26 activates the AKT/eNOS signaling [15, 16], we tested the hypothesis that Ac2-26 could mitigate the severity of VILI in ARDS rats partially by enhancing the eNOS expression.

Materials And Methods

Animals and grouping

The protocol was approved by the Animal Research and Care Committee of our hospital. Male Sprague Dawley (SD) rats were obtained from Harbin Medical University and housed in a specific pathogen-free room with free access to foods and water. The rats were randomized into the sham (S), mechanical ventilation (V), mechanical ventilation/Ac2-26 (VA) and mechanical ventilation/Ac2-26/L-NIO groups (VAL) (n=8 per group).

Establishment of ARDS, ventilation and treatment
All animals received and were maintained for anesthesia and intubation [17] and the V, VA and VAL groups of rats were injected with lipopolysaccharides (LPS, 026:B6, Sigma-Aldrich) to induce and evaluate ARDS [1, 17], followed by receiving a large tidal volume of MV for 4 h to induce VILI [17, 18]. At the beginning of ventilation, the V, VA and VAL groups of rats were injected intravenously with saline, Ac2-26 (1 mg/kg, Sigma, USA) or Ac2-26 and N5-(1-iminoethyl)-l-ornithine (L-NIO, 10 mg/kg, Santa Cruz BioTech), respectively, as previous reports [12, 19, 20].

Sample collection
Their arterial blood samples of individual rats were obtained longitudinally at T0 (before), T1 (30 min after induction of ARDS, before ventilation), and T2 (immediately after completion of ventilation) for analysis of blood gases in a Rapidlab 348 system (Bayer Diagnostics, Germany) to calculate the PaO2/FiO2 ratios. Their venous blood samples were collected for preparing serum samples, and the rats were sacrificed. Their right lungs were obtained to analyze lung injury, apoptosis and protein analysis and their left lungs were used to prepare the bronchoalveolar lavage fluid (BALF).

The alveolar-capillary permeability
The lung tissue wet/dry weight ratios and the protein concentrations in BALF in individual rats were measured to determine the alveolar-capillary permeability, as described previously.

Local and systemic inflammation
We measured the levels of serum and BLAF TNF-α, IL-1β, IL-6 and IL-10 by enzyme-linked immunosorbent assay (ELISA) using specific kits (Wuhan Boster Bio-Engineering, Wuhan, China), per the manufacturer’s protocols. The BALF samples were centrifuged and their contained macrophages and neutrophils were quantified after Giemsa staining in a blinded manner.

Oxidative stress response
One part of the right lung of each rat was homogenized. After being centrifuged, the malondialdehyde (MDA) concentrations and myeloperoxidase (MPO) and NADPH activities were measured using specific kits, per supplier’s instructions (Nanjing Jiancheng, China).

Histopathologic evaluation of lung injury
We analyzed lung injury histologically after standard staining with hematoxylin and eosin (HE) and
evaluated the severity (scores of 0-4) of lung injury independently by two pathologists in a blinded manner.

**Apoptosis evaluation**

We assessed lung cell apoptosis by TUNEL staining with a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany), per the supplier’s protocol. Briefly, lung tissue sections (4 µm) were digested with proteinase K, and after being washed, the sections were probed with TUNEL reagents and the apoptotic cells were visualized with diaminobenzidine solution. The percentages of apoptotic endothelial and epithelial cells were identified and calculated independently by two pathologists in a blinded fashion.

**Western blotting**

We analyzed the relative levels of endothelin-1, AKT1, phosphorylated-endothelial nitric oxide synthase (p-eNOS), and phosphorylated myosin light chain (pMLC) (pSer18) in the lung tissues by Western blotting. Briefly, we collected and homogenized one part of the lung tissue from each rat. After centrifugation, we determined the concentrations of proteins with the Bradford assay and analyzed them by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using specific antibodies against endothelin-1, phosphorylated-endothelial nitric oxide synthase (p-eNOS), AKT1 and phosphorylated myosin light chain (pMLC) (pSer18, Sigma). The immunocomplex was detected with HRP-conjugated secondary antibodies and visualized by enhanced chemiluminescence.

**Cell culture and measurements**

We cultured human adenocarcinomic alveolar epithelial A549 cells (American Type Culture Collection, Manassas, USA) in Dulbecco’s modified Eagles medium (DMEM) containing GlutaMAX (Gibco, Grand Island, NY, USA), 10% fetal bovine serum (FBS, Gibco), penicillin (100 units/ml) and streptomycin (0.1 mg/ml) (Beyotime, Shanghai, China) in an atmosphere of 95% air and 5% CO₂ at 37 °C upon 80% confluency. We treated the cells for 4 h with vehicle PBS, 1.5 µg/ml of LPS (026:B6, Sigma-Aldrich) alone [21], LPS+Ac2-26 (0.3 µM) or LPS+Ac2-26+L-NIO (10 µM) as the S, L, LA or LA/L group [12, 22]. We measured their viability using the cell counting kit-8 (CCK-8). We measured the relative levels of Bax, Bcl-2 and cleaved caspase-3 by Western blotting. We investigated the frequency of apoptotic
(annexin V+ alone and annexin V+PI+) cells by flow cytometry after stained the cells with the Annexin V-FITC/PI apoptosis detection kit (BestBio, Shanghai, China).

**Statistical analysis**

Data are expressed as the mean ± standard deviation (SD) or medians and 90% of interquartile range (IQR), based on the data type and sample size was estimated by power analysis (power = 0.9; alpha = 0.05) of preliminary results. The data were analyzed by two-way repeated ANOVA and post hoc Bonferroni analysis or Friedman test where applicable using SPSS 19.0 for Windows (SPSS, Inc., USA). A P-value of < 0.05 was considered statistically significant.

**Results**

**Ac2-26 treatment ameliorates lung function and mitigates the VILI-related alveolocapillary permeability in rats**

To determine the effect of Ac2-26 on the progression of VILI, following induction of ARDS, we treated the ventilated rats with vehicle PBS, Ac2-26 or AC2-26 and L-NIO as the V, VA or VA/L group. Longitudinal measurements indicated that compared with the control S group, the PaO₂/FiO₂ ratios in the V, VA and VA/L groups of rats significantly decreased at T1 time point, demonstrating that induced ARDS impaired the lung function in rats (Fig. 1A). Following ventilation for 4 h, the PaO₂/FiO₂ ratios in the V, VA and VA/L groups of rats were further reduced. Compared with that in the V group, treatment with Ac2-26 alone significantly ameliorated the PaO₂/FiO₂ ratios, which was significantly mitigated by co-treatment with L-NIO in rats. Similarly, significantly higher wet/dry weight ratios and protein concentrations in BALF were detected in the V group, which were significantly reduced in the VA group of rats (Fig. 1B and C). However, co-treatment with Ac2-26 and L-NIO significantly mitigated the Ac2-26-decreased wet/dry weight ratios and protein concentrations in BALF of rats. Hence, Ac2-26 treatment improved the lung function and mitigated the VILI-related alveolocapillary permeability in ARDS rats.

**Ac2-26 treatment reduces the severity of VILI by decreasing epithelial and endothelial cell apoptosis in rats**

Compared with the S group, other groups of rats displayed varying degrees of typical lung histological
VILI, including interstitial edema, alveolar collapse, alveolar breakage, and thick alveolar wall and hemorrhage in rats (Fig. 2A). Quantification analyses revealed that compared with the V group, Ac2-26 treatment significantly reduced the lung injury scores, which was partially mitigated by co-treatment with L-NIO in rats. A similar pattern of the frequency of lung apoptotic cells was detected among the groups of rats (Fig. 2B).

Because endothelial cells are sensitive to ventilation-related injury, which is characterized by increased levels of ET-1 and AKT1 expression, and MLC and eNOS phosphorylation. We further examined the effect of Ac2-26 on the VILI-related endothelial cell injury in the lung of rats. We found that compared with the S group, significantly higher levels of ET-1 and AKT1 expression, and MLC and eNOS phosphorylation were detected in the lungs of the V group of rats (Fig. 3). Ac2-26 treatment significantly reduced the levels of ET-1 expression and MLC phosphorylation, but enhanced the AKT1 expression and eNOS phosphorylation in the lungs of VILI rats. However, the protective effects of Ac2-26, except for AKT1 expression, were significantly impaired by co-treatment with L-NIO in rats.

Collectively, such data indicated that Ac2-26 treatment mitigated the severity of VILI by decreasing the VILI-related lung cell apoptosis and endothelial cell injury in rats.

**Ac2-26 treatment inhibits oxidative stress and inflammation in VILI rats**

To understand the therapeutic actions of Ac2-26, we measured the levels of lung MDA, MPO and NADPH activity of the different groups of rats. Compared with the S group, the levels of MDA, MPO and NADPH activities in the lungs of ventilated rats significantly increased (Fig. 4). Compared with the V group, Ac2-26 treatment significantly reduced the levels of MDA, MPO and NADPH activities in the lungs of rats, which were significantly mitigated by co-treatment with L-NIO in rats.

Given that oxidative stress can induce inflammation, we tested the levels of TNF-α, IL-1β, IL-6 and IL-10 in BALF samples of different groups of rats. Compared with the S group, the ventilated ARDS rats displayed higher levels of those cytokines in BALF (Fig. 5). Compared with the V group, Ac2-26 treatment significantly decreased the levels of TNF-α, IL-1β, IL-6, but increased IL-10 in BALF of rats, which were significantly mitigated by co-treatment with L-NIO (Fig. 5A). Longitudinal measurements revealed that similar patterns of serum cytokines were detected among these groups of rats at T2.
time point although the levels of these cytokines were significantly higher in all ventilated rats than the S group of rats at T1 time point (Fig. 5B). Thus, Ac2-26 treatment mitigated the ventilation-related oxidative stress and inflammation in rats.

**Ac2-26 protects against LPS-induced apoptosis in human alveolar epithelial cells in vitro.**

Finally, we examined the effect of Ac2-26 on the LPS-impaired cell viability in A549 cells. We found that LPS impaired the viability of A549 cells, suggesting that LPS caused cytotoxicity against A549 cells (Fig. 6A). Ac2-26 treatment significantly ameliorated the cytotoxicity of LPS against A549 cells, which was mitigated by co-treatment with L-NIO. Western blotting indicated that while LPS enhanced the Bax, BcL-2 and cleaved caspase-3 expression Ac2-26 treatment significantly decreased the LPS-stimulated Bax and cleaved caspase-3, but increased BcL-2 expression in A549 cells, suggesting that Ac2-26 treatment protected from LPS-induced apoptosis of A549 cells (Fig. 6B). In contrast, co-treatment with L-NIO significantly mitigated the protection of Ac2-26 in A549 cells. Flow cytometry analysis revealed that LSP alone triggered 22.7% of A549 cells undergoing apoptosis and necrosis while Ac2-26 treatment significantly reduced the frequency of apoptotic and necrotic A549 cells to 18.3%, consistent with its cell protection (Fig. 7). In contrast, co-treatment with L-NIO failed to alter the frequency of apoptotic and necrotic A549 cells (17.1%). Therefore, Ac2-26 treatment significantly protected from the cytotoxicity of LPS against A549 cells in vitro.

**Discussion**

The results from this study indicated that Ac2-26 treatment ameliorated the severity of VILI in ARDS rats. Evidently, Ac2-26 treatment significantly mitigated the ventilation-related alveolocapillary permeability, oxidative stress, local and systemic inflammation and reduced VILI-induced lung cell apoptosis to improve endothelial function in the rat model of ARDS. Because co-treatment with L-NIO partially attenuated the protective effects, the enhanced eNOS activity by Ac2-26 may partially contribute to its protection against VILI in ARDS rats.

Previous studies have shown that Ac2-26 has potent anti-inflammatory activity to reduce lung injury [12, 23]. During the pathogenic process of ARDS and VILI, activation of the NF-κB signaling in endothelial and epithelial cells releases chemokines and cytokines, including TNF-α, IL-1β, and IL-6,
which damage the lung tissue [24]. Those chemokines can recruit macrophages and neutrophils into the injured lung tissues to release more cytokines, driving a cascade of systemic inflammatory responses [8, 10]. In this study, we found that Ac2-26 ameliorated lung tissue histological injury, and reduced lung cell apoptosis and alveolar-capillary permeability in ARDS rats. Furthermore, Ac2-26 significantly decreased the levels of pro-inflammatory cytokines and the numbers of macrophage and neutrophil infiltrates in the lungs of rats. Moreover, Ac2-26 treatment increased the levels of anti-inflammatory IL-10 in rats, restoring the balance pro-inflammatory and anti-inflammatory responses [25-27]. Given that imbalance of pro-inflammatory and anti-inflammatory responses determines the progression and outcome of ARDS and VILI [28, 29] the improved balance of pro-inflammatory and anti-inflammatory responses by Ac2-26 may implicate its pharmacological action. Thus, modulation of imbalance of pro-inflammatory and anti-inflammatory responses may be a good strategy to control the progression of ARDS and VILI.

Under an inflammatory process, ventilation can damage endothelium that express high levels of ET-1 and promote MLC phosphorylation [30]. ET-1 can enhance the iNOS expression, which not only produces reactive oxidative species (ROS), but also promotes inflammatory cytokine production [31] [32]. The activated MLC can disrupt the endothelial barrier and enhance the lung edema [33, 34]. In contrast, eNOS can protect from endothelium injury to reduce the lung injury [13]. In this study, we found that Ac2-26 treatment significantly decreased ET-1 expression and MLC phosphorylation, but enhanced AKT1 expression and eNOS phosphorylation in the lungs of rats. More importantly, Ac2-26 significantly decreased the frequency of apoptotic lung cells in rats and reduced the cytotoxicity of LPS against human epithelial A549 cells in vitro, accompanied by reducing Bax and cleaved caspase-3 expression and enhancing BcL-2 expression. Conceivably, Ac2-26 activates the PI3K/AKT signaling, which phosphorylate eNOS to enhance its activity in the epithelium [35, 36]. These, together with the fact that L-NIO attenuated the protective effect of Ac2-26, suggest that the enhanced eNOS activity and relevant nitric oxide production may not only inhibit inflammation, but also protect the epithelium from inflammatory injury during the process of VILI in rats.

Hypoxia-related oxidative stress is necessary for the pathogenic process of ARDS and VILI [37, 38].
Oxidative stress can promote lipid peroxidation to produce MDA [39] and it can also be enhanced by increased NADPH oxidase and MPO [40-42]. In this study, we found that Ac2-26 significantly reduced the levels of MDA, NADPH oxidase and MPO activities in the lungs of rats, which were partially attenuated by L-NIO. These data indicated that Ac2-26 inhibited oxidative stress response and inflammation to reduce neutrophil and macrophage infiltrates in the lungs of rats by enhancing eNOS oxidase activity [43, 44].

Conclusion
The results from this study indicated that Ac2-26 ameliorated lung function and reduced the severity of VILI in ARDS rats. The potent anti-inflammatory, anti-oxidative stress and anti-apoptotic activity of Ac2-26 may be associated with enhancing the AKT signaling and eNOS activity. Given its relative safety in humans [45], Ac2-26 may be a new therapeutic reagent for prevention and inhibition of VILI in ARDS patients undergoing ventilation.

Declarations

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No applicable.

Authors' contributions
WG is the guarantor of the paper, taking responsibility for the integrity of the work as a whole, from inception to published article. Y-NJ contributed to study conception and design, drafting the article and final approval of the version to be published; Q-XZ, L-LZ, H-BS, Q-HT and G-XX contributed to experimental preparation; data acquisition, analysis, and interpretation; Q-XZ and L-LZ takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.
Ethics approval and consent to participate

The Committee for Animal Protection and Utilization of The Second Affiliated Hospital of Harbin Medical University approved this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Abbreviations

ARDS=acute respiratory distress syndrome; VILI=ventilator-induced lung injury; AnexA1=Annexin A1; LPS=lipopolysaccharides; BALF=bronchoalveolar lavage fluid; ICU=intensive care unit; MV=mechanical ventilation; Ac2-26=N-terminal peptide Ac-ANX-A1; L-NIO=N5-(1-iminoethyl)-l-ornithine; ELISA=enzyme-linked immunosorbent assay; MDA=malondialdehyde; MPO=myeloperoxidase; HE=hematoxylin and eosin; p-eNOS=phosphorylated-endothelial nitric oxide synthase; pMLC=phosphorylated myosin light chain; CCK-8=cell counting kit-8; SDS-PAGE=sodium dodecyl sulfate polyacrylamide gel electrophoresis; ROS=reactive oxidative species

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Figures
Ac2-26 treatment improves the lung function in rats. Following induction of ARDS, a the PaO2/FiO2 ratios in different groups of rats were measured at T0 (before), T1 (30 min after induction of ARDS), and T2 (immediately after completion of ventilation). b At the end of the experiment, their lung wet and dry weights were measured. c The levels of total proteins in the BALF of individual rats were quantified. Data are expressed as the mean ± SD or median ± IQR of each group (n=8 per group) from three separate experiments. * P<0.05 vs. the S group; # P<0.05 vs. the V group; & P<0.05 vs. the VA group. , S group; , V group; , VA group; , VA/L group.
Ac2-26 treatment mitigates the ventilation-related lung injury in rats. a The lung tissue injury was evaluated with HE staining. Ac2-26 significantly reduced lung histological injury, which was mitigated by co-treatment with L-NIO. b. The endothelial and epithelial cell apoptosis were evaluated by TUNEL staining. Ac2-26 treatment reduced the frequency of apoptotic endothelial and epithelial cells in the lung, which was mitigated by L-NIO. The arrows indicate the apoptotic cells. Data are representative images or expressed as the mean ± SD of each group (n=8) from three separate experiments □P<0.05 vs. the S group; # P<0.05 vs. the V group; & P<0.05 vs. the VA group. 

![Image of Western Blot](image.jpg)
Ac2-26 treatment reduces the ventilation-triggered endothelial injury in the lung of rats. The relative levels of ET-1, AKT1 expression and MLC and eNOS phosphorylation in the lungs of different groups of rats were determined by Western blot. Data are representative images or expressed as the mean ± SD of each group (n=8) from three separate experiments. a Western blot analysis of relative levels of ET-1, AKT1 expression and MLC and eNOS phosphorylation in the lungs. b Quantification analysis. □ P<0.05 vs. the S group; # P<0.05 vs. the L group; & P<0.05 vs. the LA group. , S group; , V group; , VA group; , VA/L group.
Figure 4

Ac2-26 treatment ameliorates oxidative stress in the lungs of rats. The levels of MDA, MPO and NADPH activities in lung tissues were determined. Data are expressed as the mean ± SD of each group (n=8) from three separate experiments. □ P<0.05 vs. the S group; # P<0.05 vs. the V group; &P<0.05 vs. the VA group. , S group; , V group; , VA group; , VA/L group.
Ac2-26 treatment reduces inflammatory responses in rats. The levels of serum TNF-α, IL-1β, IL-6 and IL-10 in individual rats were longitudinally measured by ELISA. The levels of serum TNF-α, IL-1β, IL-6 and IL-10 in BALF samples of individual rats at the end of the experiment were determined by ELISA. Data are expressed as the mean ± SD of each group (n=8) from three separate experiments. [] P<0.05 vs. the S group; # P<0.05 vs. the V group; & P<0.05 vs. the VA group. , S group; , V group; , VA group; , VA/L group.
Ac2-26 treatment promotes the survival of human epithelial cells by modulating apoptosis-related protein expression. Human lung epithelial cancer A549 cells were treated with vehicle or LPS in the presence or absence of Ac2-26 or Ac2-26 and N-LIO. a The cell viability was measured by MTT. b The relative levels of Bax, BcL-2 and cleaved caspase-3 expression in individual groups of cells were determined by Western blot and quantified. Data are representative images or expressed as the mean ± SD of each group from three separate experiments. □ P<0.05 vs. the S group; # P<0.05 vs. the L group; &P<0.05 vs. the LA group. 

Flow cytometry analysis of apoptotic cells. The frequency of apoptotic cells in individual groups of cells was determined by flow cytometry using FITC-Annexin V and PI staining. Data are representative flow cytometry charts from three separate experiments.