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Ibudilast, a Phosphodiesterase-4 Inhibitor, Ameliorates Acute Respiratory Distress Syndrome in Neonatal Mice by Alleviating Inflammation and Apoptosis

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Acute respiratory distress syndrome (ARDS) is a sudden and serious disease with increasing morbidity and mortality rates. Phosphodiesterase 4 (PDE4) is a novel target for inflammatory disease, and ibudilast (IBU), a PDE4 inhibitor, inhibits inflammatory response. Our study investigated the effect of IBU on the pathogenesis of neonatal ARDS and the underlying mechanism related to it.

Material/Methods: Western blotting was performed to analyze the expression levels of PDE4, CXCR4, SDF-1, CXCR5, CXCL1, inflammatory cytokines, and proteins related to cell apoptosis. Hematoxylin-eosin staining was performed to observe the pathological morphology of lung tissue. Pulmonary edema score was used to assess the degree of lung water accumulation after pulmonary injury. Enzyme-linked immunosorbent assay (ELISA) was used to assess levels of inflammatory factors (TNF- α , IL-1 β , IL-6, and MCP-1) in serum. TUNEL assay was used to detect apoptotic cells.

Results: Increased expression of PDE4 was observed in an LPS-induced neonatal ARDS mouse model, and IBU ameliorated LPS-induced pathological manifestations and pulmonary edema in lung tissue. In addition, IBU attenuated the secretion of inflammatory cytokines by inactivating the chemokine axis in the LPS-induced neonatal ARDS mouse model. Finally, IBU significantly reduced LPS-induced cell apoptosis in lung tissue.

Conclusions: IBU, a PDE4 inhibitor, protected against ARDS by interfering with pulmonary inflammation and apoptosis. Our findings provide a novel and promising strategy to regulate pulmonary inflammation in ARDS.

MeSH Keywords: **Apoptosis • Cyclic Nucleotide Phosphodiesterases, Type 4 • Inflammation • Severe Acute Respiratory Syndrome**

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Background

Acute respiratory distress syndrome (ARDS) is a sudden and serious disease with increasing morbidity and mortality rates; it is characterized by refractory hypoxemia and progressive respiratory failure [1]. Accompanied by alveolar interstitial edema syndrome or diffuse alveolar inflammatory response, ARDS is usually caused by dangerous infection in the body or lungs, severe injury, shock, pancreatitis, various intrapulmonary or external pathogenic substances, and other serious diseases [2]. Previous studies reported that neonates are especially susceptible to pulmonary injury, which results in high mortality rates in preterm infants [3]. Recent studies also suggested that excessive inflammation is involved in the pathogenesis of neonatal ARDS [4]. Pulmonary epithelial cell apoptosis induced by inflammatory stress aggravate the progression of ARDS and pulmonary damage [5]. Unfortunately, several therapies and pharmacological approaches have failed to decrease the mortality rate and improve outcomes of ARDS patients, and more effective clinical therapies are needed.

Phosphodiesterase 4 (PDE4) is an enzyme that serves as the novel target for inflammatory disease due to its mediation function in the process of cyclic adenosine monophosphate (cAMP) breakdown [6], and PDE4 inhibition suppresses inflammatory response accompanied with increased levels of intracellular cAMP [7]. Several PDE4 inhibitors have been approved as effective therapeutic agents for chronic bronchitis [8], chronic obstructive pulmonary disease (COPD) [9], and psoriatic arthritis [7]. Ibudilast (IBU, 3-isobutyl-2-isopropylpyrazolo [1, 5-a] pyridine) is a PDE4 inhibitor that inhibits astroglial reactivity and inflammatory cytokines expression in the striatum in a Parkinson's disease mouse model [10]. IBU was demonstrated to improve established arthritis by reducing the levels of inflammatory mediators [7]. Moreover, IBU is an antiasthma drug that suppresses the release of neuropeptides from airway sensory nerve terminals [11]. IBU was also reported to attenuate acute the pro-inflammatory effects of methamphetamine administration [12]. However, the effect of IBU on LPS-induced ARDS has been unclear.

In the present study, based on the evidence presented above, we hypothesized that IBU would have a therapeutic effect on neonatal ARDS. Neonatal C57BL/6 mice at 6 days old were selected and injected intraperitoneally with lipopolysaccharides (LPS) to establish the ARDS model. Our study aimed to investigate the effect of IBU on the pathogenesis of neonatal ARDS and the underlying mechanism related to it to provide novel insight into effective clinical therapies for neonatal ARDS.

Material and Methods

Animals

Forty 6-day-old neonatal C57BL/6 mice [13] were obtained from Oriental Bio Service, Inc. (Nanjing) and housed in standard cages on a 12-h light/dark cycle (temperature, $22\pm 2^{\circ}\text{C}$; humidity, 55–65%) and were provided food and water *ad libitum*. Animal care and experiment procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals approved by Shijingshan Hospital of Traditional Chinese Medicine Animal Center (IACUC Issue No. 20180907, Date: 20180905).

Mouse model of neonatal acute respiratory distress syndrome (ARDS) and IBU treatment

The neonatal ARDS murine model was induced as previously described with minor revision [14]. Forty neonatal C57BL/6 mice were randomly allocated to 4 groups with 10 mice in each group, including 1 normal saline control group, 1 LPS-induced group, and 2 IBU groups (3.75, 7.5 mg/kg) [15]. Mice in the LPS-induced group were injected intraperitoneally with LPS (2 mg/kg) purchased from Sigma-Aldrich (St. Louis, MO). Mice in the IBU group received intraperitoneal injection of IBU (Sigma, St. Louis, MO). Mice in the normal saline control group received an equal volume of vehicle (Veh; 35% vegetable oil and 65% physiological saline).

Western blotting

Lung tissues (50 mg) were washed with cold PBS and homogenized with RIPA lysis buffer (500 μL) supplemented with Phenylmethanesulfonyl fluoride (PMSF). Total proteins were extracted, and the concentration of proteins were determined using the BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific). Then, 25 μg protein was separated by SDS-PAGE and transferred to PVDF membranes (Merck Millipore, Billerica, MA). After being blocked with 5% nonfat milk and washed with TBST 3 times, the membranes were incubated overnight at 4°C with appropriate primary antibody against PDE4 (dilution, 1: 1000), TNF- α (dilution, 1: 1000), IL-1 β (dilution, 1: 1000), IL-6 (dilution, 1: 1000), MCP-1 (dilution, 1: 1000), CXCR4 (dilution, 1: 1000), SDF-1 (dilution, 1: 1000), CXCR5 (dilution, 1: 500), CXCL1 (dilution, 1: 1000), Bcl2 (dilution, 1: 1000), bax (dilution, 1: 1000), and cleaved caspase3 (dilution, 1: 500). All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Subsequently, PVDF membranes were incubated with the corresponding anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody at room temperature for 2 h and visualized using the ECL Plus system (Amersham/GE Healthcare, Germany). The optical densities of bands are presented as their ratios to control lanes, determined with ImageJ software.

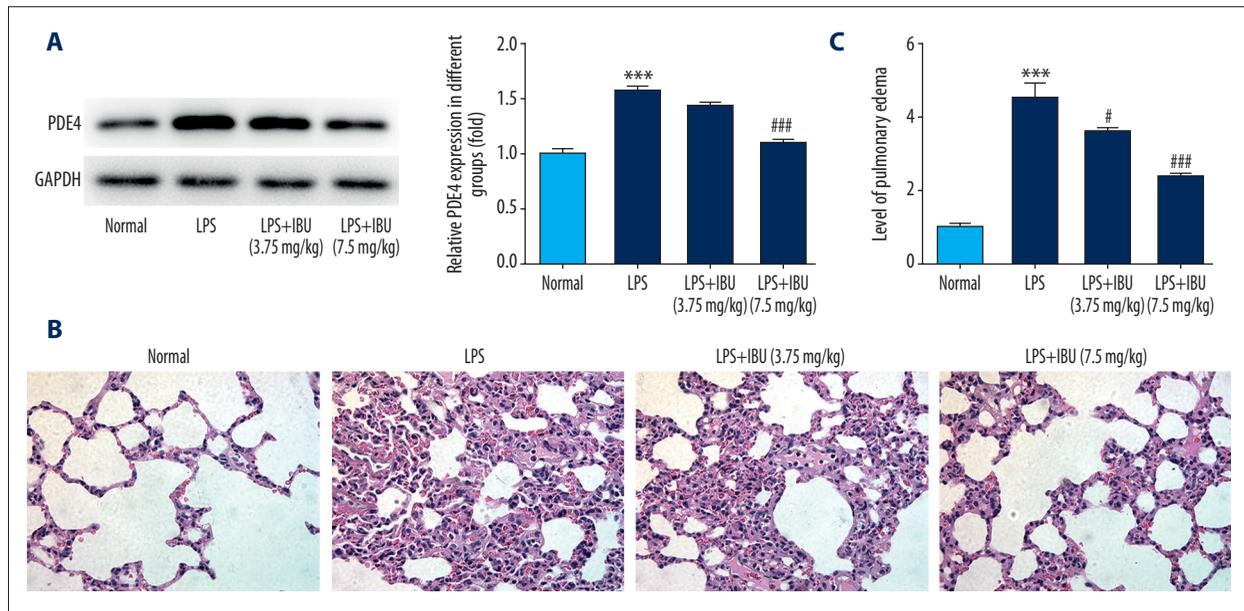


Figure 1. Influence of IBU on pathological manifestations, and pulmonary edema in lung tissue. **(A)** Expression levels of PDE4 were determined by Western blot. **(B)** Pathological manifestations were analyzed using HE staining. **(C)** Pulmonary edema was measured by pulmonary edema score. Data are presented as the mean±standard deviation (n=5). *** $P<0.001$ vs. Normal; # $P<0.05$, ### $P<0.001$ vs. LPS.

Hematoxylin-eosin (HE) staining

The samples of the left lung upper lobe were fixed in 4% paraformaldehyde for 24 h at room temperature and then embedded in paraffin. Subsequently, lung tissues were sectioned into 5- μ m-thick slices and stained with hematoxylin and eosin. Finally, the sections were transparentized with xylene for 5 min and observed using an inverted fluorescence microscope (Nikon Eclipse Ti; Nikon Corporation, Tokyo, Japan) at 200 \times magnification to analyze the morphological characteristics of lung tissues.

Pulmonary edema score

The lung wet/dry weight ratio [16] was used as the pulmonary edema score to indicate the degree of lung water accumulation after pulmonary damage. The wet weight of the right lung middle lobe was measured immediately after excision. Then, the lung tissue was dried in an oven at 80°C for 3 days or until tissues were completely dry and then were assessed instantly to determine the dry weight.

ELISA assay

Blood of mice was collected through eyeball extraction after co-treatment with LPS and IBU, and serum was separated through centrifugation at 3500 rpm at 4°C for 15 min.

The levels of TNF- α , IL-1 β , IL-6, and MCP-1 in serum were measured using ELISA assay kits (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. The color intensity was assayed at 450 nm with reference wave length using a microplate reader (Bio-Rad, Hercules, CA).

TUNEL assay

Cell apoptosis in lung tissues was detected using TUNEL assay (Millipore; Merck KGaA, Darmstadt, Germany). Lung tissues were washed with PBS and fixed with 1% paraformaldehyde. Fluorescein isothiocyanate (FITC) (green) and 4',6-diamidino-2-phenylindole (DAPI) (blue) were used to stain the apoptotic cells and nuclei. Optical microscopy (Olympus Corp., Tokyo, Japan) was performed to capture the images (magnification, $\times 200$).

Statistical analysis

GraphPad Prism 5.0 (La Jolla, CA, USA) was used for data analysis. All data are expressed as mean±standard deviation (SD). Statistical differences between the groups were measured by one-way analysis of variance (ANOVA) followed by the post hoc Bonferroni test. All experiments were repeated at least 3 times, and $P<0.05$ was considered statistically significant.

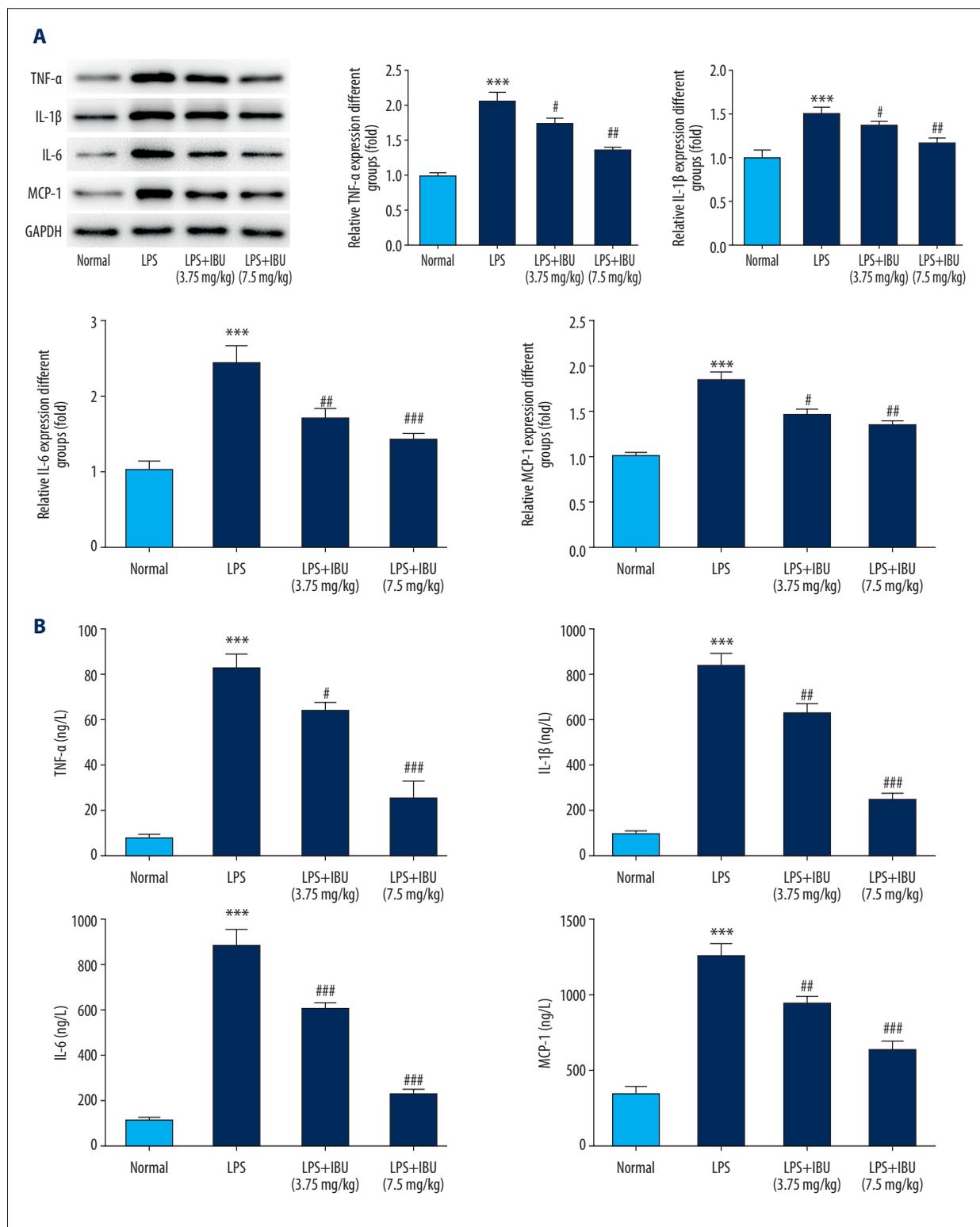


Figure 2. Influence of IBU on release of inflammatory cytokines in serum and lung tissue of LPS-induced neonatal ARDS mouse model. **(A)** Expression levels of TNF- α , IL-1 β , IL-6, and MCP-1 were determined by Western blot. **(B)** The levels of inflammatory cytokines, including TNF- α , IL-1 β , IL-6, and MCP-1 were determined by ELISA. Data are presented as the mean \pm standard deviation (n=5). *** $P < 0.001$ vs. Normal; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. LPS.

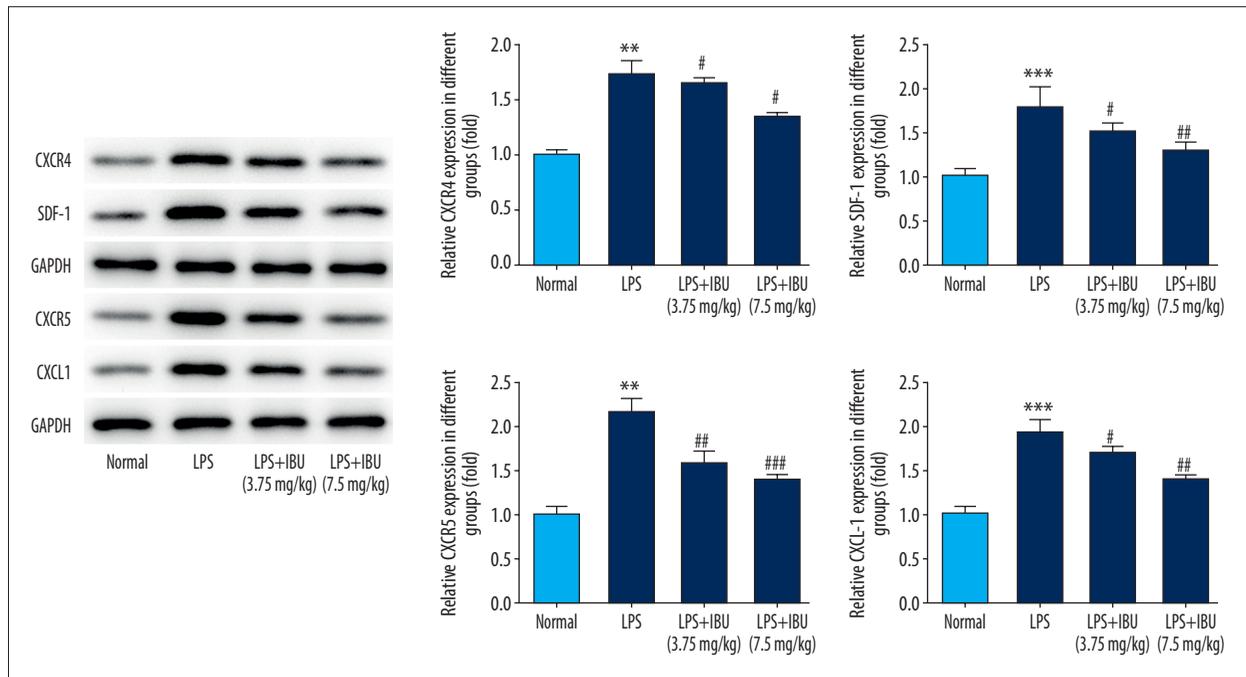


Figure 3. Influence of IBU on expression of chemokine axis in lung tissue of LPS-induced neonatal ARDS mouse model. Expression levels of CXCR4, SDF-1, CXCR5, and CXCL1 were determined by Western blot. Data are presented as the mean±standard deviation (n=5). ** $P<0.01$, *** $P<0.001$ vs. Normal; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs. LPS.

Results

Increased expression of PDE4 in the LPS-induced neonatal ARDS mouse model, and the PDE4 inhibitor ameliorated pathological manifestations, and pulmonary edema in lung tissue

Neonatal C57BL/6 mice at 6 days old were selected to establish the ARDS mouse model. The PDE4 expression in lung tissue was assessed via Western blot. As shown in Figure 1A, the expression level of PDE4 proteins was significantly upregulated by LPS stimulation. The PDE4 inhibitor, ibudilast (IBU), obviously decreased the abnormal overexpression in lung tissue of neonatal ARDS mice.

We performed hematoxylin-eosin staining to analyze the pathological morphology of lung tissue. Compared with normal mice, histological changes such as inflammation, hemorrhage, alveolar congestion, and alveolar wall edema were observed in the lung tissue of neonatal ARDS mice (Figure 1B). Interestingly, IBU treatment effectively reversed the histological changes. The pulmonary edema score was used to assess the degree of lung water accumulation after pulmonary injury. Compared with the control group, obvious pulmonary edema was observed in the LPS-induced ARDS group, but the pulmonary edema was reversed by IBU treatment (Figure 1C). These results suggested that IBU protected against the pulmonary injury induced by LPS stimulation.

Influence of ibudilast on release of inflammatory cytokines in serum and lung tissue of LPS-induced neonatal ARDS mouse model

Western blotting and ELISA kits were used to determine the expression of the inflammatory factors TNF- α , IL-1 β , IL-6, and MCP-1 in lung tissue and serum, respectively. Consistent with the control group, the expression levels of TNF- α , IL-1 β , IL-6, and MCP-1 were significantly increased in lung tissue and serum of the LPS-induced ARDS neonatal mouse model. Interestingly, IBU remarkably suppressed inflammatory cytokines release in a dose-dependent manner (Figure 2A, 2B), indicating that IBU suppressed the inflammatory response.

Influence of ibudilast on expression of chemokine axis in lung tissue of LPS-induced neonatal ARDS mouse model

The chemokine axis plays major roles in inflammation of injured tissues. The chemokine CXCL1, stromal-derived factor-1 (SDF-1), chemokine receptor4 (CXCR4), and CXCR5 have been demonstrated to be involved in induction and maintenance of inflammatory disorders [17]. Western blotting was used to determine the expression levels of the proteins CXCL1, SDF-1, CXCR4, and CXCR5 in lung tissue. As shown in Figure 3, the expression levels of CXCL1, SDF-1, CXCR4, and CXCR5 were obviously increased in the LPS-induced ARDS group compared with controls, but IBU reversed the overexpression of these proteins in a dose-dependent manner. Therefore, our results

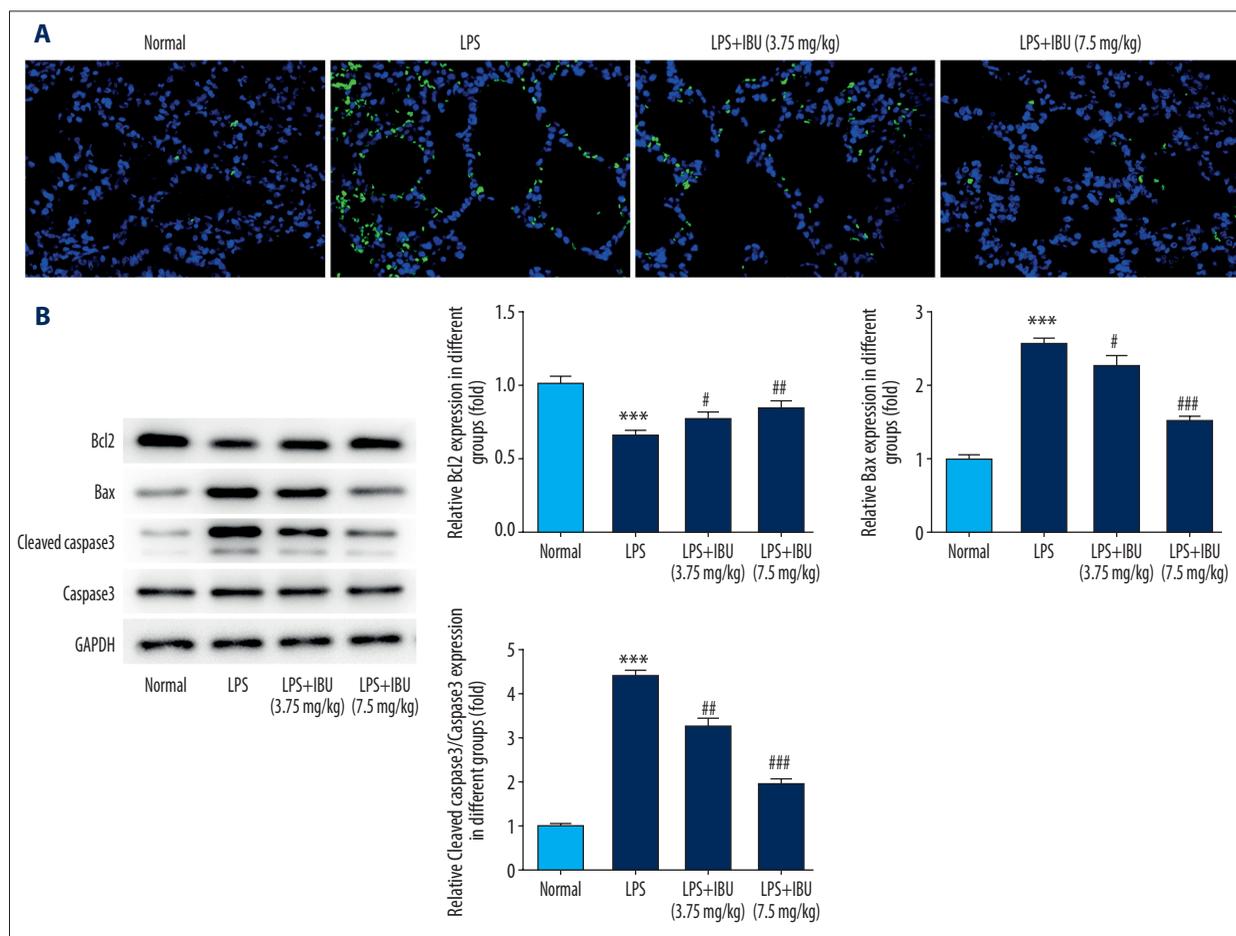


Figure 4. Influence of IBU on cell apoptosis in lung tissue of LPS-induced neonatal ARDS mouse model. **(A)** Cell apoptosis was analyzed by TUNEL assay. **(B)** Expression levels of Bcl2, Bax, cleaved-caspase3, and caspase3 were determined by Western blot. Data are presented as the mean±standard deviation (n=5). *** $P<0.001$ vs. Normal; # $P<0.05$, ## $P<0.01$, ### $P<0.001$ vs. LPS.

show that IBU suppressed the inflammatory response by inhibition of the chemokine axis.

Influence of ibudilast on cell apoptosis in lung tissue of LPS-induced neonatal ARDS mouse model

To investigate the effect of IBU on cell apoptosis, TUNEL staining and Western blot analysis were performed. As shown in Figure 4A, high FITC positivity was exhibited in lung tissue in the LPS-induced neonatal ARDC mouse model. Compared with the LPS model group, IBU notably reduced the cell apoptosis rate. Furthermore, the proteins related to cell apoptosis were assessed via Western blot (Figure 4B). The expression level of Bcl2 in lung tissue in the neonatal ARDC mouse model was downregulated compared with the control group. IBU treatment significantly increased the Bcl2 expression in a dose-dependent manner. In contrast, the expression levels of Bax and cleaved caspase3 in lung tissue in the neonatal ARDC mouse model were significantly upregulated compared with the control group. IBU treatment significantly reduced

the expression levels of Bax and cleaved caspase3 in a dose-dependent manner. Caspase3 expression was not significantly changed. All these results suggest that IBU protects against pulmonary injury by attenuating cell apoptosis in lung tissue.

Discussion

Acute respiratory distress syndrome (ARDS) is a severe disease characterized by acute respiratory failure [4]. Accompanied with high mortality and morbidity worldwide, ARDS is a serious threat to human health and has huge economic costs [18]. A large body of evidence shows that inflammation contributes to the occurrence and progression of ARDS [19]. In the present study, 6-day-old neonatal C57BL/6 mice were used to establish an RDS model. All our results revealed that the PDE4 inhibitor, ibudilast (IBU), protected against pulmonary damage through suppressing inflammatory response and cell apoptosis, suggesting that IBU can have a therapeutic effect on ARDS.

The histological changes in lung tissue (Figure 1B), such as inflammation, hemorrhage, alveolar congestion, and alveolar wall edema, show that the PDE4 inhibitor IBU has a potential therapeutic effect on pulmonary damage induced by LPS challenge. Previous studies have reported that histological changes, including alveolar wall thickening, alveolar congestion, infiltration of inflammatory cells, and intra-alveolar exudates and edema, were observed by histological analysis following LPS stimulation [16,20], which is consistent with our study. As a driving factor of ARDS, inflammation contributes to the occurrence and development of ARDS. Phosphodiesterase 4 (PDE4) is a pro-inflammatory enzyme involved in hydrolyzing and inactivating cyclic adenosine monophosphate (cAMP). Apremilast, a PDE4 inhibitor, attenuated murine ulcerative colitis via suppressing oxidative stresses and inflammatory factors production [21]. In addition, Zuo et al. reported that various PDE inhibitors can be used in the treatment of chronic obstructive pulmonary disease (COPD) and asthma [22]. In the present study, IBU markedly attenuated the PDE4 expression (Figure 1A) and clinical features of ARDS. Furthermore, the inflammatory cytokines TNF- α , IL-1 β , IL-6, and MCP-1 in lung tissue and serum were assessed to further confirm the role of IBU in the inflammatory response. It is well established that the process of inflammatory response is amplified by recruitment of neutrophils, transmigration of monocytes, and activation of resident mast cells, producing pro-inflammatory mediators like TNF- α , IL-1 β , and IL-6 [23]. Our results demonstrated that IBU significantly suppressed the secretion of inflammatory factors (Figure 2). Consistent with our findings, Audard et al. found that inhibition of alveolar inflammation reversed lung injury in a piglet model of ARDS [24]. Additionally, Kosutova et al. demonstrated that the PDE4 inhibitor roflumilast decreased concentrations of inflammatory cytokines and oxidative products in the lung and plasma of rabbits with acute lung injury [25]. CHF6001 is a novel inhaled PDE4 inhibitor, which acts as an anti-inflammatory in the lungs of patients with COPD [6].

The chemokine receptor4 (CXCR4)/stromal-derived factor-1 (SDF-1) chemokine axis plays key roles in inflammation of injured tissues. Activation of the CXCR4/SDF-1 axis can lead

to interstitial lung fibrosis or other lung disease [26]. CXCR5, expressed by vascular endothelium, is reported to regulate inflammation by affecting leucocyte migration [27]. Insufficient zinc intake resulted in increased CXCL1 hyper-responsiveness in airway inflammation [28]. Davino-Chiovatto et al. also suggested that montelukast, a leukotriene inhibitor, reduces LPS-induced acute lung inflammation by attenuating expression levels of IL-6 and CXCL1 proteins [29]. Our results reveal that IBU suppresses chemokine axis activation (Figure 3), indicating that IBU can attenuate LPS-induced lung inflammation by inactivating the chemokine axis. It has been generally accepted that uncontrolled inflammation exacerbates tissue injury [30,31]. Recent studies have demonstrated that ARDS is characterized by alveolar epithelial destruction, and lipoxinA4 ameliorated pulmonary damage by reducing epithelial cell apoptosis and promoting epithelial cell proliferation [32]. In addition, Sun et al. demonstrated that dexmedetomidine inhibited epithelial cell apoptosis in LPS-induced animal models of ARDS [33], which is consistent with our study. In the present study, IBU decreased the LPS-induced cell apoptosis in lung tissue (Figure 4), suggesting that IBU can prevent pulmonary inflammatory damage. The present study has certain limitations, and further experiments will be carried out to investigate the underlying mechanisms of IBU in ARDS.

Conclusions

In summary, inhibition of PDE4 by IBU protected against ARDS by interfering with pulmonary inflammation and apoptosis. Our findings suggest a novel and promising strategy for regulating pulmonary inflammation in ARDS.

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