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HDAC6 inhibition alleviates acute pulmonary embolism: a possible future therapeutic option

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Abstract

Introduction. Acute pulmonary embolism (APE) is a clinical syndrome of pulmonary circulation disorder caused by obstruction of the pulmonary artery or its branches. Histone deacetylase 6 (HDAC6) has been reported to play an important role in lung-related diseases. However, the functional role of HDAC6 in APE remains unclear.

Material and methods. Male Sprague Dawley rats were used. The APE model was constructed by inserting an intravenous cannula into the right femoral vein and injecting Sephadex G-50 microspheres (12 mg/kg; 300 μm in diameter). After 1 h, the control and APE rats were intraperitoneally injected with tubastatin A (TubA) (40 mg/kg, an inhibitor of HDAC6) and sampled at 24 h after modeling. H&E staining, arterial blood gas analysis, and wet/dry (W/D) weight ratio were used to evaluate the histopathological changes and pulmonary function in APE rats. ELISA, Western blot, and immunohistochemistry were used to explore the potential mechanism of HDAC6-mediated inflammation in APE.

Results. The results indicated that HDAC6 expression was significantly increased in lungs of APE rats. TubA treatment *in vivo* decreased HDAC6 expression in lung tissues. HDAC6 inhibition alleviated histopathological damage and pulmonary dysfunction, as evidenced by decreased PaO_2/FiO_2 ratio and W/D weight ratio in APE rats. Furthermore, HDAC6 inhibition alleviated APE-induced inflammatory response. Specifically, APE rats exhibited increased production of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-18, however, this increase was reversed by HDAC6 inhibition. Meanwhile, the activation of the NLRP3 inflammasome was also observed in lungs of APE rats, while HDAC6 inhibition blocked this activation. Mechanically, we demonstrated that HDAC6 inhibition blocked the activation of the protein kinase B (AKT)/extracellular signal-regulated protein kinase (ERK) signaling pathway, a classic pathway promoting inflammation.

Conclusions. These findings demonstrate that the inhibition of HDAC6 may alleviate lung dysfunction and pathological injury resulting from APE by blocking the AKT/ERK signaling pathway, providing new theoretical fundamentals for APE therapy. (*Folia Histochemica et Cytobiologica 2023, Vol. 61, No. 1, 56–67*)

Keywords: rat; acute pulmonary embolism; lung injury; histone deacetylase 6 inhibition; tubastatin A; AKT/ERK pathway

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Introduction

Pulmonary embolism is a common venous embolism associated with tremendous morbidity and mortality [1]. Statistically, approximately 20% of patients with pulmonary embolism die within two hours after the onset of the acute phase [2]. Acute pulmonary embolism (APE) has been recognized as a pathophysiological syndrome of severe respiratory and circulatory disorders characterized by thrombosis blocking the main trunk or branch of the pulmonary artery [3]. Clinical manifestations of APE include the triad of dyspnea, chest pain, and hemoptysis, further leading to pulmonary hypertension, right heart failure, obstructive shock, circulatory failure, and even death [4, 5]. Notably, APE is the third most common disease of the cardiovascular system following coronary heart disease and hypertension, which dramatically endangers the physical and mental health of patients [6]. To date, the methods for drug treatment of APE are thrombolytic therapy and anticoagulant therapy [7]. Although diagnostic techniques and therapy treatment for APE are constantly improving, APE mortality is still as high as 50% or even more [8]. Therefore, it is of clinical significance to explore more effective drugs and methods to treat APE.

Histone deacetylase 6 (HDAC6), a cytoplasmic enzyme unlike many other HDACs, contains two catalytic domains and a zinc finger ubiquitin binding protein domain [9]. HDAC6 can bind to various substrate proteins in the cytoplasm, and participate in the regulation of important biological processes [10]. The acetylation level of α-tubulin is a substitutive marker of HDAC6 activity. HDAC6 inhibition protected rat cardiac myocytes from cellular injury by α-tubulin acetylation [11]. Treatment with ACY1215, a selective HDAC6 inhibitor, has been shown to alleviate myocardial ischemia-reperfusion injury by reducing heart infarct size in rats [12]. In the diabetic rat model, inhibition of HDAC6 activity was found to reduce myocardial ischemia/reperfusion injury [13]. Additionally, honokiol treatment mitigated angiotensin II-induced hypertension and endothelial dysfunction by inhibiting HDAC6-mediated cystathionine γ-lyase degradation [14]. CM-695, a small molecule compound, increased HSP70 expression by inhibiting HDAC6 expression, thereby effectively reducing thromboses and bleeding risk in mice [15]. Furthermore, HDAC6 inhibition attenuated inflammatory indices in lipopolysaccharide (LPS)-induced acute lung injury in C57BL/6 mice [16]. Studies have reported that HDAC6 inhibition alleviated inflammatory responses *via* the activation of the protein kinase B (AKT) and the extracellular signal-regulated protein kinase (ERK) pathways *in vitro* [17, 18]. Tubastatin A (TubA), a highly selective HDAC6 inhibitor, has received extensive attention [19, 20]. Indeed, TubA alleviated the destruction of the pulmonary endothelial cell barrier and LPS-induced pulmonary edema by inhibiting tumor necrosis factor-alpha (TNF-α)-induced microtubule disassembly in mice [21]. Accumulating evidence has demonstrated that HDAC6 plays an important role in regulating pulmonary function. A previous study showed that HDAC6 inhibition alleviated endothelial barrier dysfunction and acute lung injury by inhibiting lipopolysaccharide-mediated heat shock protein 90 phosphorylation in mice [22]. Importantly, HDAC6 is implicated in the regulation of pulmonary hypertension. For instance, TubA treatment promoted cell survival and proliferation and relieved pulmonary injury in arterial hypertension rat models [23]. However, the effects of HDAC6 on APE-induced pulmonary injury remain unclear. Based on the above research background, we speculated that inhibition of HDAC6 might alleviate inflammatory reactions and pulmonary injury in the APE process.

Material and methods

Animal experiments. Healthy male Sprague Dawley rats (aged 8–9 weeks; 300–320 g) were maintained at 21–23° and 45–55% humidity on a 12 h light/dark cycle for one week. All experimental procedures were performed in strict conformity with the Ethics Committee of Wuxi 9th People's Hospital (KT2021017 certificate). Rats were stochastically separated into 4 groups: Control group, Control + TubA group, APE group, and APE + TubA group. To establish the APE model, rats in APE and APE + TubA groups were injected with 12 mg/kg suspension of Sephadex G-50 microspheres (300 μm in diameter, Macklin Biotechnology, Shanghai, China) by inserting an intravenous cannula into the right femoral vein based on the previous study [24]. Rats in Control and Control + TubA groups were injected with the equivalent volume of normal saline instead of the microspheres. After 1 h, rats in Control + TubA and APE + TubA groups were intraperitoneally injected with TubA (40 mg/kg, MedChemExpress, Shanghai, China). Then, at 24 h after modeling, the pulmonary function of rats in different groups was measured. Afterward, all rats were euthanized by carbon dioxide asphyxiation, and the lung tissues were collected. Part of the lung tissues was fixed in 4% paraformaldehyde, whereas the remaining lung tissues were frozen at –70° for subsequent experimental detection.

Blood gas analysis. Before euthanizing the rats, arterial blood was extracted from the right common carotid artery, and then a blood gas analysis was performed. The partial pressure of oxygen (PaO_2), fraction of inspired oxygen (FiO_2), and the partial pressure of carbon dioxide ($PaCO_2$) were measured using a blood gas analyzer (RAPIDPoint500, Siemens, UK). Subsequently, the values for the PaO_2/FiO_2 ratio were calculated.

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Table 1. Scoring system of lung injury

Score	Description
0	Normal appearance
1	Mild interstitial congestion
2	Perivascular oedema and moderate pulmonary structural damage
3	Massive cell infiltration and moderate alveolar structure destruction
4	Massive cell infiltration and severe lung structural damage

Inflammatory cells detection in bronchoalveolar lavage fluid (BALF). BALF was collected as previously described [25]. Briefly, a tracheostomy was performed to expose the trachea, and the lungs were gently injected with 8 mL of bolus of sterile saline three times to collect a total of 5.0–6.5 mL of BALF. Subsequently, the smear was prepared by dropping the resuspension liquid onto a clean glass slide and allowed to air–dry. After fixing with methanol, cells in BALF were stained with Giemsa staining solution (Jiancheng Bioengineering Institute, Nanjing, China). After depigmentation, the slides were washed and naturally dried. Finally, total and differential cell counts (eosinophils, neutrophils, lymphocytes, and macrophages) were evaluated in BALF under a microscope (Olympus, Tokyo, Japan).

Wet/dry (W/D) weight ratio of lung tissues. After euthanizing the rats, the lung tissues were weighed immediately to obtain the wet lung weight. Subsequently, the tissues were oven-dried at 80° until constant weight to obtain the dry lung weight. The W/D weight ratio was used to evaluate the degree of pulmonary edema.

Morphological analysis of lung tissue. The fixed lung samples were embedded in paraffin as previously described [26], and sectioned serially at 5 μm slices by using a rotary microtome (Leica, Nussloch, Germany). Subsequently, the samples were stained with hematoxylin and eosin (H&E) [27, 28]. Finally, the stained sections were observed and photographed under a BX53 microscope (Olympus, Tokyo, Japan). According to previous studies [29], the scoring system was used to estimate the severity of acute lung injury as shown in Table 1.

Immunohistochemistry. For immunohistochemistry, the lung tissue sections were permeabilized in a 3% hydrogen peroxide solution (Sinopharm, Shanghai, China) for 15 min after antigen repair. Subsequently, the sections were blocked with 1% bovine serum albumin (BSA) (Sangon Biotechnology, Shanghai, China) for 15 min, and incubated with primary antibodies against HDAC6 (1:100, ABclonal Biotechnology, Wuhan, China) and NLRP3 (1:100, Affinity Biosciences, Changzhou, China) overnight at 4°. On the next day, the sections were incubated with the secondary antibody (1:500, ThermoFisher Scientific, Pittsburgh, PA, USA) at 37° in a humid chamber for 1 h. Afterward, images were observed and captured under a microscope (BX-53, Olympus).

Table 2. The primary antibodies used in this study

Antibody name	Dilution ratio	Source
HDAC6	1:1000	ABclonal Biotechnology, Wuhan, China
α-tubulin	1:1000	Affinity Biosciences, Changzhou, China
ace-α-tubulin	1:500	Affinity Biosciences, Changzhou, China
NLRP3	1:1000	ABclonal Biotechnology, Wuhan, China
ASC	1:1000	ABclonal Biotechnology, Wuhan, China
cleaved caspase-1	1:1000	Affinity Biosciences, Changzhou, China
AKT	1:1000	Affinity Biosciences, Changzhou, China
p-AKT	1:500	Affinity Biosciences, Changzhou, China
ERK	1:1000	Affinity Biosciences, Changzhou, China
p-ERK	1:500	Affinity Biosciences, Changzhou, China
β-actin	1:50000	ABclonal Biotechnology, Wuhan, China

Determination of myeloperoxidase (MPO) activity. The activity of MPO was measured using the MPO ELISA Kits (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, wet lung tissues were weighed, and the homogenization buffer (1:19) was added to prepare a 5% tissue homogenate by a glass-glass homogenizer according to the manufacturer's instructions. Subsequently, the tissue homogenate (0.9 mL) was mixed with MPO assay buffer (0.1 mL) at 37° for 15 min. Thereafter, according to the manufacturer's instructions, MPO activity was calculated according to the formula.

MPO activity (U/g tissue wet weight) = (measured OD value – control OD value)/[11.3 × sample volume (g)]

Determination of proinflammatory cytokines content in rat lungs. Lung tissues were weighed, and normal saline was added at a ratio of weight (g)/volume (mL) = 1:9. Subsequently, 10% homogenate was prepared by homogenizing with a glass-glass homogenizer under an ice water bath, centrifuged for 10 min, and then the supernatant was collected for detection. The levels of TNF-α, interleukin (IL)-6, IL-1β, and IL-18 in the lungs were detected with specific kits. TNF-α, IL-6, and IL-1β were provided by MultiSciences Biotechnology (Hangzhou, China). IL-18 was provided by Wuhan Fine Biotechnology (Wuhan, China). All experimental steps were carried out referring to the manufacturer's instructions.

Western blot analysis. Western blot was applied to measure the expression levels of proteins. Total proteins were extracted from rat lungs by using Cell lysis buffer for Western and IP (Beyotime

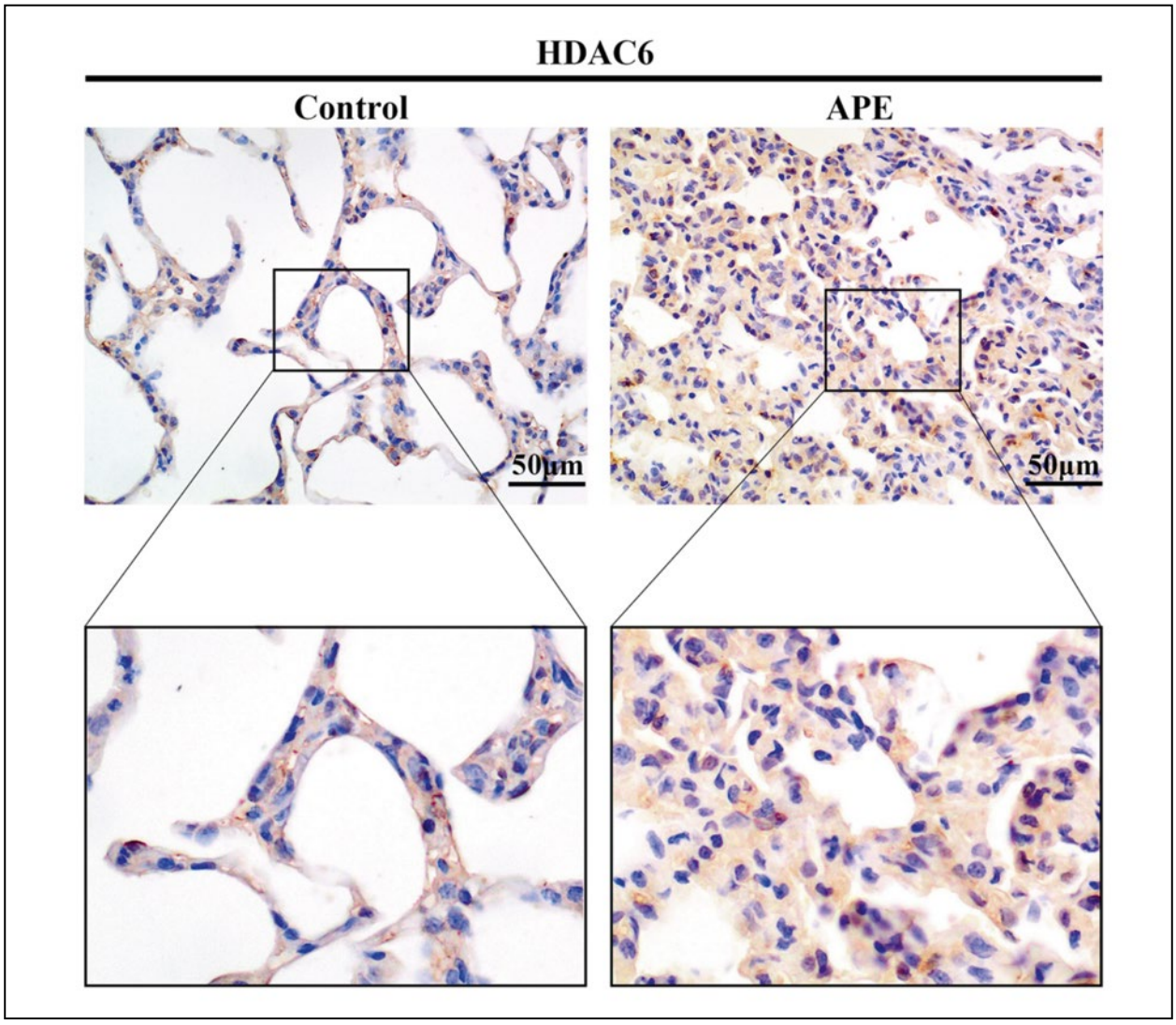

Figure 1. HDAC6 was highly expressed in lungs of rats with induced APE. Representative images of HDAC6 immunoreactivity were assessed as described in Methods. Scale bar = 50 μm. Abbreviations: APE — acute pulmonary edema; HDAC6 — histone deacetylase 6.

Biotechnology, Shanghai, China) together with phenylmethanesulfonyl fluoride (PMSF; Beyotime). Subsequently, a BCA kit assay was used to quantify concentrations of the protein. Protein samples were separated on 12% SDS-PAGE gels, blocked with 5% nonfat milk for 1 h, and incubated with primary antibodies overnight at 4°C. The primary antibodies, their sources, and dilution concentrations were listed in Table 2. β-actin was the reference protein. On the second day, samples were incubated with the secondary antibody (1:10000, ABclonal Biotechnology, Wuhan, China) for 40 min at 37°C. Finally, the protein bands were visualized by an enhanced chemiluminescence reagent (ECL; Beyotime).

Statistical analyses. Statistical analysis was performed using GraphPad Prism (version 8.0). The data analysis for the two groups was performed *via* an unpaired t-test. Additionally, data from four groups were submitted to one-way ANOVA with Tukey's multiple comparison test. The results were presented as the means ± standard deviations. Data were considered statistically significant at $P < 0.05$.

Results

HDAC6 was highly expressed in the lung tissues of APE rats

Initially, we detected HDAC6 expression in rat lung tissues under APE conditions. As shown in Fig. 1, immunohistochemical analysis revealed that HDAC6 was predominantly localized in the cytoplasm. In addition, it was shown that HDAC6 expression was increased in the lung tissues of APE rats compared with the control. Therefore, these data demonstrated that high expression of HDAC6 might participate in the progression of APE.

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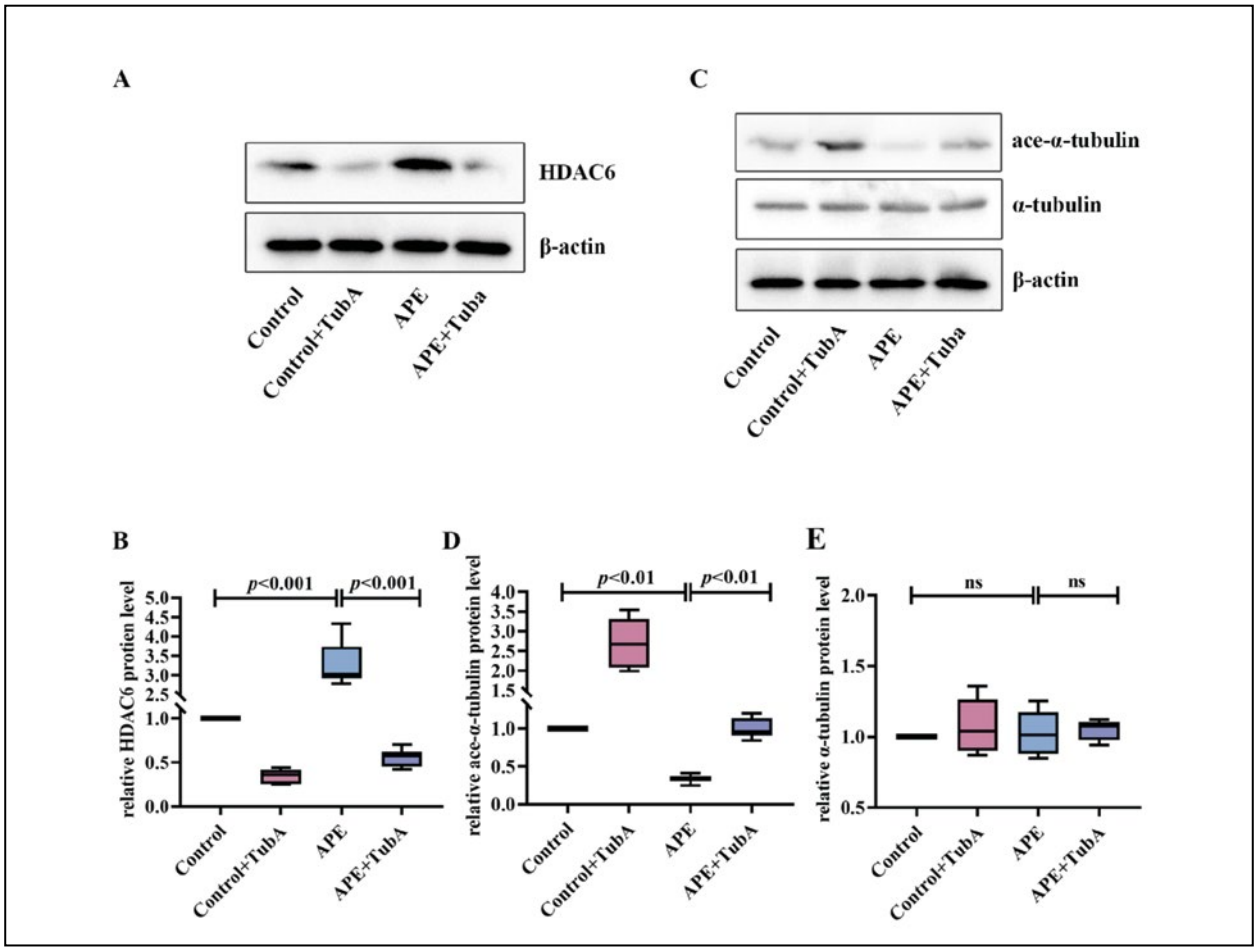

Figure 2. TubA suppressed the increased expression of HDAC6 in APE lung tissues. **A, B.** Western blot and quantitative analysis for the expression of HDAC6 protein in relation to the expression of b-actin. **C–E.** Western blot and quantitative analysis for the expression of ace-α-tubulin and α-tubulin. Abbreviations: APE — acute pulmonary edema; HDAC6 — histone deacetylase 6; TubA — Tubastatin A.

Effects of TubA on HDAC6 expression in APE lung tissues

Subsequently, we detected whether TubA is sufficient to downregulate HDAC6 expression in the rat lung. As shown in Fig. 2A and B, western blot analysis showed that TubA inhibited the increased expression of HDAC6. Acetylation of α-tubulin reflected the activity of HDAC6. Therefore, we detected α-tubulin and ace--α-tubulin at the protein level. Notably, ace-α-tubulin expression was decreased in APE-treated rats, but this decrease was restored by TubA treatment. There was no significant difference in α-tubulin expression between APE and control rats (Fig. 2C–E). Therefore, the above results suggested that the HDAC6 inhibitor successfully suppressed the increased expression of HDAC6 in APE rats.

Effects of HDAC6 inhibition on APE in rats

PaO_2/FiO_2 represents the efficiency of inhaled oxygen delivered to blood, which is an important indicator to judge the severity of lung injury. As shown in Fig. 3A, the ratio of PaO_2/FiO_2 was decreased in the APE group, and this decrease was restored by TubA treatment (Fig. 3A). Further, the $PaCO_2$ level was detected under APE conditions. As shown in Fig. 3B, APE rats exhibited increased $PaCO_2$ levels, while TubA treatment reversed the level in $PaCO_2$. These results indicated that HDAC6 inhibition alleviated pulmonary dysfunction in APE rats.

Effects of HDAC6 inhibition on lung injury in APE rats

The lung W/D ratio of rats was used to evaluate the degree of pulmonary edema. Compared with the control group, the W/D ratio in the APE group was significantly increased but HDAC6 inhibition decreased the ratio (Fig. 4A). MPO is regarded as marker of neutrophil infiltration [30]. As shown in Fig. 4B, MPO activity increased significantly under APE conditions, which was blocked by TubA treatment. Subsequently, the number of inflammatory cells in BALF was detected.

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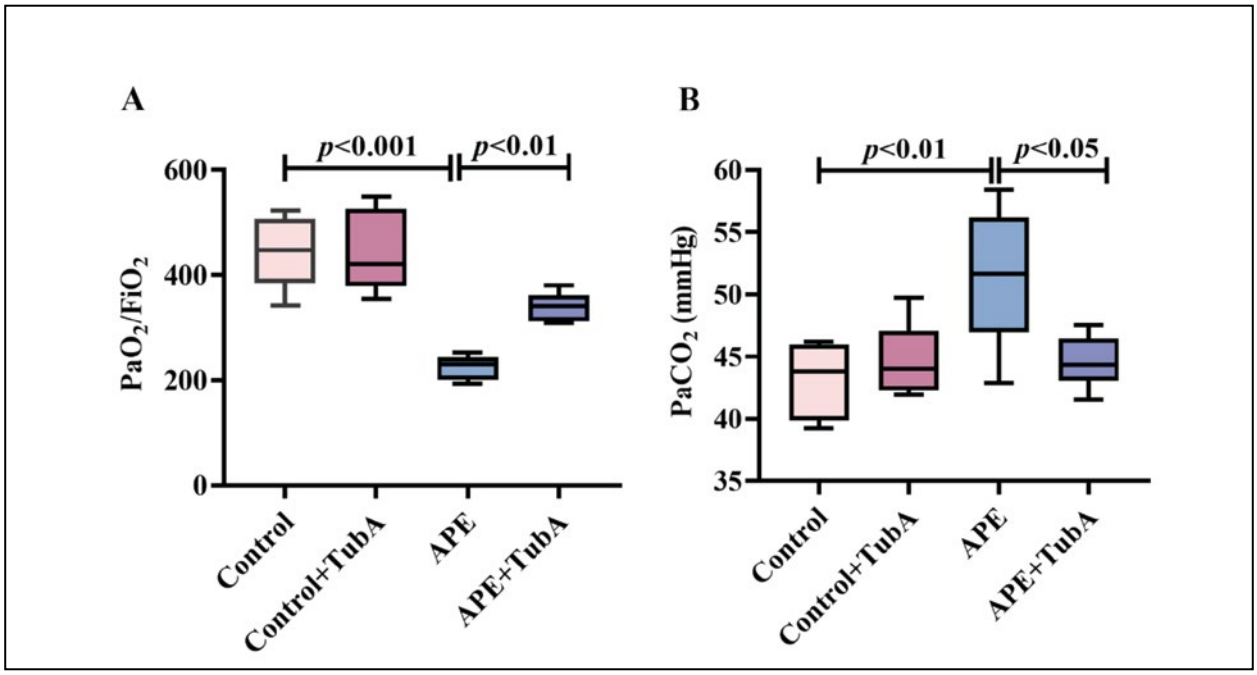

Figure 3. HDAC6 inhibition alleviated APE-induced pulmonary dysfunction. **A.** The PaO_2/FiO_2 ratio in the rat lung. **B.** The level of $PaCO_2$ in the rat lung. Abbreviations: APE — acute pulmonary edema; HDAC6 — histone deacetylase 6.

The number of total cells, eosinophils, neutrophils, macrophages, and lymphocytes was increased in BALF of APE rats. TubA treatment reversed this increase, indicating that HDAC6 inhibition alleviated the infiltration of inflammatory cells (Fig. 4C–G). Lung H&E staining results showed normal and complete structure without obvious inflammatory cell infiltration in the control group (Fig. 4H).

In APE lung tissues, destructive alveolar structure, massive inflammatory cell infiltration, and visible pulmonary congestion were observed. Remarkably, TubA treatment relieved the pathological changes induced by APE. Likewise, the lung injury score in the APE group was higher than that in the control group, but the score was decreased with TubA treatment (Fig. 4I). Thus, HDAC6 inhibition alleviated inflammatory injury and pathological damages in the lung tissues of APE rats.

Effects of HDAC6 inhibition on lung inflammatory response in APE rats

To further illustrate the effect of HDAC6 inhibition on the inflammatory response, the levels of inflammatory cytokines were detected in the rat lung. The contents of TNF-α, IL-6, IL-1β, and IL-18 in the APE group were markedly increased, while those factors in the APE + TubA group were decreased (Fig. 5A–D). As the core of the inflammatory process, the protein expression of NLRP3 was detected in the lungs by immunohistochemistry (Fig. 5E). The results revealed that NLRP3 was intensely expressed in the APE group compared with the control group, which was attenuated with TubA treatment. The NLRP3 inflammasome is executed through cleaved caspase-1 and apoptosis-associated speck-like protein (ASC). The protein levels of NLRP3, cleaved caspase-1, and ASC were upregulated under APE conditions, but HDAC6 inhibition reversed the progression (Fig. 5F–I). These results suggested that HDAC6 inhibition exerted an inhibitory role in pulmonary inflammation in APE rats.

Effects of HDAC6 inhibition on the AKT/ERK signaling pathway in APE rats

Subsequently, we further detected the specific mechanism of HDAC6 inhibition in alleviating APE-induced lung inflammation. The AKT/ERK signaling pathway is a classical pathway that activates inflammation. The protein levels of p-AKT and p-ERK were increased in APE rats, and were blocked by TubA treatment (Fig. 6A–D). No significant difference in the expression of AKT and ERK was observed in all experimental groups. The downregulation of HDAC6 expression suppressed the activation of the AKT/ERK signaling pathway in APE rats.

Discussion

APE is a potentially life-threatening disease that induces pulmonary inflammation and further causes function dysfunction [31]. A high level of HDAC6 has been shown to cause inflammatory injury in lung tissue, thus, HDAC6 downregulation apparently reverses pulmonary

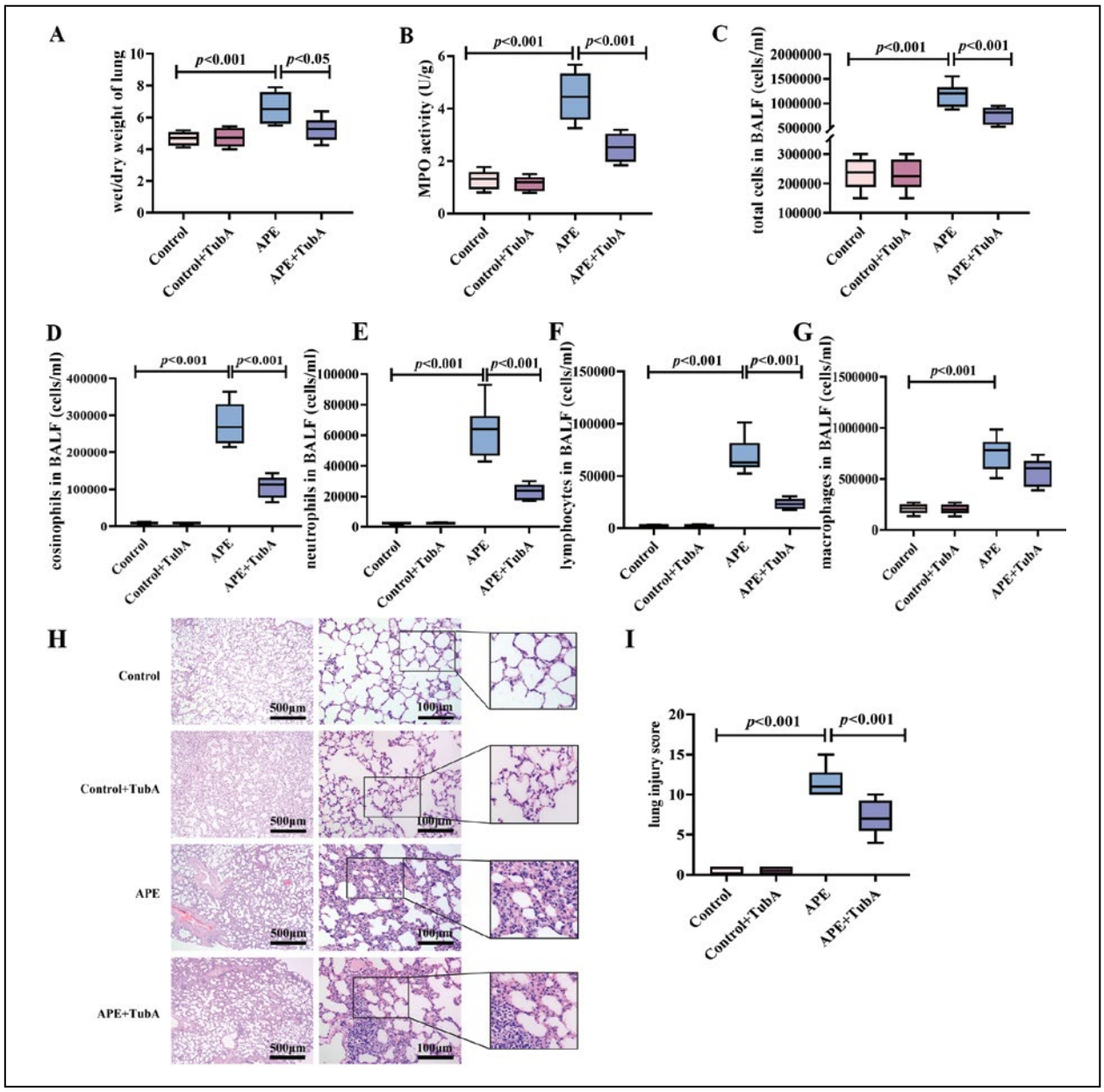

Figure 4. HDAC6 inhibition alleviated APE-induced pulmonary injury. **A.** The ratio of wet/dry weight in the rat lung. **B.** MPO activity in the rat lung. C–G. Counts for total inflammatory cells, eosinophils, neutrophils, lymphocytes, and macrophages in bronchoalveolar lavage fluid **(**BALF). **H.** Representative H&E staining images of rat lung sections. Scale bar = 100 μm or 500 μm. **I.** Lung injury scoring system in rat. Abbreviations: APE — acute pulmonary edema; HDAC6 — histone deacetylase 6.

injury [32]. Whether the injury caused by APE may be affected by HDAC6 inhibition in the lung tissue remains to be solved [33]. In the present study, we demonstrated the protective effect of HDAC6 inhibition on APE-induced lung injury. Data from the rat model suggested that APE rats showed pulmonary dysfunction and pathological damage in lung tissues, whereas HDAC6 inhibition alleviated those symptoms by regulating the AKT/ERK pathway.

HDAC6 is a microtubule-associated deacetylase that regulates post-translational modification and in this way may modulate gene expression. HDAC6 has been reported to be involved in a variety of inflammatory diseases, such as myocarditis and cardiac dysfunction [34], rheumatoid arthritis [35], atopic dermatitis [36], and other diseases. As a special member of HDAC enzymes, HDAC6 also plays a vital role in other physiological functions. Research has found that HDAC6 possessed tumor-suppressing activities by affecting the immune system, providing a promising option for cancer treatment [37, 38]. Pulya *et al*. reported that HDAC6 inhibition had an anti-proliferative effect on multiple

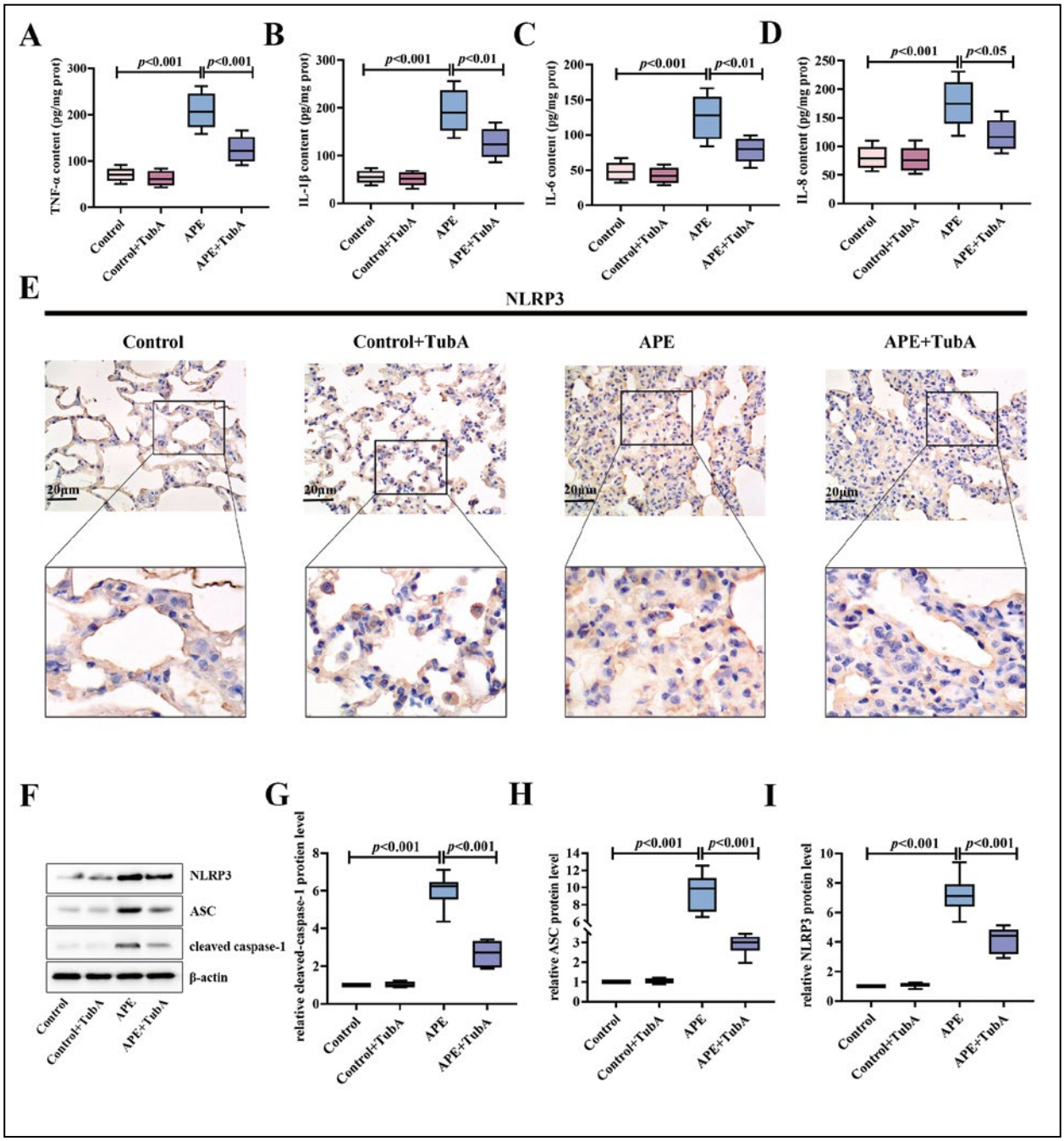

Figure 5. TubA prevented APE-induced inflammatory response in rat lung. **A–D.** The contents of TNF-α, IL-6, IL-1β and IL-18 in the rat lung were measured as described in Material and methods. **E.** Immunoreactivity of NLRP3 in the rat lung. Scale bar = 50 μm. **F–I.** Western blot and quantitative analysis for the expression of NLRP3, apoptosis-associated speck-like protein (ASC) and cleaved caspase-1 in the rat lung. Abbreviations: APE — acute pulmonary edema; TubA — Tubastatin A.

myeloma [39]. Furthermore, HDAC6 has been reported to be involved in the progression of neurological diseases, such as Alzheimer's disease (AD). More importantly, evidence has demonstrated that HDAC6-induced hypoacetylation was harmful to neuronal transport and HDAC6 inhibitors might serve as therapeutic candidates for AD [40]. TubA, a selective inhibitor of HDAC6, can protect the physiological functions of critical organs [41]. TubA relieved cigarette smoke-associated pulmonary dysfunction and infections of the lung [42]. The results in our present study were consistent with the aforementioned studies.

Herein, we established an APE rat model and showed that HDAC6 expression was significantly increased in the lung tissues of APE rats. HDAC6 inhibition relieved the pathological changes and pulmonary function

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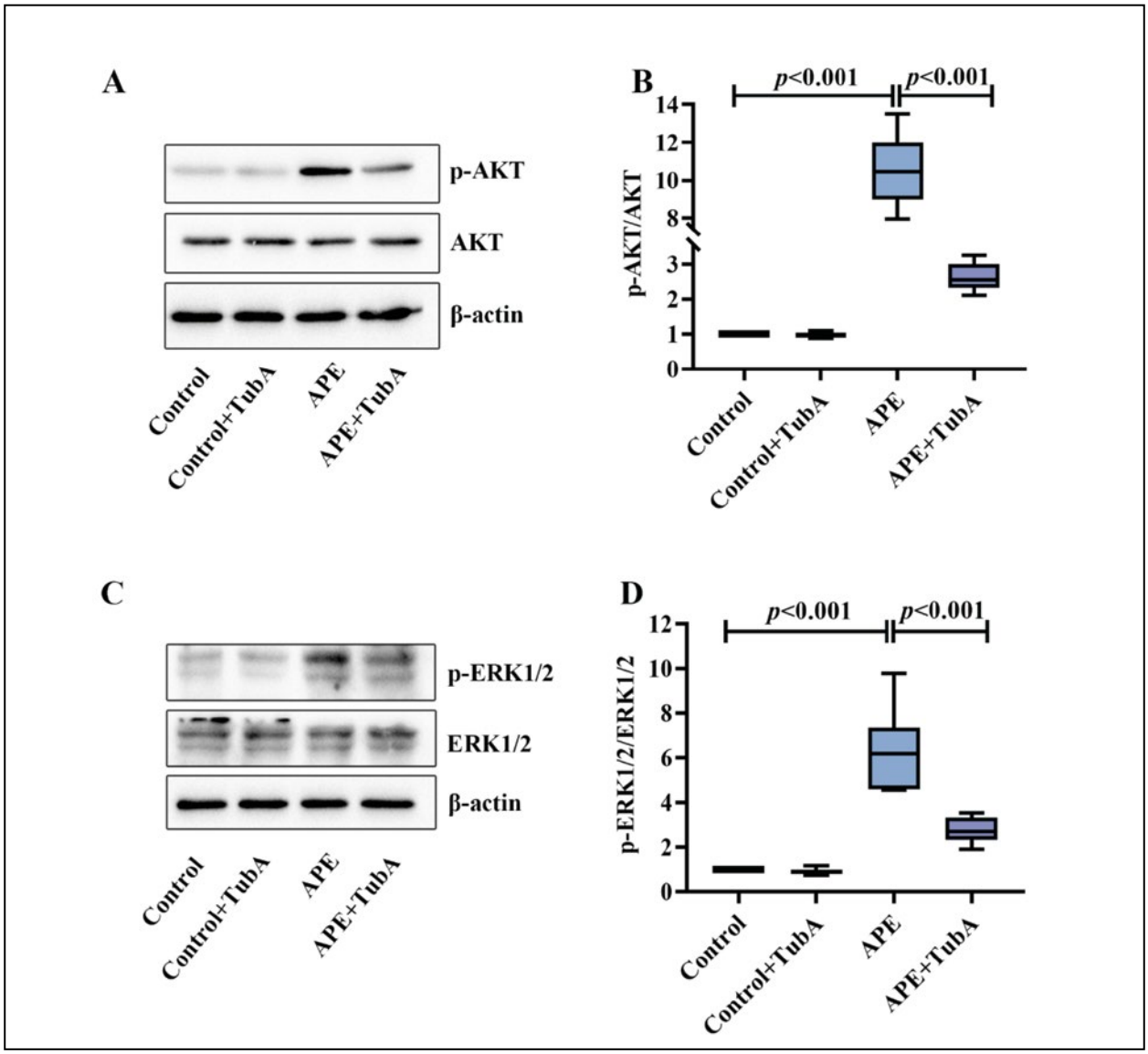

Figure 6. Tubastatin A inhibited AKT/ERK signaling pathway in APE-induced pulmonary injury in rat. **A, B.** Western blot and quantitative analysis for the expression of AKT and p-AKT in the rat lung. **C, D.** Western blot and quantitative analysis for the expression of ERK and p-ERK in the rat lung. Abbreviations: APE — acute pulmonary edema; TubA — Tubastatin A.

in APE rats, proving that HDAC6 inhibition may be an effective method to treat and prevent APE. Therefore, the function of other HDAC6 inhibitors needed further exploration. Previous studies indicated that several other members of the HDAC family, such as HDAC1 and HDAC11, could play a major role in the regulation of inflammatory response [43, 44]. Therefore, exploring the role of other members of the HDAC family in APE is a promising direction for further study [45].

The AKT/ERK signaling pathway is a classic signaling pathway mediating inflammation, which further contributes to tissue injury. In the human primary gallbladder cell line, cadmium exposure increased the activities of phosphorylated AKT and ERK1/2, further elevating the levels of pro-inflammatory cytokines [46]. Wang *et al.* suggested that the AKT and ERK pathways were involved in lung injury in APE rat models [47]. The inhibition of baicalin is an essential strategy for preventing inflammation-induced pulmonary injury through the AKT/ERK pathway [48]. The NLRP3 inflammasome assembles an ASC adaptor and recruits pro-caspase-1 to exert an inflammatory cascade reaction [49]. Following the above-mentioned studies, we found that the expression levels of proinflammatory cytokines were significantly increased in APE and that inflammatory reaction occurred through activating the AKT/ERK signaling pathway. Inhibiting HDAC6 reversed inflammatory injury to maintain the stability of the pul-

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monary environment. The AKT/ERK signaling pathway is a classic signaling pathway of inflammation. To note, the AKT and ERK pathways could be regulated by other cytokines during inflammation. Co-treatment of TNF-α and interferon-gamma enhanced AKT phosphorylation during intestinal inflammation in mice [50]. In addition, the ERK pathway could regulate inflammation by binding to PARP-1 *in vitro* [51]. It is certainly possible that other inflammatory signaling pathways are involved in the development of APE. Therefore, how inflammation regulates APE needs to be further explored.

In conclusion, our study elaborated a novel mechanism of therapeutic options in APE. TubA treatment alleviated pathological injury and lung dysfunction in the lungs. We further showed that pulmonary inflammation was activated by APE through the AKT/ERK signaling pathway, whereas HDAC6 inhibition blocked this activation. Moreover, selective HDAC6 inhibition exerts a protective function against pulmonary injury and provides a novel approach to the treatment of APE.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The rat model of graphical abstract was created by Figdraw (www.figdraw.com).

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