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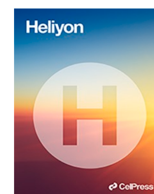

Research article

3-methyladenine ameliorates acute lung injury by inhibiting oxidative damage and apoptosis

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ABSTRACT

Background: Acute lung injury (ALI) is a condition characterized by inflammation and oxidative damage. 3-methyladenine (3-MA) has great potential for regulating apoptosis, but its regulatory role in ALI is unknown.
Methods: Lipopolysaccharide (LPS)-treated mice and *tert*-butyl hydroperoxide (TBHP)-treated bronchial epithelial cells were used to simulate in vivo and in vitro ALI models, respectively. In vivo, lung injury was assessed by histopathological analysis and lung injury scoring. The total cell count, protein content, and inflammatory factors in bronchoalveolar lavage fluid (BALF) were examined. The level of apoptosis in lung tissue was assessed through TUNEL staining. In the vitro ALI model, cell viability and levels of reactive oxygen species and apoptosis were assessed.
Results: 3-MA pretreatment ameliorated lung injury, including intra-alveolar hemorrhage and inflammatory cell accumulation, both in vitro and in vivo. 3-MA pretreatment also decreased inflammatory factor levels in the BALF. 3-MA pretreatment alleviated oxidative damage, decreased reactive oxygen species levels, and attenuated morphological changes. TUNEL and Annexin V-FITC/PI staining revealed that pretreatment with 3-MA reduced the level of apoptosis. 3-MA pretreatment significantly decreased the expression of caspase-3 and Bax but increased the expression of Bcl-2 in ALI. Mechanistically, 3-MA pretreatment also affected the PKCα/NOX4 and Nrf2 pathways, which decreased the level of apoptosis in ALI.
Conclusions: 3-MA pretreatment inhibited inflammation and oxidative damage in ALI and inhibited apoptosis to mitigate ALI in part by inhibiting the PKCα/NOX4 pathway and activating the Nrf2 pathway. Based on these results, 3-MA might be a viable medication to treat with ALI.

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1. Introduction

Acute lung injury (ALI) is a condition characterized by alveolar epithelial and capillary endothelial cell injury that can lead to pulmonary edema and secondary hypoxemia [1]. Patients with uncontrollable lung inflammation may experience a significant mortality rate from acute respiratory distress syndrome [2]. In recent years, several drugs are applied in the clinic for the treatment of ALI, such as β2 agonists and corticosteroids, which have not only insignificant benefits but also significant side effects [3]. Therefore, the development of drugs for its treatment is extremely urgent.

Apoptosis resulting from oxidative damage is a key pathological feature in ALI and is therefore a critical target for treatment [4]. Targeting reactive oxygen species (ROS) attenuates oxidative damage and can reduce inflammation and mitigate apoptosis in ALI [5]. Mesenchymal stem cell extracellular vesicles and exosomes also promote tissue regeneration by inhibiting oxidative stress to mitigate apoptosis in tissues such as the lungs [6]. In addition, some traditional Chinese medicines have been reported to alleviate ALI by inhibiting apoptosis [7,8]. Reducing apoptosis in lung epithelial cells to alleviate acute lung injury is a promising future treatment.

The deleterious effect of overactivated autophagy on the disruption of lung homeostasis in ALI has also been a concern [9,10]. Inhibiting autophagy to alleviate ALI was evidenced by many traditional Chinese medicines [11,12]. 3-methyladenine (3-MA) is commonly utilized as an autophagy inhibitor and can alleviate pulmonary microvascular endothelial cell damage by decreasing apoptosis [13]. In addition, 3-MA was also shown to reduce oxidative damage and apoptosis induced by ultraviolet B radiation in cells [14]. Because 3-MA can reduce apoptosis, the utilization of 3-MA may alleviate apoptosis in lung epithelial cells, thereby alleviating ALI.

Many signaling pathways are involved in apoptosis. PKCα and nuclear factor erythroid 2-related factor 2 (Nrf2) play important roles in ALI [15,16]. Recently, PKCα has been shown to be closely related to apoptosis [17]. Targeting PKCα to inhibit inflammation and apoptosis has been reported in acute hepatitis [18]. In addition, PKCα and NOX4 can prevent obesity-induced kidney injury by regulating apoptosis [19]. Proinflammatory factors lead to apoptosis in lung epithelial and endothelial cells [20], whereas activation of the Nrf2 pathway attenuates cell apoptosis [21]. Nrf2 also has anti-inflammatory and antiapoptotic properties in chronic obstructive pulmonary disease [22]. However, whether 3-MA can alleviate ALI by modulating the levels of apoptosis through the PKCα and Nrf2 pathways has not been fully elucidated.

Therefore, we explored the effects of 3-MA in ALI models. The levels of apoptosis and the effects on the PKCα and Nrf2 pathways were further investigated. The results of our experiments illustrated that inhibiting autophagy to alleviate ALI might be a viable therapy.

2. Materials and methods

2.1. Animals and treatments

Specific Pathogen Free (SPF)-grade male C57BL/6J mice aged 6–8 weeks and weighing 20 g–25 g were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China) and housed at a temperature of 23 ± 1 °C in an environment with readily available laboratory pellet food and water.

2.2. Cell culture

Human bronchial epithelial cells (BEAS-2B) (Cell Bank of the Chinese Academy of Science, China) cultured (DMEM/F-12) (8123482, Gibco, USA) supplemented with fetal bovine serum (FBS) (10 %) (16000044, Gibco, USA) at 37 °C in a 5 % carbon dioxide atmosphere.

2.3. Experimental design

ALI model was created by instilling Lipopolysaccharide (LPS) (10 mg/kg, L2880, Sigma−Aldrich, USA) into the trachea. Based on previous literature, 3-MA (15 mg/kg, HY-19312, MedChemExpress, USA) was administered intraperitoneally 2 h prior to the LPS [23]. Briefly, sixty mice were split into four groups at random according to blind trials: control, 3-MA, LPS, and 3-MA + LPS. After anesthetization with pentobarbital sodium, the LPS group was administered LPS by intratracheal instillation, while the control group and 3-MA pretreatment group received an equivalent dose of sterile phosphate-buffered saline (PBS). The mice were euthanized by intraperitoneal injection of excess pentobarbital sodium after 24 hours.

In ALI, excessive ROS and inflammation are the main pathogenic mechanisms [24]. Among the ROS, H_2O_2 is most directly associated with inflammation, and ROS-responsive materials have been reported to treat ALI [25]. TBHP (*tert*-butyl hydroperoxide solution) (B802372, Macklin, China) is commonly used to cause oxidative damage to cells and has also been used to simulate ALI injury models and inflammatory lung disease in vitro [26,27]. The pathophysiological mechanisms of ALI induced by LPS are complex. In this study, we used TBHP to model ALI oxidative stress environments to assess the efficacy of 3-MA. BEAS-2B cells were pretreated with 3-MA (5 mmol/L) for 2 hours before TBHP treatment (3-MA + TBHP group) and then exposed to TBHP (30 μmol/L) for 24 hours (TBHP group). Cells in the control group received no treatment.

2.4. Hematoxylin-eosin (HE) staining and lung injury score

The lung tissue was cut into 3 μm slices, embedded (paraffin), and fixed (4 % paraformaldehyde). The lung injury score assessment was based on our previous study [28]. In brief, we examined alveolar and interstitial edema, inflammatory cell infiltration, and hemorrhage in each lung. A score of 0–4 was assigned based on severity, where 0 represented no injury, 1 represented less than 25 % injury, 2 represented at least 25 % but less than 50 % injury, 3 represented at least 50 % but less than 75 % injury, and 4 represented diffuse injury (at least 75 % injury).

The lung tissue was obtained after 24 hours, and the left lung lobe was removed to assess the wet weight. Dry weight was obtained by drying the lung tissue at 60 °C for 48 hours. The protein levels in the BALF were determined with the BCA Protein Assay Kit (23225, Thermo Fisher Scientific, USA).

2.5. Immunohistochemical staining

Bovine serum albumin (3 %) was used to seal the paraffin-embedded sections, and primary antibodies against phospho-PKCα (P-PKCα) (1:5000, ab180848, Abcam, UK) and NOX4 (1:100, ab133303, Abcam, UK) were incubated overnight at 4 °C. Next, secondary antibody was incubated at room temperature for an hour. After the sections were dehydrated and restained with hematoxylin, photos were taken (Leica, Germany).

2.6. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay

The paraffin sections were subjected to a series of washes with xylene and anhydrous ethanol solutions, followed by washing with distilled water. A TUNEL test kit (11684817910, Roche, CHE) was used to stain the paraffin sections and subsequently stained with DAPI before being sealed. Images were then obtained (Leica, Germany).

2.7. Bronchoalveolar lavage fluid (BALF) collection and analysis

The mouse lungs were slowly irrigated three times with 1 ml of PBS via the tracheal tube to obtain approximately 0.9 ml of fluid (a fluid recovery rate of approximately 90 %). Next, the obtained samples were immediately centrifuged, after which the upper supernatant and the lower precipitate were collected. The levels of IL-6, TNF-α, and IL-1β (Shanghai Boyun, China) were then measured.

The erythrocytes in the lower sediment were lysed with erythrocyte lysis buffer, washed once with cold PBS, and then centrifuged. Cell count plates were used to count all the cells in the BALF after resuspension in PBS.

2.8. Cell cytotoxicity and viability assessment

The toxicity of different concentrations of TBHP (dissolved in PBS) and 3-MA (diluted in PBS) on cell viability was determined using the Cell Counting Kit-8 (CCK-8) (C0037, Beyotime, China). Cells were plated at a density of 8×10^4 per well. Various concentrations of TBHP and 3-MA were treated for 24 hours and the test solution was then added. Finally, the absorbance at 450 nm was determined.

2.9. Cell apoptosis assessment

Apoptosis Assay Kit (C1062L, Beyotime, China) was used to assess the apoptosis. Necrotic and late apoptotic cells bind both annexin V-FITC and PI, early apoptotic cells only bind annexin V-FITC (green fluorescence) and do not bind PI (red fluorescence). We assessed the results through a high content screening (HCS) system (PerkinElmer, USA).

2.10. ROS assessment

Cells were seeded at a density of 8×10^4 cells per well and exposed to TBHP (30 μmol/L) for 24 hours 3-MA (5 mmol/L) was added for 2 hours before TBHP treatment. The ROS Detection Kit (MAK145, Sigma−Aldrich, USA) was used for detection. The images were visualized with the HCS system.

2.11. Western blot analysis

The proteins were separated using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) after a total of 20 μg of protein was added to the wells of a 12 % polyacrylamide gel. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes and blocked with 5 % skim milk, after which they were incubated with primary antibodies against various proteins, including PKCα (1:1000, ab32376, Abcam, UK), phospho-PKCα (P-PKCα) (1:1000, ab180848, Abcam, UK), NOX4 (1:1000, ab133303, Abcam, UK), Nrf2 (1:1000, A1244, ABclonal, China), NQO1 (1:1000, A22290, ABclonal, China), HO-1 (1:1000, A1346, ABclonal, China), Bcl-2 (1:1000, A0208, ABclonal, China), Bax (1:1000, A0207, ABclonal, China), cleaved caspase-3 (1:1000,9661, CST, USA), Beclin-1 (1:1000, 3595T, CST, USA), LC3B (1:1000, 2775s, CST, USA), and GAPDH (1:1000, AF0006, Beyotime, China). The membrane was washed three times with PBST for 10 min and then exposed to the secondary antibody for an hour. The proteins on the membrane were then detected using an imaging system. Finally, the results were carried out by Image J software.

2.12. Statistical analysis

The data were collected from three different experiments. Our results were analyzed using GraphPad Prism 9.4.0 and presented as the mean ± standard deviation (SD). Brown-Forsythe test detected the normality of the data. One-way ANOVA followed by post hoc Bonferroni correction for multiple comparisons. A *p*-value <0.05 was considered significant.

3. Results

3.1. 3-MA pretreatment alleviates oxidative damage in a TBHP-induced ALI model

First, we investigated whether 3-MA could protect BEAS-2B cells from TBHP-induced oxidative damage. Cell viability decreased when the TBHP concentration increased, based on the CCK-8 assay results (Fig. 1A). 3-MA also affected cell viability (Fig. 1B).

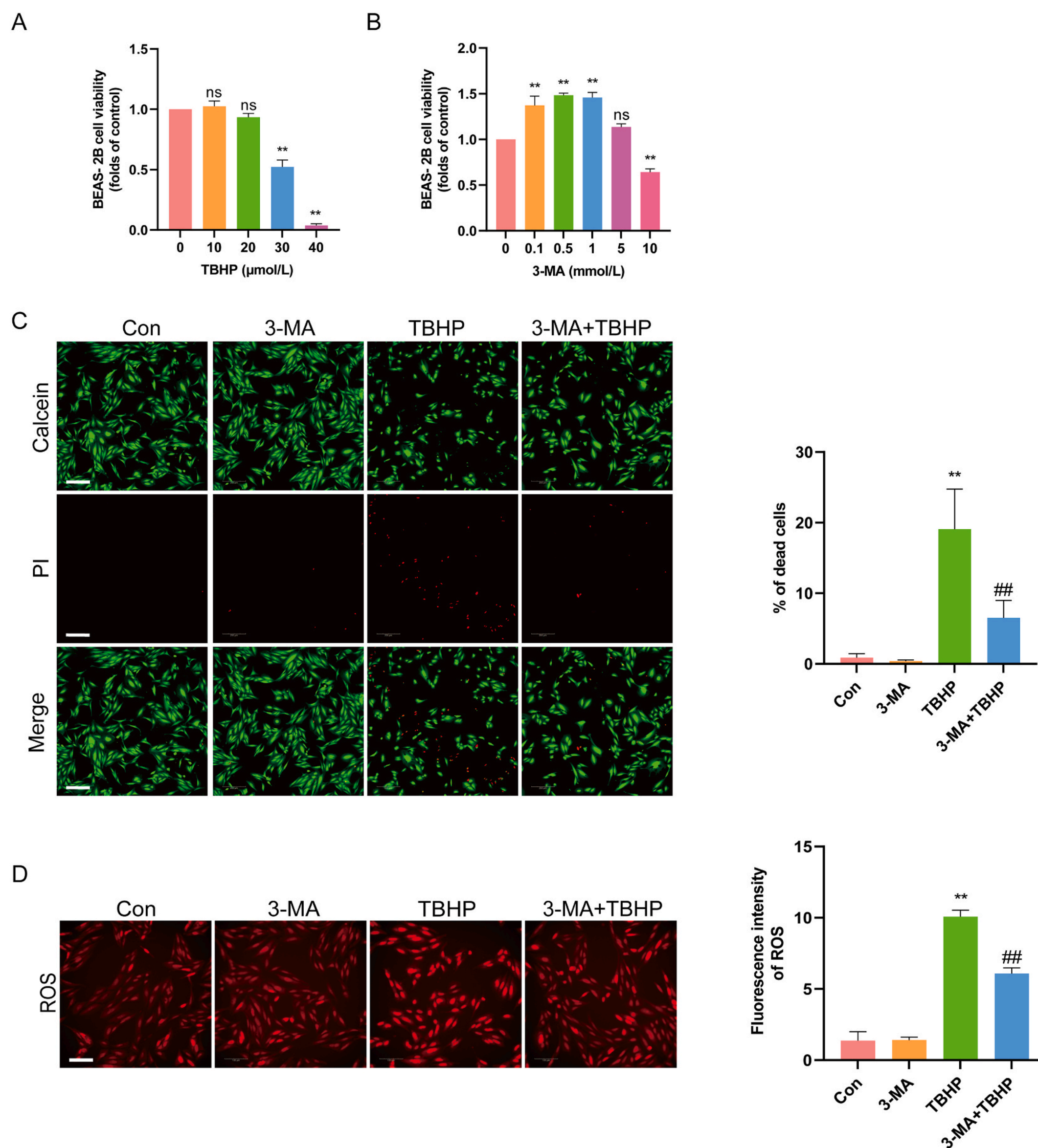

Fig. 1. 3-MA pretreatment alleviates oxidative damage in a TBHP-induced ALI model. (A–B) The viability of BEAS-2B cells treated with different concentrations of TBHP and 3-MA for 24 h. (C) BEAS-2B cells were stained with Calcein/PI and analyzed. Living cells appeared as green, and dead cells appeared as red (scale bar = 200 μm). (D) Typical images of ROS levels in BEAS-2B cells and quantitative assessment of the ROS fluorescence intensity (scale bar: 100 μm). The results are presented as the mean ± SD (n = 3); ns, not significant, * vs. control group, # vs. LPS group, *p < 0.05, **p < 0.01, #p < 0.05, ##p < 0.01.

Therefore, we used 30 μmol/L TBHP and 5 mM 3-MA for follow-up experiments. Calcein-AM/PI (live/dead) staining demonstrated that 3-MA pretreatment significantly reduced the percentage of cell death caused by TBHP (Fig. 1C). In addition, 3-MA pretreatment greatly reduced TBHP-induced ROS generation in BEAS-2B cells (Fig. 1D).

3.2. 3-MA pretreatment alleviates MPO activity and inflammation in a mouse model of ALI

As demonstrated in Fig. 2A, MPO activity was greater in the LPS group than in the control group but significantly lower in the 3-MA pretreatment group. In addition, LPS led to significant pathological damage to lung tissue, including intra-alveolar hemorrhage, inflammatory cell accumulation, and thickening of the alveolar wall (Fig. 2B), but these changes were significantly reversed by 3-MA pretreatment. As displayed in Fig. 2C–D, LPS treatment significantly not only increased the lung injury score but also increased the wet/dry weight ratio, which was decreased by 3-MA pretreatment. The total protein content, the number of cells, and inflammation factors in the BALF were significantly lower in the 3-MA pretreatment group than in the LPS group (Fig. 2E–I).

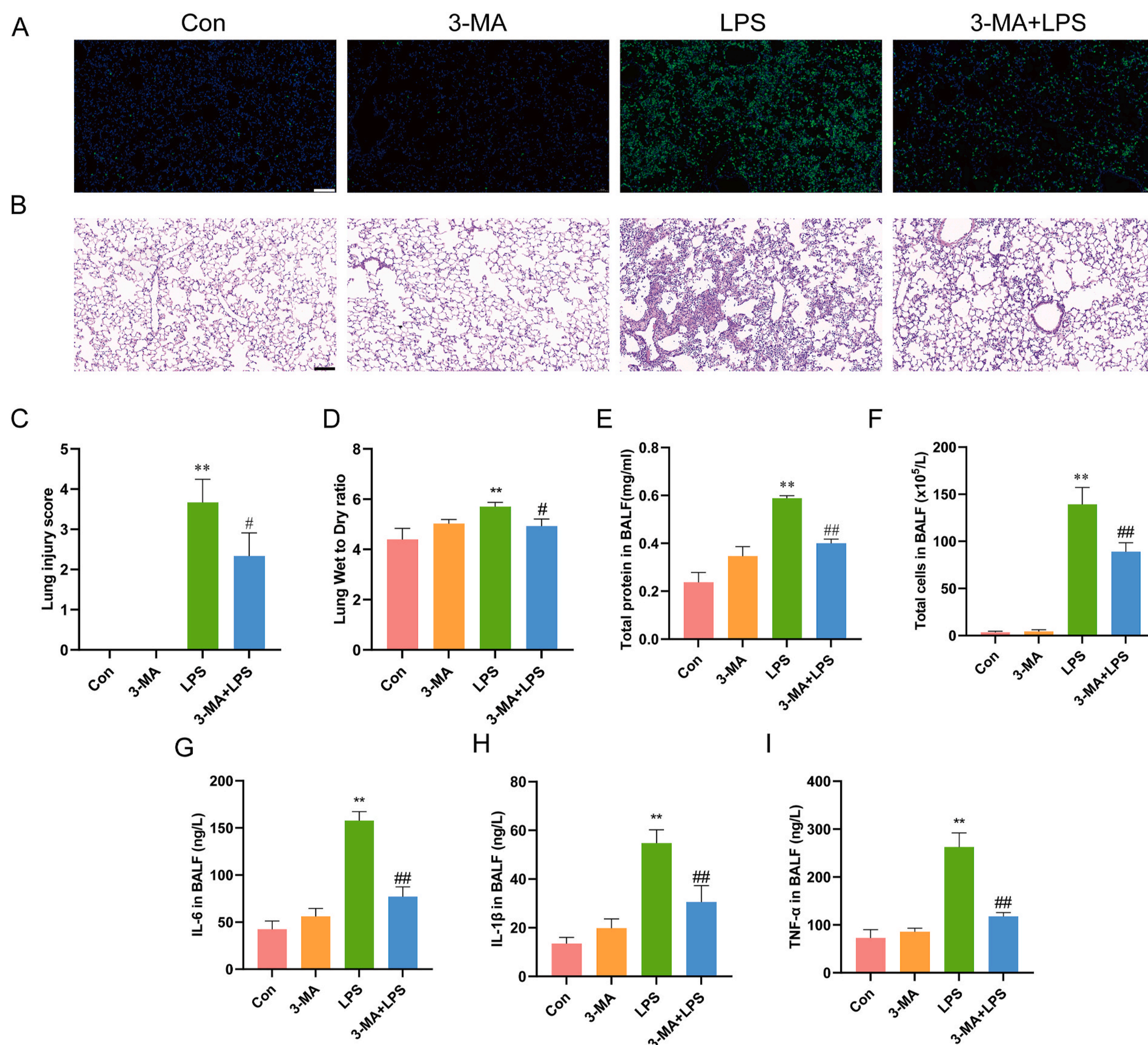

Fig. 2. 3-MA pretreatment alleviates MPO activity and inflammation in a mouse model of ALI. (A–B) Representative MPO activity images and HE-stained images of lung tissue (scale bar = 100 μm). (C) Pathological scoring of lung injury in lung tissue. (D) The wet/dry ratio of the lung tissue. (E–I) Measurement of total protein, total cell count, and the levels of IL-6, IL-1β, and TNF-α in BALF. The results are presented as the mean ± SD (n = 3); * vs. the control group, # vs. the LPS group, *$p < 0.05$, **$p < 0.01$, #$p < 0.05$, ##$p < 0.01$.

3.3. 3-MA pretreatment inhibits apoptosis in an in vitro ALI model

The level of apoptosis was also assessed. Cells bound annexin V-FITC appeared as green, while cells bound both annexin V-FITC and PI appeared as red. 3-MA pretreatment reduced the apoptosis induced by TBHP (Fig. 3A). The western bolt shows that cleaved caspase-3 and Bax were reduced by 3-MA pretreatment, while the Bcl-2 was increased (Fig. 3B). TBHP treatment of BEAS-2B cells enhanced the expression of Beclin-1 and LC3 II/I, whereas 3-MA pretreatment substantially blocked this phenomenon (Fig. 3C).

3.4. 3-MA pretreatment inhibits apoptosis in a mouse ALI model

In addition, there was a greater number of TUNEL-positive cells (red) in the LPS-treated lung tissue than in the control tissue, but 3-MA pretreatment effectively inhibited the apoptosis (Fig. 4A). The Western blot revealed that 3-MA pretreatment inhibited the

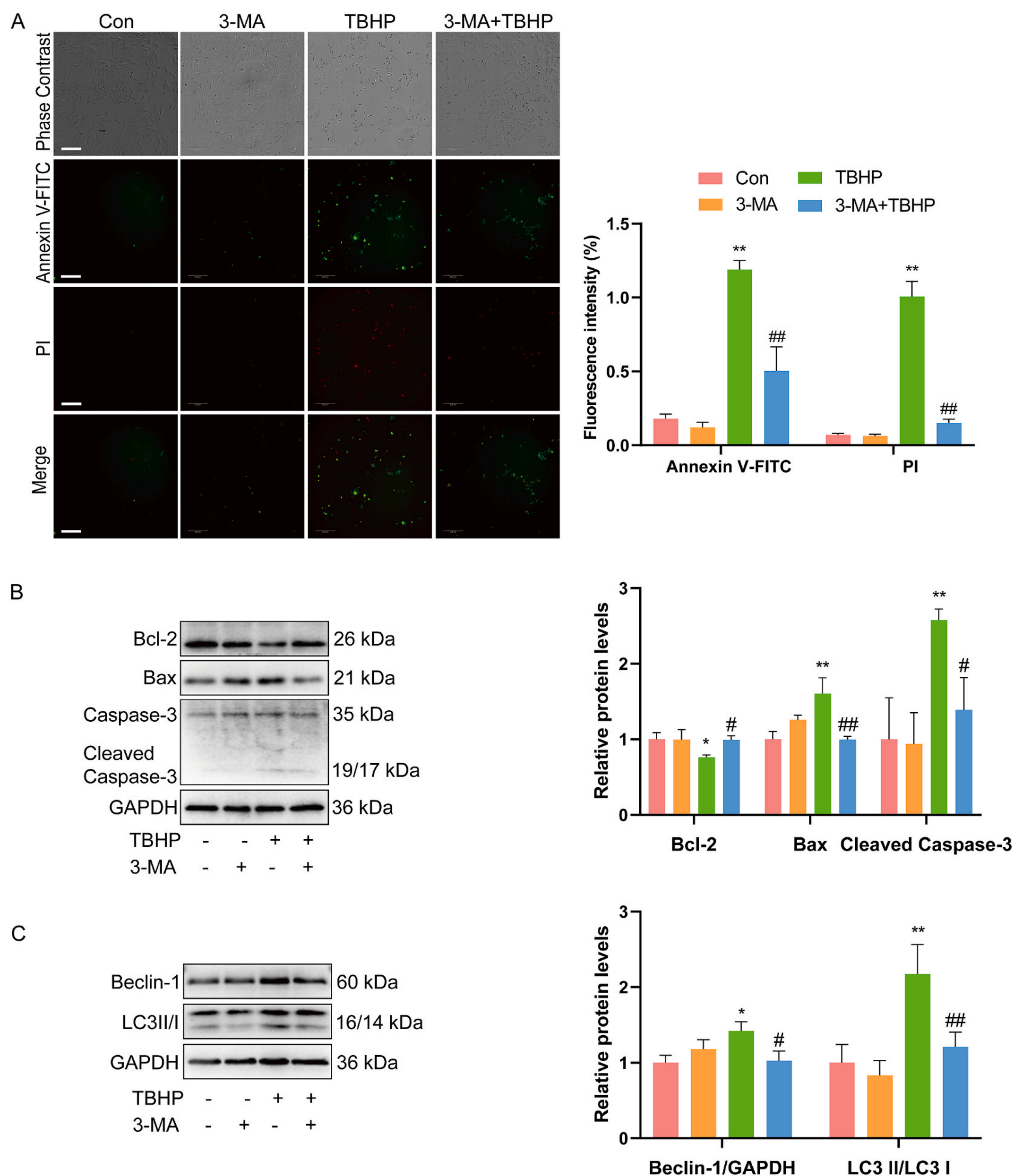

Fig. 3. 3-MA pretreatment inhibits cell apoptosis in an in vitro ALI model. (A) Representative images of annexin V-FITC/PI staining and quantitative analysis of BEAS-2B cells (scale bar = 20 μm). (B) Representative Western blot images and quantitative analysis of the expression of Bcl-2, Bax, and cleaved caspase-3. (C) Representative Western blot images for quantitative analysis of Beclin-1 and LC3 II/I expression. The results are expressed as the mean ± SD; * vs. the control group, # vs. the LPS group, *$p < 0.05$, **$p < 0.01$, #$p < 0.05$, ##$p < 0.01$.

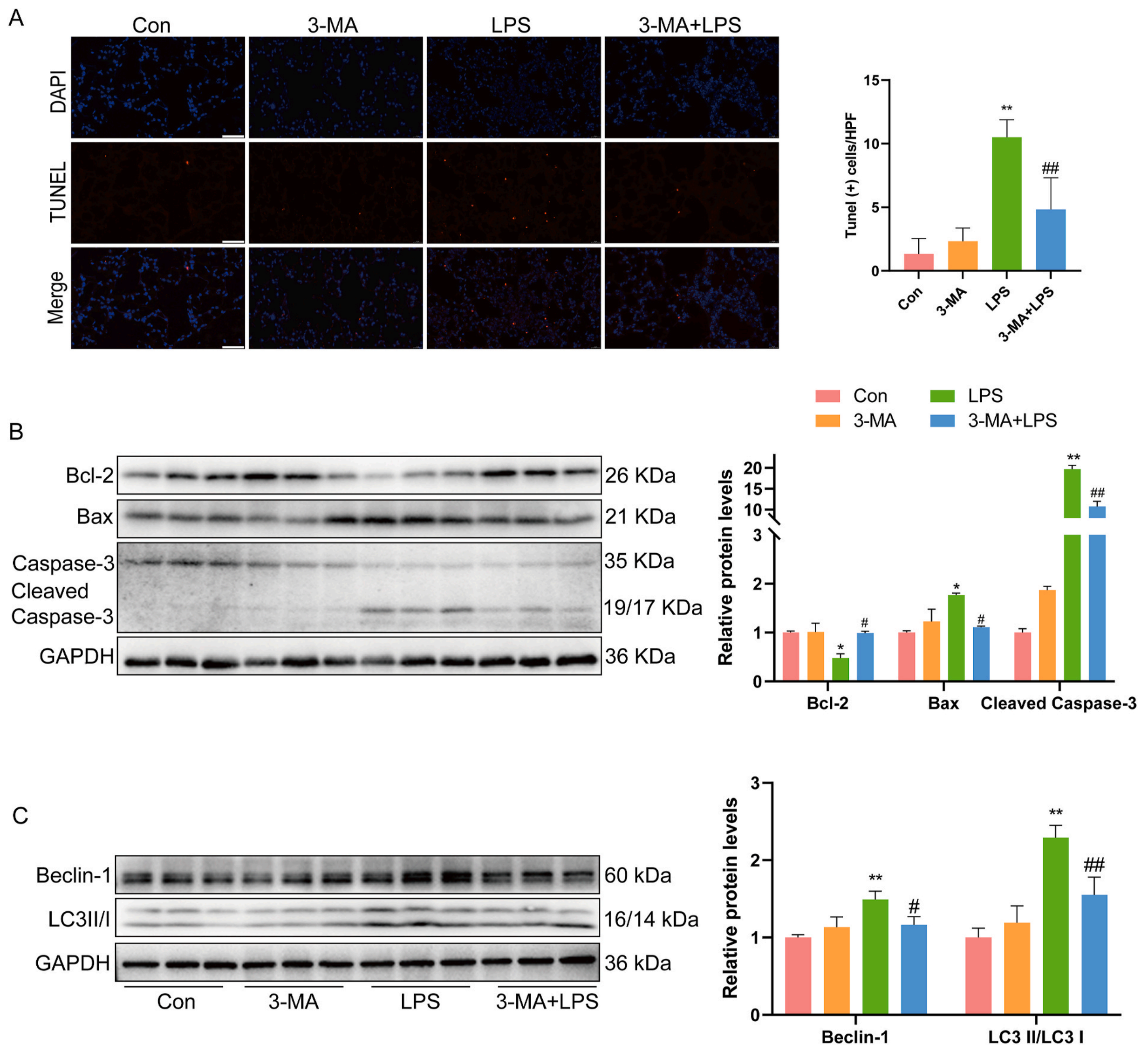

Fig. 4. 3-MA pretreatment inhibits apoptosis in a mouse ALI model. (A) Representative immunofluorescence images and quantitative analysis of TUNEL staining (red) in lung tissue (scale bar = 50 μm). (B) Representative Western blot images and quantitative analysis of the expression of Bcl-2, Bax, and cleaved caspase-3. (C) Representative Western blot images for quantitative analysis of Beclin-1 and LC3 II/I expression. The data are expressed as the mean ± SD (n = 3); * vs. the control group, # vs. the LPS group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$.

expression of cleaved caspase-3 and Bax caused by LPS-treated (Fig. 4B) as well as increasing the expression of Bcl-2. Beclin-1 and LC3 II/I were also expressed higher in the LPS-treated group, while 3-MA pretreatment successfully suppressed these effects (Fig. 4C).

3.5. 3-MA pretreatment affects apoptosis via the PKCα/NOX4 pathway

In addition, we further investigated the pathways that might be involved in 3-MA pretreatment. P-PKCα and NOX4 expression in BEAS-2B cells were enhanced in the TBHP-treated group but suppressed by 3-MA pretreatment (Fig. 5A). Next, Gö 6983, an inhibitor of PKC, was used to validate whether the effects of 3-MA pretreatment were associated with the PKCα/NOX4 signaling pathway. Gö 6983 clearly inhibited the expression of PKCα/NOX4 (Fig. 5B). Apoptosis level in the 3-MA + TBHP + Gö 6983 group also decreased, with a shift in JC-1 from red to green fluorescence as an indicator of early apoptosis (Fig. 5C).

3.6. 3-MA pretreatment affects apoptosis via the Nrf2 pathway

The ML385 (Nrf2 inhibitor) was used to validate whether the effects of 3-MA pretreatment were associated with the Nrf2 pathway. 3-MA pretreatment increased the expression levels of Nrf2, HO-1, and NQO1 compared with those in the TBHP-treated group (Fig. 6A).

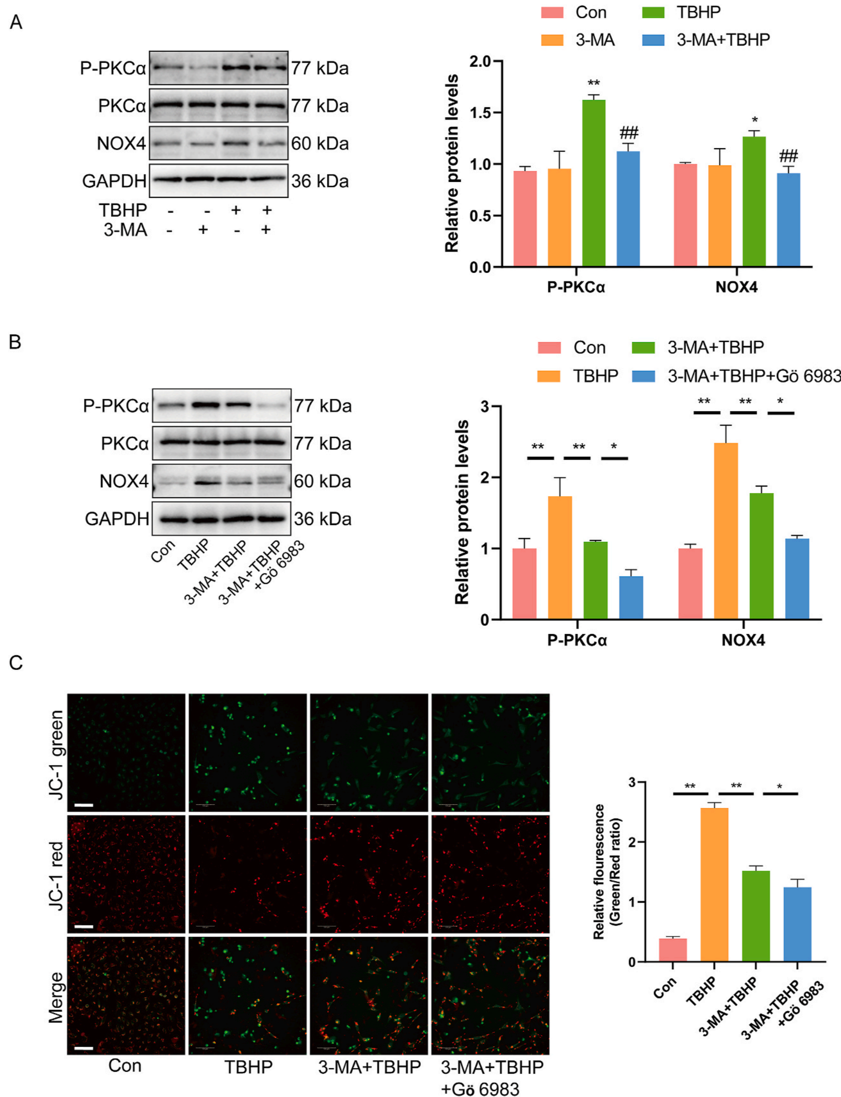

Fig. 5. 3-MA pretreatment affects apoptosis via the PKCα/NOX4 pathway. (A–B) Representative Western blot images and analysis of P-PKCα and NOX4 in BEAS-2B cells. (C) JC-1 staining images and assessment of the percentage of apoptotic cells (red/green ratio) (scale bar = 100 μm). The results are expressed as the means ± SD (n = 3); *p < 0.05, **p < 0.01, #p < 0.05, ##p < 0.01.

The ML385 successfully inhibited the expression of Nrf2 pathway (Fig. 6B). Additionally, apoptosis also increased after ML385 treatment (Fig. 6C). Our results revealed that 3-MA pretreatment alleviated ALI and might affect the PKCα/NOX4 and Nrf2 signaling pathways.

Furthermore, 3-MA pretreatment inhibited the expression of PKCα and activated the Nrf2 pathways in the LPS-induced ALI mouse model (Figs. S1A–C in the Supplementary Material).

4. Discussion

4.1. Main results

In our study, oxidative damage and apoptosis led to cell and lung tissue damage, which ultimately resulted in ALI. 3-MA pretreatment played a protective role in ALI. Pretreatment with 3-MA effectively inhibited LPS-induced pathological changes in the lungs, reduced MPO levels, and decreased inflammation. In addition, the results also revealed that 3-MA pretreatment inhibited apoptosis via the PKCα/NOX4 and Nrf2 signaling pathways. In summary, we discovered that 3-MA might be a promising medication for treating ALI, but its pharmacological activity and underlying mechanisms still need to be further investigated.

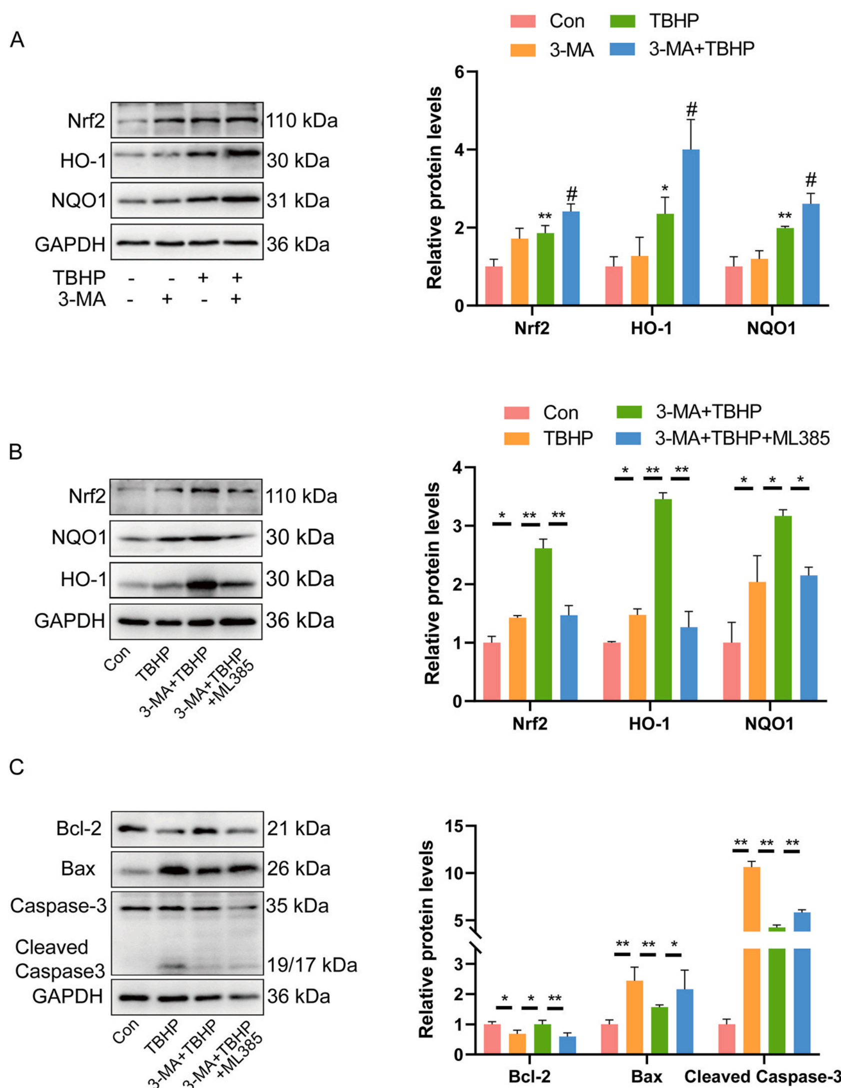

Fig. 6. 3-MA pretreatment affects apoptosis via the Nrf2 pathway. (A–B) Representative Western blot images and analysis of Nrf2, HO-1, and NQO1 in lung tissue. (C) Representative Western blot images and analysis of Bcl-2, Bax, and caspase-3. The results are expressed as the means ± SD (n = 3); *p < 0.05, **p < 0.01, #p < 0.05, ##p < 0.01.

4.2. Comparison and interpretations

Excessive inflammation caused by cytokine storms is the main contributor to the worsening of ALI [29]. MPO is predominantly expressed in neutrophils [30] and can alleviate ALI by reducing the activities and levels of its associated factors [31]. Limiting neutrophil activation is beneficial in the treatment of ALI [31,32]. A previous study revealed that the inhibition of ferroptosis alleviated LPS-induced ALI mainly through a decrease in inflammatory factors [33]. Therefore, inhibiting inflammation is the key to treating ALI. According to our research, 3-MA pretreatment reduced inflammatory factor and MPO levels and might be used to treat ALI. Our results revealed that inhibition of autophagy might contribute to reducing inflammatory responses. However, in contrast to our results, overexpression of HO-1 has been reported to enhance autophagy, thereby alleviating the level of inflammation [34]. In our study, autophagy was elevated in the ALI models and accompanied by changes in pathology and inflammation, which were reversed by 3-MA. It was reasonable to consider that autophagy played a disruptive role in ALI in the study. However, it is undeniable that the presence of some antioxidant molecules does influence autophagy, but the exact pathways are not clear. However, it should be noted that excessive inhibition of autophagy may produce other unfavorable results in future applications.

We also investigated two signaling pathways related to apoptosis to determine the possible mechanisms by which 3-MA pretreatment alleviated ALI. Our results revealed that 3-MA effectively inhibited the PKCα/NOX4 pathway and further activated the Nrf2 pathway. The inflammatory factor TNF-α increases PKCα activity [35]. Inhibition of PKCα/NOX4 can inhibit ferroptosis [36], which is associated with the inflammatory response, oxidative stress, and mitochondrial dysfunction [37]. PKCα can modulate leukotriene B4

production and promote neutrophil reverse transendothelial migration into circulation to exacerbate ALI [38]. LPS-induced inflammatory cells accumulate and produce excessive ROS, leading to oxidative stress. The Nrf2 signaling pathway can attenuate oxidative stress to alleviate ALI [39,40]. Increasing Nrf2 expression can alleviate oxidative damage and ferroptosis in ALI [41], enhance the antimicrobial capacity of macrophages, and improve patient prognosis [42]. Activating Nrf2 signaling also reduced the inflammatory factors [43]. PKCα can activate Nrf2 to induce HO-1 expression, which further inhibits the inflammation [44]. Inhibition of PKCα-related inflammasome activation and induction of Nrf2 expression can suppress inflammatory responses [45]. Consistent with previous studies, activation of PKCα was indeed accompanied by the expression of Nrf2 and its downstream HO-1 and NQO1, but how activation of PKCα induced the expression of Nrf2 was unclear. However, it has been shown that Nrf2 mediates the function of autophagic vesicles in the lung recently [46]. In our study, the enhancement of autophagy was accompanied by PKCα activation and Nrf2 alteration. It is reasonable to speculate that altered autophagy accompanied by PKCα activation stimulates Nrf2 regulation, but there is no evidence for this yet. Nrf2 activation in the LPS group showed that antioxidant capacity was increased in ALI, and Nrf2 was activated even more markedly in the 3-MA-pretreated group, which indicated that the antioxidant capacity might be further enhanced. The same effect was observed in TBHP-treated BEAS-2B cells.

Additionally, airway epithelial cell injury is the key hallmark of ALI [47]. Alveolar epithelial cell injury ultimately leads to alveolar type II cell apoptosis [48]. The LPS-induced apoptosis may be decreased by activation of the Nrf2 signaling pathway [49]. Increasing the proportion of Nrf2-positive cells can decrease the level of oxidative stress [50]. A previous study reported that 3-MA attenuated ROS production and blocked PKCα/NOX4, which alleviated dysfunction in human umbilical vein endothelial cells [51]. Our results revealed that 3-MA pretreatment inhibited apoptosis in both lung tissue and TBHP-treated BEAS-2B cells to alleviate ALI. The ability of 3-MA to target pulmonary epithelial dysfunction to alleviate ALI has been previously reported, but the specific mechanism was not described [52]. Based on the results, 3-MA might attenuate barrier dysfunction by alleviating apoptosis. However, there was literature reporting that autophagy activators alleviate ALI [53]. It was worth noting that we use different doses of LPS. However, it is not clear that the use of LPS dose caused different degrees of autophagy. In summary, activation of Nrf2 and inhibition of PKCα attenuated apoptosis to alleviate ALI was demonstrated in our results, providing a well-understood point. Similar to recent findings, inhibition of NOX4 accompanied the increase in Nrf2, which inhibited apoptosis [54]. These findings revealed that 3-MA might have the potential to protect alveolar and airway epithelial cells from injury during ALI. Apoptosis is likely regulated through the PKCα/NOX4 and Nrf2 pathways. However, our study did not investigate the PKCα/NOX4 or Nrf2 pathway in mice, and we believed that validation in knockout mice was necessary in the future. Future studies in network pharmacology and molecular docking might lead to a more definitive understanding of the role of 3-MA.

4.3. Strengths and limitations

However, the limitations of the study need further exploration. First, we were concerned about the effect of 3-MA on ALI, this study only used one concentration of 3-MA to treat mice. Future studies should explore whether different concentrations of 3-MA and time effect on ALI, facilitating detailed illustration of the dynamic changes in the effects of 3-MA treatment on ALI. Second, there are many cell deaths, such as ferroptosis and pyroptosis, which are closely related to ALI. The study insufficiently explored these pathways. Whether 3-MA could regulate these pathways to regulate the ALI was worth exploring. Since 3-MA was also recently reported to be able to affect cell pyroptosis [55]. Third, pathophysiological changes during ALI were complex, some of the results of TBHP-induced cell experiments might not fully mimic the ALI condition. Potential mechanisms need to be further explored. However, strong oxidative stress is a very important feature in the ALI, while TBHP can reproduce such an environment. TBHP is also widely used in the development of antioxidant drugs for the treatment of ALI. Additionally, cellular experiments were not an adequate substitute for animal experiments. The mechanisms should be further studied in depth in knockout mice. The 3-MA might pose a nonspecific effect in autophagy, and knocking out autophagy genes could provide stronger evidence. 3-MA might have different effects on different cell types, such as primary human alveolar epithelial cells. Identification of the specific cell types and the main pathways involved in 3-MA treatment is necessary. Last but not least, different statistical methods to control errors in the statistical process may produce different bias, and more data are needed to support the results in the future.

5. Conclusions

These findings demonstrated that the oxidative damage and apoptosis during ALI were detrimental. 3-MA pretreatment attenuated excessive inflammation and oxidative damage. Mechanistically, 3-MA pretreatment reduced apoptosis in part by inhibiting PKCα/NOX4 and activating the Nrf2 signaling pathway in ALI. These findings provide new strategies for treating ALI. Precise regulation of the degree of autophagy might be the future research trend.

Data availability statement

Data will be made available on request.

Ethics statement

All our treatments were gentle and ensured minimal suffering and protocols on animals had passed the review of Institutional Animal Care and Use Committee (IACUC) of The First Affiliated Hospital of Wenzhou Medical University (WYYY-IACUC-AEC-2023-

026).

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CRediT authorship contribution statement

Xiong Lei: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Xiling Liu:** Writing – original draft, Methodology, Formal analysis. **Jia Yu:** Methodology, Formal analysis. **Kai Li:** Methodology, Formal analysis. **Lijing Xia:** Methodology, Formal analysis. **Shanshan Su:** Methodology, Formal analysis. **Pengcheng Lin:** Methodology, Formal analysis. **Dan Zhang:** Writing – review & editing, Conceptualization. **Yuping Li:** Writing – review & editing, Conceptualization.

Declaration of competing 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.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33996.

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