



## Original

# Sirtuin3 confers protection against acute pulmonary embolism through anti-inflammation, and anti-oxidative stress, and anti-apoptosis properties: participation of the AMP-activated protein kinase/mammalian target of rapamycin pathway

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**Abstract:** An increasing number of studies have suggested that oxidative stress and inflammation play momentous roles in acute pulmonary embolism (APE). Honokiol, a bioactive biphenolic phytochemical substance, is known for its strong anti-oxidative and anti-inflammatory effects, and it served as an activator of sirtuin3 (SIRT3) in the present study. The purposes of the study were to explore the effects of honokiol on APE rats and investigate whether the function of honokiol is mediated by SIRT3 activation. In the study, the rats received a right femoral vein injection of dextran gel G-50 particles (12 mg/kg) to establish the APE model and were subsequently administered honokiol and/or a selective SIRT3 inhibitor 3-(1H-1,2,3-triazol-4-yl)pyridine (3-TYP; 5 mg/kg) intraperitoneally. The results showed that SIRT3 activation by honokiol attenuated the loss in lung function, ameliorated the inflammatory response and oxidative damage, and inhibited apoptosis in lung tissues of the rats with APE but that this was reversed by 3-TYP. In addition, we found that the AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway might be activated by honokiol but restrained by 3-TYP. These results indicated that honokiol was capable of suppressing the adverse effects of APE and that this was diminished by SIRT3 suppression, implying that activation of SIRT3 might serve as a therapeutic method for APE.

**Key words:** acute pulmonary embolism (APE), apoptosis, inflammation, oxidative stress, sirtuin3 (SIRT3)

## Introduction

Acute pulmonary embolism (APE) is the third most common acute cardiovascular disease [1]. In the clinical setting, deep vein thrombosis (DVT) is the most common risk factor for APE [2]. APE normally causes chest pain, breathlessness, and cough [3, 4]. Inflammation and oxidative stress play significant roles in APE progression. On the one hand, APE is capable of inciting inflammation

induced by multiple stimuli [5, 6], including pulmonary artery hypertension [7–9], hypoxia [10], and ischemia [11]. On the other hand, APE causes perturbations in the redox balance, thereby inducing oxidative stress [12–14]. Here, we attempted to explore a molecule associated with inflammation and oxidative stress, so as to further study the mechanism related to APE, which has an important guiding significance for treatment of this disease.

Mammalian sirtuins (SIRTs), NAD<sup>+</sup>-dependent pro-

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tein deacetylases, comprise seven isoforms (SIRT1 to SIRT7) based on distinct differences in subcellular localization and substrate specificity [15–18]. Numerous studies have shown that SIRT3 possess protective effects against aging, apoptosis, inflammation, and oxidative stress [19, 20]. SIRT3, especially SIRT3, are linked to several pathologies, including cancer, heart disease, fibrosis, acute kidney injury, and neurodegeneration diseases [21–25].

SIRT3 mostly controls mitochondrial acetylation [26, 27], which plays important roles in regulating systemic inflammation, cellular energy metabolism, oxidative stress, and apoptosis [28–30]. Previous studies have indicated that mitochondrial SIRT3 is highly expressed in a variety of metabolic tissues, such as the heart, kidney, and liver [26, 31–33]. SIRT3 is associated with cardiovascular disease and aging [34–36]. Global SIRT3 depletion (*Sirt3*<sup>-/-</sup>) induces oxidative stress, increases vascular inflammation, and accelerates vascular senescence and age-dependent hypertension in mice. Nevertheless, genetic SIRT3 overexpression prevents these deleterious effects [37]. Similarly, SIRT3 serves as a novel therapeutic molecule in endotoxin-induced acute lung injury (ALI) by repressing macrophage metabolic reprogramming and its pro-inflammatory phenotype [38]. Besides, a study has pointed out that melatonin treatment protects the heart from myocardial ischemia/reperfusion injury by reducing oxidative stress and apoptosis via activation of the SIRT3 signaling pathway [39]. SIRT3 deficiency also promotes pulmonary fibrosis by augmenting mitochondrial DNA damage and apoptosis in alveolar epithelial cells (AEC) [40]. These studies provide hints about the anti-inflammatory, anti-oxidative, and anti-apoptotic properties of SIRT3 in diseases. However, there is a lack of evidence demonstrating the effects of SIRT3 on APE.

Therefore, we used the SIRT3 activator honokiol and inhibitor 3-(1H-1,2,3-triazol-4-yl)pyridine (3-TYP) to appraise the effects of SIRT3 on inflammation, oxidative stress, and apoptosis induced by APE in lung tissues of rats and explore the underlying molecular mechanisms.

## Materials and Methods

### Animals

This study used male Sprague-Dawley (SD) rats, which were housed under a 12-h light/dark cycle at 22 ± 1°C with 45–55% humidity and free access to food and water. SD rats were treated with a dextran gel G-50 particles (diameter 300 μm; Macklin, Shanghai, China) suspension (12 mg/kg) via the right femoral vein to establish the APE model. Some of the APE rats were also

administered an intraperitoneal injection of 5 mg/kg honokiol (MedChemExpress, Shanghai, China) as described in the previous studies [41, 42]. Furthermore, some of the APE rats administered honokiol were also injected with 5 mg/kg 3-TYP (MedChemExpress) intraperitoneally [39, 43, 44]. Subsequently, arterial blood was drawn from the common carotid artery of the rats under oxygen inhalation. The blood samples were immediately analyzed using a blood gas analyzer. The levels of arterial partial pressure of oxygen (PaO<sub>2</sub>), fraction of inspiration O<sub>2</sub> (FiO<sub>2</sub>), oxygenation index (PaO<sub>2</sub>/FiO<sub>2</sub>), and arterial partial pressure of carbon dioxide (PaCO<sub>2</sub>) were recorded. The lung tissues were obtained from the euthanized rats for subsequent experiments. All animal experiments were carried out in strict adherence with the Guidelines of Laboratory Animals Center of Soochow University.

### Real-time PCR

Total RNA was extracted by TRIPure Reagent (BioTeke Corp., Beijing, China) according to manufacturer's instructions. The corresponding cDNA was obtained by reverse transcription using BeyoRT II M-MLV Reverse Transcriptase (Beyotime Biotechnology, Shanghai, China). Real-time quantitative PCR was performed using SYBR Green (Solarbio Science & Technology, Beijing, China) to measure the *Sirt3* levels of the control and APE groups with specific primer (SIRT3 forward primer, 5'-GCCCAATGTCGCTCACTA-3'; SIRT3 reverse primer, 5'-CGTCAGCCCCGTATGTCTT-3'). β-actin was used as the endogenous control (β-actin forward primer, 5'-GGAGATTACTGCCCTGGCTCCTAGC-3'; β-actin reverse primer, 5'-GGCCGGACTCATCGTACTCCTGCTT-3'). The relative expression levels of *Sirt3* were calculated using the 2<sup>-ΔΔC<sub>t</sub></sup> method.

### Immunohistochemistry (IHC)

Pulmonary tissues from the rats were embedded in paraffin, and the paraffin-embedded blocks of pulmonary tissues from the control and APE rats were processed to obtain 5-μm-thick histologic sections. The samples were incubated with anti-SIRT3 (1:100; Abclonal Technology, Hubei, China) overnight at 4°C, followed by incubation with HRP-linked goat anti-rabbit IgG (1:500; Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for 1 h. Eventually, 3,3-diaminobenzidine (DAB; Maixin Biotech, Fujian, China) and hematoxylin (Solarbio Science & Technology) were used for staining and counterstaining, respectively. The results were observed under a microscope (×400 magnification; Olympus, Tokyo, Japan).

### Immunofluorescence (IF) staining

Lung tissues obtained from the rats were processed into 5- $\mu$ m-thick sections and incubated with anti-SIRT3 overnight at 4°C, as in the case of the IHC. Next, the tissues were kept in Cy3-labeled goat anti-rabbit IgG (1:200; Invitrogen, Thermo Fisher Scientific) for 1 h. Finally, all samples were stained with 2-(4-amidinophenyl)-6-indolecarbamide dihydrochloride (DAPI; Aladdin, Shanghai, China) and visualized with a fluorescence microscope ( $\times 400$  magnification; Olympus).

### Hematoxylin-eosin (HE) staining

Briefly, 5- $\mu$ m-thick sections of lung tissues from the rats were stained with hematoxylin (Solarbio Science & Technology) and eosin (Sangon Biotech, Shanghai, China) and then photographed ( $40\times$  and  $200\times$  magnification). Severity of lung injury was evaluated by referring to the criteria of previous reports [45–47].

### Inflammatory cell count

Bronchoalveolar lavage was performed, and the collected bronchoalveolar lavage fluid was stained with Giemsa stain (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). Then, inflammatory cells, including eosinophils, neutrophils, lymphocytes, and monocyte macrophages, were counted under a microscope ( $400\times$  magnification; Olympus).

### Reactive oxygen species (ROS) determination

Lung tissues were embedded using optimal cutting temperature (OCT) compound. The embedded lung tissues were cut into 10- $\mu$ m-thick slices. ROS levels were detected with an ROS Assay Kit (BestBio, Shanghai, China). Images were captured with a microscope ( $400\times$  magnification; Olympus).

### Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining

Apoptotic cells in lung tissues were analyzed by TUNEL staining. In short, 5- $\mu$ m-thick sections of lung tissues were permeabilized with 0.1% Triton X-100 (Beyotime Biotechnology) for 8 min. The sections were then placed in a TUNEL solution mixture for 1 h. An In Situ Cell Death Detection Kit (Roche, Basel, Switzerland) was used following the manufacturer's instructions. The results were observed under a fluorescence microscope ( $400\times$  magnification; Olympus).

### Assessment of pulmonary injury markers

The dry and wet weights of lung tissues were determined, and then the wet/dry weight ratios (W/D) were calculated. Moreover, a Rat TNF- $\alpha$  ELISA Kit (Multi-

sciences Lianke Biotech, Zhejiang, China) and IL-6 ELISA Kit (Multisciences Lianke Biotech, Zhejiang, China) were utilized to measure the levels of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-6. Myeloperoxidase (MPO) was quantified in accordance with the instructions of an MPO Assay Kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The above biomarkers were used to evaluate the effect of SIRT3 on lung injury in the rats.

### Assessment of pulmonary oxidative stress markers

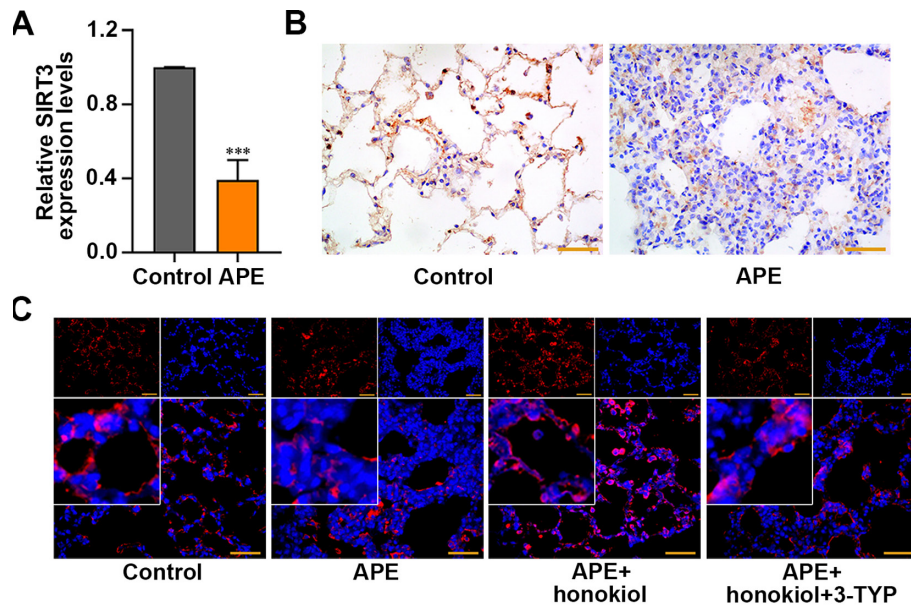
The influence of SIRT3 on oxidative stress in the APE rats was assessed by measuring the content of malondialdehyde (MDA) in lung tissues as well as the activity of serum lactic dehydrogenase (LDH) and superoxide dismutase (SOD) with assay kits from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

### Western blot

Total proteins were collected from lung tissues and separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels (Beyotime Biotechnology). After being blocked by 5% skim milk, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (MilliporeSigma, St. Louis, USA). Then the membranes were incubated overnight at 4°C with anti-cleaved caspase-3 (1:1,000), B-cell lymphoma 2 (Bcl-2; 1:1,000), Bcl-2-associated X (Bax; 1:500), p-AMPK (1:1,000), AMPK (1:1,000), p-mTOR (1:500), and mTOR (1:500) from Affinity Biosciences (Jiangsu, China) as well as  $\beta$ -actin (1:50,000) from Abclonal Technology (Hubei, China). Subsequently, the membranes were exposed to secondary antibodies, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000, Abclonal Technology, Hubei, China), at 37°C for 40 min. Protein signals were observed with an enhanced chemiluminescence (ECL; Beyotime Biotechnology) system.

### Statistical analysis

All experiments were repeated at least six times. The data are presented as the mean  $\pm$  SD. Statistical significance was determined by Student's *t*-test for comparisons between two groups and by one-way ANOVA followed by Tukey's test for comparisons among multiple groups. The data were analyzed using the GraphPad Prism 8.0 software. A *P*-value  $< 0.05$  indicated statistical significance.



**Fig. 1.** Sirtuin3 (SIRT3) expression in the lung tissues of rats with acute pulmonary embolism (APE). (A) The levels of SIRT3 were assessed by real-time quantitative PCR and (B) immunohistochemistry. (C) Immunofluorescence (IF) staining showed the expression of SIRT3 (red) in the lung tissues of APE rats after honokiol and/or 3-TYP treatment. Scale bar=50  $\mu$ m. Data are presented as the mean  $\pm$  SD (n=6 in each group). \*\*\* $P$ <0.001 versus the control group.

## Results

### SIRT3 was decreased in the pulmonary tissues

As shown in Figs. 1A and B, the levels of SIRT3 expression were downregulated in the pulmonary tissues of APE rats ( $P$ <0.001). Furthermore, IF staining assays demonstrated that honokiol elevated the expression of SIRT3 (Fig. 1C).

### SIRT3 alleviated pulmonary function loss and lung injury

Honokiol enhanced the  $\text{PaO}_2/\text{FiO}_2$  ratio and reduced the  $\text{PaCO}_2$  of the APE rats. These results were reversed by 3-TYP (Figs. 2A–D,  $P$ <0.01). Measurement of pulmonary injury revealed that the W/D ratio was reduced by honokiol but was restored by 3-TYP (Fig. 2E,  $P$ <0.001). We performed HE staining to estimate the degrees of inflammation, and the results were consistent with the above (Figs. 2F and G,  $P$ <0.001). These results suggested that SIRT3 preserved pulmonary function and reduced the lung injury induced by APE.

### SIRT3 reduced inflammation

The increases in inflammatory cells, including eosinophils, neutrophils, lymphocytes, and monocyte macrophages, caused by APE were suppressed by honokiol in the APE rats (Figs. 3A–E,  $P$ <0.05).  $\text{TNF-}\alpha$ , IL-6, and MPO, known as pro-inflammatory cytokines, were augmented in the rats with APE compared with the controls.

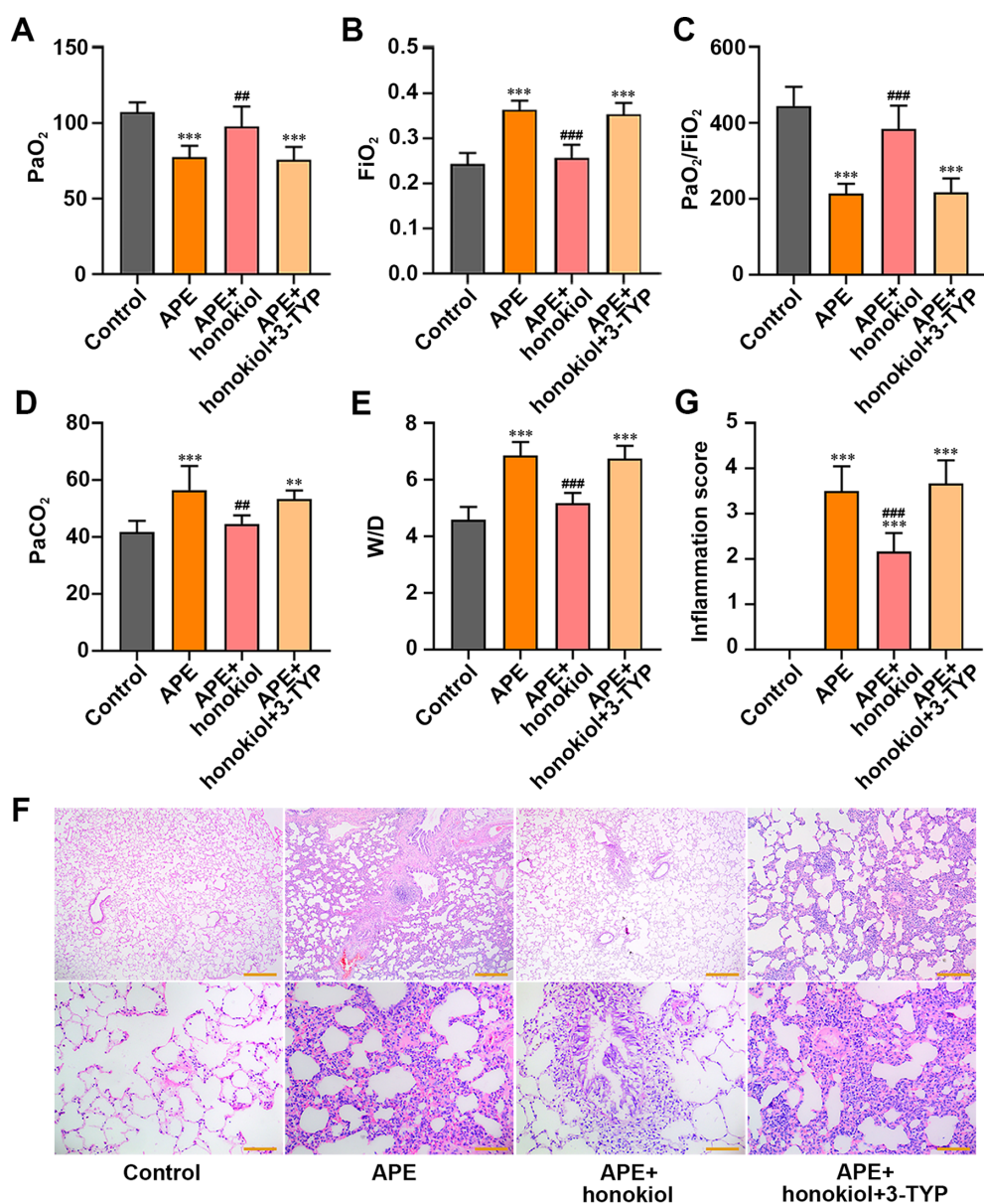
The levels of these markers were reduced after honokiol treatment in the rats with G-50 particle-induced APE (Figs. 3F–H,  $P$ <0.01). On the other hand, 3-TYP reversed the changes (Fig. 3). Our results proved that SIRT3 might play an anti-inflammatory role in the rats suffering from APE.

### SIRT3 decreased oxidative stress and apoptosis

The results shown in Fig. 4A revealed that honokiol prevented elevation of ROS. Similarly, the growth of MDA was inhibited by honokiol (Fig. 4B,  $P$ <0.001). In addition, the activities of LDH and SOD were lower in the APE rats than in the controls. Honokiol was able to recover their activities (Figs. 4C and D,  $P$ <0.05). However, 3-TYP reversed these effects (Figs. 4A–D). Besides, honokiol restrained cell apoptosis, and 3-TYP mitigated this effect (Fig. 4E). Moreover, honokiol-induced decreases of pro-apoptosis genes, including cleaved caspase-3 and Bax, and elevation of the anti-apoptotic gene Bcl-2 were reversed by 3-TYP treatment (Figs. 4F–I,  $P$ <0.001). Taking into consideration all the above results, SIRT3 might protect the rats from oxidative stress and apoptosis induced by APE.

### SIRT3 enhanced the AMPK/mTOR signaling pathway

The results of the western blot assay revealed that the p-AMPK/AMPK ratios, which were reduced by APE, were augmented by honokiol, whereas 3-TYP removed

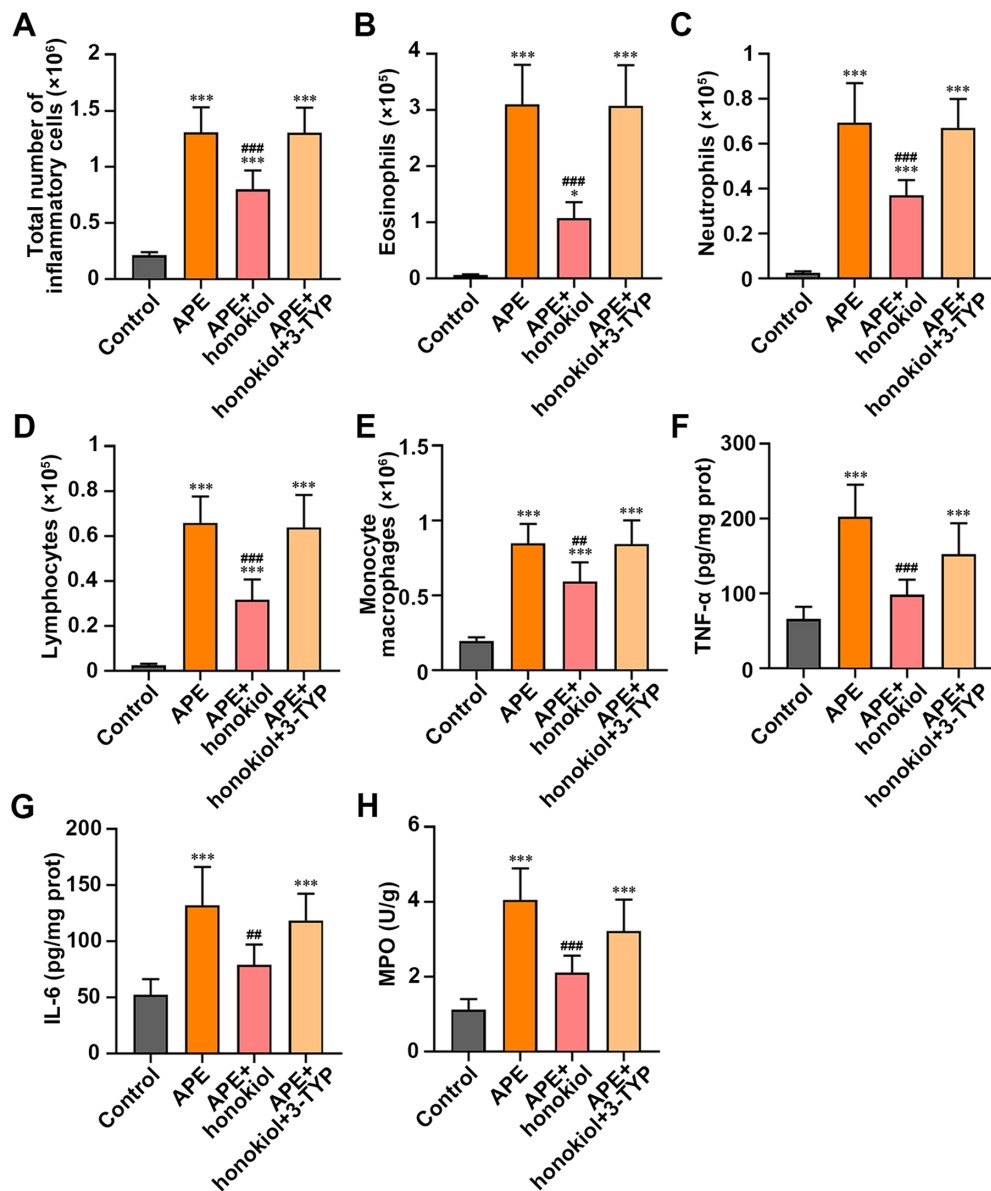


**Fig. 2.** Effect of honokiol or 3-TYP on pulmonary function and injury in the rats with acute pulmonary embolism (APE). (A) Arterial partial pressure of oxygen (PaO<sub>2</sub>). (B) Fraction of inspiration O<sub>2</sub> (FiO<sub>2</sub>). (C) Oxygenation index (PaO<sub>2</sub>/FiO<sub>2</sub>). (D) Arterial partial pressure of carbon dioxide (PaCO<sub>2</sub>). (E) Wet/dry weight ratio (W/D). (F, G) Hematoxylin-eosin (HE) staining was used to observe the pathological changes at 40× (Scale bar=500 μm) and 200× magnification (Scale bar=100 μm) and determine the inflammation grade. Data are presented as the mean ± SD (n=6 in each group). \*\*\**P*<0.001 versus the control group. \*\**P*<0.01 versus the control group. ###*P*<0.001 versus the APE group. ##*P*<0.01 versus the APE group.

this effect in the APE rats (Figs. 5A and B, *P*<0.001). Similarly, Fig. 5C shows that the p-mTOR/mTOR ratios, which were increased by APE, were restored by honokiol in the rats with APE, and this was reversed by 3-TYP (Figs. 5C and D, *P*<0.001). Based on the above results, honokiol might be capable of activating the AMPK/mTOR signaling pathway, whereas 3-TYP might inhibit this. In other words, SIRT3 might protect rats from APE-induced the deleterious effects by activating the AMPK/mTOR signaling pathway.

## Discussion

APE is typically caused by a thrombus that travels from a vein in a lower limb [2], and most deaths from APE occur within the first several hours to days. Therefore, early diagnosis and intervention are of vital importance [48–50]. In the present study, we investigated the effects of SIRT3 on rats with G-50 particle-induced APE via its modulators and explored the possible mechanisms. Previous studies have found that the abnormal

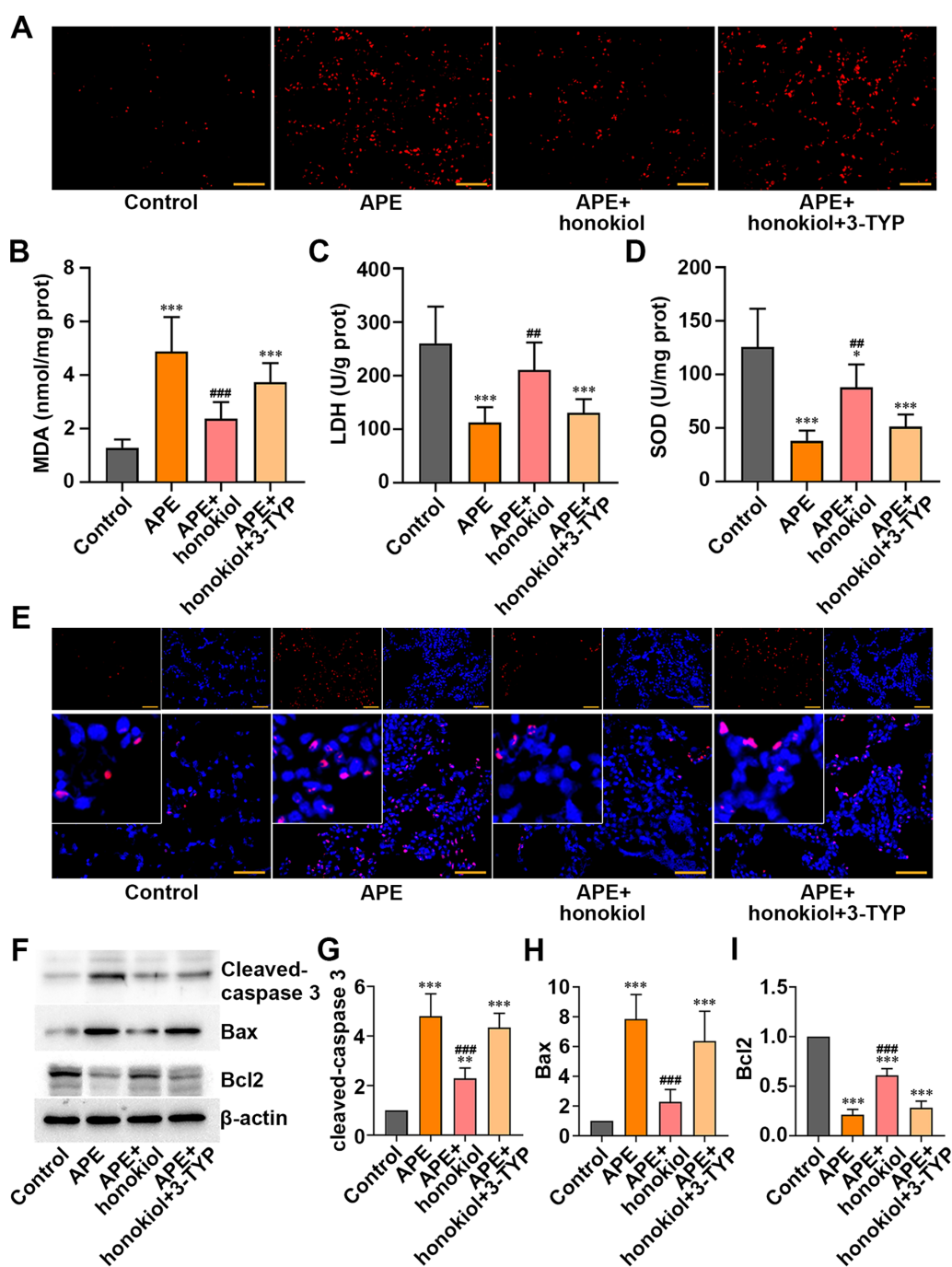


**Fig. 3.** Effect of honokiol or 3-TYP treatment on inflammation in the acute pulmonary embolism (APE) rats. (A) Total number of inflammatory cells. (B) Eosinophils. (C) Neutrophils. (D) Lymphocytes. (E) Monocyte macrophages. (F) Measurement of the inflammation-linked indices tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), (G) IL-6, and (H) myeloperoxidase (MPO). Scale bar=50  $\mu$ m. Data are presented as the mean  $\pm$  SD (n=6 in each group). \*\*\* $P$ <0.001 versus the control group. \* $P$ <0.05 versus the control group. ### $P$ <0.001 versus the APE group. ## $P$ <0.01 versus the APE group.

expression of SIRT3 is closely pertinent to cardiovascular diseases [28, 37, 51]. We provided the evidence that SIRT3 was downregulated in the rats suffering from APE. Our data showed that SIRT3 activation induced by honokiol alleviated the abnormality of lung function in the APE rats, while SIRT3 suppression induced by 3-TYP exacerbated it. Furthermore, the increased anti-inflammatory, anti-oxidative, and anti-apoptotic capacities induced by honokiol were inhibited by 3-TYP, indicating that SIRT3 exhibited a protective effect in the APE rats. Notably, we suggested that this phenomenon might involve the AMPK/mTOR signaling pathway.

In our investigation of the anti-inflammatory effect of SIRT3 in APE rats, HE staining revealed that the lung tissues of APE rats suffered serious damage, including inflammatory cells infiltration and alveolar structure destruction. Significantly, SIRT3 activation diminished, but SIRT3 suppression aggravated these symptoms of APE. Besides, honokiol administration effectively decreased the levels of eosinophils, neutrophils, lymphocytes, and monocyte macrophages. This was sufficient evidence to suggest that SIRT3 had anti-inflammatory effects.

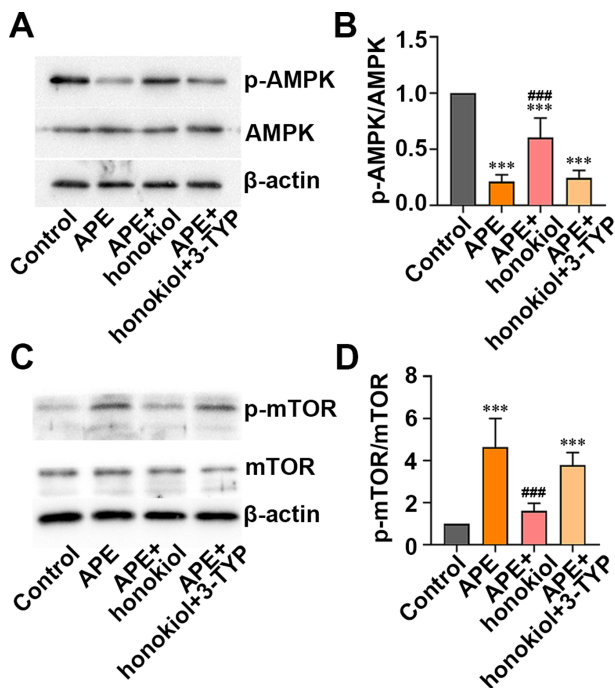
Oxidative stress, defined as a disturbance in the bal-



**Fig. 4.** Effect of honokiol or 3-TYP on oxidative stress and apoptosis in the acute pulmonary embolism (APE) rats. (A) Reactive oxygen species (ROS). (B) Measurement of the oxidative stress-linked indices malondialdehyde (MDA), (C) lactic dehydrogenase (LDH), and (D) superoxide dismutase (SOD). (E) Apoptotic cells were detected by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) (red), and nuclei were detected by 2-(4-amidinophenyl)-6-indolecarbamide dihydrochloride (DAPI) (blue). (F-I) Apoptosis-linked indices (cleaved caspase-3, B-cell lymphoma 2 (Bcl-2)-associated X (Bax), Bcl2) were assessed by western blot. Scale bar=50  $\mu$ m. Data are presented as the mean  $\pm$  SD (n=6 in each group). \*\*\* $P$ <0.001 versus the control group. \*\* $P$ <0.01 versus the control group. \* $P$ <0.05 versus the control group. ### $P$ <0.001 versus the APE group. ## $P$ <0.01 versus the APE group.

ance between oxidation and anti-oxidant defense, is able to cause excessive accumulation of ROS [52, 53]. SIRT3 is reported to rapidly balance ROS levels via deacetylation and activation of superoxide dismutase 2 (SOD2) [31, 54, 55]. In addition to oxidative stress, SIRT3 plays

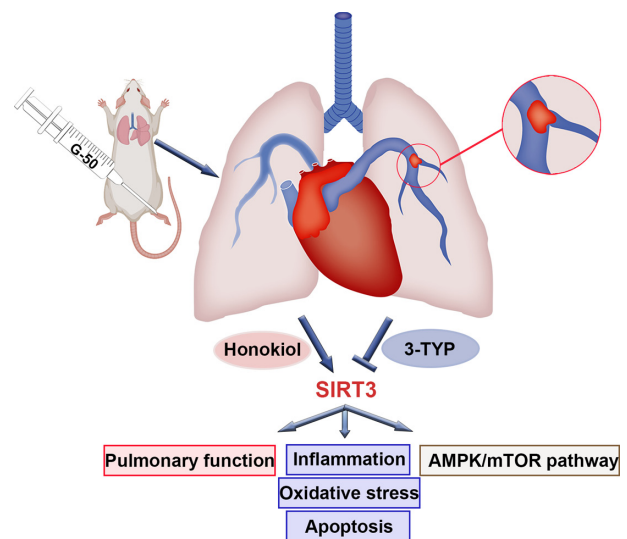
a regulatory role in controlling the inflammatory response to diseases [37, 38]. Honokiol ( $C_{18}H_{18}O_2$ ), a SIRT3 activator [56], possesses anti-oxidative, anti-inflammatory, and anti-apoptotic properties [57–59]. Zheng *et al.* [60] found that honokiol attenuates oxida-



**Fig. 5.** Effect of honokiol or 3-TYP on AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway in the rats with acute pulmonary embolism (APE). (A, B) The levels of p-AMPK/AMPK were determined by western blot. (C, D) The levels of p-mTOR/mTOR were analyzed by western blot. Data are presented as the mean  $\pm$  SD ( $n=6$  in each group). \*\*\* $P<0.001$  versus the control group. ### $P<0.001$  versus the APE group.

tive stress injury by deacetylating SOD2 and then scavenging excess ROS after intracerebral hemorrhage in diabetic rats. As shown in a study by Chiang *et al.* [42], honokiol effectively protects skeletal muscle from eccentric exercise-induced damage by repressing oxidative stress and inflammation in rats. Additionally, honokiol ameliorates the cognitive decline induced by laparotomy under sevoflurane anesthesia in mice through the inhibition of neuronal apoptosis, oxidative stress, and inflammation in the hippocampus [44]. In contrast to honokiol, 3-TYP ( $C_7H_6N_4$ ) is a selective SIRT3 inhibitor that removes honokiol's therapeutic effects. A previous study reported that 3-TYP increases hepatic damage caused by oxidative stress and endoplasmic reticulum stress and facilitates the hepatocyte apoptosis in mice with acute liver failure [61]. Interestingly, it has been reported that 3-TYP administration abolishes the neuroprotective effects of honokiol [44]. In the present study, we found that the anti-oxidative, anti-inflammatory, and anti-apoptotic capacities provided by honokiol were eliminated by 3-TYP in the lung tissues of the APE rats, which was consistent with the previous studies.

AMPK, a regulator of cellular energy homeostasis [62], plays a crucial role in cellular physiology and the patho-



**Fig. 6.** Graphical abstract.

logical development of chronic diseases [63]. It is noteworthy that high expression of SIRT3 ameliorates sepsis-related cardiomyocyte injury by facilitating AMPK activity [64]. mTOR is one of the downstream targets of AMPK. It functions as an intracellular nutrient sensor to control protein synthesis, cell growth, and metabolism [65–67]. A stress-inducing protein, Sestrin2, serves as a guardian by activating AMPK, reducing mTOR, and maintaining the redox balance under the conditions of various stress environments [68]. A previous study clarified that dapagliflozin activates AMPK and suppresses the phosphorylation of mTOR in Zucker diabetic fatty rats, thereby mitigating hepatic steatosis [69]. Given these studies, we conjectured that SIRT3 might exert a protective effect on the lung tissues of APE rats by inducing anti-inflammation, anti-oxidative stress, and anti-apoptosis effects through the AMPK/mTOR pathway.

## Conclusions

Collectively, we demonstrated that SIRT3 was downregulated in the lung tissues of APE rats. We investigated the effects of SIRT3 on the APE rats using the SIRT3 activator honokiol and inhibitor 3-TYP and showed that honokiol diminished APE's negative influence by alleviating oxidative stress, inflammation, and apoptosis. However, 3-TYP treatment eradicated the rescuing effects (Fig. 6). Our study indicated that SIRT3 might serve as a promising molecular target in APE therapy.

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