RESEARCH ARTICLE

NF-κB affected the serum levels of TNF-α and IL-1β via activation of the MAPK/NF-κB signaling pathway in rat model of acute pulmonary microthromboembolism

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Abstract
Pulmonary thromboembolism caused by thrombi blocking major pulmonary artery and its branches, is a frequently encountered phenomenon and an important cause of high morbidity and mortality in lung diseases and may develop into persistent pulmonary hypertension (PH). Nuclear factor-κB (NF-κB) signaling pathway had been reported participated in the formation and development of PH by promoting inflammatory response. The aim of this study was to investigate the effects of NF-κB activation on the serum levels of tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) in acute pulmonary microthromboembolism (APMTE) rats. Rats were randomized into five groups. APMTE group received jugular vein injection of autologous thrombus, while control group rats received normal saline injection. Pulmonary hemodynamic parameters were measured through ECHO-guided transthoracic puncture. Pulmonary vascular morphological changes were analyzed by HE. The expression changes of NF-κB and serum TNF-α、 IL-1β levels were detected by enzyme-linked immunosorbent assay. Protein expression of the MAPK/NF-κB signaling pathway including p-IκBα, p-p38 MAPK, p-NF-κB p65, IκBα, p38 MAPK, and NF-κB p65 was determined using western blot analysis. Compared with control group, the expression of NF-κB in lung tissue and the levels of serum TNF-α and IL-1β rats were higher, a significant reduction in IκBα and elevation in the phosphorylation of IκBα, p38 MAPK, and NF-κB p65 were found in APMTE group rats. And UK administration reversed the APMTE-induced increase in TNF-α, IL-1β, p-IκBα, p-MAPK, and p-NF-κB protein. Furthermore, the levels of NF-κB, TNF-α, and IL-1β were positively correlated with mean pulmonary artery. And

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Abbreviations: APMTE, acute pulmonary microthromboembolism; IL-1β, interleukin-1β; NF-κB, nuclear factor-κB; PAH, pulmonary arterial hypertension; PTE, pulmonary thromboembolism; TNF-α, tumor necrosis factor α.

Yanfen Zhong and Binbin Liang contributed equally to this study.

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the levels of TNF-α and IL-1β were positively correlated with NF-κB. These findings suggest that the activation of MAPK/NF-κB pathway as a critical driver of increasing TNF-α and IL-1β level in APMTE rats and UK exerted protective effects against APMTE-induced PH may be related to the down-regulation of the MAPK/NF-κB signaling pathway.

INTRODUCTION

Pulmonary thromboembolism (PTE), a syndrome with pulmonary and cardiac dysfunction, caused by thrombi blocking major pulmonary artery and its branches, was a frequently encountered phenomenon and an important cause of high morbidity and mortality in lung diseases.[1] Thrombi include detached thrombi from deep vein or right heart and in situ thrombus, in which detached thrombi from deep vein was dominant.[2] Symptoms and prognosis of PTE depended on the site of embolism. Embolism of main pulmonary artery and important branches can be treated with timely and effective reperfusion therapy because it starts and progresses rapidly and easily observed. However, PTE has a poor prognosis and is prone to relapse. Residual thrombus which leads to long term existence and recurrence of thromboembolism may be associated with chronic thromboembolic pulmonary hypertension (PH) due to incomplete thrombolysis.[3]

PH, characterized by increased pulmonary blood pressure, is a devastating respiratory and circulatory system disease, with increased afterload in the right ventricle (RV) and right heart failure, which can lead to death. Inflammation, driven in part by the activation of nuclear factor-κB (NF-κB), previous studies had indicated which played an important role in PH pathogenesis.[4,5] In recent years, relevant studies have shown that reducing the inflammatory response can significantly reduce the production of PH and improve the prognosis of patients.[6] Controlling the inflammatory may be a promising new therapeutic tool for treating PH. NF-κB was a kind of nuclear transcription with multidirectional transcriptional regulation function factor which can promote local inflammatory reaction, endothelial cell dysfunction, thrombosis, oxidative stress, and other pathophysiological processes participate in the formation and development of PH.[7] Recent reports advocated that uncontrolled NF-κB activation aggravated the progression of PH and NF-κB inhibition reduced pulmonary arterial

obliteration.[8] Thus elaborate negative regulatory mechanisms to terminate NF-κB activation and the inflammatory response might provide new therapeutic ideas and targets to prevent PH. Most studies had focused on hypoxic or monocrotaline induced PH in animal models.[9,10] However, there were few reports on other types of PH, such as acute thromboembolic PH. The relationship between acute thromboembolic PH and NF-κB was unclear.

Although the causes and mechanisms of PH remain unclear, it was recognized that inflammation in lung tissues initiates, maintains, or participates in PH as well as other cardiovascular diseases.[11] The inflammation status in pulmonary arterioles can injure pulmonary arterial endothelial cell. Common vascular insults associated with these diseases lead to activation of endothelial cells and resident leukocytes of the vessel wall. These activated cells then promote secretion of major proinflammatory cytokines. More specifically, certain cytokines were important mediators of inflammation such as tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β). They were observed to be increased in PH lung and could contribute to the development of PH.[12] In recent years, the NF-κB and TNF-α signaling pathways have become hotspots for research, and they play a predominant role in the pathogenesis of PH.[12,13] The importance of inflammation was illustrated by successful therapies using an IL-1β receptor antagonist and antibodies to monocyte chemotactic protein-1 in a monocrotaline (MCT)-induced PH model.[14]

Thus, this study intended to establish a rat acute pulmonary microthromboembolism (APMTE) model, observe the expression of NF-κB, TNF-α, and IL-1β, evaluated the effect of NF-κB activation on the serum levels of TNF-α and IL-1β, and explored the relationship NF-κB/MAPK signaling pathway and chronic thromboembolic PH which could provide basic research evidence and new ideas for APMTE thrombolytic therapy. The anti-inflammatory effects and interference of NF-κB/

MAPK signaling pathway may serve as a therapeutic target for PH inhibition and provide the new theory basis of pathogenesis of PH and the potential therapeutic opinion in the future.

METHODS

Materials

Urokinase (UK) for injection (250,000 U; Guangdong Tianpu Biochemical Pharmaceutical Co., Ltd) was obtained from Central Pharmacy of the First Affiliated Hospital of Guangxi Medical University. Sodium pentobarbital used in anesthesia was purchased from Beijing Solarbio Science & Technology Co., Ltd. Antibodies against p65 along with horseradish peroxidase-conjugated goat anti-rabbit antibodies and bicinchoninic acid (BCA) assay were purchased from Shanghai Biyuntian Biotechnology Co., Ltd. Anti-TNF-α antibodies and anti-IL-1β antibodies were purchased from Beijing Solarbio Technology Co., Ltd.

Anti-IκB alpha antibody (ab32518) and anti-phospho-NF-κB p65 (S536) antibody (ab86299) were purchased from Abcam. Anti-NF-κB p65 antibody (10745-1-AP) and anti-beta-actin antibody (66009-1-Ig) were purchased

from Proteintech. Anti-phospho-p38 MAPK antibody (AP0526), anti-p38 MAPK antibody (A4771), and goat anti-rabbit IgG (AS014) were purchased from ABclonal Technology Co., Ltd. Anti-phospho-IκBα (S32) antibody (2859T) was purchased from Cell Signaling Technology. BL-420F biological signal acquisition and analysis system was purchased from Chengdu Taimeng Software Co., Ltd. The needle of a 5 mL syringe is used for puncture.

Animal models

Eighty healthy 8-week-old male SD rats were provided by the Animal Experiment Center of Guangxi Medical University (license number: SCXK GUI 2020–0003). Animal feeding environment in line with China's national standard, Laboratory Animal-Requirements of Environment and Housing Facilities (GB14925-2010). All rodent models were housed on a 12 h light/dark cycle and fed ad libitum with chow diet and water. Animals were matched for age and body weight. The SD rat were randomly divided into five groups ($n = 16$ per group, Figure 1).

(1) Normal control group: received no treatment.
(2) Sham operation group: right common jugular vein injected with 0.7 mL saline for 1 h.
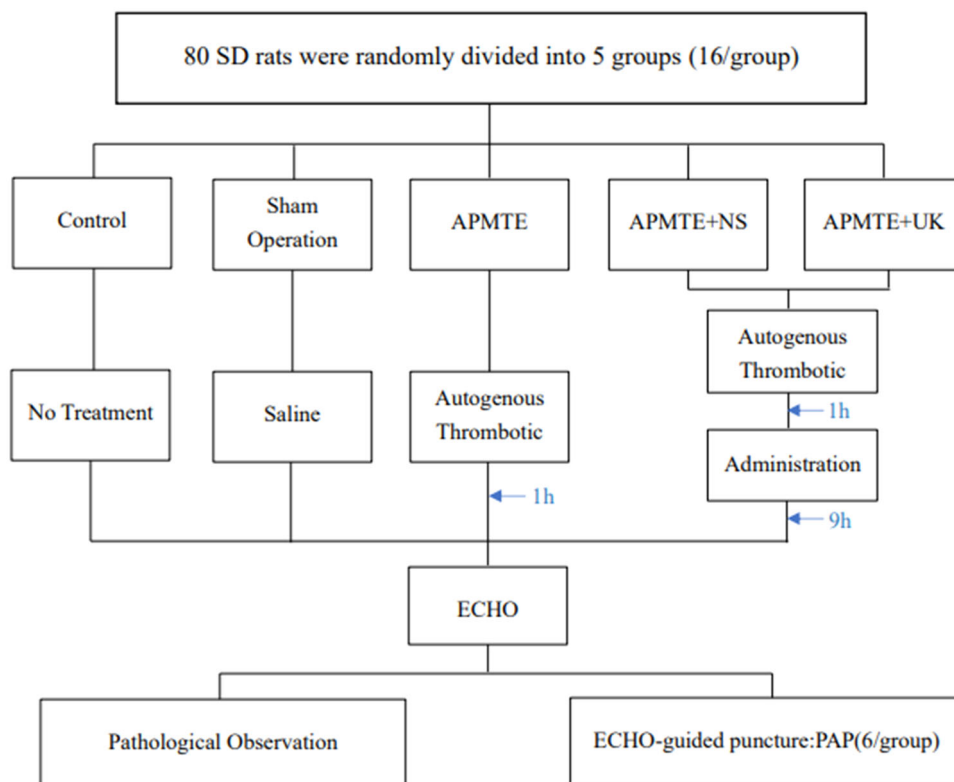

FIGURE 1 Flowchart of grouping and administration of rats in each group. APMTE, acute pulmonary microthromboembolism; ECHO, echocardiography; NS, normal saline; PAH, pulmonary artery pressure; UK, urokinase.

(3) APMTE group: right common jugular vein injected with autogenous thrombi (0.5 mL autogenous thrombotic suspension + 0.2 mL saline) to induce acute pulmonary microthrom-boembolism for 1 h.

(4) APMTE + NS group (normal saline [NS] treatment group): right common jugular vein injected with autogenous thrombi (0.5 mL autogenous thrombotic suspension + 0.2 mL saline) to induce APMTE for 1 h + administrated with NS (100 U/g) via tail vein and then observed for 9 h.

(5) APMTE + UK group (UK treatment group): right common jugular vein injected with autogenous thrombi (0.5 mL autogenous thrombotic suspension + 0.2 mL saline) to induce APMTE for 1 h+ administrated with UK (100 U/g) via tail vein and then observed for 9 h.

After modeling, six rats in each group were randomly selected for transthoracic puncture measurement of PAP under the guidance of echocardiography (ECHO). Finally, only lung tissues of unpunctured rats were collected for pathological observation and immumo-histochemical staining, because of the possibility of accidental injury to both lungs during puncture process.

Preparation of autologous thrombus

The inner diameter of pulmonary small vessels in five normal SD rats were observed and measured, and found that the inner diameter was about 100 μm. Use a capillary pipet (about 100 μm in diameter) to collect blood from the post-glomus vein of the rat, and place it in a 37°C constant temperature water bath box overnight. Flushing the thrombus with NS to prepare thrombus particles autogenous thrombotic particles (about 100 μm × 100 μm × 100 μm) and suspending them in NS.

Establishment of rat models of APMTE

The rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital (50 mg/kg). After anesthesia, the rats were fixed on the experimental table, and the right common jugular vein was exposed. About 0.5 mL saline suspension containing 30 autogenous thrombotic particles was injected at a constant and slow speed (0.5 mL/min, 1 embolus was inserted in about 2 s, as far as possible to ensure that the emboli did not cluster) through the right common jugular vein with a 1 mL syringe. And then 0.2 mL saline was used to flush the tube (Figure 2). The blood volume of rat was 60 mL/kg, and each rat used in the experiment was about 250−300 g (blood volume 15−18 mL). The amount of fluid injected into the rats during modeling accounted for 4.7%−4% of the total blood volume. After injection, the general condition of heart rate and respiration were observed and the thrombus were confirmed in pulmonary arterioles by pathological observation (Figure 3).

Measurement of pulmonary artery pressure

Traditional pulmonary artery pressure measurement adopts the right heart catheter method. The catheter passes through the peripheral vein-right atrium-RV-pulmonary artery and needs to undergo many
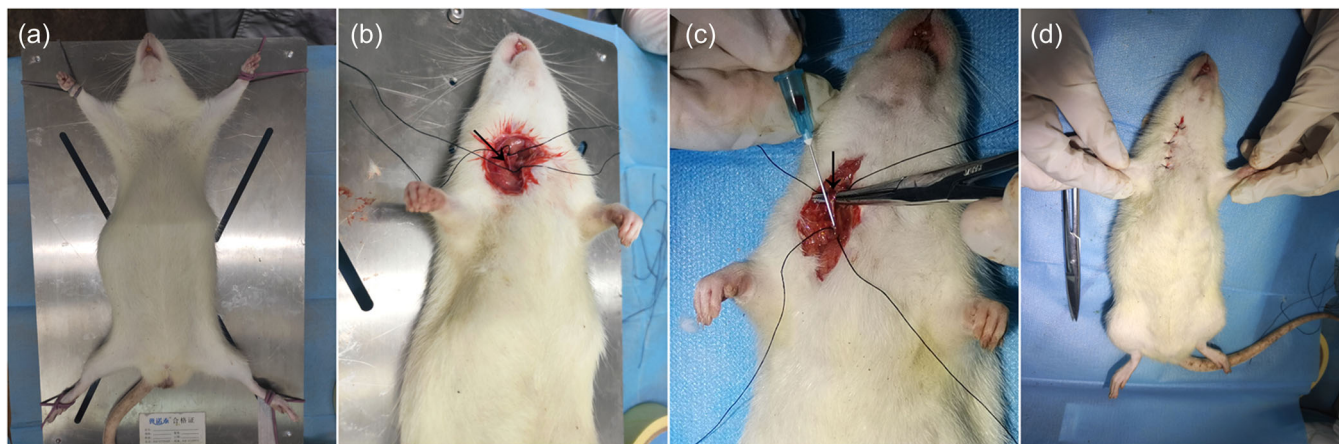

FIGURE 2 Establishment of rat models of APMTE. (a) The rat was anesthetized and fixed on the operating table. (b) Exposed the right common jugular vein (black arrow). (c) About 0.5 mL saline suspension containing 30 autogenous thrombotic particles was injected. (d) After the operation, the rat skin was sutured. APMTE, acute pulmonary microthromboembolism.
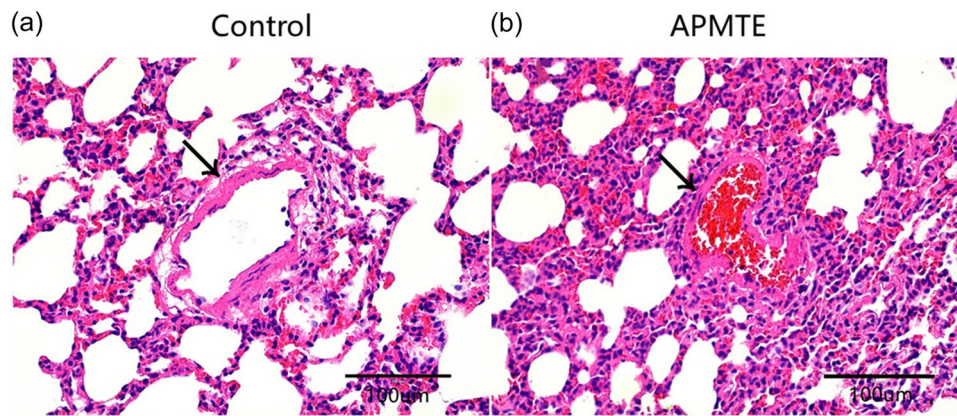
FIGURE 3 APMTE model were confirmed established successfully by pathological section observation (high-magnification [×400] H&E-stained). (a) Pulmonary sections of normal control rats. (b) Pulmonary sections of APMTE rats, thrombus (black arrow) were observed in pulmonary arterioles. APMTE, acute pulmonary microthromboembolism; H&E, hematoxylin and eosin.
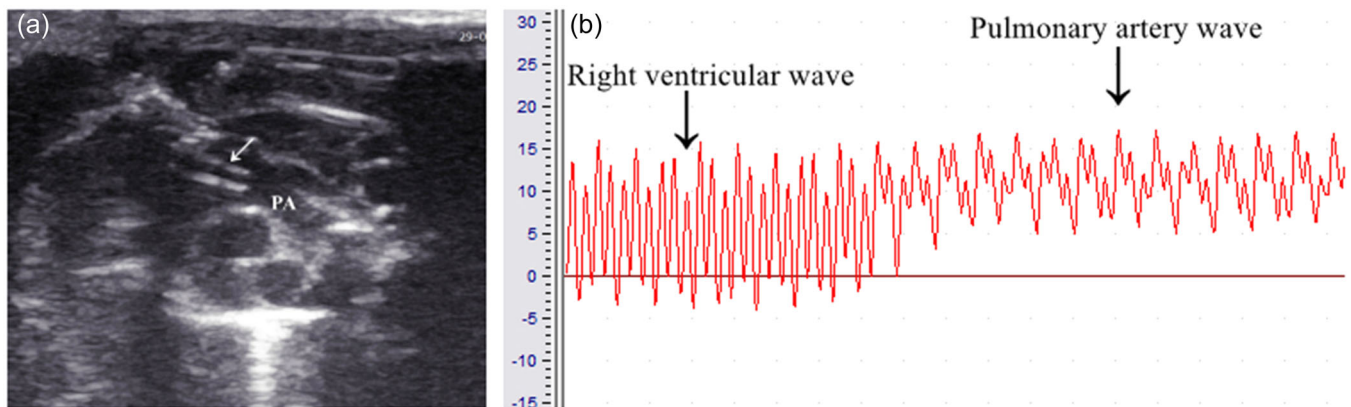

FIGURE 4 (a) ECHO-guided transthoracic puncture measurement of PAP. Parasternal short-axis section: puncture needle (white arrow) was inserted into pulmonary artery. (b) Pressure curve of catheter entering right ventricular outflow tract and pulmonary artery. ECHO, echocardiography; PA, pulmonary artery; PAP, pulmonary artery pressure.

physiological bends. The catheter is easy to pierce the vein and heart, causing hemorrhage or death and failure of pressure measurement in rats. In recent years, ultrasound-guided puncture technology had made significant progress in the field of diagnosis and treatment. With the application of high-resolution ultrasound in small animal imaging, the puncture area could be accurately identified and located. After anesthesia, the puncture needle was connected to BL-420F system, and prefilled with heparin NS (20 U/mL). Under the guidance of ECHO, the surgeon carefully inserted the puncture needle into the right ventricular outflow tract in parasternal short-axis section. After the RV pressure curve appeared, the surgeon continued to slowly advance the puncture needle to reach the main pulmonary artery and recorded the PAP curve (Figure 4).

Histopathological and immunohistochemical staining

Before the rats were killed under excessive anesthesia, heparin (10 U/100 g) was injected through the tail vein to prevent blood coagulation. Tissues of the lung were removed quickly and washed up with NS. Then the entire lung tissues were fixed in 10% formalin solution for 48 h, dehydrated with alcohol, transparent with xylene, embedded in paraffin and sliced. Sections were stained with hematoxylin and eosin (H&E) for observed in general. For immunohistochemistry, after the tissue sections were blocked with goat serum, they were incubated with anti-NF-κB-phospho-p65 (1:200) at 37°C for 90 min. Then, an anti-rabbit IgG antibody with HRP-horseradish peroxidase labeled (1:200) were added to the

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sections and incubated at 37°C for 30 min. Immuno-reactivity was visualized using diaminobenzidine.

Enzyme-linked immunosorbent assay (ELISA)

Before the rats were killed under excessive anesthesia, blood was collected through the abdominal aorta, and then centrifuged at 4°C, retained the supernatant and store at −20°C for later use. TNF-α and IL-1β levels and in rat was determined using rat TNF-α ELISA kit and rat IL-1β ELISA kit according to manufacturer's instructions (Solarbio).

Western blot analysis

Lung tissues (100 mg) were homogenized and sonicated in a lysis buffer containing a protease and phosphatase inhibitor cocktail. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C to obtain the supernatant.

A BCA assay was performed to examine the protein concentration in the supernatant. Equal amounts of protein (50 μg) were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12%) after being mixed with Laemmli sample buffer. The separated proteins were transferred to a polyvinylidene fluoride membrane. After blocking with 5% nonfat milk in tris buffered saline with tween 20 for 1 h, the membranes were incubated with primary antibodies against anti-p38 MAPK (1:1000), anti-phospho-p38 MAPK (1:1000), phospho-IκBα (1:1000), IκBα (1:1000), phospho-NF-κB p65 (1:2000), NF-κB p65 (1:1000), and GAPDH (1:1000) overnight (12−18 h) at 4°C, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000) for 60 min at 25°C after washing. The protein bands were visualized using the enhanced chemiluminescence method (AI600; General Electric Company) after washing and quantified using Image Studio Lite Software.

Statistical analysis

Statistical Product and Service Solutions software Version 26.0 (SPSS; IBM) was used for statistical analysis. Results were expressed as mean ± standard deviation (mean ± SD). Statistical analysis was carried out by ANOVA and LSD test was used for further pairwise comparison. Pearson's correlation was used for correlation analysis. $p < 0.05$ was considered statistically significant. $r > 0.6$ was considered a strong positive correlation.

RESULTS

Characteristics of rat

During modeling process, a certain proportion of rats in each group died (mortality rate 6%−25%), most of which were due to anesthesia accidents, only two rat in the APMTE group and APMTE + NS group died of right heart failure (Table 1). None of rats died after administration as planned. Six rats in each group were successfully punctured to measure PAP under ECHO guidance, and the remaining rats were killed according to the norms and pathological specimens of them were taken.

PAP was increased in APMTE group which were lowered by UK thrombolytic therapy

To determine whether acute PTE can cause PH, six rats in each group were randomly selected for transthoracic puncture measurement of PAP under the guidance of ECHO. The results are presented in (Figure 5a). PAP in normal control group were not significantly different from that in sham operation group. PAP was significantly higher in the APMTE group than in the normal control or sham operation group (sPAP, dPAP, mean pulmonary

TABLE 1 Characteristics of rats in each group.

Group	Case (n)	Death	PAP measurement	Pathological observation
Control group	16	1	6	9
Sham operation	16	2	6	8
APMTE	16	2	6	8
APMTE + NS	16	4	6	6
APMTE + UK	16	1	6	9

Abbreviations: APMTE, acute pulmonary microthromboembolism; NS, normal saline; UK, free urokinase.
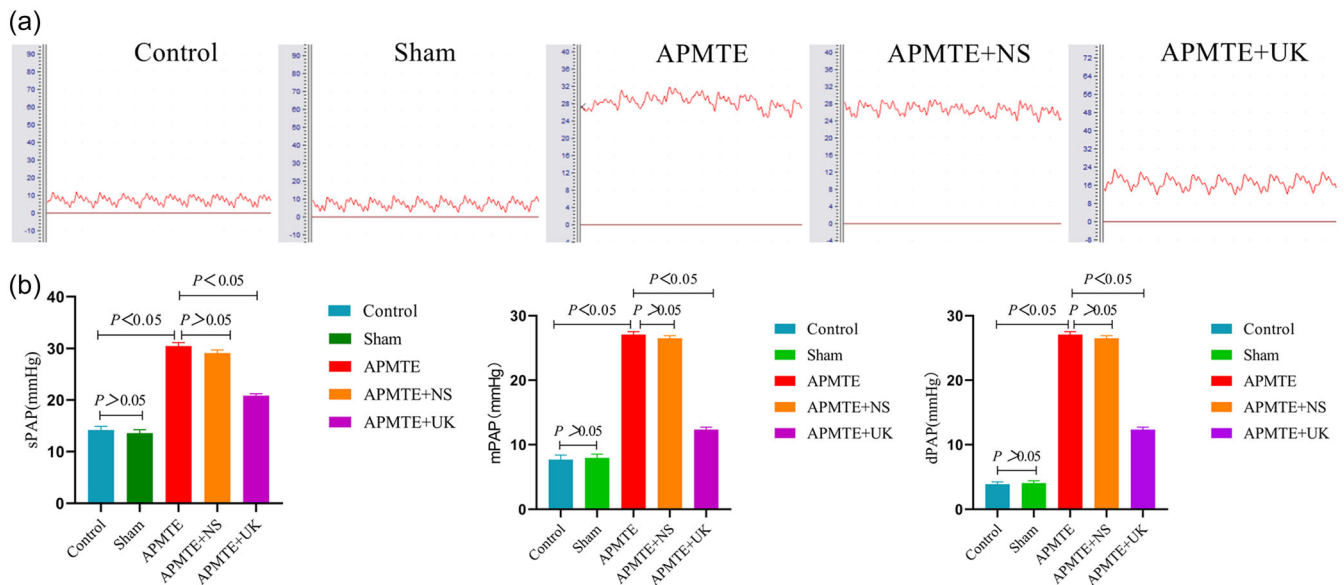
(a)

(b)

FIGURE 5 Pulmonary artery pressure in every group. One hours after injection of saline/autologous thrombus, rats' PAP in normal control, sham operation, and APMTE groups (six rats/group) were measured. Nine hours after administration, rats' PAP in APMTE + NS, APMTE + UK groups (six rats/group) were measured. PAP was higher in the APMTE group than in the normal control/sham group. Compared with APMTE + NS and APMTE groups, PAP of APMTE + UK groups decreased gradually. APMTE, acute pulmonary microthromboembolism; dPAP, diastolic pulmonary artery pressure; mPAP, mean pulmonary artery; NS, normal saline; PAP, pulmonary artery pressure; Sham, sham operation; sPAP, systolic pulmonary artery pressure; UK, urokinase.

artery pressure [mPAP]: APMTE group vs. normal control/sham operation group, $p < 0.05$). There was no significant difference in PAP between APMTE + NS group and APMTE group. PAP in APMTE + UK group was significantly lower than that in APMTE/APMTE + NS group (sPAP, dPAP, mPAP: APMTE + UK group vs. APMTE/APMTE + NS group, $p < 0.05$) (Figure 5b). These data suggested that APMTE can cause PH, which were lowered significant by APMTE + UK thrombolytic therapy.

Histological observation of the lung tissues shows thrombi were found in pulmonary arterioles of APMTE rat, which was loose, and arteriole lumens were slightly clear by UK thrombolytic therapy

Except for the normal control group and the sham operation group, thrombi were found in pulmonary arterioles of the other groups, with different shapes and sizes. Large emboli with diameters from 80 to 120 μm were found in the arterioles, similar in size to injected autologous thrombus. Emboli with diameters of about 50 μm or smaller could be seen in arterioles. Due to the large number and unfixed location, these vessels and thrombus were not counted. In APMTE and APMTE +

NS group, the pulmonary thrombus structure was relatively intact and arteriole lumens were almost occluded. The alveoli around the embolism site collapsed and many erythrocytes exuded. Thrombus structure in APMTE + UK group was loose, and arteriole lumens were slightly clear. The collapse degree of alveoli around embolism decreased and erythrocyte exudation was reduced (Figure 6a).

NF-κB activity was increased in APMTE-induced PH, but decreased with UK treatment

We investigated NF-κB p65, an indicator of NF-κB activity, by immunohistochemical staining expression in lung tissue of rats. Under the microscope (high power ×400), the immunohistochemical staining of NF-κB in lung tissues was brownish yellow or brown, and it was mainly expressed in cell nucleus and cytoplasm (Figure 6b). Five 400-fold visual fields were randomly selected from each section (the average number of positive cells in 100 such cells were counted), and the average percentage of positive cells in each visual field was taken. We detected staining in lung tissues of both APMTE group rats and APMTE + NS group rats significantly increased. The average percentage of positive cells
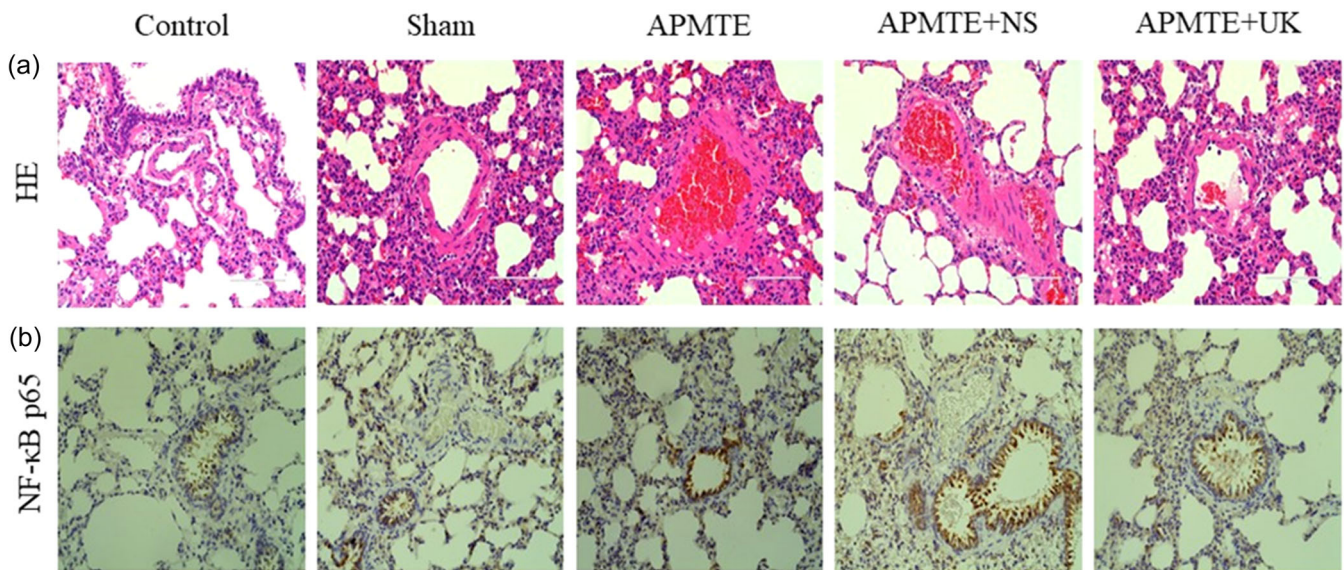
FIGURE 6 (a) Photomicrographs of pulmonary tissue sections (H&E, ×400). 1 h after injection of saline/autologous thrombus, lungs of unpunctured rat in normal control, sham operation, and APMTE groups were collected for further observation microscopically. Nine hours after administration, lungs of unpunctured rat in APMTE + NS, APMTE + UK groups were collected. There was no obvious pulmonary thrombosis in normal control and sham operation groups. Thrombi were observed in APMTE, APMTE + NS, and APMTE + UK groups. Thrombus was relatively intact in APMTE + NS groups. Thrombus structure in APMTE + UK group was loose. (b) NF-κB activity in pulmonary tissue (×400). The expression of NF-κB p65 in lung tissues were brownish yellow in normal control and sham operation groups and brown in APMTE, APMTE + NS, and APMTE + UK groups, and it was mainly expressed in cell nucleus and cytoplasm. APMTE, acute pulmonary microthromboembolism; H&E, hematoxylin and eosin; NF-κB, nuclear factor-κB; NS, normal saline; Sham, sham operation; UK, urokinase.

was significantly higher in the APMTE/APMTE + NS groups than in the normal control or sham operation group (the average percentage of positive cells: APMTE/APMTE + NS group vs. normal control/sham operation group, $p < 0.05$). There was no significant difference between APMTE + NS group and APMTE group, nor do the normal control group and sham operation group. While the expression of NF-κB p65 in lung tissue decreased of APMTE + UK group rats than that in APMTE/APMTE + NS group (the average percentage of positive cells: APMTE + UK group vs. APMTE/APMTE + NS group, $p < 0.05$) (Figure 7a). These data suggested that NF-κB activity was increased in APMTE-induced PAH, but decreased with UK treatment.

Activation of MAPK/NF-κB signaling in APMTE-induced PH rats

We found a significant reduction in IκBα and elevation in the phosphorylation of IκBα, p38 MAPK, and NF-κB p65 in both APMTE group rats and APMTE + NS group rats compared with those in the control rats ($p < 0.05$ for all) (Figure 8). UK administration reversed the APMTE-induced increase in p-IκBα, p-MAPK, and p-NF-κB protein levels compared with those in the APMTE + NS

group rats ($p < 0.05$) (Figure 8). These results suggest that APMTE could drive the activation of the MAPK/NF-κB signaling pathway.

Serum TNF-α and IL-1β levels was increased in APMTE-induced PH rats, but decreased with UK treatment

We detected the serum TNF-α and IL-1β levels of rats by ELISA. Both the serum TNF-α and IL-1β levels of rats were significantly higher in the APMTE/APMTE + NS groups than in the normal control/sham operation group (the serum TNF-α and IL-1β levels: APMTE/APMTE + NS group vs. normal control/sham operation group, $p < 0.05$). There was no significant difference between APMTE + NS group and APMTE group, nor do the normal control group and sham operation group. While the serum TNF-α and IL-1β levels of rats decreased of APMTE + UK group rats than that in APMTE/APMTE + NS group (the serum TNF-α and IL-1β levels of rats: APMTE + UK group vs. APMTE/APMTE + NS group, $p < 0.05$) (Figure 7b, c). These data suggested that the serum TNF-α and IL-1β levels of rats increased in APMTE-induced PH, but decreased with UK thrombolysis.
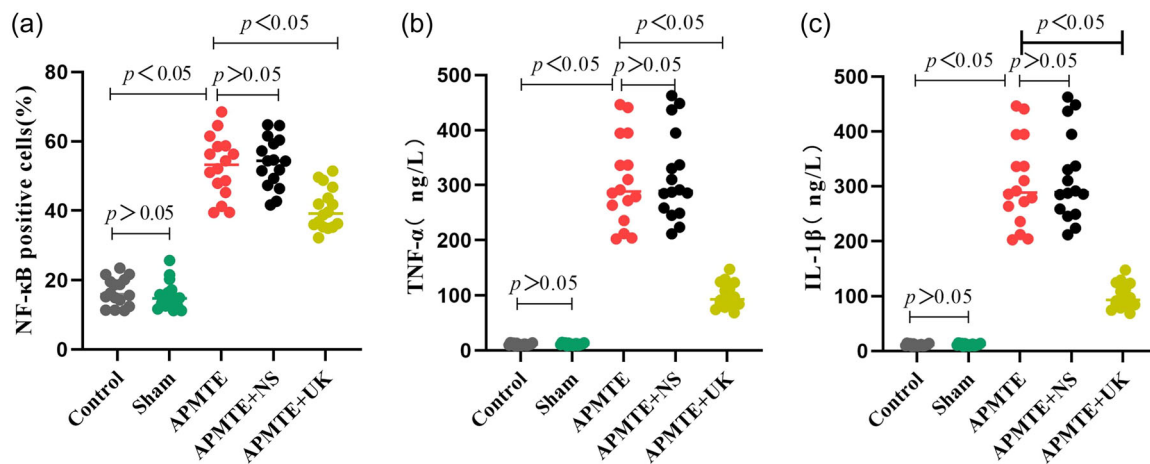
FIGURE 7 (a) NF-κB activity in each group. The average percentage of positive cells was significantly higher in the APMTE/ APMTE + NS groups than in the normal control or sham operation group. While the expression of NF-κB p65 in lung tissue decreased of APMTE + UK group rats than that in APMTE/APMTE + NS group. (b) Serum TNF-α level in each group. (c) Serum IL-1β level in each group. Both the serum TNF-α and IL-1β levels of rats were significantly higher in the APMTE/APMTE + NS groups than in the normal control/sham operation group. While the serum TNF-α and IL-1β levels of rats decreased of APMTE + UK group rats than that in APMTE/ APMTE + NS group. APMTE, acute pulmonary microthromboembolism; IL-1β, interleukin-1β; NF-κB, nuclear factor-κB; NS, normal saline; Sham, sham operation; TNF-α, tumor necrosis factor α; UK, urokinase.
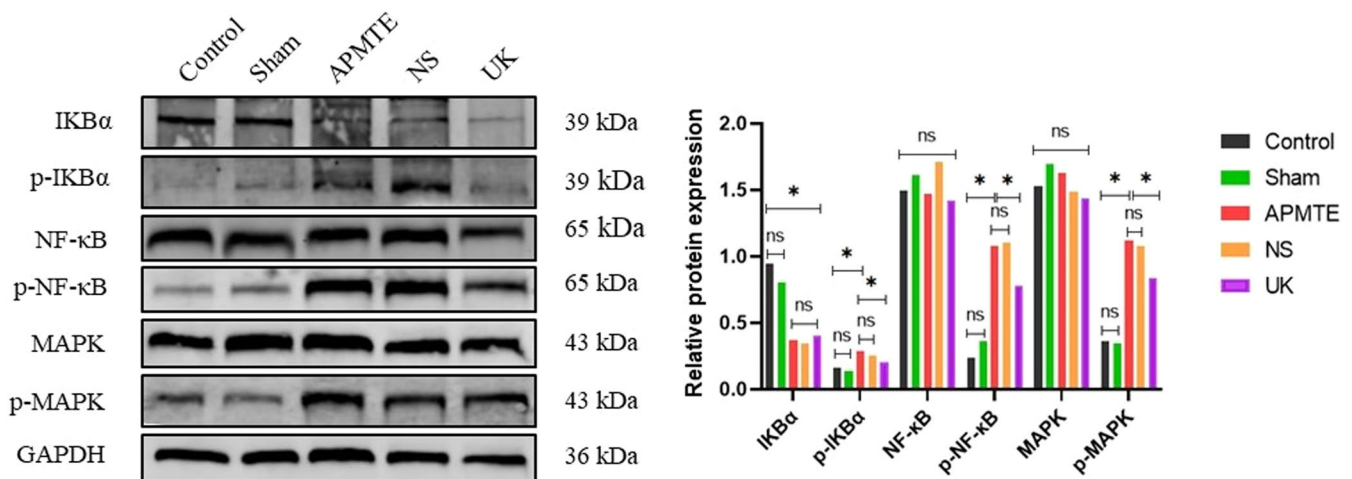

FIGURE 8 The activation of the MAPK/NF-κB signaling in APMTE-induced PH rats. Representative western blots analysis and quantification of IκBα/GAPDH, p38 MAPK/GAPDH, NF-κB p65/GAPDH, p-IκBα/GAPDH, p-p38 MAPK/GAPDH, and p-NF-κB p65/ GAPDH ratios in the lung tissues in each group. Data were expressed as mean ± SD. *$p < 0.05$. APMTE, acute pulmonary microthromboembolism; NF-κB, nuclear factor-κB; PH, pulmonary hypertension; Sham, sham operation; UK, urokinase.

NF-κB activation effects of serum TNF-α and IL-1β levels in rat model of APMTE-induced PH

Based on our finding of increased NF-κB activity and serum TNF-α and IL-1β levels in rat model of APMTE-induced PH, we analyzed the correlation between NF-κB, TNF-α, IL-1β, and pulmonary artery pressure. We investigated the levels of NF-κB, TNF-α, and IL-1β were positively correlated with mPAP (Figure 9). Moreover, the levels of TNF-α and IL-1β were positively correlated with NF-κB (Figure 10). We believed that NF-κB may participate in the occurrence and development of PH in APMTE by mediating inflammation.

DISCUSSION

MAPK/NF-κB signaling has been identified as an important pathway in inflammation. Previous studies have demonstrated that inflammatory cytokines aggravate vascular
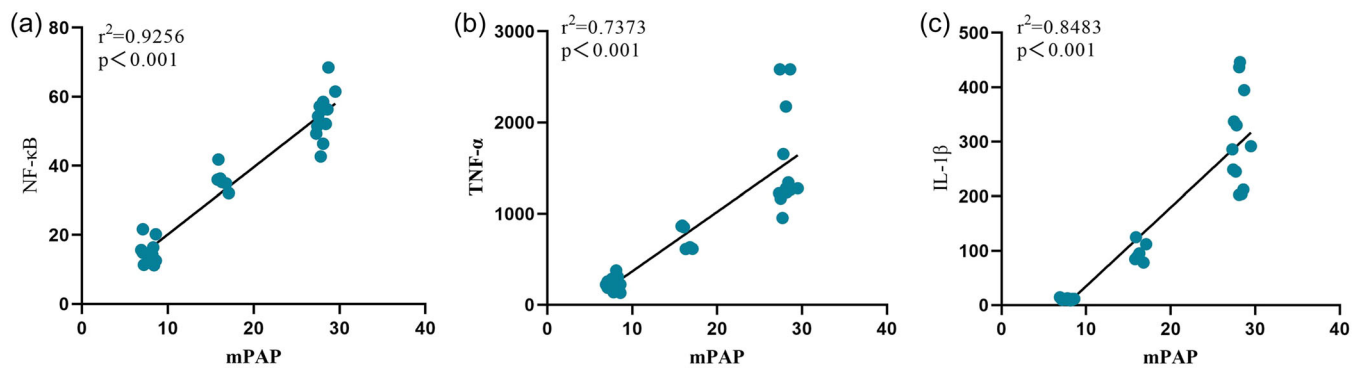
FIGURE 9 (a) The correlation between NF-κB and pulmonary artery pressure (mPAP). (b) The correlation between TNF-α and mPAP. (c) The correlation between IL-1β and mPAP. The expression of NF-κB and the levels of serum TNF-α and IL-1β were positively correlated with mPAP. IL-1β, interleukin-1β; mPAP, mean pulmonary artery pressure; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor α.
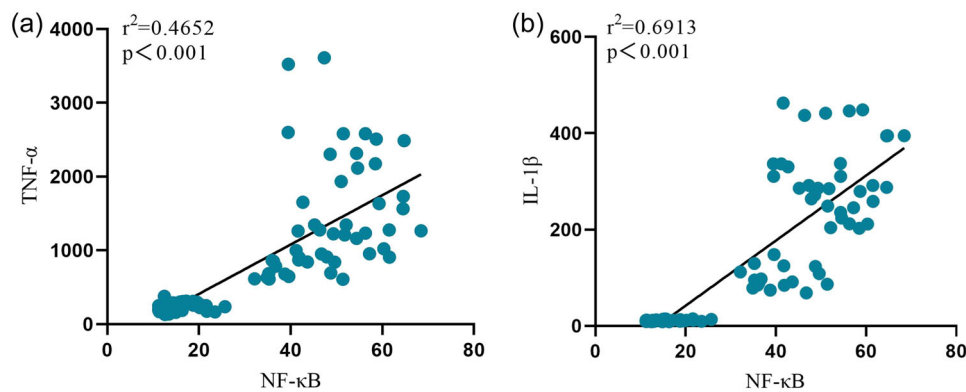

FIGURE 10 (a) The correlation between TNF-α and NF-κB. (b) The correlation between IL-1β and NF-κB. The levels of TNF-α and IL-1β were positively correlated with NF-κB. IL-1β, interleukin-1β; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor α.

remodeling by binding to their receptors, thereby activating MAPK/NF-κB signaling.[15] We investigated NF-κB activity, serum TNF-α and IL-1β levels was increased in APMTE, but decreased with UK treatment. And we found a significant reduction in IκBα and elevation in the phosphorylation of IκBα, p38 MAPK, and NF-κB p65 in APMTE group, and UK administration reversed the APMTE-induced increases in p-IκBα, p-p38 MAPK, and p-NF-κB protein levels. These results suggest that NF-κB has an effect on serum TNF-α and IL-1β levels via activation of the MAPK/NF-κB signaling pathway in rat model of APMTE.

Thrombi in pulmonary arterioles of APMTE rat

There are two common methods to establish animal models of PTE, in vivo and in vitro embolus formation The method of in vitro embolus formation is to prepare emboli outside the body and inject them into the venous system or right atrium, and then emboli travel to the lung and cause pulmonary embolism.[16] To simulate the natural process of acute pulmonary microembolism, the method of in vitro embolus formation was used to establish animal models.[17]

Fragmentation of large thromboembolus and in situ thrombosis in small vessels were two dominating causes of pulmonary microthromboembolism. The formation of microthrombus in situ, related to endothelial dysfunction, was a chronic process.[18] APMTE was associated with large thrombus disruption.[19] After large thromboembolus formation, most clots undergo fibrinolysis and organization that dissipates the thrombus and recanalizes the vessel.[20] Thromboemboli may break up into numerous tiny fragments, either in the process of endogenous thrombolysis or passing through the heart, thus blocking small ramifications of pulmonary arteries. After the recanalization of the vessel, the fibrin and platelets covering the surface of thrombus were carried to a distance because of blood flow. Then the coagulation process were reactivated to produce microthrombus to jam up the micro branches of pulmonary vessel.[21] When

the micro branches of pulmonary vessel blocked up, pulmonary vascular resistance, pulmonary arterial pressure, and right ventricular afterload increase, because the total cross-sectional area of pulmonary vessels decreases which attribute to neuro-modulation, humoral regulation, and mechanical obstruction of the pulmonary vasculature.[22] Eventually, PH occurred.

Compared with normal control group, pulmonary artery pressure was increased in APMTE rats and lung tissue of them showed post-thromboembolic appearance. Based on the results above, this study successfully established a rat model of APMTE, which provided a solid experimental basis for the experiment of the relationship between APMTE-induced PH and NF-κB.

MAPK/NF-κB signaling activity was activated in APMTE

NF-κB was a transcription factor that regulates multiple genes associated with inflammatory responses, cell growth control, and apoptosis.[23] Activation of the NF-κB signaling pathway was related to the dissociation of IκBα from the P65/P50 dimer by external factors. When stimulated, IκBα was phosphorylated and then degraded, and P65 and P50 dimers were released into the nucleus, and the NF-κB signaling pathway was activated. Therefore, the change in the expression level of p-IκBα was also used as a NF-κB signal activated. In the resting state, NF-κB was localized in the cytoplasm and binds to IκBα. Once IκBα was phosphorylated by the outer boundary signal, it was ubiquitinated and degraded, releasing NF-κB to translocate to the nucleus, where it binds to specific response elements in the DNA. And NF-κB get activated finally.[24] Accumulating evidence suggests that NF-κB may be involved in the formation and development of PH. It was confirmed that NF-κB in macrophages lymphocytes, pulmonary artery endothelial cells and smooth muscle cells of pulmonary tissue increased activation in patients with idiopathic PH than those in normal controls.[25] Recent studies have been conducted using NF-κB inhibitors or specific blocks of NF-κB pathway in animal PH models found to be effective in slowing down PH progression and improved right ventricular remodeling.[8,26]

p38-mitogen activated protein kinase (p38 MAPK) is a subgroup of the MAPK family. It can recruit white blood cells, activate related inflammatory cytokines, and mediate the process of adhesion and migration of inflammatory response. At the same time, studies have shown that p38 MAPK was closely related to vascular smooth muscle cell proliferation, pulmonary artery endothelial cells apoptosis and calcium ion regulation.

p38 MAPK is inactive in the dephosphorylated state. When subjected to various stimuli, p38 MAPK is rapidly phosphorylated, which activates a series of substrates and leads to the occurrence of inflammation.[27]

It has been reported that MAPK/NF-κB signaling pathway involved in inflammatory response. In recent years, many studies have reported that inflammatory response plays a very important role in the pathophysiology of PH.[28] Here, we present evidence for increased activity of NF-κB pathway predominantly in the pulmonary tissue of APMTE rats and sustained elevation of NF-κB levels was associated with pulmonary artery pressure. What's more, we identified that the phosphorylation levels of p38 MAPK, IκBα, and NF-κB p65 were elevated in APMTE-induce PH rats. UK administration reversed the APMTE-induced increase in p-IκBα, p-MAPK, and p-NF-κB protein levels. Above the results suggested that MAPK/NF-κB signaling was activated in APMTE rats and MAPK/NF-κB signaling was involved in the protective mechanism of UK in APMTE-induced PH. Consistent with our results, Nalban et al. reported that arbutin significantly reduced the phosphorylation of IκBα and NF-κB in isoproterenol-treated mice.[29] In addition Chen et al. confirmed that the activation of NF-κB-dependent proinflammatory cytokines as a critical driver of vascular remodeling and a promising target in patients with PH and SBT exerts protective effects against MCT- and hypoxia-induced PH by ameliorating inflammation and vascular remodeling, which is related to the downregulation of the MAPK/NF-κB signaling pathway.[30]

Serum TNF-α and IL-1β levels was increased in APMTE

TNF including TNF-α and TNF-β were first discovered in 1975 as a substance that causes necrosis in a variety of tumors occurs in the serum.[31] In terms of biological activity, TNF-β only accounts for 5%−30% of the total biological activity of TNF. Therefore, TNF-α is usually referred to as TNF. And data suggested that as an important inflammatory factor, TNF-α played an important role in the development of PH.[32,33] TNF-α involved in systemic inflammatory response, which can directly stimulate pulmonary vascular endothelial cells, activate the proinflammatory phenotype of endothelial cells, cause vascular endothelial cell injury. What's more, TNF-α involved in the occurrence and development of PH by promoting thrombosis. The pro-thrombotic effect of TNF-α on arteriolar thrombosis was mainly mediated by the expression of TNF receptor TNFR2 (TNF-RP75) on hematopoietic cells and endothelial cells.[34] TNF-α can

also promote thrombosis by promoting the shedding of protein C receptor, inhibiting the production of thrombomodulin, inducing complement activation, stimulating endothelial cells, and mononuclear phagocytes to produce tissue factors, resulting in an imbalance of the coagulation system.[35] Previous study showed that the elevated levels of serum TNF-α were closely related to the pathophysiological process of PH.[36] And we present evidence for increased of serum TNF-α in APMTE rats. The results suggested TNF-α levels had important value for closely monitoring development of PH and anti-inflammatory therapies may help achieve positive outcomes in PH.

IL-1β has been reported can directly or indirectly lead to the injury of vascular endothelial cells through a variety of ways, and was one of the markers of inflammatory response.[37] The hypoxia caused by pulmonary embolism can separate NF-κB from IKB and activate NF-κB. And activated NF-κB can induce increased expression of IL-1β.[38,39] Previous study showed that IL-1β/IL-1R1/MyD88 signaling pathway was one of the important mechanisms for the formation of PH.[40] IL-1β can induce the synthesis of IL-1, IL-6, and TNF-α by activating myeloid differentiation protein 88 (My D88), which was involved in IL-1R1 and toll-like receptor signal transduction, and then activating NF-κB. It can promote the aggregation of inflammatory cells to the damaged part of the body tissue, promote the adhesion, deformation and migration of white blood cells, and cause inflammatory damage to vascular endothelial cells in the end. What's more, it played an important role in the proliferation of pulmonary smooth muscle cells and inflammatory damage. In addition, the present study revealed that the expression of IL-1β was closely related to the severity of thrombosis.[41] IL-1β can induce vascular endothelial cells to produce a lot of reactive oxygen species, induce vascular endothelial cells to secrete various cytokines including chemokines, selectin, adhesion molecules, and induce white blood cells, platelets to gather to vascular endothelial cells, which playing an important role in thrombosis. And we found sustained elevation of IL-1β and NF-κB levels in APMTE rats associated with injury of vascular endothelial cells and increased pulmonary artery pressure.

NF-κB activation effects of serum TNF-α and IL-1β levels in APMTE

In deeded, as an early transcription factor, NF-κB can rapidly respond to harmful stimulant of lung vascular cells and activate the NF-κB signaling pathway without the need to translate new proteins, thus to regulate gene expression. And the NF-κB pathway can be activated by

the metabolic products of bacterial infection and the activation of receptors on the surface of pulmonary vascular endothelial cells due to various causes. Previous studies had shown that TNF-α and IL-1β activated ERK and JNK MAPK through activated FAK/Pyk2, which mediated the expression of proinflammatory genes and the occurrence of vascular inflammation, and then activated the NF-κB pathway.[42] What's more, TNF-α and IL-1β can initiate the downstream NF-κB signaling pathway by binding to the receptors on the surface of vascular endothelial cells, and NF-κB dimer was further activated after various posttranslational modifications. In the nucleus, NF-κB united to the corresponding genes and regulated the expression of TNF-α and IL-1β, respectively promoted the expression of TNF-α and IL-1β, forming a positive feedback effect, which further magnified the inflammatory reaction and aggravated the injury of pulmonary vascular endothelial cells.

Limitation

Most of the data in this experiment were consistent with theory and expectations, but there were still some parameters that did not have suppositional performance. PAP measurement by transthoracic puncture is a new method combined with ECHO, which is not perfect and mature. Length and inner diameter of the puncture needle affected the measurement results. Even if this method was highly sensitive, it could only reflect the relative changes in the pressures of each group and failed to accurately measure the PAP under standard atmospheric pressure. Safer and more accurate pressure measurement methods are still worth further exploration. Secondarily, albeit the correlation between the expression level of NF-kB and the mPAP and the relationship between NF-kB and the expression level of TNF-α and IL-1β were demonstrated, data using the specific inhibitor for NF-kB was lack in this study. The metabolism of MAPK/NF-kB signaling in the pathogenesis of PH still unclear. More in-depth research is urgently needed.

Conclusion

In summary, this study implicates the activation of MAPK/NF-κB pathway as a critical driver of increasing TNF-α and IL-1β level in APMTE rats. We demonstrated for the first time that UK exerts protective effects against APMTE-induced PH may be related to the downregulation of the MAPK/NF-κB signaling pathway. Hence, inhibition of MAPK/NF-κB activation and thus controlling the inflammatory be a promising new therapeutic tool for treating PH.

AUTHOR CONTRIBUTIONS

Among the authors in the list, Ji Wu is as the teacher of drafting the article and revising it critically for important intellectual content. Yan fen Zhong and Binbin Liang mainly takes charge of writing and participate in all experimental sections. Xiaofeng Zhang and Decai Zeng paly a role in measuring pulmonary artery pressure by echocardiography-guided transthoracic puncture. Jingtao Li and Tongtong Huang are mainly responsible for animal model. After consultations, all the authors agreed with the arrangement of the names. All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data sets used and/or analyzed during the current study available from the corresponding author on reasonable request.

ETHICS STATEMENT

All animal procedures and protocols were approved by the Experimental Animal Ethics Committee of Guangxi Medical University (NO: 2022-KT-国基-175). All methods were carried out in accordance with relevant guidelines and regulations. This study was carried out in compliance with the ARRIVE guidelines. Institutional Animal Care and Use Committee at the Affiliated Hospital of Guangxi Medical University and were in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, 8th Edition, 2011).

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