BAY11-7082 inhibits the expression of tissue factor and plasminogen activator inhibitor-1 in type-II alveolar epithelial cells following TNF-α stimulation via the NF-κB pathway

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Abstract. Pulmonary inflammation strongly promotes alveolar hypercoagulation and fibrinolytic inhibition. NF-κB signaling regulates the expression of molecules associated with coagulation and fibrinolytic inhibition in type-II alveolar epithelial cells (AECII) stimulated by lipopolysaccharide. However, whether TNF-α-induced alveolar hypercoagulation and fibrinolysis inhibition is also associated with the NF-κB pathway remains to be determined. The aim of the present study was to determine whether BAY11-7082, an inhibitor of the NF-κB pathway, inhibits the expressions of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) in AECII in response to TNF-α. Rat AECII were treated with BAY11-7082 for 24 h and stimulated with TNF-α for 1 h. The expression of TF and PAI-1 were determined using western blotting and reverse transcription-quantitative PCR. The concentrations of TF and PAI-1 in culture supernatant were also measured by ELISA. Moreover, levels of NF-κB p65 (p65), phosphorylated (p)-p65 (p-p65), inhibitor of NF-κB α (IκBα) and p-IκBα were also evaluated. Immunofluorescence was used to detect p65 levels in cell nuclei. TNF-α significantly promoted TF and PAI-1 expression either at the mRNA or protein level in AECII cells. Concentrations of TF and PAI-1 in supernatant also significantly increased upon TNF-α stimulation. Furthermore, TNF-α upregulated the levels of p-IκBα, p65, and p-p65 in the cytoplasm. Immunofluorescence analysis indicated that TNF-α increased p65 translocation from the cytoplasm to the nucleus. However, AECII pre-treated with BAY11-7082 expressed lower levels of TF and PAI-1 following TNF-α treatment. Levels of p-IκBα, p65 and p-p65 in the cytoplasm also decreased, and translocation of p65 from cytoplasm into the nucleus was inhibited by BAY11-7082 pretreatment. These findings suggest that BAY11-7082 improves the hypercoagulation and fibrinolytic inhibition induced by TNF-α in alveolar epithelial cells via the NF-κB signaling pathway. BAY11-7082 might represent a therapeutic option for alveolar hypercoagulation and fibrinolytic inhibition in acute respiratory distress syndrome.

Introduction

Hypercoagulability and fibrinolytic inhibition, either systemic or in lung tissue, are important characteristics of acute respiratory distress syndrome (ARDS) (1-4). Both are associated with decreased lung compliance, diffusion dysfunction and imbalance of the ventilation/perfusion ratio, resulting in refractory hypoxemia, very small lung volume or even pulmonary fibrosis (3). It has been proposed that correcting coagulation defects would benefit critically-ill patients who develop lung injury (5). A previous study demonstrated that alveolar hypercoagulation and fibrinolytic inhibition are associated with pulmonary inflammation in ARDS (6). Pulmonary inflammation can damage pulmonary vascular endothelial cells and type-II alveolar epithelial cells (AECII), which further increases the expression of tissue factor (TF) and plasminogen activator inhibitor-1 (PAI-1) in these cells, thus activating the exogenous coagulation system. Moreover, activation of the coagulation system can in turn result in or aggravate pulmonary inflammatory responses (3,7).

NF-κB is a nuclear transcription factor that participates in several physiopathological processes, such as apoptosis, inflammation and various autoimmune diseases (8,9). Previous studies suggested that the NF-κB pathway could mediate hypercoagulation, fibrinolysis inhibition and pulmonary inflammation (10-13). It was suggested that AECII serves a pivotal role in alveolar hypercoagulation and fibrinolysis inhibition in ARDS (14). Our previous studies indicated that knockdown of the p65 gene or IKKβ inhibited lipopolysaccharide (LPS)-induced upregulation of TF and PAI-1 in rat AECII (15,16), indicating that the NF-κB signaling pathway participates in coagulation and fibrinolysis regulation in LPS-treated AECII cells. Given that pulmonary inflammation is a strong inducer for hypercoagulation and fibrinolytic inhibition and that NF-κB is also involved in the onset of

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inflammation in ARDS, it was hypothesized that the NF-κB signaling pathway is the mechanism underlying the cross-talk between hypercoagulation, fibrinolysis inhibition and pulmonary inflammation in ARDS. To confirm the present hypothesis, AECII were pre-treated with BAY11-7082, an inhibitor of IκBα (17), then stimulated with the pro-inflammatory factor TNF-α. The expressions of coagulants, such as TF, PAI-I and fibrinolysis inhibition factor, and activation of the NF-κB pathway were also determined in AECII.

Materials and methods

Cell culture. The RLE-6TN rat ACEII cell line was cultured in M199 medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Hyclone; Cytiva), penicillin (100 units/ml) and streptomycin (100 µg/ml) (Hyclone; Cytiva) at 37˚C with 5% CO_2. For experiments, cells in the control group received culture medium, whereas cells in the BAY group were treated BAY11-7082 (5 µM; purity: 99.73%; cat. no. 19542-67-7; MedChem Express) for 24 h. In the model group, cells were stimulated with TNF-α (10 ng/ml; Peprotech, Inc.) for 1 h. In the BAY + TNF group, cells were treated with BAY11-7082 for 24 h, then with TNF-α for 1 h.

Detection of BAY11-7082 cytotoxicity using cell counting Kit-8 (CCK-8). RLE-6TN cells were seeded into a 96-well plate ($5x10^3$ cells/well in 100 µl volume) and pre-incubated for 24 h at 37˚C with 5% CO_2. Different concentrations of BAY11-7082 were added into the wells (0, 1, 2, 3, 4 and 5 µM). PBS was used as a blank control. The cells were cultured for 24 h in the incubator, and CCK-8 reagent (Dojindo Molecular Technologies, Inc.) was added at 10 µl/well for 1 h. The absorbance was measured at a wavelength of 450 nm using a microplate reader.

Reverse transcription-quantitative (RT-q) PCR. The mRNA expression of p65, TF and PAI-1 were measured using RT-qPCR. GAPDH was used as an internal reference. Briefly, cells were collected, and total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA concentration was assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). The A260/A280 ratio of the extracted RNA was adjusted to 1.8-2.0, then RT was performed on 2 µg RNA with oligo (dT) primers in 20-µl reactions using the RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Primers were designed according to the sequence of the rat NF-κB p65 (*Rela*) gene in the National Center for Biotechnology Information database. The primers were synthesized by Shanghai Bioengineering Co., Ltd. (Table I). The reactions were set up as follows: 10 µl SYBR Green mix (Thermo Fisher Scientific, Inc.), 0.8 µl forward primer, 0.8 µl reverse primer, 0.8 µl cDNA template and 7.6 µl ddH_2O, for a total reaction volume of 20 µl. The entire reaction system was preheated at 95˚C for 10 min. qPCR was then performed using the following thermo-cycling procedure: 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec, for 40 cycles. After that, dissolution and amplification curves of the target gene were recorded following gene amplification. Specificity of the reaction was evaluated, and the Ct value was calculated according to the dissolution and amplification curve. Expressions of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct, target - Ct, GAPDH)_{sample} - (Ct, target - Ct, GAPDH)_{control}$ (18).

Western blotting. The levels of p65, phosphorylated (p)-p65, IκBα, p-IκBα, TF and PAI-1 were determined by western blot analysis. Following treatment with TNF-α for 1 h, the cells were washed with cold PBS. Total protein from the cells was extracted using RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd.). Protein concentration measured with a BCA assay kit according to the manufacturer's instructions. 10 µg of protein from each sample was resolved on 12% Tris-glycine gel using SDS-PAGE. Subsequently, protein bands were blotted into nitrocellulose membranes. After incubation for 3 h in blocking solution (5% skim milk powder diluted with TBST solution, containing 0.05% Tween-20) at room temperature, the membrane was incubated for 24 h with antibodies targeting p65 (1:1,000; cat. no. ab16502; Abcam), p-p65 (1:1,000; cat. no. ab86299; Abcam), IκBα (1:1,000; cat. no. ab32518; Abcam), TF (1:1,000; cat. no. ab151748; Abcam) and PAI-1 (1:1,000; cat. no. ab66705; Abcam) at 4˚C. The secondary antibody (horseradish peroxidase-conjugated goat ant-rabbit immuno-globulin; 1:5,000; cat. no. ZB-2301; OriGene Technologies, Inc.) was added and incubated with horseradish blocking solution for 1 h at room temperature using the membrane chemiluminescence detection system (EMD Millipore). Relative band densities were quantified using ImageJ software 1.4.3 (National Institutes of Health).

ELISA. Cell supernatants were harvested and stored at -80˚C. TF (Rat; cat. no. CSB-E07914r; Cusabio Technology LLC) and PAI-1 (Rat; cat. no. CSB-E07948r; Cusabio Technology LLC) levels in cell supernatants were determined using ELISA kits according to the manufacturer's instructions.

Immunofluorescence staining. Briefly, cells of each groups were fixed at room temperature with 4% formaldehyde in PBS for 30 min, permeabilised with 0.5% Triton X-100 for 30 min and blocked with 1% BSA (Beijing Solarbio Science & Technology Co., Ltd.) at room temperature for 30 min. After that, these cells were incubated with primary antibodies targeting rabbit anti-rat p65 (1:100; cat. no. ab16502; Abcam) overnight at 4˚C. Subsequently, they were incubated with FITC-labeled secondary antibodies (1:200; cat. no. ZF-0311; OriGene Technologies, Inc.) for 1 h at room temperature. Each step was followed with 5 min of washes in PBS, three times. The prepared specimens were counterstained with DAPI for 10 min at room temperature and observed with a fluorescence microscope (Carl Zeiss AG) and were captured under an original magnification of x20.

Statistical analysis. Data are presented as the mean ± SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

BAY11-7082 inhibits the expressions of TF and PAI-1 in TNF-α-stimulated AECII cells. To determine the effects of BAY11-7082 on TF and PAI-1 expression in AECII

Table I. Primer sequences.

Gene	Forward (5'-3')	Reverse (3'-5')	Size (bp)
p65	AGCAAGCCATTAGCC	ACCGCATTCAAGTCAT	91
TF	AATGGGCAGATAGAGTGT	TCTGATTGTGGGTTTGTA	182
PAI-1	ACCAACTTCGGAGTAAAA	TTGAATCCCATAGCATCT	158
GAPDH	CAAGTTCAACGGCACAG	CCAGTAGACTCCACGACAT	138

TF, tissue factor; PAI-1, plasminogen activator inhibitor 1.

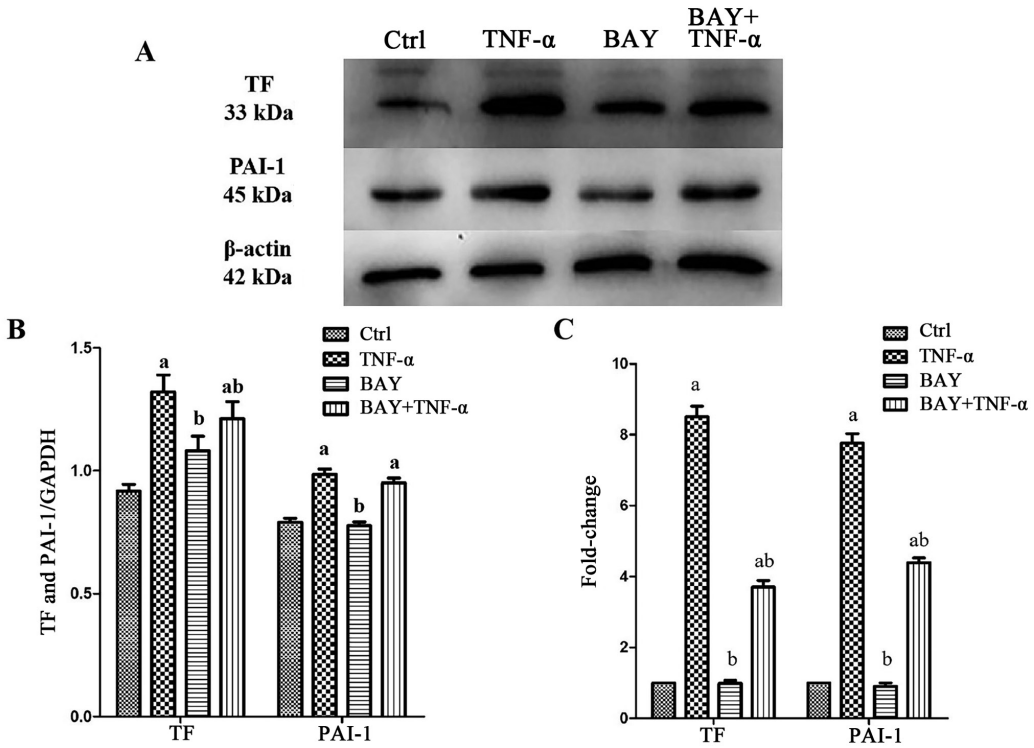

Figure 1. TF and PAI-1 expression in TNF-α-stimulated cells is reduced following BAY11-7082 treatment. (A and B) Western blot analysis of TF and PAI-1 levels after TNF-α-stimulation. Data are presented as the mean ± SEM. n=3. (C) Reverse transcription-quantitative PCR of TF and PAI-1 mRNA levels following TNF-α-stimulation. [a]P<0.05 vs. Ctrl. [b]P<0.05 vs. TNF-□. TF, tissue factor; PAI-1, plasminogen activator inhibitor 1; Ctrl, control; BAY, BAY11-7082.

following TNF-α stimulation, TF and PAI-1 mRNA and protein levels were determined using RT-qPCR and western blotting, respectively. The expressions of TF and PAI-1, both at the mRNA and the protein levels, was significantly upregulated in AECII after TNF-α stimulation compared with controls. However, the expression levels of TF and PAI-1 significantly decreased in the BAY and BAY + TNF-α groups (Fig. 1).

BAY11-7082 inhibits TF and PAI-1 secretion from TNF-α stimulated AECII. The levels of TF and PAI-1 were measured in cell supernatants using ELISA. The secretion of TF and PAI-1 significantly increased following TNF-α stimulation compared with controls. In the cells in the BAY group, this difference was not obvious. However, pretreatment with BAY11-7082 (i.e, BAY + TNF-α group), partially reduced the increase of TF and PAI-1 secretions induced by TNF-α stimulation, although they are significantly higher than the control group (Fig. 2).

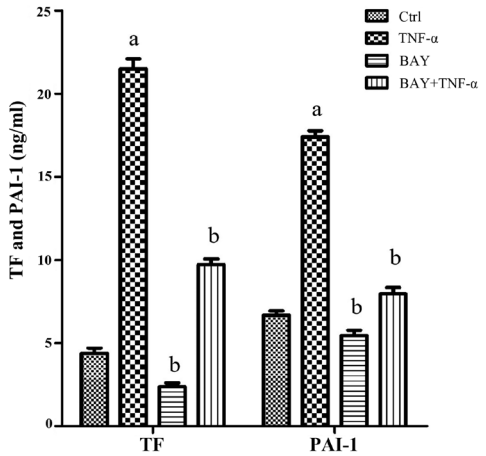

Figure 2. BAY11-7082 significantly inhibits TF and PAI-1 secretion in TNF-α-induced cells. ELISA results of TF and PAI-1 concentrations following 1-h TNF-α-stimulation. Data are presented as the mean ± standard error of the mean. [a]P<0.05 vs. ctrl. [b]P<0.05 vs. TNF-α. TF, tissue factor; PAI-1, plasminogen activator inhibitor 1; ctrl, control; BAY, BAY11-7082.

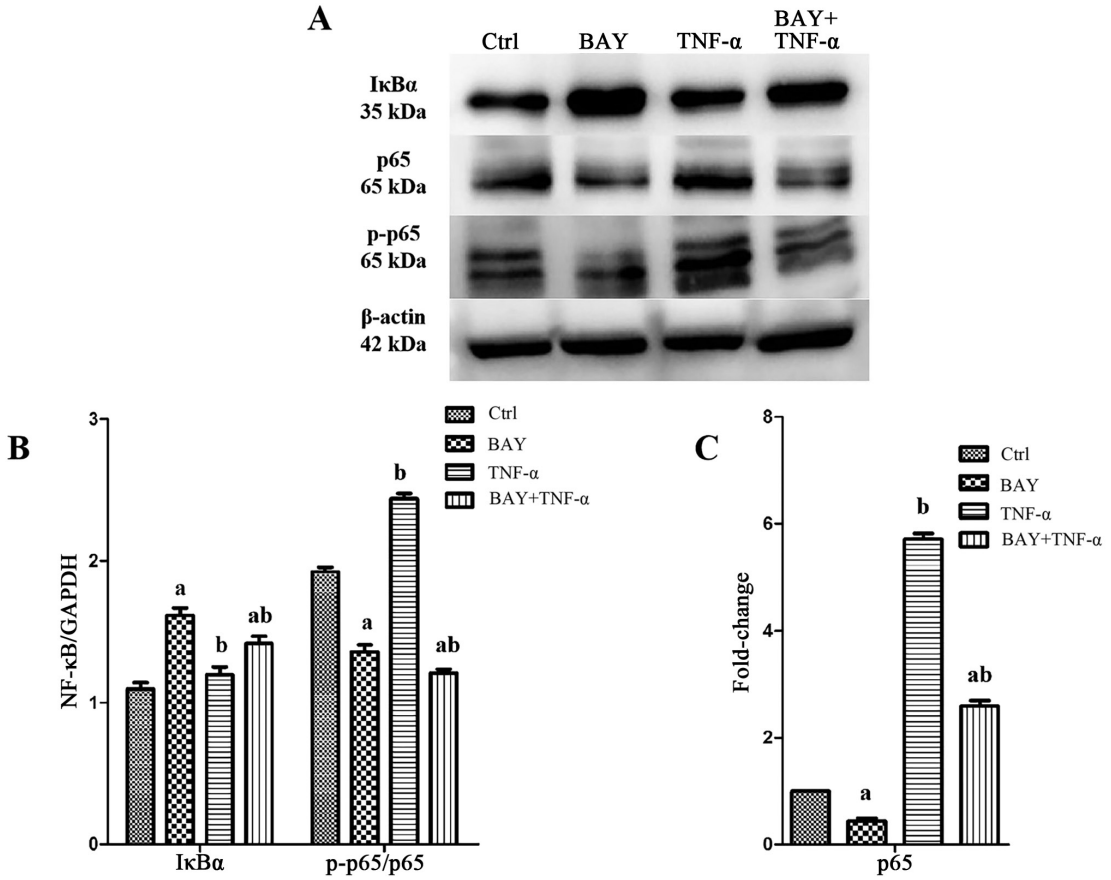

Figure 3. BAY11-7082 inhibits the NF-κB signaling pathway. (A) Western blot analysis and (B) quantification of the levels of IκBα, p65 and p-p65 after 1-h TNF-α stimulation. (C) p65 mRNA expression. Data are presented as the mean ± standard error of the mean. n=3. [a]P<0.05 vs. Ctrl. [b]P<0.05 vs. TNF-α. p-, phosphorylated; ctrl, control; BAY, BAY11-7082.

BAY11-7082 suppresses NF-κB pathway activation following TNF-α stimulation in AECII. TNF-α stimulation caused significant activation of the NF-κB pathway in AECII, as evidenced by the significantly increased ratio of p-p65/p65. However, BAY11-7082 pretreatment significantly inhibited p65 mRNA, decreased the p-p65/p65 ratio and increased the IκBα levels, indicating that the activation of NF-κB signaling pathway was inhibited by BAY11-7082 (Fig. 3).

BAY11-7082 blocks the translocation of p65 from the cytoplasm to the nucleus in TNF-α-stimulated AECII. Immunofluorescence staining was used to determine the cellular localization of p65. The results indicated that TNF-α stimulation resulted in increased green fluorescence staining in the nucleus, indicating an increase in translocation of p65 from the cytoplasm to the nucleus. In the cells in the BAY group, this difference was not obvious compared with cells in the ctrl group. However, in the cells that were pretreated with BAY11-7082 (the BAY + TNF-α group), the degree of green fluorescence staining of p65 in the nucleus of AECII was weakened, demonstrating that nuclear translocation of p65 was inhibited by BAY11-7082 pre-treatment (Fig. 4).

Discussion

AECII plays a pivotal role in the regulation of alveolar hypercoagulation and fibrinolytic inhibition through several important mediators, such as TF and PAI-1 (14,19-22). In the present study, following TNF-α stimulation, AECII either expressed or secreted large concentrations of TF and PAI-1, which was consistent with our previous studies on LPS stimulation (15,16).

TF is a key coagulation factor that initiates the exogenous coagulation pathway and plays an important role in regulating coagulation during ARDS (23). PAI-1 is a key factor regulating fibrinolysis inhibition (24). These two molecules were used as indicators of coagulation and fibrinolytic inhibition respectively in the present study. TF and PAI-1 were not only highly expressed in AECII cells, but also excessively secreted from AECII following TNF-α stimulation, indicating that TNF-α induced a state of hypercoagulation and fibrinolytic inhibition in AECII.

TNF-α is a potent mediator of the inflammatory response (25). Given the functional characteristics of TF and PAI-1, the present findings suggested that TNF-α induced a hypercoagulation and fibrinolysis inhibition mediated by AECII, which confirmed that inflammatory stimuli can provoke hypercoagulation and fibrinolysis inhibition in ARDS.

Preclinical and clinical studies have demonstrated abnormalities in coagulation and fibrinolytic activity in pulmonary tissue during ARDS (3,21). However, no satisfactory therapeutic drugs for alveolar hypercoagulation and fibrinolysis inhibition are available. This may be related to the crosstalk

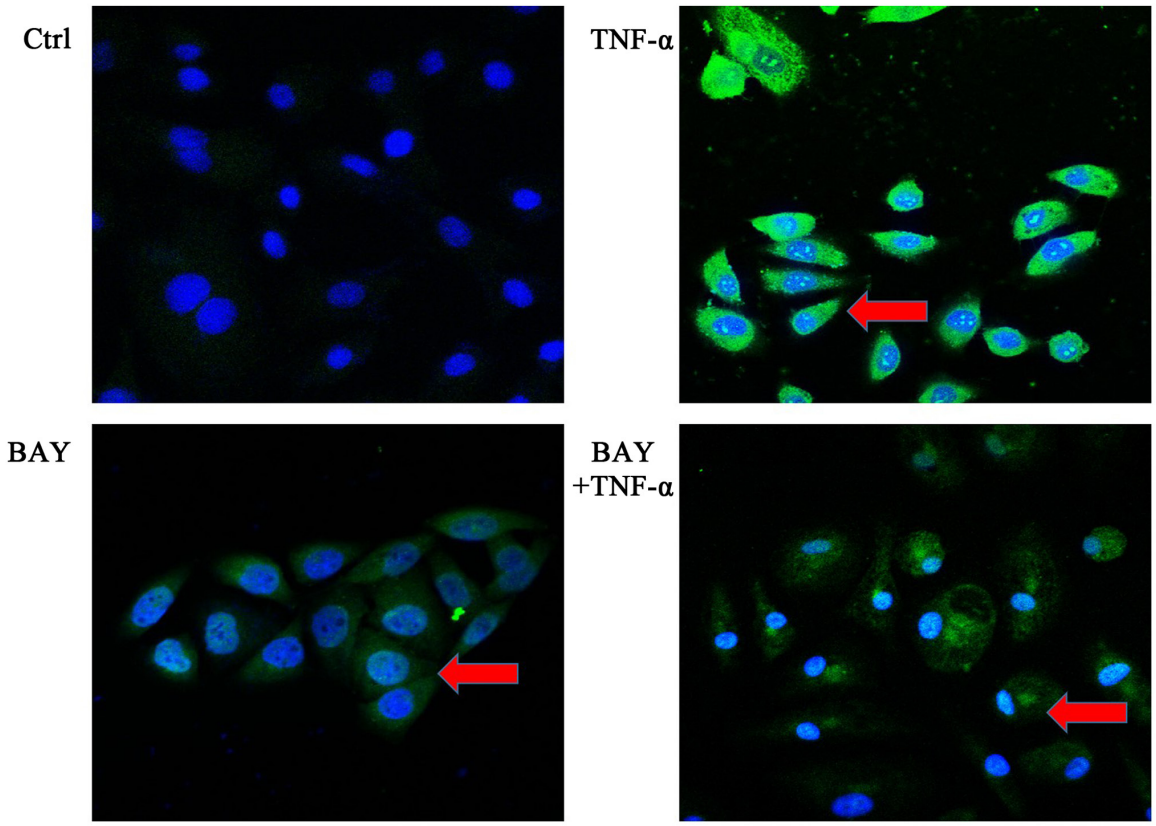

Figure 4. Treatment with BAY11-7082 inhibits the translocation of p65 from the cytoplasm to the nucleus following TNF-α stimulation. p65 localization was detected by immunofluorescence. p65 protein is detectable with green fluorescent protein marker and the nuclei were dyed blue with DAPI. p65 nuclear translocation is indicated by red arrows. Magnification, x400. ctrl, control; BAY, BAY11-7082.

between alveolar hypercoagulation, fibrinolysis inhibition and pulmonary inflammation, three pivotal characteristics in ARDS (26). As a result, treatments targeting coagulation or inflammation alone often do not achieve satisfactory therapeutic results. Thus, it is crucial to explore the mechanism simultaneously regulating coagulation/fibrinolysis inhibition and inflammation, which is the basis of effective treatment for ARDS. Given that the NF-κB pathway participates in alveolar hypercoagulation, fibrinolytic inhibition and in pulmonary inflammation (15,27), disrupting the NF-κB pathway may prove beneficial. The present study showed that BAY11-7082 significantly decreased TF and PAI-1 expression, as well as their production in cell culture supernatant following TNF-α stimulation in AECII. In addition, BAY11-7082 reduced the levels of p65 and p-p65 in the cytoplasm and prevented p65 nuclear translocation. Therefore, BAY11-7082 might attenuate TNFα-induced expression and secretion of TF and PAI-1 in AECII through NF-κB pathway inactivation. These research findings demonstrated that BAY11-7082 may prove potentially effective in ARDS treatment.

BAY11-7082 is a commonly used inhibitor of the NF-κB pathway, which irreversibly and specifically inhibits TNF-α-induced phosphorylation and degradation of IκBα (28), preventing NF-κB from entering the nucleus, thus inhibiting the transcription of target genes (29,30). IκBs are essential upstream regulatory molecules of the NF-κB pathway, bind to NF-κB p65 and maintain the pathway in an inactivated state. IκBs are activated by IκB kinase complex (IKKs) inducing degradation and dissociation of IκBs from p65 (31). IκBs consist of IκBα, IκBβ and IκBγ, among which IκBα is the key regulating molecule (32). In the present study, BAY11-7082 promoted the upregulation of IκBα in the cytoplasm, which was consistent with the hypothesis that BAY11-7082 inhibited IκBα phosphorylation and degradation, whereby this is the mechanism in which BAY11-7082 affects the NF-κB pathway. However, whether BAY11-7082 also inhibits TF and PAI-1 expression and secretion through other mechanisms remains to be determined.

The present study has its limitations. First, only one cell line and one NF-κB pathway inhibitor were used. Second, the inflammatory response caused by excessive coagulation and fibrinolysis inhibition was not evaluated in the present study. Third, only a single observation timepoint was used in the present study, resulting in a lack of dynamic results. Finally, the experiments were only performed at the cellular level, and the results of the present study still needs to be further verified using ARDS animal models in the future.

In summary, BAY11-7082 ameliorated the expression and secretion of TF and PAI-1 in AECII induced by TNF-α via NF-κB signaling pathway inactivation. BAY11-7082 is expected to be an effective therapeutic target in ARDS.

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Availability of data and materials

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

Authors' contributions

YC and BL performed the experiments, analyzed the data and wrote the manuscript. HQ, HY, YWa and YWu analyzed the data. FS designed the study, analyzed the data and organized the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments in the present study conformed to the Guide for the Care and Use of Laboratory Regulations and were approved by the Institutional Experiment Committee of Guizhou Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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