IKKβ regulates the expression of coagulation and fibrinolysis factors through the NF-κB canonical pathway in LPS-stimulated alveolar epithelial cells type II

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Abstract. Aim: Hypercoagulation and fibrinolysis inhibition in the alveolar cavity are important characteristics in acute respiratory distress syndrome (ARDS). Alveolar epithelial cells type II (AEC II) have been confirmed to have significant role in regulating alveolar hypercoagulation and fibrinolysis inhibition, but the mechanism is unknown. Nuclear factor-κB (NF-κB) signaling pathway has been demonstrated to participate in the pathogenesis of these two abnormalities in ARDS. The purpose of the present study is to explore whether controlling the upstream crucial factor IκB kinase (IKK)β could regulate coagulation and fibrinolysis factors in LPS-stimulated AEC II. Materials and methods: An IKKβ gene regulation model ($IKK\beta^{+/+}$ and $IKK\beta^{-/-}$) was prepared using lentiviral vector transfection. The models with wild type cells were all stimulated by lipopolysaccharide (LPS) or saline for 24 h. Expression of the related proteins were determined by western-blotting, ELISA and revere transcription-PCR respectively. Tissue factor (TF) procoagulant activity and nuclear p65 protein level were also detected. Results: IKKβ increased in $IKK\beta^{+/+}$ cells but decreased in $IKK\beta^{-/-}$ cells. LPS stimulation promoted the expression of p-IκBα, p65, p-p65 and p-IKKβ as well as TF and plasminogen activator inhibitor (PAI)-1, at the mRNA or protein level, and this was significantly enhanced by IKKβ upregulation but weakened by IKKβ downregulation. TF procoagulant activity presented the same changes as the molecules above. ELISAs showed additional increases in the concentrations of as thrombin antithrombin, procollagen III propeptide, thrombomodulin and PAI-1 in $IKK\beta^{+/+}$ cell supernatant under LPS stimulation, however they decreased in $IKK\beta^{-/-}$. The level of as antithrombin III however, appeared to show the opposite change to those other factors. Immunofluorescence demonstrated a greatly enhanced expression of p65 in the nucleus by IKKβ upregulation, which was reduced by IKKβ downregulation. Conclusions: IKKβ could regulate the expression and secretion of coagulation and fibrinolysis factors in LPS-stimulated AEC II via the NF-κB p65 signaling pathway. The IKKβ molecule is expected to be a new target for prevention of coagulation and fibrinolysis abnormalities in ARDS.

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Introduction

Acute respiratory distress syndrome (ARDS), a common cause of death in intensive care units (1,2), is a devastating clinical syndrome characterized by non-cardiogenic pulmonary edema, respiratory distress and hypoxemia (3-5). There are ~190,000 newly diagnosed ARDS cases in the United States each year (6). Although a great deal of progress has been made in ARDS management in previous years, the mortality rate caused by ARDS is still as high as 35-50% (7,8), while the survivors suffer from significant physical and psychological impairments (3-5).

Hypercoagulation and fibrinolysis inhibition, existing either systemically or locally, are important characteristics in the pathogenesis of ARDS (9,10). However abnormalities of coagulation and fibrinolysis are more obvious in the alveolar space than in systemic circulation, demonstrated by much higher levels of tissue factor (TF) and plasminogen activator inhibitor (PAI) in airspaces than in the blood, and the numerous fibrin deposits seen in the alveolar compartment in ARDS (11-14). Coagulation and fibrinolysis dysfunctions in local pulmonary tissue lead to reduced lung compliance, diffusion dysfunction, and disruption of V/Q ratios, resulting in refractory hypoxemia, very small lung volumes (baby lungs) and even pulmonary fibrosis. Lung epithelial cells have been confirmed to be the primary source of TF, contributing 60-70% of the total lung TF (15) and alveolar epithelial cells (AEC II) have been shown to express a large number of TF and PAI-1 when stimulated (16,17), indicating that AEC II has a pivotal role in regulating coagulation and fibrinolysis in airspaces, via expressing TF and PAI-1. However, until now, the specific regulatory mechanism of AEC II still remains to be elucidated.

Nuclear factor (NF)-κB is an evolutionarily conserved family of DNA binding proteins involved in transcriptional

regulation of a number of gene products, including inflammation and apoptosis. Under normal conditions, NF-κB is sequestered in the cytoplasm, bound by members of the IkB family of inhibitor proteins, which include IkBα, IkBβ and IkBε. IκB kinase (IKK) complex, containing IKKα, IKKβ and IKKγ, are important molecules that initiate the NF-κB cascade activation by promoting IκB phosphorylation (18) and liberating NF-κB from the combined state. NF-κB activation could be initiated by canonical and non-canonical pathway, among which the former is the main form, and IKKβ is the essential upstream signal in activation of NF-κB canonical pathway. Moreover, studies have shown that IKKβ is a major kinase that activates NF-κB activation induced by proinflammatory cytokines and IKKβ may be more important in NF-κB activation pathway than IKKα (19-21). The NF-κB pathway has been shown to play a key role in inflammatory processes, angiogenesis, immunity and apoptosis (22). In addition, previous studies *in vitro* or *in vivo* have demonstrated that the NF-κB pathway was also involved in regulating coagulation and fibrinolytic factors (23-26). Ding *et al* (27) reported that inhibiting Rho kinase (an upstream site of NF-κB signal pathway) significantly reduced the lung tissue inflammatory response and lung TF and PAI-1 levels by blocking the NF-κB pathway. Since IKKβ, just like Rho kinase is also an essential upstream molecule of NF-κB pathway, the present study speculated that adjusting IKKβ gene expression could impact the expression of coagulation and fibrinolysis factors in LPS-stimulated AEC II. To confirm this hypothesis, $IKK\beta^{+/+}$ and $IKK\beta^{-/-}$ AEC II models were first set up using lentiviral vector cell transfection and then observed whether coagulation, and fibrinolysis factors in LPS-stimulated AEC II would be changed during IKKβ gene up- or downregulation.

Materials and methods

Cell culture and LPS stimulation. The cell line used for lentivirus vector transfection in the experiment was the RLE-6TN cell line (The Cell Bank of Xiangya Medical College; ACE II cell line from rats). This cell line was grown in M199 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Hyclone; SH30070.03), penicillin (10,000 U/ml) and streptomycin (10,000 U/ml) (Hyclone; SV 30010). Cells were cultured in an incubator at 37˚C and 5% CO_2. The cells in the control group were not manipulated. The cells in short-hairpin (sh)-negative control (NC) group were infected using a negative control viral plasmid, while the cells in sh-IKKβ group were infected by IKKβ shRNA interference (20 μl of virus solution per well) (RNAi) virus. Cells in the NC group were infected by empty pcDNA3.1 virus (Hunan Fenghui Biotechnology Co., Ltd; 0 μl of virus solution per well) and cells in the IKKβ group were infected with the pcDNA3.1-IKKβ overexpression virus. Cells except the control group were all stimulated with LPS at a concentration of 50 μg/ml for 24 h.

Construction of $IKK\beta^{+/+}$ and $IKK\beta^{-/-}$ model by virus transfection. Based on the IKKβ gene (NM_053355), the shRNA sequence was designed (Table I) for the IKKβ gene and a negative control was designed that was verified by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to have no interference effect on other genes. Then based on the small interfering (si)RNA sequences, complementary single-stranded DNA was designed (Table II).

The lentivirus vector plasmids used in this study are pcDNA3.1+ and pLKO.1 (Tiangen Biotech Co., Ltd), among which the interference and overexpression sequence were constructed in pLKO.1 and in pcDNA3.1+ plasmid respectively. Plasmids were amplified in *Escherichia coli*., followed by the lentivirus packaging. RLE-6TN cells ($1x10^6$/well, 20 μl of virus solution per well) were infected with lentivirus which carried IKKβ-shRNA and IKKβ overexpression, by which the stable expression of sh-IKKβ and IKKβ overexpression was obtained. RLE-6TN cells infected by the virus alone was used as control.

Reverse transcription-quantitative (RT-q)PCR assay. The mRNA expression of IKKβ, p65, TF and PAI-1 was detected by qPCR. GAPDH was used as internal reference. Briefly, cells were collected after 48 h of virus transfection and total RNA was extracted using Trizol® (Takara Bio, Inc.; cat. no. 9108), and then the concentration of the total mRNA was assessed using the NanoDrop2000 Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). The A260/A280 ratio of the extracted RNA was adjusted to be 1.8-2.0, then reverse transcription 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.; K1622) according to the manufacturer's protocol. Primers were designed according to the sequence of IKKβ gene of rat in the NCBI gene database (https://www.ncbi.nlm.nih.gov/gene/84351). The primer sequences used were as follows: GADPH forward, 5'-GGGAAACCCATCACCATCTT-3' and reverse, 5'-CCAGTAGACTCCACGACATACT-3'; IKKβ forward, 5'-GTGACATAGCATCGGCTCTTAG-3' and reverse, 5'-CTCTCCTTGCTGTAGGACAATG-3'; NF-κB p65 forward, 5'-CATGCGTTTCCGTTACAAGTG-3' and reverse, 5'-CCCGTGTAGCCATTGATCTT-3'; TF forward, 5'-CCTCCAGGGAAAGCGTTTAAT-3' and reverse, 5'-GTGTAGGTATAGTTGGTGGGTTTC-3'; PAI-1 forward, 5'-GCCACCAACTTCGGAGTAAA-3' and reverse, 5'-GTAGGGAGAGAAGACCACATTTC-3'. PCR amplification was performed using the cDNA as template. The temperature protocol was as follows: 95˚C for 10 min, heating for 95˚C for 5 sec, 60˚C for 1 min for 40 cycles, 95˚C for 15 sec, 60˚C for 1 min and 95˚C for 15 sec. The reaction system was set up as follows: SYBR Green Mix (cat. no. RR820A; Takara Bio, Inc.) 10 μl, forward primer and reverse primer 0.4 μl respectively, cDNA template 2 μl, ddH_2O 7.2 μl, which were made up into a system containing 20 μl reagents. The dissolution and amplification curve of the genes were recorded following the gene amplification. The specificity of the reaction was evaluated and the Cq value was calculated according to the dissolution and amplification curve, respectively. Expression of target genes was calculated using the $2^{-\Delta\Delta Cq}$ method, $\Delta\Delta Cq=(Cq,_{target}-Cq,_{GAPDH})$ sample-$(Cq,_{target}-Cq,_{GAPDH})$ control (28).

Western blotting. After being transfected with virus and then being treated with LPS for 24 h, the cells were washed with cold PBS. The total protein was extracted with RIPA (Hunan Fenghui Biotechnology Co., Ltd). Briefly, concentration of

Table I. The short hairpin RNA sequence for the IKKβ gene and the sequence of negative control.

Negative	5'-GCCTTATTTCTATCTTACGtt-3'
siRNA	5'-GCACAATCAGGTGACAGGTtt-3'

Si, small interfering.

Table II. The shRNA sequence for the complementary single-stranded DNA.

IKKβ	F:5'-GTACCTCGCACAATCAGGTGACAGGTTC AAGAGACCTGTCACCTGATTGTGCTTTTTGG AAA-3'
	R-5'AGCTTTTCCAAAAAGCACAATCAGGTGA CAGGTCTCTTGAACCTGTCACCTGATTGTGC GAG-3'
NC	F: 5'-GTACCTCGCCTTATTTCTATCTTACGTCA AGAGCGTAAGATAGAAATAAGGCTTTTTGG AAA-3'
	R:5'-AGCTTTTCCAAAAAGCCTTATTTCTATCT TACGCTCTTGACGTAAGATAGAAATAAGGC GAG-3'

NC, negative control.

protein was measured with a BCA assay kit according to the manufacturer's protocol. An equal amount of protein (30 mg of the protein solution) from each sample was resolved in Tris-glycine 10% SDS-PAGE. Protein bands were blotted onto nitrocellulose membranes. At the end of the membrane transferring, the membrane was soaked from the bottom to the top with TBS, and then transferred to a dish containing the blocking solution (blocking solution: 5% skim milk powder diluted with TBST solution), and shaken for 3 h at room temperature on a shaker.

The membrane was incubated for 24 h with the antibody of rabbit anti-rat IKKβ (1:1,000; cat. no. ab124957; Abcam), p-IKKβ (1:1,000; cat. no. ab194519; Abcam), 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), p-IκBα (Cell Signaling Technology, Inc; 1:1,000; cat. no. 9241; 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 immunoglobulin; 1:5,000; cat. no. ZB-2301; ZSGB-BIO) was added and incubated with horseradish blocking solution for 10 min at room temperature, the membrane chemiluminescence detection system (EMD Millipore). Relative band densities were quantified by Image J software 1.4.3 (National Institutes of Health).

Detection of TF procoagulant activity. TF procoagulant activity was performed using split RLE-6TN cells for a one-step recalcification clot time assay (29).

ELISA assay. Cell supernatants were harvested and stored at -80˚C. Thrombin antithrombin (TAT) (Cusabio Biotech Co., Ltd; cat. no. CSB-E08432r), antithrombin III (ATIII) (Cusabio Biotech Co., Ltd; cat. no. CSB-E13885r), procollagen III propeptide (PIIIP) (Cusabio Biotech Co., Ltd; cat. no. CSB-E08096r), thrombomodulin (TM) (Cusabio Biotech Co., Ltd; cat. no. CSB-E07939r) and PAI-1 (Cloud-Clone Corp; cat. no. SEA532Ra) levels in cell supernatants were determined by ELISA according to the manufacturer's protocol.

Immunofluorescence. Briefly, cell each group was fixed at room temperature with 4% formaldehyde in PBS for the first 30-min and then permeabilized with 0.5% Triton X-100 for another 30-min, followed by a third blocking step of 30 min with 1% bovine serum albumin. After that, these cells were incubated with primary rabbit antibody against rat p65 and IKKβ (1:100; cat. no. ab16502; Abcam) overnight at 4˚C. And next, they were incubated with fluorescein isothiocyanate-labeled secondary antibody (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 expressed as mean ± standard deviation. Statistical significance was determined using one-way analysis of variance (ANOVA) and Student-Newman-Keuls method (SPSS 17.0; SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

$IKK\beta^{+/+}$ and $IKK\beta^{-/-}$ cell models are replicated successfully by virus transfection. The IKKβ gene overexpression and low expression models were set up by the virus transfection technique. The protein and mRNA levels of IKKβ were screened to verify the success of the transfection. Expression of mRNA and protein in $IKK\beta^{+/+}$ cells were significantly increased, and the expression in $IKK\beta^{-/-}$ cells were significantly decreased in wild type (WT) cells, respectively ($P<0.05$; Fig. 1), indicating the success of transfection.

Genetic changes in IKKβ can affect the expression of TF and PAI-1 in LPS-stimulated AEC II. To observe the impact of IKKβ gene level on expression of TF and PAI-1 in condition of LPS injury, cells were stimulated with different levels of the IKKβ gene using LPS for 24 h. Results showed that expression of TF and PAI-1, either in mRNA or in protein, were all significantly upregulated in WT cell following 24-h of LPS stimulation ($P<0.05$). In $IKK\beta^{+/+}$ cells, however, the expression was further enhanced by LPS stimulation, while TF and PAI-1 expression in $IKK\beta^{-/-}$ cells was inhibited in spite of LPS stimulation (Fig. 2).

Different IKKβ gene results in different secretions of ATIII, TAT, PAI-1, TM and PIIIP from LPS-stimulated AEC II. To further determine the secretion of coagulation and fibrinolysis related molecules from LPS-treated cells with

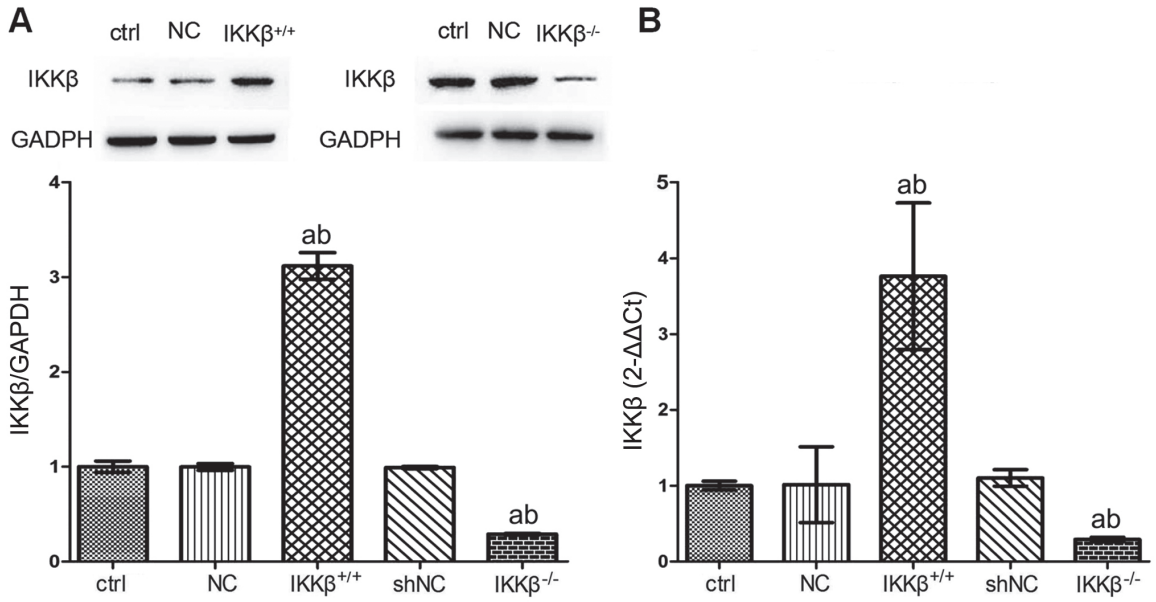

Figure 1. IKKβ gene overexpression and low expression cell models were successfully set up by the virus transfection technique. (A) Western blotting was used to detect the expression of IKKβ protein in cells and GAPDH was used as an internal reference for protein. (B) Reverse transcription-quantitative-PCR analysis of the expression of IKKβ mRNA in cells. The blank was set to normalize against the control. Each bar represents the mean ± standard deviation of 3 groups of cells. [a]P<0.05 vs. the ctrl. [b]P<0.05 vs. LPS. LPS, lipopolysaccharide; AEC II, alveolar epithelial cell type II; ctrl, control; NF, nuclear factor; NC, negative control; sh, short hairpin.

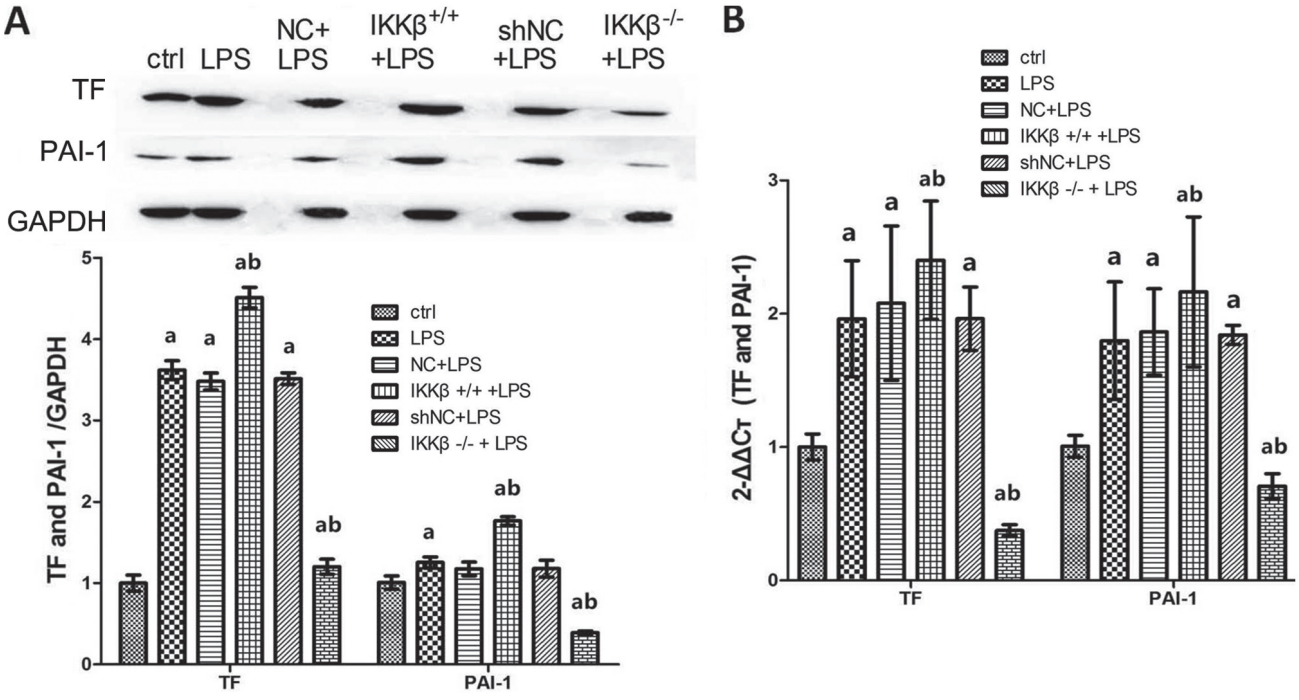

Figure 2. Over-expression of IKKβ promotes, low-expression of IKKβ inhibits the expression of TF and PAI-1 in LPS-stimulated AECII. (A) Western blotting was performed to measure TF and PAI-1protein expression in cells, GAPDH was used as an internal control for protein reference. Some bands appear obscured as some unnecessary groups, which were added at the beginning of the experiment, were removed. (B) Reverse transcription-quantitative-PCR was performed to analyze expression TF and PAI-1 mRNA in cells. Each bar represents the mean ± standard deviation of 3 groups of cells. [a]P<0.05 vs. ctrl. [b]P<0.05 vs. LPS. LPS, lipopolysaccharide; AEC II, alveolar epithelial cell type II; ctrl, control; TF, tissue factor; PAI-1, plasminogen activator inhibitor; NC, negative control; sh, shorthairpin.

different IKKβ gene levels, the concentrations of ATIII, TAT, TM, PIIIP and PAI-1 were measured. The results of the present study showed that LPS induction significantly promoted secretions of TAT, TM, PIIIP and PAI-1 from WT cell compared with WT cells induced by saline (P<0.05). More secretions of these molecules were obtained from IKKβ$^{+/+}$ cells under LPS treatment, but secretions of TAT, TM, PIIIP and PAI-1 in IKKβ$^{-/-}$ cells decreased under LPS stimulation compared with WT cells as well as IKKβ$^{+/+}$cells. The ATIII level, however, demonstrated the opposite change

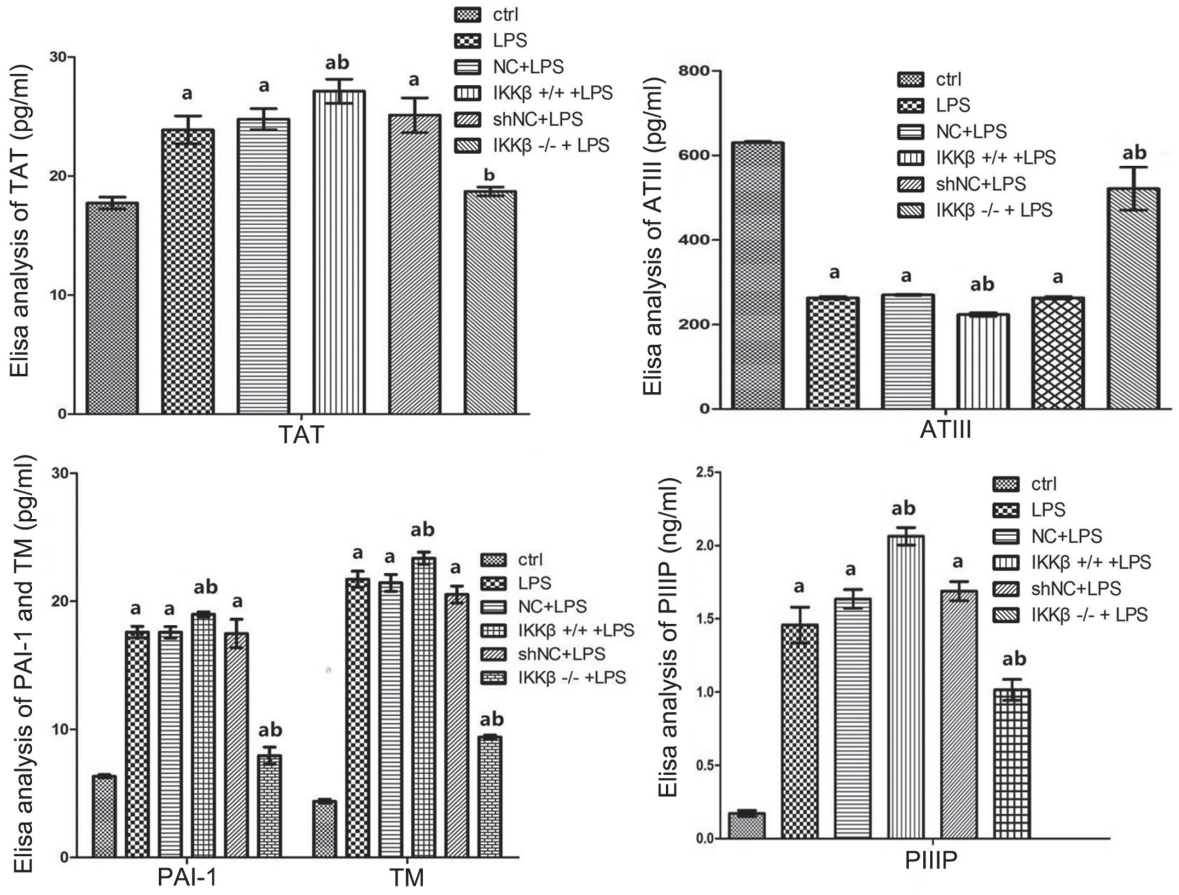

Figure 3. Over-expression of IKKβ promotes secretions of TAT, TM, PIIIP and PAI-1, and inhibits ATIII production, while low-expression has inverse effects on these molecules from LPS-stimulated AEC II. Bar graphs of concentrations about the indicators above as assessed by enzyme-linked immunosorbent assays in cell-supernatant of each group. Values are presented as the mean ± standard deviation; [a]P<0.05 vs. the ctrl, [b]P<0.05 vs. the LPS group as determined by one-way analysis of variance. TAT, thrombin-antithrombin; ATIII, antithrombin III; TM, thrombomodulin; PIIIP, procollagen III N terminal peptide; ctrl, control; AEC II, alveolar epithelial cell type II; LPS, lipopolysaccharide; NC, negative control; sh, short hairpin.

when compared with changes of TAT, TM, PIIIP and PAI-1 (Fig. 3).

IKKβ gene level impacts LPS-induces TF procoagulant activity (TF-PCA) in AEC II. TF-PCA in LPS-stimulated AEC II was examined using ELISA. The results showed that LPS stimulation resulted in increased TF-PCA in WT cells and TF-PCA was further enhanced in IKKβ$^{+/+}$ cells, and but was significantly inhibited in IKKβ$^{-/-}$ cells as compared with in WT cells (P<0.05; Fig. 4).

Conditional up-/downregulation of IKKβ gene affects the NF-κB canonical signaling pathway in AEC II. To explore the mechanism by which the IKKβ gene impacts coagulation and fibrinolysis related molecules in AEC II under the condition of LPS treatment, the role of IKKβ gene on certain important molecules in the NF-κB canonical signaling pathway in LPS-induced AEC II was observed. The results demonstrated that conditional IKKβ gene upregulation significantly improved expression of p65, p-p65, IkB and p-IkB induced by LPS (P<0.05). However, the expression of these molecules were significantly inhibited in LPS-treated AEC II if IKKβ gene was downregulated beforehand (P<0.05; Fig. 5).

The present study also determined the translocation ability of p65 from the cytoplasm into the nucleus using an

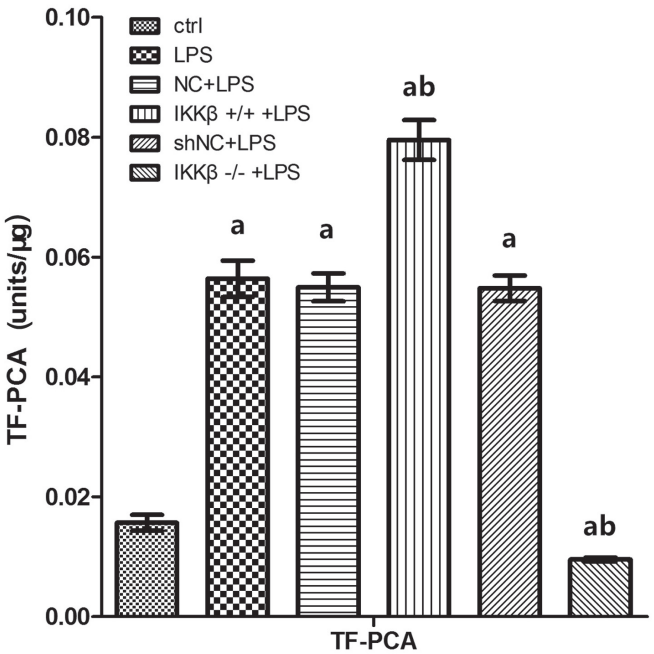

Figure 4. Over-expression of IKKβ enhances and low-expression of IKKβ inhibits TF-PCA in LPS-induced AEC II. Values are presented as the mean ± standard deviation; [a]P<0.05 vs. ctrl, [b]P<0.05 vs. the LPS group as determined by one-way analysis of variance. LPS, lipopolysaccharide; AEC II, alveolar epithelial cell type II; TF-PCA, tissue factor-procoagulant activity.

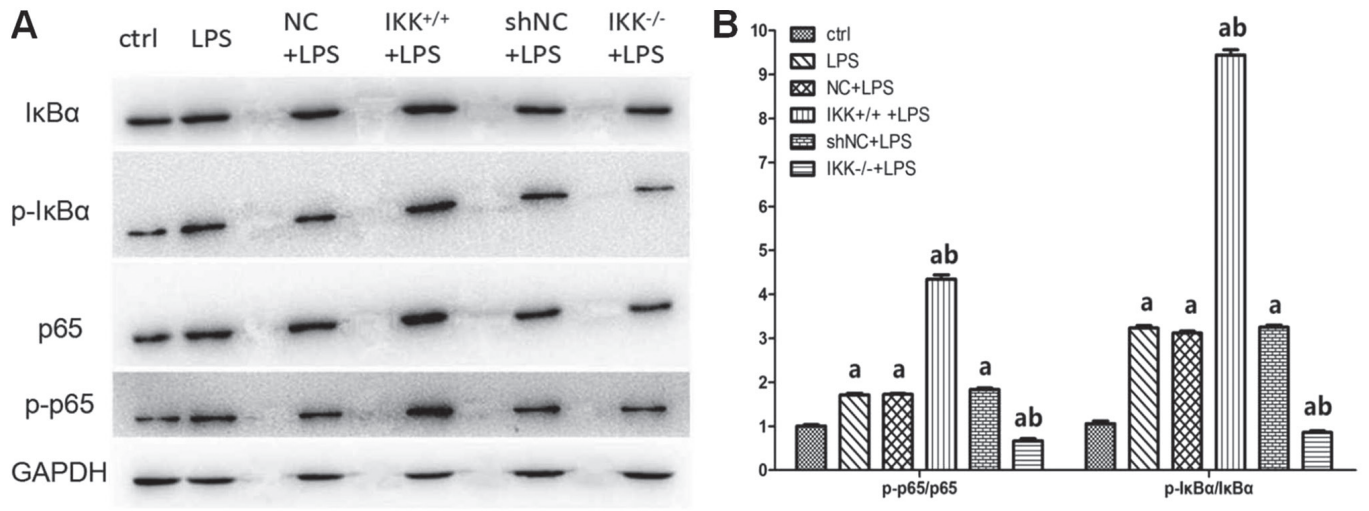

Figure 5. Western blotting is performed to measure p-p65/p65 and p-IκBα/IκBα protein expression in cells. (A and B) Over-expression of IKKβ activates and low-expression of IKKβ inhibits the canonical nuclear factor-κB signaling pathway in LPS-stimulated AEC II. Some bands appear obscured as some unnecessary groups, which were added at the beginning of the experiment, were removed. Each bar represents the mean ± standard deviation of 6 groups of cells. [a]P<0.05 vs. the ctrl. [b]P<0.05 vs. LPS. LPS, lipopolysaccharide; AEC II, alveolar epithelial cell type II; ctrl, control; p, phosphorylated; NC, negative control; sh, short hairpin.

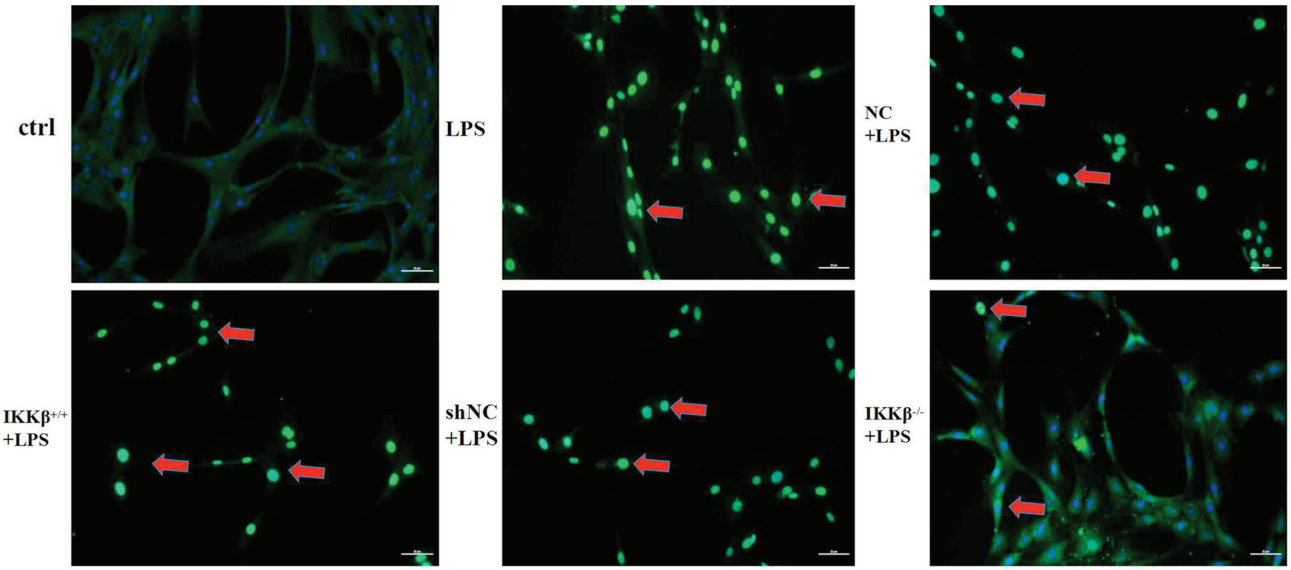

Figure 6. IKKβ overexpression increases, while IKKβ low-expression inhibits the translocation of p65 from cytoplasm to nucleus initiated by LPS stimulation. The p65 protein was marked with a green fluorescent protein marker and the nucleus was dyed blue with DAPI, p65 localization was detected by immunofluorescence. p65 nuclear translocations are indicated with red arrows. Original magnification: x200. Comparisons among the six groups were made based on the statistical analysis of the cells with nuclear localization of p65 counted in three random fields. LPS, lipopolysaccharide; AEC II, alveolar epithelial cell type II; ctrl, control; NC, negative control.

immunofluorescence assay in the LPS-stimulation state. The staining results indicated that LPS stimulation resulted in a marked increase of p65 fluorescent staining in nucleus, indicating enhanced p65 translocation from the cytoplasm into the nucleus. p65 fluorescent staining was seen to have an additional enhancement in IKKβ$^{+/+}$ cells but it was weakened in IKKβ$^{-/-}$ cells with LPS stimulation (Fig. 6).

Discussion

In biological research, the virus has become an effective carrier for delivering DNA or RNA into cells. Lentiviruses can efficiently introduce the target gene or RNAi into human or animal primary cells or cell lines, so that conditional up-/downregulation of the target gene could be set up (30,31). In the present study, up-/downregulation of IKKβ expression was achieved through lentivirus transfection therapy. The results of the present study showed that, as compared with WT cell, a higher IKKβ and a lower IKKβ gene expression were achieved by target gene or RNAi transfection respectively, indicating that the IKKβ$^{+/+}$ and IKKβ$^{-/-}$ cell models were successfully set up (32).

Previous studies have shown that the hypercoagulability and fibrinolysis inhibition in the alveolar space in ARDS is more prominent than in the systemic vascular network because the TF levels in the bronchoalveolar lavage fluid of patients

with ARDS are significantly increased compared with in the plasma (9,10,32,33). Since TF is a key coagulation factor that initiates the extrinsic coagulation pathway, it plays an important regulatory role in abnormal coagulation of ARDS (34), while PAI-1 is a key factor regulating fibrinolysis inhibition (35,36). Therefore, TF and PAI-1 were respectively selected as the coagulation and fibrinolysis factor in this experiment. The present data showed that both TF and PAI-1 are highly expressed in WT rat AEC II under LPS stimulation, either at the mRNA or protein level, which indicates the dysfunction in coagulation and fibrinolysis under LPS stimulation.

In addition to TF and PAI-1, there are other factors associated with coagulation and fibrinolysis, such as TAT, ATIII, PIIIP and TM. TAT, a complex of thrombin and anti-thrombin, reflects coagulation state, and ATIII is an anticoagulant substance in the body. TM is a glycoprotein and has anticoagulation characteristics, whose increase indicates a hypercoagulable state. PIIIP is an important marker of tissue fibrosis and a high level of PIIIP is often associated with inhibition of fibrinolysis. The results of the present study demonstrated that WT cells secreted a larger amount of TAT, TM and PIIIP and PAI-1, but just produced a little of ATIII after being induced by LPS compared with being treated by saline. Combined with the expression changes of TF and PAI-1, all the changes of these factors indicated hypercoagulation and fibrinolysis inhibition mediated by LPS-stimulated AEC IIs.

IKKβ is a crucial molecule located at upstream of the NF-κB canonical signaling pathway which was thought to be involved in regulating coagulation and fibrinolysis in ARDS (23-26), and AEC II contributes to most of alveolar coagulation and fibrinolysis abnormalities by expressing some important factors such as TF and PAI-1 (16,17,36,37), so the present study speculated that IKKβ would adjust the expression of coagulation and fibrinolysis factors. To test this hypothesis, $IKK\beta^{+/+}$ and $IKK\beta^{-/-}$ AEC cell models were set up first, and then the role of IKKβ on these factors was explored. The results of the present study demonstrated that under LPS stimulation, overexpression of IKKβ ($IKK\beta^{+/+}$ cell) not only boosted TF and PAI-1 expression, but also promoted the secretion of TAT, TM, PIIIP and PAI-1, and inhibited ATIII production from the LPS treated AEC II. When IKKβ was downregulated ($IKK\beta^{-/-}$ cells), however, the expression level or amount of production of all these factors was completely reversed. From the results of the present study, it is reasonable to think that IKKβ was involved in the regulation of AEC II-mediated coagulation and fibrinolysis abnormalities in some pathological circumstances such as ARDS.

The NF-κB cascade mainly comprises two divergent signaling pathways; the classical canonical pathway and non-canonical pathway (38). In the canonical pathway of NF-κB, stimuli such as LPS stimulates the IκB kinase (IKKα, IKKβ and IKKγ) and activation of IKKs results in phosphorylation of the IκBs proteins and degradation of IκBs follows. Degradation of IκBs proteins reveals nuclear localization sequences of NF-κB, leading to the rapid translocation of active NF-κB p65 into the nucleus, where they bind to κB binding sites in the promoters of target genes, promoting the transcription of these genes. In an alternative pathway of NF-κB, the non-canonical pathway however, it is p100 rather than p65 that is activated (38), although IKKβ is also involved in the regulation. The figures from the present study showed that the expression of IκB, p-IκB, p65 and p-p65 changed with the variation of IKKβ gene level under LPS treatment. Furthermore, conditional up- or downregulation of IKKβ could enhance or inhibit p65 translocation from cytoplasm into nucleus, demonstrated by p65 fluorescent staining in the nucleus. Therefore, it is estimated that NF-κB canonical cascade is at least one of the mechanisms by which IKKβ regulates procoagulant and fibrinolytic inhibitory factors in LPS-stimulated AEC II.

There are some limitations in the present experiment. First, the condition of this experiment *in vitro* is relatively simple as a single cell is assessed and not an entire body, and it does not necessarily represent the real situation in the whole body. Second, because of possible toxicity of the virus and the reagent itself, the technology used could not be used in a whole body study. Finally, LPS is just one of the causes of ARDS (bacterial infection), so these results do not stand for ARDS caused by other causes such as aspiration or severe acute pancreatitis.

In conclusion, the present study has demonstrated that upregulation of IKKβ enhanced hypercoagulation and fibrinolysis inhibition, while conditional deletion of IKKβ has a completely reversed role on coagulation and fibrinolysis related factors in LPS-stimulated AEC II. The NF-κB canonical signaling pathway is at least one of the mechanisms by which IKKβ impacts the coagulation and fibrinolysis function in LPS-induced AEC II. IKKβ is expected to be a new target for prevention of coagulation and fibrinolysis abnormalities in ARDS.

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Availability of data and materials

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

Authors' contributions

BL performed the whole experimental operation, finished the statistical analysis and wrote the primary manuscript. YWa, YWu, YC, HQ and HY were mainly responsible for the data collection. FS conceived the whole design of the study, analyzed the data and organized the final manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The current study was approved by the Ethics Committee of Animal and Cell Laboratory of Guizhou Medical University.

Patient consent for publication

Not applicable.

Conflicts of interest

The authors declare no conflict of interest.

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