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) could regulate coagulation and fibrinolysis factors in LPS-stimulated AEC II. Materials and methods: An IKKβ gene regulation model (IKKβwt and IKKβ−/−) 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 IKKβwt cells but decreased in IKKβ−/− cells. LPS stimulation promoted the expression of p-1κBα, 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 IKKβwt cell supernatant under LPS stimulation, however they decreased in IKKβ−/−. 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.

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 IκB family of inhibitor proteins, which include IκBα, IκBβ and IκBε. IκB kinase (IKK) complex, containing IκKα, IκKβ and IκKγ, 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 IκKβ is the essential upstream signal in activation of NF-κB canonical pathway. Moreover, studies have shown that IκKβ is a major kinase that activates NF-κB activation induced by proinflammatory cytokines and IκKβ may be more important in NF-κB activation pathway than IκKα (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).

Construction of IKKβ short-hairpin (sh)-negative control (NC) group. The cells in sh-IKKβ were infected using a negative control viral plasmid, while the cells in sh-IKKβ were infected with lentivirus which carried IκKβ-shRNA and IκKβ overexpression, by which the stable expression of sh-IKKβ and IκKβ overexpression was obtained. RLE-6TNC cells infected by the virus alone was used as control.

Reverse transcription-quantitative (RT-q)PCR assay. The mRNA expression of IκKβ, 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 IκKβ gene of rat in the NCBI database (https://www.ncbi.nlm.nih.gov/gene/84351). The primer sequences were as follows: GADPH forward, 5'-GGG AAA CCC ATC CCA CTT TAT G-3', and reverse, 5'-GGT GGT TTT AAC CAG GTC C-3'; IκKβ forward, 5'-CCG CAC ATC GGC AGT GGG CTT TGG-3' and reverse, 5'-CCG CAC ATC GGC AGT GGG CTT TGG-3'; TF forward, 5'-CCG CAC ATC GGC AGT GGG CTT TGG-3' and reverse, 5'-CCG CAC ATC GGC AGT GGG CTT TGG-3'; PAI-1 forward, 5'-CCG CAC ATC GGC AGT GGG CTT TGG-3' and reverse, 5'-CCG CAC ATC GGC AGT GGG CTT TGG-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, ddH2O 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-ΔΔCq method, ΔΔCq=(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.

| siRNA     | Sequence                        |
|-----------|---------------------------------|
| Negative  | 5'-GCCTTTATTTCTATCTACGGtty-3'   |
| siRNA     | 5'-GCACAATCAGGTGACAGGTtt-3'     |

Si, small interfering.

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

| IKKβ   | F: 5'-GTACCTCGCAAAATCACAGGTGACAGGTTC AAGAGCCTGTCACCTGTAGTTGCTTTTTGG AAA-3' |
|--------|---------------------------------------------------------------|
| NC     | F: 5'-GTACCTCGCCTTTATTTCTATCTACGGCA AGAGCGTAAGATGAAATAAAGGGTTTTTGG AAA-3' |
|        | R: 5'-AGCTTTTCCAAAAAGCACAATCAGGGTA CAGGTCTTTGAACTCTGATTGTGCG 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 μg 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), IkBα (1:1,000; cat. no. ab32518; Abcam), p-IkBα (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), procoag- lagen 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

IKKβ+/+ and IKKβ−/− 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 IKKβ−/− cells were significantly increased, and the expression in IKKβ+/+ 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 IKKβ−/− cells, however, the expression was further enhanced by LPS stimulation, while TF and PAI-1 expression in IKKβ+/+ 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
different IKKβ gene levels, the concentrations of ATIII, TAT, TM, PIIP, and PAI-1 were measured. The results of the present study showed that LPS induction significantly promoted secretions of TAT, TM, PIIP, 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, PIIP, 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.
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, IκB and p-IκB 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
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, PIHP 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. PIHP is an important marker of tissue fibrosis and a high level of PIHP 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 PIHP 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, IKKβ+/− and IKKβ−/− 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 (IKKβ−/− cell) not only boosted TF and PAI-1 expression, but also promoted the secretion of TAT, TM, PIHP and PAI-1, and inhibited ATIII production from the LPS treated AEC II. When IKKβ was downregulated (IKKβ−/− 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-ΙκB, 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 present 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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