IKKε deficiency inhibits acute lung injury following renal ischemia reperfusion injury

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Abstract. Renal ischemia reperfusion injury (IRI) after surgery may promote acute lung injury (ALI) by inducing an inflammatory response. However, the underlying molecular mechanism is still unclear. Studies have reported that inhibitor of κB kinase (IKK)ε primarily regulates inflammation and cell proliferation. The present study aimed to investigate the regulatory role of IKKε in the lung and renal IRI in mice, in order to provide an experimental basis for preventing ALI following surgery-induced renal IRI. C57BL/6J wild-type (WT) and IKKε knockout (IKKε−/−) mice underwent bilateral renal pedicle occlusion. The plasma creatinine concentration, urea nitrogen level and lung wet-to-dry ratio were measured at baseline, and at 24 and 48 h after declamping. The histological localization and protein levels of inflammatory factors, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-10, were analyzed in lung tissues. Subsequently, the interactions between IKKε and components of the nuclear factor (NF)-κB pathway were studied. The results of the present study demonstrated that the IKKε−/− groups displayed similar renal function but less pulmonary edema compared with that of the WT groups. The levels of proinflammatory factors in the lungs were significantly upregulated in WT mice compared with those in IKKε−/− mice after IRI surgery. The NF-κB pathway components and downstream factors were substantially upregulated in the WT groups after acute ischemic kidney injury, and these effects were significantly inhibited in the IKKε−/− groups. Based on these data, the present study hypothesized that IKKε may serve a negative role in kidney-lung crosstalk after renal IRI and may be a novel target for the treatment of patients with renal IRI.

Introduction

Acute kidney injury (AKI) is a frequent complication after cardiac surgery and abdominal aortic aneurysm repair (1,2). However, ~50% of AKI cases in hospitalized patients are caused by renal ischemia reperfusion injury (IRI) (3). The pathophysiology of AKI is very complex and combines major ischemia-induced cell stress, a significant burst of free radicals and pro-inflammatory cytokines [such as interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α] evoking a pro-inflammatory cascade, and subsequent injuries on distant organs, including the lung, heart, liver and brain (4-6). This organ crosstalk phenomenon is well-known in critical care medicine as multiple organ failure due to systemic inflammatory response syndrome. Acute lung injury (ALI) is the most clinically relevant remote organ dysfunction associated with AKI (5). The pathological characteristics of ALI are increased pulmonary vascular permeability, lung edema and alveolar hemorrhage (7-9). A previous study indicated that when both AKI and ALI occur, the overall morbidity is as high as 80% (4). Therefore, improved understanding of the effects of renal IRI on remote organs, especially on the lungs, is urgently needed.

Nuclear factor (NF)-κB proteins are a family of ubiquitously expressed transcription factors. In their inactive forms, NF-κB proteins are bound by members of the inhibitor of κB (IkB) family. IkB kinase (IKK)ε is a member of the IKK family, which influences NF-κB signaling and concomitant gene expression downstream of IkB (10,11). A previous study indicated that the NF-κB pathway may aggravate tubular injury and exacerbate a maladaptive inflammatory response in renal ischemia reperfusion-induced AKI (12). IKK family members may be therapeutic targets for lung injury, and the beneficial effects of IKK proteins may be mediated by inhibition of the NF-κB pathway (13).

According to Park et al (14) and Bulek et al (15), IKKε may serve an important role in enhancing lipopolysaccharide-induced and IL-17-mediated inflammatory responses, including increasing the transcription of inflammation-related genes in primary airway epithelial cells, and exacerbating the...
severity of neutrophilia and pulmonary inflammation in ALI. However, the molecular mechanism of ALI in the setting of renal IRI remains unclear. Based on the results of a previous study, NF-κB-dependent gene expression induced by TNF-α or IL-1 may be abrogated by IKKe (16); however, to the best of our knowledge, reports of the role of IKKe in kidney-lung crosstalk in renal IRI are lacking.

The present study aimed to investigate the possible molecular mechanisms by which IKKe induces ALI, and the effect of the NF-κB pathway on kidney-lung crosstalk in renal IRI by primarily focusing on the role of IKKe. The present study may improve understanding of the pathological mechanisms underlying IKKe-induced ALI and the significance of anti-inflammatory treatments for patients with IRI.

Materials and methods

Animals. IKKe knockout C57BL/6J mice (IKKe−/−) (male; age, 6-8-weeks; weight, 25-30 g) were purchased from the Jackson Laboratory and housed in the Model Animal Research Center of Nanjing University (Nanjing, China). Wild-type (WT) C57BL/6J mice (male; age, 6-8-weeks; weight, 25-30 g) were obtained from the Animal Research Center of Nanjing Medical University (Nanjing, China). All mice were housed at 2 animals/cage in a light-controlled environment at 24±1˚C, 40-80% humidity, 12-h light/dark cycles with access to food and water ad libitum throughout the experimental period. Animal experiments were performed in compliance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals of the NIH and approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Surgical procedures. A total of five experimental groups were evaluated in this study (n=6 in each group): Two IRI groups (WT and IKKe−/−), two sham groups (WT and IKKe−/−) and one control group (WT; no surgery). All procedures were performed using strict sterile techniques after inducing anesthesia with an intraperitoneal (IP) injection of pentobarbital (50 mg/kg body weight). Adequate anesthesia was assessed by pinching the paw and tail. Animals from each group were placed on a heating blanket prior to sham and IRI operations. Renal IRI was performed as previously described (17). Briefly, the back of the mouse was shaved and a 1.5-cm incision was made on both sides. Two vascular clamps were applied across both renal pedicles for 45 min. Occlusion was visually verified on both sides. Two vascular clamps were applied across both renal pedicles for 45 min. Occlusion was visually verified by monitoring the change in the color of the kidney to a paler hue. After clamp removal, the restoration of blood flow to the kidney was confirmed by the return of the original color. The incisions on the back of each mouse were closed in two layers with sutures, and mice were returned to their cages for 24 or 48 h. The animals were allowed to recover, and had free access to food and water. Animals in the sham groups underwent an identical procedure without vascular clamp placement. Blood and tissue samples were separately collected at different time points (24 and 48 h) from different groups.

Plasma parameters. At different time points after operation (24 and 48 h), mice were anesthetized with an IP injection of pentobarbital (50 mg/kg body weight), and the adequacy of anesthesia was evaluated by monitoring hind limb reflexes. Blood samples (~0.5 ml) were obtained from the retroorbital plexus and centrifuged at 1,509 x g for 15 min at 4˚C to obtain serum. The samples were stored at -80˚C until further use. Serum creatinine (SCr) and blood urea nitrogen (BUN) levels were measured as renal function markers using an Olympus AU2700 automatic biochemistry apparatus (Olympus Corporation).

Tissue collection and lung wet-to-dry ratio. Immediately after the blood samples collection procedure, mice were euthanized with an overdose of pentobarbital (150 mg/kg body weight). The inferior lobe of the left lung was harvested to measure the lung wet-to-dry ratio. First, an arteriovenous (AV) fistula needle was placed in the left atrium. Saline was slowly forced through another AV fistula needle inserted into the right ventricle using the injector until the lung changed color from red to white. The upper lobe of the left lung was harvested and preserved in liquid nitrogen for western blotting. The left main bronchus was isolated and cross-clamped. The right lung was filled with 0.5% low-melting point agarose in 10% formalin at a constant pressure of 25 cm H2O through a tracheotomy with an AV fistula needle, allowing the homogenous expansion of the lung parenchyma. The lung wet-to-dry ratio was measured by desiccating the lung at 80˚C until a constant weight was obtained. The ratio was calculated as an indicator of lung edema.

Histological analysis using hematoxylin and eosin (H&E) staining. For histopathological evaluation of lung injury, lung tissues were obtained from the mice and subsequently fixed in 10% formaldehyde at room temperature for 24 h and embedded in paraffin. The 5-μm sections were heated at 60˚C for 1 h, before being dewaxed in xylene and rehydrated using a descending ethanol series. H&E staining was then performed on sections, with hematoxylin for 10 min room temperature and eosin for 5 min at room temperature. Stained sections were visualized using a light microscope (magnification, x200) by a pathologist in a blinded manner.

Western blot analysis. IHC and western blotting were performed as previously described (12,17). Lung tissue samples were ground in liquid nitrogen and lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) for 30 min. Total protein was quantified using a bicinchoninic acid assay and 50 μg protein was separated using 12% SDS-PAGE for 90 min. The separated proteins were subsequently transferred onto polyvinylidene difluoride membranes and blocked in TBS with 5% skimmed milk for 2 h at room temperature. The membranes were incubated with primary antibodies against GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.), IKKe (1:500; cat. no. ab124766; Abcam), NF-xB phosphorylated p50 (pi-p50; 1:200; cat. no. sc-271908; Santa Cruz Biotechnology, Inc.), NF-xB phosphorylated p65 (pi-p65; 1:200; cat. no. sc-165748; Santa Cruz Biotechnology, Inc.), NF-xB p50 (1:1,000; cat. no. ab32360; Abcam), NF-xB p65 (1:800; cat. no. ab16502; Abcam) overnight at 4˚C. Following the primary antibody incubation, the membranes were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. sc-2370; 1:5,000; Santa Cruz Biotechnology, Inc.).
Biotechnology Inc.) for 1 h at 37°C. The protein-antibody complexes were visualized using Pierce™ Fast Western Blot Kit, ECL Substrate (cat. no. 35050; Thermo Fisher Scientific, Inc.) with a chemiluminescence instrument (Tanon Science and Technology Co., Ltd.). Protein expression was quantified using Imagepro plus software (version 6.0; Media Cybernetics, Inc.).

**Immunohistochemical analysis.** Immunohistochemistry was performed using paraffin-embedded tissue sections cut at 4-µm thickness mounted on glass slides. The slides were then deparaffinized and rehydrated. Then, the lung sections were incubated with primary antibodies against TNF-α (1:200; cat. no. ab9739; Abcam), Ki67 (1:2,000; cat. no. ab15580; Abcam), IL-1β (1:200; cat. no. sc-7884; Santa Cruz Biotechnology, Inc.), and IL-10 (1:400; cat. no. bs-0698R; BioSS), NF-κB phosphorylated p50 (pi-p50; 1:200; cat. no. sc-271908; Santa Cruz Biotechnology, Inc.) and NF-κB phosphorylated p65 (pi-p65; 1:200; cat. no. sc-166748; Santa Cruz Biotechnology, Inc.) and then with biotin secondary antibodies (B3640; Sigma-Aldrich; Merck KgaA) at room temperature for 30 min.

The IHC score was determined using the Fromowitz standard as previously described (18). The percentage of positive stained cells was graded as follows, 0-5%, 0; 6-25%, 1; 26-50%, 2; 51-75%, 3; >75%, 4. The intensity of staining was graded as follows: Absent or faint blush, 0; weak, 1; moderate, 2; strong, 3. Then the two scores were added.

**Statistical analysis.** The data are presented as the mean ± standard error of the mean of at least three independent repeats. The results were analyzed by one-way ANOVA followed by a Tukey’s post hoc test. The IHC scores were compared by Mann-Whitney U test. SPSS 17 software (SPPS, Inc.) was used to perform the statistical analysis. P<0.05 was considered to indicate a statistically significant difference.

**Results**

*Renal function significantly decreases following experimental renal IRI.** SCr and BUN levels were measured in IKKε-/- and WT mice at 24 and 48 h after surgical IRI to confirm the decreased renal function. Compared with the control and sham groups, the IRI group mice exhibited significant increases in SCr values and BUN levels at 24 and 48 h (P<0.05; Fig. 1A and B). Additionally, no significant difference was observed in renal function between the WT and IKKε-/- groups 24 and 48 h after surgical IRI (P>0.05; Fig. 1A and B).

*IKKε-/- mice exhibit significantly weaker acute disease and pulmonary edema compared with that of WT mice.** The lung wet-to-dry ratio was detected to evaluate the degree of pulmonary edema. The results demonstrated a gradually increasing trend after surgical IRI (Fig. 1C). In addition, the IKKε-/- group exhibited significantly weaker pulmonary edema compared with that of the WT mice in 24 and 48 h (P<0.05).
IKKe knockout attenuates experimental renal IRI-induced lung inflammation. H&E-stained lung sections were examined to further determine whether IKKe knockout affects experimental renal IRI-induced ALI. Since the present study indicated that there was no significant difference in renal function and pulmonary edema between the control and sham groups at 24 and 48 h, a 48-h timepoint was used for the sham group in subsequent experiments. The results demonstrated that in the WT group, renal IRI induced persistent interstitial edema, focal alveolar hemorrhage, alveolar wall thickening and inflammatory cell infiltration. By contrast, lung tissues from the IKKeε−/− group exhibited less damage, which manifested with a disordered and uneven distribution (Fig. 2A).

Immunohistochemical analysis of the proliferation-associated antigen Ki67 (Fig. 2B) indicated the absence of mitotic figures, and very few cells in the sham group were Ki67-positive. By contrast, compared with those in the IKKeε−/− groups, the number of Ki67-positive cells was significantly increased in the WT groups at 24 and 48 h (Fig. 2B and C); thus suggesting cellular proliferation occurred following kidney IRI in the WT group.

Inflammatory markers, including TNF-α, IL-1β and IL-10, were detected in lung tissues. The results demonstrated that the expression levels of TNF-α, IL-1β and IL-10 were significantly lower in the IKKeε−/− groups compared with those in the WT groups 24 and 48 h post-surgery (P<0.05; Fig. 3A-D). These results suggested that inflammatory activity was suppressed in the lungs of IKKeε−/− mice.

IKKe ablation blocks NF-κB activation induced by acute ischemic kidney injury. Studies have demonstrated that IKKe participates in renal IRI (12); however, the exact mechanism is still unclear. Therefore, the levels of downstream factors of the NF-κB pathway, such as pi-p50 and pi-p65, in lung tissues was examined by immunohistochemical staining. The results demonstrated that the expression levels of pi-p50 and pi-p65 were significantly lower in bronchial epithelial cells of the IKKeε−/− groups compared with those in the WT groups (P<0.05; Fig. 4A-C).

Western blotting was performed to determine the expression levels of pi-p50 and pi-p65, which are the functionally active and nuclear forms of NF-κB (11). The results demonstrated that IKKe expression levels gradually increased in the WT group after acute ischemic kidney injury (Fig. 5). In addition, the expression levels of pi-p50 and pi-p65 were significantly reduced in the IKKeε−/− group compared with those in the WT group (P<0.05), whereas no significant differences were observed in the total p50 and p65 protein expression levels (Fig. 5).

Discussion

The pathogenesis of renal IRI is complex and is still not entirely understood. However, inflammation is currently accepted as an important pathogenic component (19). Renal IRI has been reported to result in endothelial and leukocyte activation, reactive oxygen species production, tubular cell death and the release of inflammatory mediators, such as cytokines and chemokines (19). AKI has been reported to activate host innate and adaptive immune responses, and experimental data have identified both soluble and cellular mediators activated by the post-ischemic kidney that drive ALI (5,20,21). Despite the clinical association between renal IRI and ALI, little is known about the molecular mechanism of kidney-lung crosstalk following renal IRI. A previous study has observed that increased expression of TNF-α in the lungs may induce pulmonary inflammatory damage (22). The present study used an IKKeε−/− mouse model of unilateral IRI and investigated the possible roles of IKKeε−/− and related inflammatory mediators, such as TNFα, IL-10 and IL-1β. To the best of our knowledge, the present study is the first to demonstrate that the IKKe pathway may serve a role in kidney-lung crosstalk following renal IRI.

The results of the present study demonstrated the IKKeε−/− and WT groups exhibited similar decreased renal function following experimental AKI; however, the lung wet-to-dry ratio was significantly decreased in the IKKeε−/− group compared with in the WT group. In addition, this study examined the morphological and molecular alterations in lung tissues to investigate the effect of IKKe on ALI following renal IRI. A series of histopathological changes were demonstrated...
by H&E staining; renal IRI induced persistent interstitial edema, focal alveolar hemorrhage, alveolar wall thickening and inflammatory cell infiltration in the WT group, whereas lung tissues from the IKKε−/− group exhibited less damage. The present findings demonstrated that IKKε knockout may reduce lung edema after renal IRI. Thus, it was hypothesized that IKKε deficiency may contribute to the reduction of inflammation in the lungs after renal IRI. Subsequently, Ki67 levels were measured in lung tissues by IHC to explore cell proliferation following the pathological changes. The number of Ki67-positive cells was greater in the lungs of the WT group compared with that in the IKKε−/− group. Thus, lung cell proliferation was significantly reduced after inflammatory injury in the absence of IKKε. Thus, it was hypothesized that IKKε may be associated with inflammatory cell infiltration and lung tissue destruction.

In the present study, the expression of inflammatory markers (TNFα, IL-10, and IL-1β) was detected in lung tissues. The results demonstrated that TNFα expression levels were elevated after renal IRI treatment, but were inhibited by IKKε knockout. Higher TNF-α expression levels were observed in the airway epithelial cells of the WT group after renal IRI treatment, whereas lower expression levels were observed in the IKKε−/− group. Renal IRI has been reported to activate soluble TNFα, and thus induce TNFα receptor 1-dependent pulmonary cell apoptosis and microvascular barrier dysfunction (22). Thus, TNF-α may serve a key role in aggravating the downstream effects of renal IRI. IL-1β is an important protein participating in NF-κB-induced inflammatory responses, which has been shown to exhibit a biphasic distribution in IRI injury models (23,24). Previous studies...
observed that IL-1β expression levels were significantly increased and remained high during the reperfusion period compared with controls (23-25). In the present study, the expression levels of IL-1β were investigated during the reperfusion period and the results indicated that IL-1β expression levels were higher in the WT groups compared with those in the IKKε-/- groups. These results suggested that IL-1β may be related to IKKε activation. According to previous studies, TNF-α, IL-1β, IL-6 and other pro-inflammatory cytokines inhibited the anti-inflammatory effects mediated by IL-10, indicating that these factors may perform similar roles in IRI-related pathways (26,27). Consistent with other studies, the results of the present study demonstrated that the expression levels of IL-10 were higher in the lungs of the WT groups following renal IRI compared with those of the IKKε-/- groups. IKKε phosphorylates NF-κB (pi-p65), contributing to the NF-κB-dependent expression of target genes in response to pro-inflammatory signals (10,30,31). However, the inhibitory effects were not completely dependent on IKKε deficiency, indicating other signaling pathways may exist. Studies have observed that NF-κB activation is separately mediated by myeloid differentiation primary response 88 via IKKα/β and by TIR domain-containing adaptor-inducing interferon-β via IKKε (28,32,33).

Previous studies have reported that myeloperoxidase activity, neutrophil infiltration, blood oxygen saturation and pulmonary vascular permeability are important evaluation indicators of ALI (5,20). Therefore, these factors could be further explored in future studies.

In conclusion, in the present study, a novel role for IKKε in regulating renal IRI-induced inflammation in mouse lungs was identified. In the absence of IKKε, the renal IRI-associated destruction of mouse lung tissue and inflammatory responses were substantially prevented, and the expression levels of components associated with NF-κB signaling were reduced. Based on these findings and

Figure 5. Differential expression of pi-p50 and pi-p65 in lung tissues from the IKKε-/- and WT groups at 24 or 48 h following IRI. (A) Representative western blotting results of each group. (B) Relative protein expression levels were normalized to GAPDH/total protein expression (n=4). *P<0.5, **P<0.01 vs. WT group. NF-κB, nuclear factor-κB; IRI, ischemia reperfusion injury; WT, wild-type; IKKε-/-, inhibitor of κB kinase ε knockout; pi, phosphorylated.
previous studies suggesting a role for IKKe in NF-κB activation, it was hypothesized that IKKe may serve a pivotal role in the activity of NF-κB, which in turn could regulate the expression of genes involved in the inflammatory response in lungs following renal IRI.

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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

CM designed the study and performed experiments. XM and AZ designed the study. CZ and HL performed experiments. YQ analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Nanjing Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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