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SHORT ARTICLE

Induction of the NEK family of kinases in the lungs of mice subjected to cecal ligation and puncture model of sepsis

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ABSTRACT

Endothelial barrier dysfunction (EBD) is the hallmark of Acute Respiratory Distress Syndrome (ARDS), a potentially lethal respiratory disorder associated with the COVID-19 – related deaths. Herein, we employed a cecal ligation and puncture (CLP) murine model of sepsis, to evaluate the effects of sepsis-induced EBD in the expression of the never in mitosis A (NIMA)-related kinases (NEKs). Members of that family of kinases regulate the activity and expression of the tumor suppressor P53, previously shown to modulate the actin cytoskeleton remodeling. Our results introduce the induction of NEK2, NEK3, NEK4, NEK7, and NEK9 in a CLP model of sepsis. Hence, we suggest that NEKs are involved in inflammatory processes and are holding the potential to serve as novel therapeutic targets for pathologies related to EBD, including ARDS and sepsis. Further studies will delineate the underlying molecular events and their interrelations with P53.

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Introduction

ARDS is an inflammatory lung disease associated with high mortality rates, as reflected in the severe outcomes of the current pandemic (COVID-19).[1] Lung endothelial hyper-permeability to fluid, proteins, neutrophils, and red blood cells contributes to the deposition of edematous fluid into the alveolar air space, leading to severe respiratory complications.[2,3]

The family of never in mitosis A (NIMA)-related kinases (NEKs) includes eleven members (NEK1-11) involved in several cellular functions including intracellular trafficking[4] and actin remodeling.[5,6] NEK9 regulates RhoA activation via Rho guanine nucleotide exchange factor 2 phosphorylation,[7] which in turn promotes the myosin light chain 2 (MLC2) phosphorylation. Under hypoxic conditions, the promoter of NEK1 binds to the hypoxia-inducible factor 2-α, inducing hyperpermeability responses.[8] NEK7 is an important component of the NLRP3 (NLR family pyrin domain containing 3) inflammasome,[9] which enhances the lipopolysaccharides (LPS)-mediated stimulation of toll-like receptor 4/nuclear factor-kappa B (NF-κB) signaling.[10] The expression of that serine/threonine protein kinase (NEK7) is also associated with the hyperpermeability of the blood-brain barrier (BBB) and cerebral edema.[11]

The previously mentioned observations suggest the strong involvement of certain NEKs in EBD. However, the effects of sepsis on the expression of NEKs are unknown. Hence, we employed a cecal ligation and puncture (CLP) model of murine sepsis to evaluate the effects of sepsis in the pulmonary expression of NEK2, NEK3, NEK4, NEK7, and NEK9. This experimental model (CLP) replicates the clinical picture of sepsis-inflicted pulmonary edema, a principal cause of ARDS. Our results indicate for the first time that the expression of NEKs in the lungs is elevated in murine sepsis. Thus, we suggest that NEKs may serve as a potential therapeutic target in sepsis-induced ARDS. However, further studies are needed to advance our understanding toward those events.

Materials and methods

Reagents

RIPA buffer (AAJ63306-AP), sheep anti-mouse IgG HRP-linked polyclonal antibody (95,017–554), donkey anti-rabbit IgG HRP-linked polyclonal antibody (95,017–556), EZBlock™ protease

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inhibitor cocktail (75,837–938), and nitrocellulose membranes (10,063–173) were obtained from VWR (Radnor, PA). The NEK2 (sc-55,601), NEK3 (sc-390,872), NEK4 (sc-81,332), NEK7 (sc-393,539), and NEK9 (sc-100,401) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-ERK1/2 (9101S) and ERK1/2 (9102S) antibodies were obtained from Cell Signaling (Danvers, MA). The β-actin (A5441) antibody was purchased from Sigma-Aldrich (St Louis, MO).

Animals

Six-week-old male C57BL/6 mice (Envigo, Indianapolis, IN) were used in this study. They were maintained in a 12:12-h light/dark cycle, in pathogen-free conditions. All animal experiments were conducted in accordance with the approved protocol by the University of Louisiana Monroe Institutional Animal Care and Use Committee (IACUC) and were in line with the principles of humane animal care adopted by the American Physiological Society.

CLP model of sepsis

Mice were anesthetized with a subcutaneous injection of ketamine and xylazine (9:1) solution. A midline laparotomy was performed under sterile conditions. The cecum was exposed and was ligated below the ileocecal valve without disturbing the intestinal continuity. The ligated cecum was punctured twice with a 20-gauge needle. Small amount of intestinal content was extruded through the punctured holes before returning the ligated cecum to the peritoneal cavity. The abdominal and skin incision were sealed using a 3–0 silk running suture and 7 mm reflex wound clips, respectively. In the sham-operated mice, the cecum was exposed and returned to the abdominal cavity without ligation and puncture. The animals were resuscitated by subcutaneous administration of 1 ml of 0.9% NaCl solution immediately after surgery. The mice were sacrificed after 24 hours of CLP operation, and the lungs were collected and homogenized for protein analysis.

Western blot analysis

The protein expression was determined by western blots, as previously described.[12] Briefly, proteins were isolated from mice lung tissues using RIPA buffer and equal amounts (40 μg) of protein samples were separated onto sodium dodecyl sulfate polyacrylamide gel electrophoresis. Wet transfer was used to transfer the proteins onto the nitrocellulose membranes. After blocking with 5% nonfat dry milk at room temperature, the blots were exposed to appropriate primary and secondary antibodies to detect protein signals. The β-actin was used as a loading control unless indicated otherwise.

Densitometry and statistical analysis

The densitometry of the immunoblots was performed by using Image J software (NIH). All values are expressed as the mean ± SEM (standard error of the mean). Graphpad Prism (version 5.01) was used to analyze the data. Student's t-tests were performed to determine the statistical differences among groups.

Results and discussion

To evaluate the effects of sepsis in the expression of NEKs in the lungs, we subjected the mice to the CLP operation. Our results indicate that the expression of NEK2 (Figure 1a), NEK3 (Figure 1b), NEK4 (Figure 1c), NEK7 (Figure 1d), and NEK9 (Figure 1e) in the lungs of the septic mice were increased as compared to the sham group. The increase in the expression levels of NEK9 appear to be higher than NEK7, and that may be due to the fact that NEK7 is a downstream target of NEK9.[13] Moreover, the septic lungs expressed more phospho-ERK1/2 (figure 1f), as previously reported.[14] Kinases (e.g. AMPK, MLCK, PKC, FAK, PAK, ROCK) are well-known inflammatory modulators of endothelial permeability.[15] To the best of our knowledge, this is the first study to report the induction of NEKs in sepsis.

Tight junctions serve as the main regulators of fluid and ion exchange, and are involved in the regulation and maintenance of endothelial barrier function. Several tight junction proteins (TJs) have

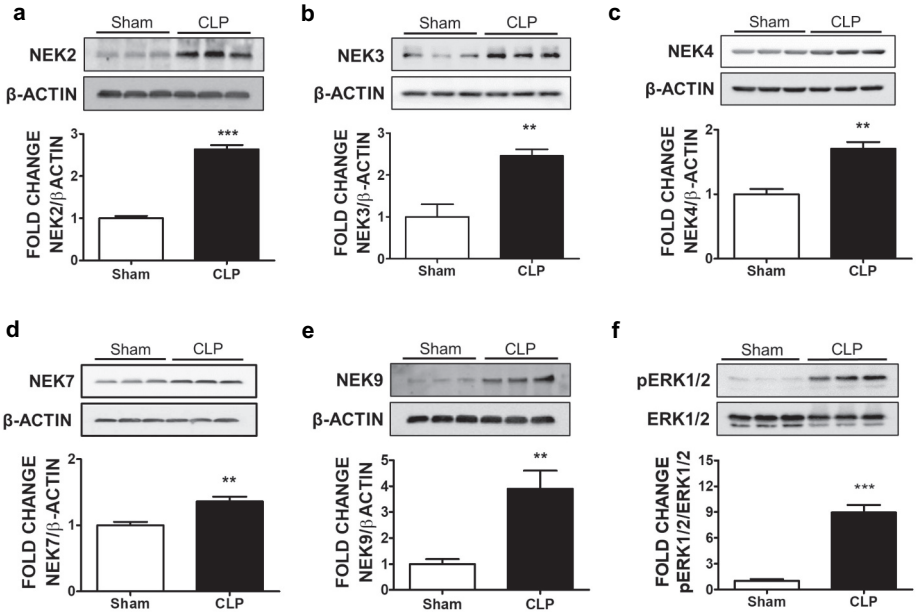

Figure 1. Effects of CLP-induced sepsis on NEK kinases expression in mice lungs. C57BL/6 mice were subjected to either sham surgery or CLP. 24 H after the surgery, mice were sacrificed and lung tissues were collected for the western blot analysis of NEK2 and β-actin (a), NEK3 and β-actin (b), NEK4 and β-actin (c), NEK7 and β-actin (d), NEK9 and β-actin (e), phospho-ERK1/2 (pERK1/2) and ERK1/2 (f). The signal intensity of the bands was analyzed by densitometry. Protein levels were normalized to β-actin unless indicated otherwise in the graph of densitometry. **P <.01, ***P <.001 vs sham, N = 3. Means ± SEM.

been identified in the lungs (e.g. occludins, claudins and zonula occludens),[16] and at least 14 different mRNAs encode for claudins in the lung epithelium. Claudin-3, claudin-4 and claudin-18 are the most abundant.[17] Indeed, claudin-4 is associated with fluid clearance from the alveolar airspace, and exerts a protective role against ventilator-induced lung injury (VILI).[17] Claudin-4 was also shown to induce alveolar barrier function, since its augmentation substantially increased transepithelial resistance.[18] In a CLP-induced model of sepsis, the expression of both claudin-4 and claudin-18 were reduced, suggesting the important role of those proteins in the maintenance of normal respiratory functions.[19]

Previous studies have associated certain members of the NEK family of kinases with the mediation of inflammatory responses. NEK2 activated the NF-κB signaling pathway and increased the expression of heparanase, an endoglycosidase responsible for bone destruction.[20] This kinase was reported to induce the interleukin-8 expression and potentiate angiogenesis.[21] It was also suggested that NEK6 targets the signal transducer and activator of transcription 3,[22] and induces the hypoxia-inducible factor 1α.[23] In lung cancers it was shown that NEK9 is recruited to the microtubules, and it is a target of EML4-ALK which in turn accelerates the NEK7-dependent migration.[24]

In recent studies NEK7 has been reported to activate the NLRP3 inflammasome by binding to its leucine-rich repeat domain.[25,26] The inflammasome is a multiprotein complex which activates caspase-1 and it is involved in the secretion of IL-1β and IL-18. This occurs in response to different stimuli, including multiple microbial products, endogenous molecules and particulate matter.[26] NEK7-induced NLRP3 activation is associated with various diseases such as inflammatory bowel disease (IBD),[10] atherosclerosis, and the development of neuroinflammation in post-traumatic brain injury (TBI).[27] In our study, we also observed the significant induction of NEK7 in CLP-induced sepsis.

NEKs have been associated with P53, a tumor suppressor and anti-inflammatory protein, which exerts protective activities in the lung microvasculature.[28,29] P53 increases the endothelial barrier integrity by deactivating cofilin; and by suppressing the formation of filamentous actin. Moreover, this endothelial defender (P53) mediates the barrier enhancing effects of heat shock protein 90 (Hsp90) inhibitors[30,31] and

growth hormone releasing hormone antagonists[28,32] in the lungs and the blood-brain barrier.[33,34] Indeed, the hydrogen peroxide-induced BBB hyperpermeability was counteracted by those unfolded protein response inducers.[35]

P53 is subjected to post-translational modifications which regulate its intracellular abundance and activity,[36] since P53 phosphorylation accelerates its degradation.[37,38] In bovine pulmonary artery endothelial cells, LPS, an endotoxin of the Gram-negative bacteria, reduced P53 via phosphorylation at Ser. 6, Ser. 15, Ser. 33, and Ser. 392.[39] Furthermore, lipoteichoic acid, an endotoxin of Gram-positive bacteria, induced P53 phosphorylation in Ser. 15, Ser. 33, Ser. 46, and Ser. 392.[40] The Hsp90 inhibitors 17-AAG, 17-DMAG, and AUY-922 counteracted those phosphorylations, to increase P53 expression.[41]

The NIMA family proteins can also phosphorylate P53. Polo-like kinase 1 (Plk1), an upstream activator of NEK6/7/9, inhibits the function of P53 by physical interaction and phosphorylation. Moreover, it shares common phosphorylation-site motif with NEK6, NEK7 and NEK9.[13] Interestingly, NEK2 and NEK10 reduce P53 by Ser315 and Y327 phosphorylation.[42] Hence, we speculate that the interrelations of P53 and NEKs in the context of the pulmonary microvasculature may dictate the severity and outcomes of the sepsis-induced cytokine storm in the lung context.[43] Further studies in endothelial specific knock-out mice that do not express NEKs and/or P53 will test our hypothesis.

To the best of our knowledge, the present study introduces the upregulation of NEKs in the lungs of septic mice. Hence, it is highly probable that certain NEKs (NEK2, NEK3, NEK4, NEK7, and NEK9) may serve as a potential therapeutic target in sepsis and other pathologies related to EBD.

Disclosure of potential conflicts of interest

No potential conflict of interest was reported by the author(s).

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