Original Article

Knockdown of NF-κB-inducing kinase prevents inflammation-sensitized hypoxic-ischemic brain injury in newborns

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Abstract: Inflammation-sensitized hypoxic-ischemic (HI) brain injury causes high mortality and severe disability in neonates. Hence, understanding the pathogenesis and developing novel therapy targets are necessary. NF-κB-inducing kinase (NIK) is a ubiquitously-expressed kinase and is activated by diverse stimuli. However, the expression and role of NIK in inflammation-sensitized HI-induced brain injury in neonates remains unclear. In the present study, western blotting and immunofluorescence were employed to determine the expression and distribution of NIK in the perinatal brain hippocampus in a rat model of LPS-HI. Specific siRNA targeting NIK was administered intracerebroventricularly to determine the potential role of siNIK in neuroprotection. The results indicated that NIK was upregulated and localized in neurons and astrocytes following HI injury, compared with the sham group. Further study demonstrated that knockdown of NIK significantly inhibited non-canonical NF-κB pathway, the related pro-inflammatory cytokine expression, and dramatically ameliorated acute inflammation-sensitized HI-induced brain injury. Collectively, our results suggest that NIK may be a potential therapeutic target for the treatment of neonates with inflammatory-sensitized HI-induced brain injury.

Keywords: NIK, inflammation, hypoxia-ischemia, neonatal, NF-κB

Introduction

About 4 to 9 million neonates suffer from birth asphyxia each year in the world, with about 1.2 million deaths and the same number of infants with severe disability, including cerebral palsy, epilepsy and mental retardation [1]. These deaths and disabilities are mostly due to hypoxic-ischemic (HI) brain injury. Meanwhile, intrauterine infection (chorioamnionitis) is a high risk factor for cerebral palsy neonates [2] with incidence ranging from 3 to 30% [3]. Clinical studies have shown that increased levels of systemic cytokines in premature infants with chorioamnionitis are associated with hemodynamic disturbances leading to cerebral HI, whereas co-morbid chorioamnionitis and placental perfusion defects put preterm infants at higher risk of abnormal neurologic outcomes than either insult alone [4-6]. Animal studies also indicate that pre-exposure to systemic lipopolysaccharide (LPS) sensitizes HI injury in the immature brain [7, 8]. These concerns highlight the need for a better understanding and a more effective therapy for inflammation-sensitized HI brain injury in newborns.

Nuclear factor-κB (NF-κB) transcription factor can be activated via the canonical and non-canonical pathway. Pro-inflammatory stimuli including TNF-α activates the NF-κB canonical pathway accompanied with the requirement of IKKβ [9]. In contrast, the non-canonical pathway is strictly dependent on NF-κB-inducing kinase (NIK) and IKKα [9]. NIK is a ubiquitously-expressed Ser/Thr kinase and its levels are very low due to rapid proteasome-mediated degradation [10]. Meanwhile, NIK is activated by diverse stimuli, including oxidative stress, saturated fatty acids, cytokines, endotoxins, as well as ligands that activate toll-like receptors, receptor tyrosine kinases, and G protein-cou-
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Pleural receptors [11, 12]. Furthermore, NIK was demonstrated to play crucial role in the pathogenesis of various diseases and widely used as a therapeutic target [13-15].

But, until now, the expression and function of NIK in inflammation-sensitized HI-induced brain injury of neonates was still unclear. Thus, in the present study, we first investigated the expression and distribution of NIK after LPS-HI insult. Knockdown of brain NIK by injection of siNIK was performed to understand the functional significance of NIK in cytokine expression and in acute LPS-HI induced cerebral injury. Our data suggests that NIK may be a potential therapeutic target for inflammation-sensitized brain damage after HI in neonates.

Materials and methods

LPS-HI rat model and treatment

Seven day old pups were purchased from the Animal Centre of Sichuan University and were used for establishing the LPS-HI model, as described previously [16]. Briefly, the 7-days old pups were first injected intraperitoneally with Escherichia coli LPS (Sigma-Aldrich, St Louis, MO, USA). The pups were then returned to their dams after LPS injection, and housed in an incubator to maintain body temperature at 36 to 37°C before HI. Three hours post LPS injection, the pups were anesthetized with diethylether and the body was maintained at 37°C using a homoisothermy bench. Following a 0.5 cm skin incision in the midline of the neck, and the right common carotid artery (CCA) was permanently ligatured with 5-0 silk. After ligation of the CCA, the pups were returned to the dam for 0.5 h to recover from anesthesia. Then, the pups were taken into a chamber at constant 37°C for hypoxia (8% O₂, 92% N₂) for 6, 12, 24 and 48 h, respectively. The sham group underwent a neck dissection with silk around the CCA, but not ligated. All animal procedures used were approved by Sichuan University Committee of Animal Use and Care and all efforts were made to minimize animal suffering and animal numbers.

siRNA targeting NIK (siNIK) and negative control (NC) were purchased from Ribobio (Guangzhou, China). 24 h prior to LPS injection, 10 nmol siNIK or NC in 100 μl PBS was injected into the right cerebral hemisphere of LPS-HI insult rats using a 30-gauge needle with 5-μl Hamilton syringe.

Immunofluorescence

To determine the expression and distribution of NIK in brain, immunofluorescence was employed. The brains were perfused and fixed in 4% paraformaldehyde for 48 h and then embedded by paraffin and sectioned 4-μm thick. The primary antibody NIK (Cell Signaling, CA, 1:400) and mouse monoclonal antibodies against NeuN (Millipore, CA; 1:150) or GFAP (Millipore, CA, 1:80) was performed and followed by incubation with 1:120 dilution of secondary antibody, either FITC- or TRITC-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology, USA). The nucleus was stained with 4',6-diamidino-2-phenylindole (DAPI, Beyotime, Beijing, China).

Western blotting

To determine the protein expression by western blotting, the hippocampus from the right hemisphere were collected and lysed with the RIPA lysis buffer (Beyotime, Beijing, China) containing a protease inhibitor cocktail (Merck Millipore, MA, USA). BCA protein assay kit (Beyotime, Beijing, China) was employed to detect the protein concentrations with bovine serum albumin (BSA, Beyotime, China) as the standard. Then 20 μg protein samples per lane were used. After blocking, the membranes were immunoblotted with various antibodies: NIK; rabbit anti-NF-κB p52 monoclonal antibody overnight at 4°C. A rabbit anti-GAPDH polyclonal antibody was used as an internal loading control. The bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibodies against rabbit or mouse IgG using the ECL advance western blotting detection kit (Merck Millipore, MA, USA). Image-Pro Plus (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA) was used to quantify the densities of the protein signals on X-ray films following scanning.

ELISA

To determine the protein expression by ELISA, the protein samples were collected as described above. The IL-6, TNF-α and IL-1β ELISA detection kit were purchased from Neobioscience (Shenzhen, China). The experiment was performed according to the manufacturer’s instructions.
**HE staining and Nissl staining**

The hematoxylin and eosin were purchased from Beyotime (Beijing, China) and the Nissl staining kit was purchased from Huayueyang Bio (Beijing, China). The paraffin embedded 4-mm thick brain sections from different group were used for H&E staining and Nissl staining. The sections were deparaffinized in xylene, treated with a graded series of alcohol [100%, 95%, and 80%] ethanol/double-distilled H$_2$O (v/v)], and rehydrated in PBS (pH 7.4). The HE and Nissl staining were performed following the instructions supplied by the manufacturer. All specimens were evaluated using Olympus B×600 microscope and Spot Flex camera.

**TUNEL assay**

To detect apoptotic cells in brain tissues, TUNEL assay using a Dead End TM Fluorometric TUNEL System (Promega, Madison, Wisc, USA) was performed and the apoptosis index was analyzed as the previous study indicated [17]. Cell nuclei with dark green fluorescent staining were defined as apoptosis cell. To quantify

![Figure 1](image-url)
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TUNEL-positive cells, the number of green fluorescence-positive cells was counted in four random fields. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). All specimens were evaluated using Olympus B×600 microscope and Spot Flex camera.

Statistical analysis

Statistical analysis was performed using the SPSS version 19.0 (SPSS Institute, Chicago). Numerical continuous data were presented as mean ± standard deviation. Statistical analysis was performed by the Student’s t-tests for comparing two groups. Statistical significance was set at a two-tailed P<0.05.

Results

Induction of NIK expression in the developing brain hippocampus of rat with LPS-HI treatment

To investigate the potential role of NIK in the pathogenic process of LPS-HI treatment induced brain damage, western blotting was employed to determine the expression of NIK in the hemispheres of rats at different time point post HI treatment. Our results indicated that NIK expression was significant upregulated in the brain hippocampus at 6 h after insult, followed by a 4-6 fold increase in NIK protein expression in the hemispheres of LPS-HI model compared with sham group at 12, 24 and 48 hours (Figure 1A and 1B). Furthermore, we employed double immunofluorescence to investigate the distribution and expression of NIK protein in the hippocampus of sham and insult groups. The astrocyte-specific marker GFAP and the neuron-specific marker NeuN were used to detect the astrocytes and neurons, respectively. As shown in Figure 1C and 1D, the upregulated expression of NIK was localized with astrocyte and neurons at 24 h after insult, compared with sham group. Statistical analysis indicated that only 21.3±4.7% astrocyte expressed NIK protein in the experimental group, whereas 72.6±12.6% neurons expressed NIK protein (Figure 1C and 1D). Collectively, NIK expression was significantly upregulated in the neurons and astrocytes in the hippocampus of rats following LPS-HI induced brain injury.

Induction of NF-κB and inflammatory cytokine expression in the hippocampus of rat brain following LPS-HI

Next, we determined the expression of NF-κB and inflammatory cytokine in the hippocampus following LPS-HI model.
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of rat brain in an LPS-HI model using Western blot and ELISA. p52 is an important subunit of NF-κB, which is the hallmark of the non-canonical pathway [18]. As shown in Figure 2A and 2B, the expression of p52 was significantly increased from 6 h to 48 h after insult, compared with the sham group. Furthermore, the expression of IL-6, TNF-α and IL-1β in the hippocampus was determined at different time points after LPS-HI insult using ELISA. Our results demonstrated that IL-6, TNF-α and IL-1β expression was dramatically increased from 6 h to 48 h after insult (Figure 2C-E). Taken together, the above results indicated that upregulation of NIK after LPS-HI insult may have induced the activation of NF-κB via the non-canonical pathway, and resulted in the upregulation of pro-inflammatory cytokine expression.

Figure 3. Expression of NIK and NF-κB after siNIK injection. A and B. Detection of NIK protein in the hippocampus of sham, LPS-HI model, NC injection and siNIK injection group by western blotting. GAPDH was used as the loading control. The relative expression of NIK was analyzed. C. Immunofluorescence detection of NIK in the hippocampus of sham, LPS-HI model, NC injection and siNIK injection group. DAPI indicated cell nucleus. Scale bar=100 μm. D and E. Detection of p52 protein in the hippocampus of sham, LPS-HI model, NC injection and siNIK injection group by western blotting. GAPDH was used as the loading control. The relative expression of p52 was analyzed. F-H. ELISA detection of IL-6, TNF-α and IL-1β expression in the hippocampus of sham, LPS-HI model, NC injection and siNIK injection group (n=4; **, P<0.01 compared with sham controls; ##, P<0.01 compared with NC injection group).
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Knockdown of NIK inhibits NF-κB and inflammatory cytokine expression

To further determine the potential neuroprotective role of NIK silencing on LPS-HI induced brain injury, siRNA targeting NIK was used injected into the brain prior to LPS-HI insult. Our results indicate that injection of siNIK dramatically inhibited NIK expression in the hippocampus, compared with negative control (NC) group (Figure 3A and 3B). Further immunofluorescence staining was used to confirm that NIK

Figure 4. Apoptosis detection by TUNEL in hippocampus. A and B. HE staining and Nissl staining were performed to determine the histomorphology of paraffin-embedded sections from the brain hippocampus of sham, LPS-HI model, NC injection and siNIK injection group. C and D. TUNEL assay was used to detect the apoptotic cell in paraffin-embedded sections from the hippocampus of sham, LPS-HI model, NC injection and siNIK injection group. DAPI indicated cell nucleus. Apoptosis cells in per frame was counted and used for apoptosis index analysis (n=4; **, P<0.01 compared with sham controls; ##, P<0.01 compared with NC injection group). Scale bar=100 μm.
expression was inhibited by siNIK in hippocampus (Figure 3C). To further determine the downstream target expression, western blotting was employed and indicated that p52 were both significantly inhibited by siNIK in the hippocampus, compared with the NC group (Figure 3D and 3E). ELISA results demonstrated that IL-6, TNF-α and IL-1β expression were also inhibited in the siNIK treatment group, compared with NC group. Collectively, we showed that knockdown of NIK by siNIK significantly inhibited NF-κB activity and the subsequent pro-inflammatory cytokine expression.

Knockdown of NIK prevents brain from LPS-HI-induced injury

Next, we determined the functional relevance of NIK inhibition in the rat model of LPS-HI injury. HE and Nissl staining were performed to determine the histology of hippocampus after LPS injection for 48 h. As shown in Figure 4A, HE staining indicated that the pyramidal neurons in the CA1 and CA3 area of LPS-HI and NC group were swollen and disorganized without nuclear and membrane structure. Whereas, in the siNIK injection group, less disorganized cells were found in the CA1 and CA3 area (Figure 4A). Furthermore, Nissl staining was performed and demonstrated that there was lesser Nissl bodies found in the LPS-HI and NC group, accompanied with cell swelling, disorganized and membrane loss (Figure 4B). Injection of siNIK significantly prevented brain damage (Figure 4B). It is known that apoptosis is one of the major effects of brain injury. Thus, we employed TUNEL staining to detect the apoptotic cell in the brain hippocampus. As shown in Figure 4C and 4D, more apoptotic cells were found in the HI and NC treatment groups, compared with sham group. But, less apoptosis was found in the siNIK group (Figure 4C and 4D). Collectively, our results indicated that knockdown of NIK markedly inhibited neuronal loss and apoptosis in brain hippocampus.

Discussion

HI and infectious events during perinatal and neonatal periods have cumulative effects on the risk of cerebral palsy in preterm infants [19]. In the current study, we aimed to investigate whether and how NIK affect LPS-sensitized HI-induced brain injury in neonates. We first showed that NIK expression in the developing brain was increased following LPS-HI treatment, suggesting that NIK may be correlated with the development of LPS-HI-induced brain damage. Furthermore, knockdown of NIK with siNIK injection significantly alleviated the brain injury and apoptosis via the non-canonical NF-κB pathway and associated pro-inflammatory cytokine expression. Our results provide evidence for better understanding the role of NIK in inflammatory-sensitized HI-induced brain injury and suggest that NIK may be a potential therapeutic target for term and near-term neonates.

Growing evidence suggests that systemic inflammation during perinatal and neonatal periods may affect cerebral vulnerability and thereby act concomitantly with HI insult to aggravate brain injury [20]. Experimental and clinical studies both demonstrate that inflammation-sensitized HI brain injury is less responsive to therapeutic hypothermia than pure-HI injury in neonates [21, 22]. In addition, it is still unclear how maternal infection aggravates neonatal HI brain injury [23] according to that newborns were often considered immunodeficient and lacking the adaptive immunity to initiate systemic anti-pathogen responses. The previous study has indicated that NF-κB activity is induced as early as 4 h past insult in LPS-sensitized HI brain injury [24, 25]. Meanwhile, inhibition of NF-κB pathway significantly prevents LPS-sensitized HI brain injury in neonates, but has no protection against pure-HI brain injury [24]. Our study firstly suggests that NF-κB transcription factors was activated via the non-canonical pathway at 6 h past insult in the LPS-sensitized HI brain injury model, accompanied with the upregulation of NIK, which is the necessary activator of NF-κB via the non-canonical pathway. Furthermore, inhibition of NF-κB activity through inhibiting the non-canonical pathway activation significantly decreased the followed pro-inflammatory cytokine expression and prevented LPS-sensitized HI brain injury.

NIK is widely expressed in most tissues [10]. Systemic deletion of NIK leads to severe immunodeficiency resulting in premature death in mice [13, 26]. Meanwhile, loss-of-function NIK mutation was accompanied with primary immunodeficiency disorders [27]. Whereas, abnormal activation of NIK increased renal tubule epithelial inflammation associated with diabe-
Elevating cellular levels of NIK increased expression and nuclear translocation of p52 through the non-canonical pathway and hence increased the transcriptional activity of NF-κB [15, 28]. Hyper activation of hepatic NIK promoted hepatocytes to secrete pro-inflammatory cytokine and triggers fatal macrophage-dependent liver injury and fibrosis leading to death in mice [14]. Thus, it is indicated that NIK plays dual role in the immunology system during the pathogenesis of various diseases. In the present study, we first demonstrated the upregulation of NIK in the hippocampus following inflammation-sensitized HI insult and indicated that the expression of NIK localized with astrocytes (about 21% of total astrocytes) and most neurons (about 72% of total neurons). Furthermore, knockdown of NIK with siNIK injection significantly inhibited the activity of NF-κB and subsequent pro-inflammatory cytokine expression, leading to the prevention of inflammation-sensitized HI brain injury. The results provide solid evidence for the functional role of NIK during the development of LPS-sensitized HI brain injury and indicate that NIK may be an ideal therapeutic target for term and near-term neonates.

Collectively, our results suggest that NIK expression was significantly increased in the hippocampus of neonates during the development of LPS-HI-induced brain damage, and activated the non-canonical NF-κB pathway. Furthermore, we show for the first time that knockdown of NIK with intracerebroventricular injections of siRNA targeting NIK significantly attenuated acute LPS-sensitized HI brain injury by inhibiting the non-canonical NF-κB pathway, associated pro-inflammatory cytokine expression, prevented apoptosis and neuronal loss, leading to the reduced HI-induced brain damage. In light of the above, NIK may be considered as a therapeutic target for the treatment of inflammation-sensitized HI-induced brain injury in neonates.

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Disclosure of conflict of interest

None.
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