Published in final edited form as:

Neurobiol Dis. 2017 July ; 103: 133–143. doi:10.1016/j.nbd.2017.04.006.

DLK silencing attenuated neuron apoptosis through JIP3/MA2K7/JNK pathway in early brain injury after SAH in rats

Cheng Yin, MD, PhD^{1,2}, Guang-fu Huang, MD², Xiao-chuan Sun, MD, PhD³, Zongduo Guo, MD, PhD³, and John H. Zhang, MD, PhD¹

¹Departments of Anesthesiology and Physiology, Loma Linda University School of Medicine, Loma Linda, California

²Department of Neurosurgery, Hospital of the University of Electronic Science and Technology of China, Sichuan Provincial People's Hospital, Chengdu, China

³Department of Neurosurgery, First Affiliated Hospital of Chongqing Medical University, Chongqing, China

Abstract

Objective—Dual leucine zipper kinase (DLK/MA3K12) has been reported involved in apoptosis and neuronal degeneration during neural development and traumatic brain injury. This study was designed to investigate the role of DLK with its adaptor protein JNK interacting protein-3 (JIP3), and its downstream MA2K7/JNK signaling pathway in early brain injury (EBI) after subarachnoid hemorrhage (SAH) in a rat model.

Design—Controlled in vivo laboratory study.

Setting—Animal research laboratory.

Subjects—Two hundred and twenty-three adult male Sprague-Dawley rats weighing 280–320 g.

Interventions—SAH was induced by endovascular perforation in rats. The SAH grade, neurological score, and brain water content were measured at 24 and 72 hours (hrs) after SAH. Immunofluorescence staining was used to detect the cells that expressed DLK. The terminal deoxynucleotid transferase-deoxyuridine triphosphate (dUTP) nick end labeling (TUNEL) was used to detect the neuronal apoptosis. In mechanism research, the expression of DLK, JIP3, phosphorylated-JNK (p-JNK)/JNK, and cleaved caspase-3 (CC-3) were analyzed by western blot at 24 hrs after SAH. The DLK small interfering RNA (siRNA), JIP3 siRNA, MA2K7 siRNA and recombinant DLK protein which injected intracerebroventricularly were given as the interventions.

Measurements and Main Results—The DLK expression was increased in the left cortex neurons and peaked at 24 hrs after SAH. DLK siRNA attenuated brain edema, reduced neuronal

#Correspondence to John H. Zhang, MD, PhD, Department of Physiology and Pharmacology, Loma Linda University, School of Medicine, 11041 Campus St, Risley Hall, Room 219, Loma Linda, CA 92354, USA, johnzhang3910@yahoo.com.

Disclosure: None

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

apoptosis, and improved the neurobehavioral functions after SAH, but the recombinant DLK protein deteriorated neurobehavioral functions and brain edema. DLK siRNA decreased and recombinant DLK protein increased the expression of MA2K7/p-JNK/CC-3 at 24 hrs after SAH. The JIP3 siRNA reduced the level of JIP3/MA2K7/p-JNK/CC-3, combined DLK siRNA and JIP3 siRNA further decreased the expression of DLK/MA2K7/p-JNK/CC-3, and MA2K7 siRNA lowered the amount of MA2K7/p-JNK/CC-3 at 24 hrs after SAH.

Conclusions—As a negative role, DLK was involved in EBI after SAH, possibly mediated by its adaptor protein JIP3 and MA2K7/JNK signaling pathways. To reduce the level of DLK may be a new target as intervention for SAH.

Graphical Abstract

Keywords

subarachnoid hemorrhage; early brain injury; DLK; neuron; apoptosis

Introduction

Subarachnoid hemorrhage (SAH) is a severe and devastating cerebrovascular disease with high mortality and disability rates (Etminan, 2015; Grunwald et al., 2014). After SAH, early brain injury (EBI) and delayed cerebral vasospasm contribute to the poor clinical outcome (Chen et al., 2013a; Hasegawa et al., 2015). EBI begins immediately after aneurysm rupture and lasts to 72 hrs. Alleviating EBI has become the primary interfering target to reduce SAH brain injury (Kusaka et al., 2004). Among different pathological process, neuronal apoptosis

had been confirmed occurring in EBI and regarded as an apparently negative role (Cahill et al., 2006).

Dual leucine zipper kinase (DLK/MA3K12) is a mitogen-activated protein kinase kinase (MA3K). Accumulative evidences have shown that DLK regulates neuronal apoptosis during neural development (Ghosh et al., 2011) and neurodegeneration (Chen et al., 2008). For example, injury of optic nerve led to DLK elevation and retinal ganglion cells (RGCs) apoptosis; in contrast, deletion of DLK improved mouse RGCs survival rate after optic nerve injury (Watkins et al., 2013; Welsbie et al., 2013). In addition, survived cells were accompanied with decreased MA2K7, p-JNK, c-Jun phosphorylation and cleaved caspase-3 (CC-3) (Itoh et al., 2014; Tedeschi and Bradke, 2013). Further studies indicate DLK can form a signal complex with its adaptor protein JIP3 to activate the downstream signal proteins (Ghosh et al., 2011).

However, the potential role of DLK in stroke, especially in EBI after SAH has not been investigated. In this study, we assessed the effect of DLK on EBI in a rat endovascular perforation SAH model by evaluating behavioral function and neuronal apoptosis as well as DLK, its adaptor protein JIP3 and downstream MA2K7/JNK signaling pathways.

Material and Methods

1. Experimental Design and SAH Model

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Loma Linda University. The schematic illustration of the timeline was displayed (Fig. 1) and total 223 male Sprague-Dawley rats were randomly assigned:

Experiment I (group): Sham (n=8), Different time points SAH (n=36).

Experiment II (group): Sham (n=8+6), SAH (n=6+6), SAH+siRNA vehicle (n=11+7), SAH+scramble siRNA (n=10+7), SAH+DLK siRNA (n=9+6), SAH+recombinant DLK vehicle (n=6+7), SAH+recombinant DLK (n=8+8).

Experiment III (group): Sham (n=6), SAH (n=7), SAH+siRNA vehicle (n=8), SAH+scramble siRNA (n=7), SAH+DLK siRNA (n=7), SAH+recombinant DLK vehicle (n=7), SAH+recombinant DLK protein (n=9), SAH+JIP3 siRNA (n=8), SAH+MA2K7 siRNA (n=8), SAH+DLK siRNA+JIP3 siRNA (n=7).

The endovascular perforation SAH model was performed as previously described (Song et al., 2015; Zhan et al., 2015). Briefly, after anesthesia with isoflurane and intubation, a sharpened 4-0 nylon suture was inserted into left internal carotid artery and advanced until puncturing the bifurcation of the anterior and middle cerebral artery. The suture was withdrawn, resulting in SAH. Sham rats underwent the same procedures without perforation. All rats were euthanized. Briefly, rats were lethally anesthetized, and the heads of rats were got by the decapitation guillotine, then the whole brains were quickly taken out from the skulls (Song et al., 2015).

3. SAH Grade

After euthanasia, rat's brain base was divided into six areas on ice, each area was given a score (0–3). The total SAH grade was calculated by adding all area scores (maximum SAH grade =18)(Sugawara et al., 2008). Twenty-one rats whose SAH grades ≥ 7 were excluded.

4. Neurological Score

Neurological function was assessed with blinded fashion, by the modified Garcia score (Garcia et al., 1995) and beam balance test (Chen et al., 2013b) at 24 and 72 hrs after SAH.

The modified Garcia score included six subtests: spontaneous activity, reaction to side stroking, vibrissae touch, limb symmetry, forelimb walking, and climbing ability. The total score (maximum score =18) was determined by accumulative each subtest score (0–3).

For the beam balance test, rats were arranged to walk on a 15 mm-wide wooden beam for 1 minute. The score (0–4) was decided by the walking distance. The average score of three consecutive trials was calculated.

5. Brain Water Content

Every rat's brain was removed at 24 and 72 hrs after SAH for brain water content test. The left/right hemisphere, cerebellum, and brain stem were separately weighed (wet weight). After drying in 105°C for 72 hrs, every part was weighed again (dry weight). The percentage of water content = (wet weight - dry weight)/wet weight (Liu et al., 2014; Sherchan et al., 2016).

6. Intracerebroventricular Injection Administration

Intracerebroventricular injection was operated as previously described (Chen et al., 2015; Tang et al., 2015). Briefly, rats were placed in a stereotaxic apparatus under anesthesia. The needle of a 10 μ L Hamilton syringe (Microliter 701; Hamilton Company, Reno, NV) was exactly inserted into the left lateral ventricles.

DLK/JIP3/MA2K7 siRNA (500 pmol/5 μ L, Life Technologies, Grand Island, NY) all were a pool of three different siRNA duplexes for improving the knockdown efficiency.

DLK siRNA

- a. sense, GCUCAGGCGAGAGCAAGCUUUAGAA,
antisense, UUCUAAAGCUUGCUCUCGCCUGAGC;
- b. sense, CCCUCAUGUUGCAACUAGAACUCAA,
antisense, UUGAGUUCUAGUUGCAACAUGAGGG;
- c. sense, CCAAUAGUGUCCUGCAGCUACAUGA,
antisense, UCAUGUAGCUGCAGGACACUAUUGG;

JIP3 siRNA

- a. sense, AGCGUCCCACCUCUCUGAAUGUCUU,
antisense, AAGACAUUCAGAGAGGUGGGACGCU;

- b. sense, UGGCAGUUCUUUAGCCGCCUCUUA,
antisense, UGAAGAGGCGGCUAAAGAACUGCCA;
- c. sense, CAGCUGGCUUUAGCCAGCGUCGCAA,
antisense, UUGCGACGCUGGCUAAAGCCAGCUG;

MA2K7 siRNA

- a. sense, CCUUGUUCACACCUCGCAGTT,
antisense, CUGCGAGGUGUGAACAAGGTT;
- b. sense, GGATCGACCTCAACCTGGATAT, antisense,
CCAGCGTTATCAGGCAGAAATC;
- c. sense, CCCTACATTGTTCAGTGCTTTG, antisense,
CAGTGCTTTGGTACCTTCATCA.

SiRNA vehicle—Sterile phosphate buffered saline (PBS) 5 μ L served as siRNA vehicle control. Scramble siRNA: Scramble siRNA (500 pmol/5 μ L) also be used. Above liquid of DLK/JIP3/MA2K7 siRNA, siRNA vehicle and scramble siRNA were injected into intracerebroventricularly by a microinfusion pump (Harvard Apparatus, Holliston, MA) at a rate of 0.5 μ L/minute at 24 hrs before SAH.

Recombinant DLK vehicle—PBS 5 μ L served as recombinant DLK vehicle control. Recombinant DLK: The recombinant DLK (1 μ g, Cedarlane, Burlington, NC) dissolved by 5 μ L PBS. Above liquid of recombinant DLK vehicle and recombinant DLK were injected intracerebroventricularly at 2 hrs after SAH.

7. Immunofluorescence Staining

This double staining was performed as previously described (Tang et al., 2015; Thatipamula et al., 2015). Briefly, brain specimens were fixed in formalin, histological slices 10 μ m thick were subjected to antigen retrieval by microwaving in the citrate buffer at pH 6.0 for 10 minutes. After incubation with blocking serum, brain slices were treated with the primary antibody overnight at 4°C. The primary antibody included mouse anti-neuronal nuclei (NeuN, marker for neuron, Abcam, Cambridge, MA), rabbit anti-glial fibrillary acidic protein (GFAP, marker for astrocyte, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-ionized calcium binding adaptor molecule 1 (Iba1, marker for microglia, Abcam, Cambridge, MA), and goat anti-DLK (Santa Cruz Biotechnology, Santa Cruz, CA). The microphotographs of left cerebral tissue-facing blood clots were analyzed by fluorescent microscope (Olympus OX51, Japan).

8. Quantification of Neuronal Cell Death

For double staining of NeuN (red) and TUNEL (green, Roche, Mannheim, Germany), a TUNEL kit was used after the slices were incubated with anti-NeuN primary antibody and Texas Red-conjugated secondary antibody. TUNEL-positive neurons were counted in left cortex-facing blood clots (3 sections per rat, 4 different areas in 500 $\times$ 500 μ m grids per

section) by a blinded investigator. Data were expressed as TUNEL+Neurons/mm² (Broad et al., 2016; Topkuru et al., 2013).

9. Western Blot

The left cerebral cortex (perforation side) were collected at 24 hrs after SAH, and western blot was performed as described previously (Li et al., 2014). Briefly, brain specimens were homogenized in the RIPA buffer containing protease/phosphatase inhibitors. The protein was extracted and resolved with the SDS-PAGE, then transferred onto nitrocellulose membranes, which were blocked and subsequently incubated at 4°C overnight with primary antibodies: DLK, p-JNK, JNK, actin (Santa Cruz Biotechnology, Santa Cruz, CA), JIP3, MA2K7 (MyBioSource, San Diego, CA), CC-3 (Cell Signaling Technology, Danvers, MA).

10. Statistical Analysis

Data are expressed as means±SD. Statistical significance was verified with ANOVA analysis of variance, followed by Tukey test for multiple comparisons. The probability levels $p<0.05$ were considered as statistically significant. The analysis of mortality was done with χ^2 test. Nonparametric analysis of variance was used for categorical variables.

Results

1. SAH Grade and Mortality Rates

SAH severity scores were measured at 24 hrs after SAH in survived rats, and the mean SAH grading was 12.2 ± 2.5 . There was no significant difference in the severity scores among groups ($p>0.05$).

Mortality rates in experimental groups: Sham 0% (0 of 22); SAH 9% (7 of 51); SAH +siRNA vehicle 23% (5 of 19); SAH+scramble siRNA 17% (3 of 17); SAH+DLK siRNA 9% (2 of 16); SAH+recombinant DLK vehicle 10% (1 of 13); SAH+recombinant DLK 28% (5 of 17); SAH+JIP3 siRNA 25% (2 of 8); SAH+MA2K7 siRNA 25% (2 of 8); SAH+DLK siRNA and JIP3 siRNA 14% (1 of 7). No statistical significance was detected among groups ($p>0.05$).

2. The DLK expression increased in the left cortex and peaked at 24 hrs after SAH

Immunofluorescence showed that DLK expressed in the cortex and co-localized with NeuN. The DLK level was markedly increased in neurons after SAH (Fig. 2A). No marked DLK expression was observed in astrocytes and microglia in sham and SAH groups (data not shown).

Western blot showed that DLK was increased in the cortex after SAH and peaked at 24 hrs ($p<0.05$) before declined at 72 hrs ($p<0.05$) (Fig. 2B).

3. DLK siRNA improved neurobehavioral function and attenuated brain edema, while recombinant DLK protein deteriorated above outcome at 24 hrs after SAH

At 24 hrs after SAH, DLK siRNA obviously improved the modified Garcia score ($p<0.05$; Fig. 3A) but not bean balance score ($p>0.05$) (Fig. 3B). DLK siRNA decreased brain water

content in left and right hemispheres ($p<0.05$; Fig. 3C), but did not reduce brain water content of cerebellum and brain stem ($p>0.05$, data not shown). The recombinant DLK protein significantly deteriorated the modified Garcia score ($p<0.05$; Fig. 3A) and bean balance score ($p<0.05$) (Fig. 3B). Besides, it also increased brain water content in left and right hemispheres ($p<0.05$; Fig. 3C).

At 72 hrs after SAH, DLK siRNA significantly improved the modified Garcia score ($p<0.05$; Fig. 3D) and bean balance score ($p<0.05$) (Fig. 3E). DLK siRNA decreased brain water content in left and right hemispheres ($p<0.05$; Fig. 3F), but did not relieve brain water content of cerebellum and brain stem ($p>0.05$, data not shown). The recombinant DLK protein significantly deteriorated the bean balance score ($p<0.05$) (Fig. 3E) rather than modified Garcia score ($p>0.05$; Fig. 3D). Besides, it also increased brain water content in left hemispheres ($p<0.05$; Fig. 3F).

4. DLK siRNA decreased neuronal apoptosis at 24 hrs after SAH

Immunofluorescence discovered the increased number of TUNEL and NeuN double-stained cells (TUNEL+neurons) in the left cortex after SAH. DLK siRNA reduced the number of TUNEL+neurons, when compared to the SAH+siRNA vehicle and SAH+scramble siRNA groups ($p<0.05$; Fig. 4A,B).

5. The expression of DLK and its adaptor JIP3, downstream signaling proteins in the left cortex at 24 hrs after SAH

Western blot showed that the expression of DLK, its adaptor JIP3 and downstream MA2K7, p-JNK and CC-3 were increased at 24 hrs after SAH (Fig. 5).

6. DLK siRNA reduced and Recombinant DLK protein raised the expression of MA2K7/p-JNK/CC-3 at 24 hrs after SAH

DLK siRNA decreased the level of DLK compared with the SAH+siRNA vehicle and SAH+scramble siRNA group at 24 hrs after SAH ($p<0.05$; Fig. 6A,B), but the amount of JIP3 was increased ($p<0.05$; Fig. 6A,C). MA2K7, p-JNK and CC-3 levels were significantly decreased in SAH+DLK siRNA group compared to the SAH+siRNA vehicle and SAH+scramble siRNA group ($p<0.05$; Fig. 6A,D,E, F).

On the contrary, recombinant DLK protein increased the expression of DLK, MA2K7, p-JNK and CC-3 when compared with SAH+ recombinant DLK vehicle group ($p<0.05$; Fig. 7A,B,D,E,F), but JIP3 expression was not changed when compared to the SAH+ recombinant DLK vehicle group ($p>0.05$; Fig. 7A,C).

7. JIP3 siRNA decreased the expression of JIP3/MA2K7/p-JNK/CC-3 at 24 hrs after SAH

JIP3 siRNA decreased the level of JIP3, MA2K7, p-JNK, and CC-3 but not DLK when compared with the SAH+siRNA vehicle group and SAH+scramble siRNA group ($p<0.05$; Fig. 8A,B,C,D,E,F).

8. MA2K7 siRNA reduced the level of MA2K7/p-JNK/CC-3 at 24 hrs after SAH

MA2K7 siRNA decreased the expression of MA2K7, as well as p-JNK and CC-3 but not DLK and JIP3 when compared with the SAH+siRNA vehicle group and SAH+scramble siRNA group in the left cortex at 24 hrs after SAH ($p<0.05$; Fig. 9A,B,C,D,E,F).

9. Combined DLK siRNA and JIP3 siRNA further decreased the expression of MA2K7/p-JNK/CC-3 at 24 hrs after SAH

Gene silencing of DLK and JIP3 simultaneously further decreased the level of DLK, MA2K7, p-JNK and CC-3 but not JIP3 ($p<0.05$; Fig. 10A,B,C,D,E,F).

Discussion

Parenchymal hematoma compresses surrounding cerebral tissue and usually leads to intracranial hypertension and hydrocephalus (Marbacher et al., 2012). Different from above intracerebral hemorrhage, the blood clots of SAH distributes in subarachnoid space and produces global brain injury after the initial aneurysm rupture (Suwatcharakoon et al., 2016) which is defined as EBI (Kusaka et al., 2004). The mechanisms of EBI mainly include rapid increasing of intracranial pressure (Bederson et al., 1995), reduction in cerebral perfusion pressure (Ayer and Zhang, 2010), and decreasing of cerebral blood flow (Bederson et al., 1998). Therefore, the resulted cerebral ischemia following SAH affects different cerebral regions and causes sporadic infarcts and mostly displays in the form of neuronal apoptosis (Cahill et al., 2006).

DLK protein was found distributed in most regions of the rat brain, including cortex, cerebellum, brain stem, corpus callosum, and optic nerve, as well as spinal cord and dorsal root ganglion (Mata et al., 1996). The expression of DLK was detected predominantly in neurons in adult rat cortex (Merritt et al., 1999). Some experimental studies demonstrated that DLK participated in regulation of neural development, axon degeneration and apoptosis (Bloom et al., 2007; Ghosh et al., 2011; Hirai et al., 2011; Hirai et al., 2006; Hirai et al., 2002; Itoh et al., 2011). Other studies found that down-regulation of DLK improved the survival and the function of RGCs (Watkins et al., 2013; Welsbie et al., 2013). For example, DLK protein was up-regulated and peaked at about 30 hrs in response to optic nerve crush in RGCs (Welsbie et al., 2013) but not in other cells (Watkins et al., 2013). Nevertheless, the potential neuroprotective function of DLK in stroke especially SAH has not been paid enough attention.

In this study, we observed that SAH enhanced the expression of DLK, accompanied by brain edema, neuronal apoptosis and neurological deficits. DLK siRNA reduced brain edema, decreased neuronal apoptosis and improved neurobehavioral function after SAH. On the other side, the recombinant DLK protein deteriorated above benign effect of DLK siRNA. DLK with its adaptor JIP3 and downstream factors MA2K7/p-JNK/CC-3 were all upregulated by SAH, and DLK/JIP3/MA2K7 siRNA reduced downstream factors especially reduced p-JNK and CC-3 levels. On contrary, recombinant DLK protein enhanced the levels of DLK and its downstream factors. Interestingly, knock down JIP3 had no effect on DLK expression but knock down DLK enhanced JIP3 expression.

The JIP belongs to scaffold protein family, joined in aggregation and subcellular localization of various JNK signaling (Gupta et al., 1996). DLK binds with its adaptor JIP1, JIP2 and JIP3 (Ghosh et al., 2011; Merritt et al., 1999; Yasuda et al., 1999) to form a signal complex to activate downstream proteins, yet DLK bound with JIP3 rather than JIP1 and JIP2 to protect neurons of dorsal root ganglions (Ghosh et al., 2011). Therefore, we decided to study JIP3 and its functional relationship with DLK. We observed that JIP3 protein level was increased after gene silencing of DLK, even though silencing both JIP3 and DLK markedly reduced other downstream factors MA2K7/p-JNK/CC-3. Without further evidences to demonstrate the interactions between DLK and JIP3, we speculate that since knock down of DLK will reduce combined DLK-JIP3, among of free JIP3 may be increased (Fig. 6A). It may also be possible that the expression of JIP3 was compensatory increasing after DLK level was reduced by gene silencing.

As a member of the MA2K, MA2K7 is a downstream of DLK. Previous studies showed that MA2K4 and MA2K7 have interactions with DLK, and multiple MA3Ks have capability to activate JNK via MA2K4/MA2K7 (Itoh et al., 2014; Robitaille et al., 2008; Xu et al., 2001). It has been suggested that DLK and MA2K7 were localized in similar nuclear and extranuclear compartments of neurons, but MA2K4 located distinctly from those of DLK and MA2K7. Moreover, DLK was overexpressed by plasmid transfection in cells and activated MA2K7 rather than MA2K4 (Merritt et al., 1999). Therefore, we selected MA2K7 as the downstream of DLK in this study. Besides, the Merritt, S.E., et al research found that the DLK regulated its downstream molecule MA2K7 by changing its expression levels in vitro and vivo (Merritt et al., 1999), so we chose to assess the MA2K7 expression levels in our research and got the positive results.

In a prior study, we focused on the effect of drug tozasertib on neuronal apoptosis after SAH in rats, and the results showed DLK participated into above process (Yin et al., 2016). However, whether DLK independently took part in neuronal apoptosis and the potential molecular signaling pathway have not been elucidated. In this study, the results discovered that directed intervention of against DLK attenuated the harmful outcome after SAH. It indicated DLK played an essential role in neuronal apoptosis in SAH rats, and not just a small participant of tozasertib effectiveness. Besides, the administration of JIP3 (DLK adaptor) siRNA clarified the relationship between DLK and JIP3 in this model. The MA2K7 siRNA was used to confirm MA2K7 belonged to DLK downstream signaling pathway. After applying each siRNA, the target protein and the other proteins (DLK/JIP3/MA2K7/p-JNK/CC-3) were detected for proving the relation to each other.

There were several limitations in our study. Firstly, previous studies revealed that DLK could be activated in injured axons and promoted axonal regeneration (Nix and Bastiani, 2012; Valakh et al., 2015), but other studies suggested the role of DLK in facilitating neuronal apoptosis (Ghosh et al., 2011; Itoh et al., 2011; Itoh et al., 2014). It is probable that DLK and its signaling pathway like a double-edged sword in pathological processes of nervous system (Tedeschi and Bradke, 2013) and participate in both axonal regeneration and cell death. In our study, we observed that DLK contributes to neuronal apoptosis and abolition of DLK was beneficial for neuronal surviving after SAH. Secondly, in this research we focused on MA2K7-JNK pathway, which was an established pathway in neuronal apoptosis. But we

could not rule out if DLK contribute to neurological injury by other signaling pathways. Thirdly, in view of this study was designed to examine EBI, so the long term effects of DLK inhibition had not been examined. Overall, perhaps other potential signaling pathways of DLK exist in neurological injury and long term effects of silencing DLK and its downstream factors warrant further investigation.

Conclusions

We demonstrated that DLK protein expression was enhanced after SAH. By gene silencing DLK relieved brain edema, decreased neuronal apoptosis, and improved neurobehavioral functions, but the recombinant DLK protein deteriorated above effect. It is likely that DLK produced these effect via its adaptor protein JIP3 and downstream MA2K7/JNK signaling pathways. Thus, targeting DLK maybe a novel strategy to decrease neuronal apoptosis and ameliorate EBI after SAH.

Acknowledgments

This study is partially supported by the National Institutes of Health grants NS081740 and NS084921 to JHZ.

References

- Ayer R, Zhang J. Connecting the early brain injury of aneurysmal subarachnoid hemorrhage to clinical practice. *Turk Neurosurg.* 2010; 20:159–66. [PubMed: 20401843]
- Bederson JB, et al. Cortical blood flow and cerebral perfusion pressure in a new noncraniotomy model of subarachnoid hemorrhage in the rat. *Stroke.* 1995; 26:1086–91. discussion 1091–2. [PubMed: 7762027]
- Bederson JB, et al. Acute vasoconstriction after subarachnoid hemorrhage. *Neurosurgery.* 1998; 42:352–60. discussion 360–2. [PubMed: 9482187]
- Bloom AJ, et al. The requirement for Phr1 in CNS axon tract formation reveals the corticostriatal boundary as a choice point for cortical axons. *Genes Dev.* 2007; 21:2593–606. [PubMed: 17901218]
- Broad KD, et al. Inhaled 45–50% argon augments hypothermic brain protection in a piglet model of perinatal asphyxia. *Neurobiol Dis.* 2016; 87:29–38. [PubMed: 26687546]
- Cahill J, et al. Mechanisms of early brain injury after subarachnoid hemorrhage. *J Cereb Blood Flow Metab.* 2006; 26:1341–53. [PubMed: 16482081]
- Chen Q, et al. Chronic hydrocephalus and perihematoma tissue injury developed in a rat model of intracerebral hemorrhage with ventricular extension. *Transl Stroke Res.* 2015; 6:125–32. [PubMed: 25167916]
- Chen S, et al. Controversies and evolving new mechanisms in subarachnoid hemorrhage. *Prog Neurobiol.* 2013a; 115:64–91. [PubMed: 24076160]
- Chen S, et al. P2X7 receptor antagonism inhibits p38 mitogen-activated protein kinase activation and ameliorates neuronal apoptosis after subarachnoid hemorrhage in rats. *Crit Care Med.* 2013b; 41:e466–74. [PubMed: 23963136]
- Chen X, et al. Antiapoptotic and trophic effects of dominant-negative forms of dual leucine zipper kinase in dopamine neurons of the substantia nigra in vivo. *J Neurosci.* 2008; 28:672–80. [PubMed: 18199767]
- Etmann N. Aneurysmal subarachnoid hemorrhage--status quo and perspective. *Transl Stroke Res.* 2015; 6:167–70. [PubMed: 25860440]
- Garcia JH, et al. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. *Stroke.* 1995; 26:627–34. discussion 635. [PubMed: 7709410]

- Ghosh AS, et al. DLK induces developmental neuronal degeneration via selective regulation of proapoptotic JNK activity. *J Cell Biol.* 2011; 194:751–64. [PubMed: 21893599]
- Grunwald IQ, et al. Aneurysmal SAH: current management and complications associated with treatment and disease. *J Invasive Cardiol.* 2014; 26:30–7. [PubMed: 24402809]
- Gupta S, et al. Selective interaction of JNK protein kinase isoforms with transcription factors. *Embo Journal.* 1996; 15:2760–2770. [PubMed: 8654373]
- Hasegawa Y, et al. Characteristics of Cerebrovascular Injury in the Hyperacute Phase After Induced Severe Subarachnoid Hemorrhage. *Transl Stroke Res.* 2015; 6:458–66. [PubMed: 26358229]
- Hirai S, et al. Axon formation in neocortical neurons depends on stage-specific regulation of microtubule stability by the dual leucine zipper kinase-c-Jun N-terminal kinase pathway. *J Neurosci.* 2011; 31:6468–80. [PubMed: 21525288]
- Hirai S, et al. The c-Jun N-terminal kinase activator dual leucine zipper kinase regulates axon growth and neuronal migration in the developing cerebral cortex. *J Neurosci.* 2006; 26:11992–2002. [PubMed: 17108173]
- Hirai S, et al. MAPK-upstream protein kinase (MUK) regulates the radial migration of immature neurons in telencephalon of mouse embryo. *Development.* 2002; 129:4483–95. [PubMed: 12223406]
- Itoh A, et al. ZPK/DLK, a mitogen-activated protein kinase kinase kinase, is a critical mediator of programmed cell death of motoneurons. *J Neurosci.* 2011; 31:7223–8. [PubMed: 21593306]
- Itoh T, et al. ZPK/DLK and MKK4 Form the Critical Gateway to Axotomy-Induced Motoneuron Death in Neonates. *J Neurosci.* 2014; 34:10729–42. [PubMed: 25100604]
- Kusaka G, et al. Signaling pathways for early brain injury after subarachnoid hemorrhage. *J Cereb Blood Flow Metab.* 2004; 24:916–25. [PubMed: 15362722]
- Li H, et al. Evaluation of the protective potential of brain microvascular endothelial cell autophagy on blood-brain barrier integrity during experimental cerebral ischemia-reperfusion injury. *Transl Stroke Res.* 2014; 5:618–26. [PubMed: 25070048]
- Liu F, et al. Recombinant Milk Fat Globule-EGF Factor-8 Reduces Oxidative Stress via Integrin beta3/ Nuclear Factor Erythroid 2-Related Factor 2/Heme Oxygenase Pathway in Subarachnoid Hemorrhage Rats. *Stroke.* 2014
- Marbacher S, et al. A new rabbit model for the study of early brain injury after subarachnoid hemorrhage. *J Neurosci Methods.* 2012; 208:138–45. [PubMed: 22595025]
- Mata M, et al. Characterization of dual leucine zipper-bearing kinase, a mixed lineage kinase present in synaptic terminals whose phosphorylation state is regulated by membrane depolarization via calcineurin. *J Biol Chem.* 1996; 271:16888–96. [PubMed: 8663324]
- Merritt SE, et al. The mixed lineage kinase DLK utilizes MKK7 and not MKK4 as substrate. *J Biol Chem.* 1999; 274:10195–202. [PubMed: 10187804]
- Nix P, Bastiani M. DLK: the “preconditioning” signal for axon regeneration? *Neuron.* 2012; 74:961–3. [PubMed: 22726825]
- Robitaille K, et al. Calphostin C-induced apoptosis is mediated by a tissue transglutaminase-dependent mechanism involving the DLK/JNK signaling pathway. *Cell Death Differ.* 2008; 15:1522–31. [PubMed: 18497756]
- Sherchan P, et al. Recombinant Slit2 attenuates neuroinflammation after surgical brain injury by inhibiting peripheral immune cell infiltration via Robo1-srGAP1 pathway in a rat model. *Neurobiol Dis.* 2016; 85:164–73. [PubMed: 26550694]
- Song J, et al. Correlating Cerebral (18)FDG PET-CT Patterns with Histological Analysis During Early Brain Injury in a Rat Subarachnoid Hemorrhage Model. *Transl Stroke Res.* 2015; 6:290–5. [PubMed: 25833084]
- Sugawara T, et al. A new grading system evaluating bleeding scale in filament perforation subarachnoid hemorrhage rat model. *J Neurosci Methods.* 2008; 167:327–34. [PubMed: 17870179]
- Suwatcharakoon S, et al. Loss of Consciousness at Onset of Subarachnoid Hemorrhage as an Important Marker of Early Brain Injury. *JAMA Neurol.* 2016; 73:28–35. [PubMed: 26552033]
- Tang J, et al. Neuroprotective Role of an N-acetyl Serotonin Derivative via Activation of Tropomyosin-related Kinase Receptor B after Subarachnoid Hemorrhage in a Rat Model. *Neurobiol Dis.* 2015

- Tedeschi A, Bradke F. The DLK signalling pathway--a double-edged sword in neural development and regeneration. *EMBO Rep.* 2013; 14:605–14. [PubMed: 23681442]
- Thatipamula S, et al. Genetic deletion of neuronal pentraxin 1 expression prevents brain injury in a neonatal mouse model of cerebral hypoxia-ischemia. *Neurobiol Dis.* 2015; 75:15–30. [PubMed: 25554688]
- Topkora BC, et al. Nasal administration of recombinant osteopontin attenuates early brain injury after subarachnoid hemorrhage. *Stroke.* 2013; 44:3189–94. [PubMed: 24008574]
- Valakh V, et al. Cytoskeletal disruption activates the DLK/JNK pathway, which promotes axonal regeneration and mimics a preconditioning injury. *Neurobiol Dis.* 2015; 77:13–25. [PubMed: 25726747]
- Watkins TA, et al. DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury. *Proc Natl Acad Sci U S A.* 2013; 110:4039–44. [PubMed: 23431164]
- Welsbie DS, et al. Functional genomic screening identifies dual leucine zipper kinase as a key mediator of retinal ganglion cell death. *Proc Natl Acad Sci U S A.* 2013; 110:4045–50. [PubMed: 23431148]
- Xu Z, et al. The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. *Mol Cell Biol.* 2001; 21:4713–24. [PubMed: 11416147]
- Yasuda J, et al. The JIP group of mitogen-activated protein kinase scaffold proteins. *Mol Cell Biol.* 1999; 19:7245–54. [PubMed: 10490659]
- Yin C, et al. Tozasertib attenuates neuronal apoptosis via DLK/JIP3/MA2K7/JNK pathway in early brain injury after SAH in rats. *Neuropharmacology.* 2016; 108:316–23. [PubMed: 27084696]
- Zhan Y, et al. Imatinib preserves blood-brain barrier integrity following experimental subarachnoid hemorrhage in rats. *J Neurosci Res.* 2015; 93:94–103. [PubMed: 25196554]

Highlights

The level of DLK was increased in the left cortex and peaked at 24 hours after SAH.

Reduction of DLK by siRNA led to decreased brain edema, neuronal apoptosis and improved neurological functions.

Reducing DLK is neuroprotective and this effect may be mediated by JIP3 and MA2K7/JNK signal pathway.

Fig. 1.
The schematic illustration of the timeline in all experiments.

Fig. 2.

The expression of DLK was increased in cortex neurons and peaked at 24 hrs after SAH. **A**, Immunofluorescence showed a few neurons expressed DLK in sham group, but in SAH group, the number of neuron which expressed DLK was increased after SAH at 24 hrs. **B**, DLK at different time points in sham and SAH group. Scale bar, 30 μ m. * p <0.05 vs sham, # p <0.05 vs SAH 24 hrs.

Fig. 3.

DLK siRNA improved the neurobehavioral deficits and attenuated brain edema, while recombinant DLK protein deteriorated above outcome at 24 and 72 hrs after SAH. **A,D**, Modified Garcia score test was significantly improved by DLK siRNA at 24 and 72 hrs after SAH, but only was significantly worsened by the recombinant DLK protein at 72 hrs after SAH. **B,E**, Beam balance score only was markedly improved by DLK siRNA at 72 hrs after SAH, but was significantly worsened by the recombinant DLK protein at 24 and 72 hrs after SAH. **C,F**, DLK knock-down significantly decreased brain water content in the bilateral hemispheres, while the recombinant DLK protein reversed above effect at 24 and 72 hrs after SAH. * $p < 0.05$ vs sham, # $p < 0.05$ vs SAH/SAH+siRNA vehicle/SAH+scramble siRNA, ## $p < 0.05$ vs SAH+recombinant DLK vehicle.

Fig. 4.

DLK siRNA reduced neuronal apoptosis at 24 hrs after SAH. **A**, Immunofluorescence showed the TUNEL+neurons in sham group, SAH+ siRNA vehicle group, SAH+scramble siRNA group and SAH+DLK siRNA group. **B**, DLK siRNA administration obviously decreased the TUNEL-positive cells. Scale bar, 30 μ m, * p <0.05 vs sham, # p <0.05 vs SAH +siRNA vehicle/SAH+scramble siRNA.

Fig. 5.
The level of DLK, its adaptor JIP3 and downstream signaling proteins MA2K7/p-JNK (JNK)/CC-3 were increased in the left cortex at 24 hrs in SAH group.

Fig. 6.

DLK siRNA potentiated JIP3 but decreased the expression of DLK/MA2K7/p-JNK and CC-3 in the left cortex at 24 hrs after SAH. **A**, All western blot. **B, C, D, E, F**, Quantitative analysis of DLK/JIP3/MA2K7/p-JNK (JNK)/CC-3. * $p < 0.05$ vs sham, # $p < 0.05$ vs SAH +siRNA vehicle/SAH+scramble siRNA.

Fig. 7.

Recombinant DLK protein enhanced the expression of DLK/MA2K7/p-JNK/CC-3 except JIP3, in the left cortex at 24 hrs after SAH. **A**, All western blot. **B, C, D, E, F**, Quantitative analysis of DLK/JIP3/MA2K7/p-JNK (JNK)/CC-3. * $p < 0.05$ vs sham, # $p < 0.05$ vs SAH+ recombinant DLK vehicle.

Fig. 8.

JIP3 siRNA decreased the expression of JIP3/MA2K7/p-JNK/CC-3 except DLK in the left cortex at 24 hrs after SAH. **A**, All western blot. **B**, **C**, **D**, **E**, **F**, Quantitative analysis of DLK/JIP3/MA2K7/p-JNK (JNK)/CC-3. * $p < 0.05$ vs sham, # $p < 0.05$ vs SAH+siRNA vehicle/SAH+scramble siRNA.

Fig. 9.

MA2K7 siRNA decreased the amount of MA2K7/p-JNK/CC-3 except the DLK and JIP3 in the left cortex at 24 hrs after SAH. **A**, All western blot. **B**, **C**, **D**, **E**, **F**, Quantitative analysis of DLK/JIP3/MA2K7/p-JNK (JNK)/CC-3. * $p < 0.05$ vs sham, # $p < 0.05$ vs SAH+ siRNA vehicle group/SAH+scramble siRNA group.

Fig. 10.

DLK siRNA combined with JIP3 siRNA further decreased the expression of DLK, MA2K7/p-JNK/CC-3 in the left cortex at 24 hrs after SAH. No additional synergistic effect was observed on JIP3 expression. **A**, All western blot. **B, C, D, E, F**, Quantitative analysis of DLK/JIP3/MA2K7/p-JNK (JNK)/CC-3. * $p < 0.05$ vs SAH+DLK siRNA, # $p < 0.05$ vs SAH +JIP3 siRNA.