Original Paper

Raf Kinase Inhibitor Protein Attenuates Ischemic-Induced Microglia Cell Apoptosis and Activation Through NF- κ B Pathway

Li Su^a Rongrong Zhang^b Yuanyuan Chen^a Chao Ma^c Zhenyu Zhu^a

^aSchool of Pharmacy, Second Military Medical University, Shanghai, ^bDepartment of Gynecology, Xinhua Hospital, School of Medicine, Shanghai Jiao Tong University, Shanghai, ^cDepartment of Radiology, Changhai Hospital, Second Military Medical University, Shanghai, China

Key Words

RKIP • MCAO • NF- κ B pathway • Ischemic stroke • OGD

Abstract

Background: Acute ischemic stroke is one of the most important factors leading to disability and death with the characterization of accumulated neuron death and injured supportive neurovascular structures. Raf-1 kinase inhibitory protein (RKIP) is a key molecule in cell response to survival or death stimuli. However, the role of RKIP in stroke is worthy to be further studied.

Methods: We used lentivirus mediated RKIP knockdown and overexpression to investigate the effect of RKIP on animal models of focal cerebral ischemia. Cell Counting Kit-8 assay, lactate dehydrogenase release analysis, and Annexin V-APC apoptosis assay were used to detect the effect of RKIP on microglial cell apoptosis and survival. Transwell migration assay was carried out to evaluate the migration of microglia cells. The releases of inflammatory cytokines were determined by ELISA. The activation of NF- κ B signaling pathway was determined by western blot. **Results:** Overexpression of RKIP reduced focal cerebral ischemia injury. RKIP knockdown and overexpression regulated survival, activation, and motility via the NF- κ B pathway. NF- κ B inhibitor BAY 11-7082 blocked the changes caused by RKIP down-regulation after oxygen-glucose deprivation (OGD). RKIP overexpression inhibited the upregulation of phosphorylation of NF- κ B induced by OGD and cerebral ischemia. **Conclusions:** The present study showed that RKIP protects against ischemic stroke through inhibition of microglial excessive activation, inhibits its motility, and promotes neuronal survival partly through IKK β -I κ B α -NF- κ B signaling axis and indicate that RKIP is a new target for the treatment of ischemic stroke.

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L. Su and R. Zhang contributed equally to this work.

Zhenyu Zhu,
and Chao Ma

School of Pharmacy, Second Military Medical University, Shanghai, China. 325 Guo-He Road, Shanghai, 200433 (China) and Department of Radiology, Changhai Hospital, Second Military Medical University, Shanghai, 168 Chang-Hai Road, Shanghai, 200433 (China); E-Mail zzyzfyh@163.com / machaohdsd@126.com

Introduction

Stroke is a leading cause of death in the world, and ischemic stroke is the main subtype [1]. Ischemic stroke occurs when an acute obstruction of arterial blood flow to the brain. The American Association of Neurology clarified that the defense mechanism against stroke is considered key to the development of new treatments for stroke treatment and prevention.

Raf-1 kinase inhibitory protein (RKIP), belongs to the family of phosphatidylethanolamine-binding protein, is widely expressed in various tissues of mammals [2]. Since the first time discovered in brain and designed as a physiological inhibitor for Raf kinase protein, RKIP is likely to be a key molecule for cellular response to stimuli. In cancer, Alzheimer's disease, and other pathological conditions, the interrupt of RKIP has been proved. For example, RKIP has a variety of physiological functions in brain, including its function in the synaptic plasticity and circadian clock [3]. As a precursor protein of hippocampal cholinergic neurons, RKIP is important to synthesis acetylcholine and develop septal cholinergic neurons for learning and memory [4]. Furthermore, RKIP expression disorders can lead to diabetic nephropathy and Alzheimer's disease [5]. In addition, RKIP involved in the process of neuronal apoptosis and cognitive dysfunction in neurodegenerative diseases [6]. It is recognized that RKIP plays an important role in many key intracellular signaling pathways that control cell growth, mobility, survival, and therapeutic resistance [7, 8]. Through interaction with NIK and TAK1, RKIP negatively regulates the NF- κ B pathway [9, 10]. RKIP downregulation and the activation of NF- κ B and AP-1 pathways could lead to cell apoptosis [11, 12]. Moreover, RKIP has been shown to be a metastasis suppressor gene related to cell proliferation, differentiation, migration, motility and mitosis, as well as neural development, cardiac function and spermatogenesis [13-15]. These reports suggest RKIP may be an interesting target for individualized treatment and disease specific intervention.

In our previous study, we demonstrated that RKIP plays a key role in PC12 cells apoptosis caused by OGD partly via regulating NF- κ B and ERK pathways [16]. However, the role of RKIP in the brain has been poorly investigated. In the present study, we show that RKIP plays a defensive role against ischemic stroke and the neuroprotection of RKIP is partly regulated by NF- κ B pathway.

Materials and Methods

Middle cerebral artery occlusion

Sprague-Dawley rats (male, 180-200 g) were purchased from Shanghai SLAC Lab. Animal Co., Ltd. Middle cerebral artery occlusion (MCAO) surgery was performed as described [17]. Briefly, chloral hydrate at 350 mg/kg was injected intraperitoneally in rats for anesthesia. A heating lamp and a homeothermic heating pad were used to maintain 37°C. A silicone rubber-coated nylon monofilament was introduced into the internal carotid artery. After two hours, the filament was withdrawn. At 24 hours after MCAO, rats were sacrificed for subsequent examinations. Neurological deficit score was recorded according to Longa's method [18]. Rats were sacrificed 24 h after reperfusion in ischemia brain and brains were cutted coronally into 2-mm slices. Slices were immersed in 1% 2,3,5-triphenyl tetrazolium chloride (Sigma Chemical Co., St. Louis, MO). After 4% paraformaldehyde preservation, the infarcted regions areas were calculated by Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

The present protocol and animal experiments were approved by the local institutional review board in accordance with the use of animal care and established institutional guidelines at the Second Military Medical University.

Oxygen-glucose deprivation (OGD)

One day after the separation of the cerebral cortex of newborn rats, the primary neuronal cells were replenished and produced cultured cells with >90% neurons. Neurons were confirmed by staining of NeuN (Cell Signaling, Beverly, MA). After 7 days, cells were transfected with lentivirus for overexpression or knockdown of RKIP. After transfection with RKIP overexpression or shRNA lentivirus for 48 h, microglia

cells were replaced with glucose free medium and subjected to OGD. Cultures were switched from the normal feeding medium to the oxygen-depleted, glucose-free medium. Cells were incubated in a hypoxia chamber (Forma Scientific, Marietta, OH) previously flushed for 15 min with 5% CO₂/95% N₂. After that, valve was closed and then chamber was incubated for 12 h. Control cultures were maintained under normal oxygen atmospheric conditions.

Cell viability assay

Cell viability was assayed by Cell Counting Kit-8 according to the manufacturer's instructions (Dojindo, Kumamoto, Japan). Lactate dehydrogenase (LDH) release test was performed as instructed by the manufacturer (Promega, Madison, WI). Cell apoptosis assay was analyzed by APC-Annexin V/PI kit using flow cytometry BD FACSCalibur (BD Biosciences, San Jose, CA, USA).

Molecular cloning and lentivirus production

The coding sequence of RKIP and the primers used were generated as described previously [19]. RKIP cDNA was constructed into the vector by Shanghai R&S Biotechnology Co. Ltd. Virus particles were packaged in 293T cells with the constructed plasmid. After 48 h transfection, the virus particles were collected and titered on 293T cells. For RNA interference, short hairpin RNA (shRNA) targeted RKIP were designed (GenBank accession no. NM_178812.3) and cloned into pLenti6.3-MCS/V5 DEST lentiviral vector by Shanghai R&S Biotechnology Co. Ltd. The infectious lentivirus was produced by 293T cells. Packaging efficiency was determined by a fluorescence microscopy. At 60% confluence in six well plates, cells were transfected with LV-GFP mixed with sh-Scramble, LV-RKIP and sh-RKIP, respectively. Transduction efficiency was determined to be greater than 80% by measuring the green fluorescent protein.

Lentivirus administration in vivo

For lentivirus infusion, saline, LV-GFP, or LV-RKIP (2 µl per site; 5×10⁸ transduction units/ml) was injected into the rat hippocampus and cortex at 4 different locations stereotactically according to the reference [17].

Immunoblotting

Immunoblotting assays were performed as previously described [20]. Samples were harvested and washed with PBS and lysed for electrophoresis. Protein was determined by a BCA kit (Pierce, Rockford, IL, USA), electrophoresed on 10% SDS polyacrylamide gels after boiling for 5 min, and then transferred to PVDF membranes (Millipore, Bedford, MA). The membranes were incubated with p-IKKβ, p-IκBα, p-p65, and IKKβ antibodies (Cell Signaling, Beverly, MA) diluted according to the manufacturers' instructions. Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE) was used to capture the images. Protein densitometry was scanned and normalized against GAPDH (Beyotime, Jiangsu, China).

Transwell migration assay

Migration assay was performed using transwell chambers containing 8 µm pore filters (Corning, Lowell, MA). Cells were inoculated at a density of 1×10⁵ in the upper chamber with 200 µl serum free medium. The lower chamber was added 600 µl DMEM supplemented 10% fetal bovine serum. After 12-h incubation at 37°C, cells that migrated to the lower surface of the membrane were stained in 0.5% crystal violet for 20 min. Migration of cells from five random fields were photographed and counted to estimate microglia cell migration.

Immunohistochemistry

Brain slices were immobilized on 4% poly formaldehyde, 10% goat serum closed, and incubated with anti-RKIP (Abcam, 1:400) anti-IBA-1 (Abcam, 1:200), and anti-NeuN (CST, 1:200), respectively. After rinsed in PBS, slices were incubated with secondary antibodies (Alexa 488-conjugated or Cy3-conjugated). TUNEL assay was performed according to the manufacturer's instructions (Roche, Penzberg, Germany). Images were captured by fluorescence microscope (Leica Microsystems, Mannheim, Germany) with camera. Digital images were recorded and analyzed using Image-Pro Plus 5.0 software (Media Cybernetics, Silver Spring, USA).

Cytokines ELISA and real-time PCR assays

IL-1 β , IL-6, iNOS and TNF- α levels were assayed according to the manufacturer's instructions (eBioscience, San Diego, CA). Total RNA of microglia cells was extracted with TRIzol according to the manufacturer's instructions (Invitrogen). cDNA was subjected with denaturing at 95°C for 10 s, annealing at 55°C for 15 s, and extension at 72 °C for 30 s for 45 cycles. A SYBR Green RT-PCR kit (Takara) was used for real-time RT-PCR analysis. The quantitative data were normalized to GAPDH expression. PCR amplification was performed using the following primers: RKIP sense 5'-GACTACGGCGGAGTAACGGTG-3', antisense 5'-GGGGTCCTTCCTGCTGGG-3'; GAPDH sense 5'-GGAGTCCACTGGCGTCTTCAC-3'; GAPDH antisense 5'-GAGGCATTGCTGATGATCTTGAGG-3'.

Statistical analysis

Data were expressed as mean $\pm$ SEM. The comparison between two groups was analyzed by unpaired Student *t*-test and multiple comparisons were compared by one-way ANOVA analysis of variance followed by Dunnett's test. *P* value of 0.05 or less was considered statistically significant.

Results

RKIP overexpression protects against ischemic stroke

The effect of RKIP on ischemic stroke was further studied by local overexpression in the brain. LV-RKIP or the empty vector encoding green fluorescent protein (LV-GFP) was injected into cerebral cortex and hippocampus at 4 locations. MCAO was carried out after 3 weeks of injection and 24 hours later, animals were killed for subsequent experiments (Fig. 1A). Injection of LV-RKIP led to an approximately 5-fold increase of protein levels in the targeted

Fig. 1. Lentivirus (LV)-mediated RKIP overexpression protects against ischemic stroke. (A) Time schedule for lentivirus injection, MCAO operation, and sacrifice for examinations. (B) Immunoblots and quantification showing efficient overexpression of RKIP by LV-RKIP. (C) Immunohistochemistry staining of RKIP, IBA-1, microglia marker IBA-1, and neuron marker NeuN in brain tissue (magnification 200 $\times$). Immunohistochemistry quantification showing efficient overexpression of RKIP (D) and increase the number of neurons (E) by LV-RKIP. RKIP overexpression by LV-RKIP in local brain reduces ischemic infarction (F, G), neurological deficit (H), and peri-infarct penumbral cell injury (I) 24 hours after MCAO in rats. **P* < 0.05 and ***P* < 0.01 vs vector control (LV-GFP); *n* = 3.

region of the brain (Fig. 1B). Injection of LV-RKIP led to an approximately 5-fold increase of RKIP protein levels in targeted brain regions (Fig. 1B). Immunohistochemistry shown efficient overexpression of RKIP by LV-RKIP and activation of microglia was inhibited by IBA-1 expression (Fig. 1C, 1D). The number of neurons was increased in the LV-RKIP group indicated by neuronal marker NeuN staining (Fig. 1E). LV-RKIP was significantly reduced infarct size in rats (Fig. 1F, 1G) and ameliorated neurological deficit score (Fig. 1H). Brain cells injury in penumbra was significantly decreased in LV-RKIP group (Fig. 1I).

OGD downregulates RKIP in microglia cells

Firstly, RKIP expression was determined in microglia cells. Quantitative PCR results demonstrated that RKIP mRNA was downregulated after OGD (Fig. 2A). RKIP lentivirus overexpression (LV-RKIP) and interference (sh-RKIP) significantly caused increase and decrease of RKIP mRNA expression, respectively. Western blot results confirmed RKIP overexpression after LV-RKIP transfection. In contrast, RNA interference reduced RKIP expression after OGD. In addition, the LV-GFP and sh-Scramble vector (vector) had not obvious effect on the expression of RKIP after exposure to OGD (Fig. 2B, 2C). Together, these results show that the downregulation of RKIP was occurred after OGD and transfection with RKIP lentivirus in microglia cells was effective.

RKIP protects microglia cells from OGD-induced injuries

We further investigated the effect of RKIP on OGD-induced microglia cells apoptosis. As shown in Fig. 3A and 3B, after treatment with sh-RKIP for 24 h, the survival rate of microglia cells was significantly reduced while RKIP overexpression significantly increased after OGD. Furthermore, RKIP overexpression significantly attenuated microglia cells apoptosis while RKIP knockdown showed the reverse effect. Briefly, OGD-induced microglia cells displayed a cell apoptosis profile with an elevated necrotic and apoptotic cells rates (Fig. 3C). In addition, the LV-GFP and sh-Scramble vector had no obvious effect on microglia cells apoptosis after OGD. Together, these data indicated that RKIP regulates OGD-induced microglia cells apoptosis.

RKIP overexpression inhibits microglia cell motility

We next investigated whether RKIP may affect the migration of microglia cells. Microglia cells were infected with lentiviral vector to express RKIP and cell migration ability was assessed by a transwell migration assay. The results showed that overexpressing RKIP

Fig. 2. The expression of RKIP in microglia cells was determined by real-time PCR. (A) and western blot (B) after OGD. GAPDH acted as the internal control. For real-time PCR and western blot, the data was from three independent experiments (n=3), and quantitative comparisons (C) were made by densitometry relative to GAPDH. ** $P < 0.01$ versus control and ## $P < 0.01$ versus OGD.

Fig. 3. RKIP regulated OGD-induced microglia cells apoptosis. The effect of RKIP overexpression and knock-down on the cell viability (A) and LDH (B) release of normal and OGD-induced microglia cells. (C) Apoptosis assay of RKIP overexpression and knockdown to OGD-induced microglia cells by flow cytometry. * $P < 0.05$ and ** $P < 0.01$ versus LV-GFP; # $P < 0.05$ and ## $P < 0.01$ versus sh-Scramble.

Fig. 4. RKIP regulated OGD-induced microglia cells motility. The migration ability of microglia cells following infection with RKIP overexpression and knockdown lentivirus and non-infected cells were analyzed by migration assay. All experiments were repeated three times with identical results. ** $P < 0.01$ versus LV-GFP and ## $P < 0.01$ versus sh-Scramble.

in microglia cells decreased cell transwell migration while RKIP knockdown showed the reverse effect (Fig. 4).

Regulation on NF- κ B pathway by RKIP contributes to its neuroprotection in microglia cells after OGD

We also investigated the role of RKIP on NF- κ B pathway. RKIP overexpression and BAY 11-7082 significantly increased the survival rate of OGD induced microglia cells (Fig. 5A,

Fig. 5. RKIP regulates neuro cells survival induced by OGD partly via NF- κ B Pathway. The effect of RKIP overexpression, knockdown and BAY 11-7082 on the cell viability (A), LDH (B) release and cytokine assays (E) of normal and OGD-induced microglia cells. (C) The phosphorylation expression of IKK β , I κ B α , and p65 were measured by western blot. (D) Quantitative comparisons of protein bands in microglia cells were analyzed by densitometry normalized to GAPDH. (F) The phosphorylation expression of IKK β , I κ B α , and p65 were measured by western blot in LV-GFP and LV-RKIP MCAO rat brains. (G) Quantitative comparisons of protein bands in rat brains were analyzed by densitometry normalized to GAPDH. (H) The expression of cytokine was assayed after LV-RKIP injection by ELISA. Data shown are the results of three different experiments. * $P < 0.05$ and ** $P < 0.01$ versus vector control.

Fig. 6. Proposed mechanisms of the effect of RKIP in ischemic stroke and the neuroprotection of RKIP through NF- κ B pathway. RKIP inhibits of microglia excessive activation, inhibits its motility, and promotes neuronal survival partly through suppressing the IKK β -I κ B α -NF- κ B signaling axis. KD, knockdown; OE, overexpression.

5B). Furthermore, the phosphorylation of IKK β , I κ B α , and p65 were increased obviously, indicating that NF- κ B is excessively activated by OGD. RKIP overexpression inhibited the upregulation of phosphorylation of NF- κ B induced by OGD. While RKIP downregulation promoted the activation of NF- κ B pathway (Fig. 5C, 5D). Moreover, the expression of IL-1 β , IL-6, iNOS, and TNF- α were decreased after RKIP overexpression (Fig. 5E). In addition, IKK β , I κ B α , and p65 phosphorylation were inhibited in LV-RKIP rat brain group (Fig. 5F, 5G). It was also shown that the expression of IL-1 β , IL-6, iNOS, and TNF- α were decreased after LV-RKIP injection (Fig. 5H). Taken together, the results demonstrate that regulation of cell apoptosis by RKIP is partly dependent on the NF- κ B pathway.

Discussion

RKIP has been identified as an inhibitor of the NF- κ B pathway [9, 10]. RKIP affects cell differentiation, apoptosis, and migration by regulating NF- κ B pathways [7, 21]. NF- κ B pathway is also known to alter the expression of proinflammatory factor such as IL-6, iNOS, IL-1 β , and TNF- α by regulating the activity of AP-1 transcription factors [22, 23]. Therefore, RKIP is considered to play a pivotal role in cell apoptosis, activation, and motility. However, it remained to be solved that how RKIP regulates these process in cerebral ischemia.

In the present study, we found that the NF- κ B pathway regulated by RKIP play an important role in microglial apoptosis, excessive activation, and motility induced by OGD. BAY 11-7082, a NF- κ B pathway inhibitor, can protect microglia cells induced by OGD, which further indicates that NF- κ B pathway play a pro-apoptotic role in OGD-induced injury of microglia cells. Microglia, a kind of glial cells, constitutes the first and most important immune defense line in the CNS. Microglia can clear damaged nerves, plaques and infectious substances in the CNS [24]. Numerous clinical and neuropathological studies have shown that activated microglia play a very important role in the pathogenesis of degenerative diseases, such as Parkinson's disease, multiple sclerosis and Alzheimer's disease [25-27]. But too much activation or uncontrolled microglia can cause neurotoxicity [28]. We investigated whether RKIP may affect the migration of microglia cells by Transwell assay. We found that overexpressing RKIP in microglia cells decreased cell transwell migration while RKIP knockdown showed the reverse effect.

Previous reports have shown that NF- κ B activation may act as a pro-apoptotic factor, although it has been described as an anti-apoptotic factor in vitro [29, 30]. Much evidence indicates that the long-term activation of NF- κ B pathway can induce neural cells apoptosis [31-33]. Furthermore, activation of NF- κ B pathway has been proved to be one of characteristics of neuronal degeneration diseases [34-36]. Moreover, the capacity of NF- κ B to regulate pro-survival and anti-apoptosis signaling makes it a promising target for neuronal survival. In various human neuronal cells, there is constitutive activation of NF- κ B and then leads to uncontrolled growth, motility, and immune evasion. Activation of the NF- κ B signaling pathway by OGD, ranging from inflammation cytokines to DNA damage induce its target gene expression to promote either the growth and survival of microglia cells [37]. In the present study, RKIP knockdown lead to microglia cells injury after OGD and may further cause neuronal degeneration. Therefore, overexpression of RKIP may be useful for the treatment of injury induced by OGD. In addition, the recent reports suggest that RKIP is a potential biomarker for prognosis of cancer and neurodegenerative disorders [38], and its dysfunction may play an important role in certain cancer cells [39-41]. Overall, this study may provide insights into the effect of RKIP in neuronal apoptosis in ischemic stroke. Further studies are needed to elucidate the exact mechanism of RKIP in cell apoptosis.

Conclusions

In conclusion, this study demonstrated that upregulation of RKIP in ischemic stroke protects neurons against energy stress. RKIP inhibits of microglial excessive activation, inhibits its motility, and promotes neuronal survival partly through inhibiting NF- κ B pathway (Fig. 6). Although further precise mechanism studies are needed, the present results provide new endogenous defense mechanisms in ischemic stroke and highlight that RKIP is a new target for the treatment of ischemic stroke.

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Disclosure Statement

There are no competing interests.

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