Original Research

Raf-kinase inhibitor protein attenuates microglia inflammation in an *in vitro* model of intracerebral hemorrhage

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Abstract: Microglia mediated neuroinflammation plays a crucial role in intracerebral hemorrhage (ICH). Raf kinase inhibitor protein (RKIP), a member of the phosphatidylethanolamine-binding protein (PEBP) family, is a negative regulator of inflammatory responses. However, the expression and anti-inflammatory effects of RKIP in microglia after ICH have not been reported. Therefore, in the current study, we investigated the effects of RKIP on inflammatory responses in erythrocyte lysate-treated BV2 microglia. Furthermore, we analyzed the detailed molecular mechanisms underlying the anti-inflammatory effects of RKIP in microglia. Our results showed that the expression level of RKIP was significantly decreased by erythrocyte lysate treatment in BV2 microglia. Overexpression of RKIP inhibited the production of pro-inflammatory molecules. In addition, overexpression of RKIP attenuated neuronal cell death induced by activated microglia. Moreover, RKIP suppressed the activation of NF-κB signaling pathway in erythrocyte lysis-treated BV2 cells. In conclusion, these data suggest that overexpression of RKIP attenuated microglia inflammation through inhibiting the NF-κB signaling pathway in erythrocyte lysis-treated BV2 cells. The present study provides evidence that RKIP may be used as an effective molecular target for the treatment of ICH.

Key words: Raf kinase inhibitor protein (RKIP), intracerebral hemorrhage (ICH), microglia, neuroinflammation.

Introduction

Intracerebral hemorrhage (ICH) is a subtype of stroke with high rates of morbidity and mortality, accounting for 15–20% of all stokes and affecting more than 2 million people worldwide each year (1). Despite its importance, effective treatment remains unsatisfactory, and the pathogenesis of ICH-induced brain damage is not completely understood. Emerging data show that inflammatory response plays an important role in ICH-induced secondary brain damage (2, 3). Microglia cells are involved in the inflammatory response after ICH. ICH-induced neuronal death in the brain leads to activation of microglia cells, which release various pro-inflammatory/neurotoxic molecules, including nitric oxide (NO), reactive oxygen species (ROS) and cytokines (4-6). Therefore, the identification of inflammatory mediators in the pathogenesis of ICH is a key element in determining novel targets for therapeutic intervention.

Raf kinase inhibitor protein (RKIP) belongs to the phosphatidylethanolamine-binding protein (PEBP) family (7). Studies have demonstrated that it plays important roles in the development of the nervous system, spermatogenesis and tumorigenesis (8-11). Recent studies have also shown that RKIP interacts with multiple signaling molecules that may potentiate multiple functions during inflammatory processes. One study showed that RKIP overexpression significantly decreased the expression of interleukin (IL)-6, IL-8, matrix metalloproteinase (MMP)-1, and MMP-3 in tumor necrosis factor (TNF)-α-stimulated rheumatoid fibroblast-like synoviocytes (FLS) (12). Another study demonstrated that RKIP may play a role in the production of interferon (IFN)γ during a systemic inflammatory response syndrome (SIRS) response (13). However, the expression and anti-inflammatory effects of RKIP in microglia after ICH have not been reported. Therefore, in the current study, we investigated the effects of RKIP on inflammatory responses in erythrocyte lysate-treated BV2 microglia. Furthermore, we analyzed the detailed molecular mechanisms underlying the anti-inflammatory effects of RKIP in microglia.

Materials and Methods

Microglial and Neuronal Cell Cultures

The immortalized murine BV2 microglial cell line and Neuro-2a mouse neuroblastoma cell line were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO, USA), streptomycin (10 μg/ml) and penicillin (10 U/ml). 1×10^5 cells/well were seeded on sterile poly-Llysine (Sigma-Aldrich) coated glass cover slips in a 24-well plate and incubated at 37°C and 5% CO_2. After 1 h, culture medium was changed completely.

Erythrocyte lysate preparations

Human blood from three healthy donors was drawn into Na_2EDTA-containing tubes. The mean corpuscular hemoglobin concentration in red blood cells (RBCs) from individual adult donors was within the range of reference values (MCHC; 32–38 g/dl). RBCs were harvested by centrifugation at 1,000 rpm for 5 min. Hematocrits (Ht) were measured after a 5-min spin in a

Janetzki TH12 centrifuge. Erythrocyte suspension (Ht: 35–50 %) 20 µl was lysed in 2 ml of water.

Cell Treatment

BV2 microglia collected from culture flasks were seeded at a density of 1×10^5 cells/well onto 24-well tissue culture plates. One day after seeding, each culture well was stimulated with 10 µl erythrocyte lysate. After 72 h, the supernatants were removed and further analyzed for cytokine production with ELISA. Neuron was cultured in a 96-well plate with 1×10^4 cells per well. For the toxicity experiments, neuron was serum-starved for 4 h and then treated with a mixture of microglia-conditioned medium. For MTT and apoptosis assays, cells were treated for 24 h.

Real Time Quantitative PCR (RT-qPCR)

Total RNA from microglia was prepared with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Total RNA (500 ng) was reverse transcribed into cDNA using a PrimeScript™ 1st Strand cDNA Synthesis kit (Takara, Dalian, China). The PCR was performed using the Bio-Rad iQ5 Quantitative PCR System (Takara, Dalian, China). The specific primers for RKIP were forward 5'-ACACCCTGGTCCT-CACAGAC-3', and reverse, 5'-CTGCTCCTGCT-CATACACCA-3'; β-actin, forward 5'-ATTGGCAA-TGAGCGGTTC-3' and reverse 5'-GGATGCCACAG-GACTCCAT-3'. Relative quantities of the candidate genes were calculated with the previously described comparative threshold cycle method (14).

Western Blot

Total protein was extracted from BV-2 cells using RIPA lysis buffer (Beyotime, Nantong, China) according to the manufacturer's instructions. A total of 30 µg protein was separated by 12% SDS-PAGE electrophoresis followed by electro-blotting onto a nitrocellulose membrane (Amersham, Little Chalfont, UK). The membranes were blocked in 5% skimmed milk for 1 h at room temperature. Immunodetection of target proteins [RKIP, p-NF-κB p65, NF-κB p65 and β-actin] was performed using rabbit monoclonal antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Subsequently, they were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:1,500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature. ECL reagent was used for detection. The fluorescence was scanned using a Typhoon scanner (Amersham Biosciences, Piscataway, NJ, USA).

RKIP Expression Plasmid

RKIP recombinant adenovirus vector was constructed as described previously (15). The cDNA encoding RKIP was amplified and subcloned into the adenoviral shuttle vector pAd-CMV; green fluorescent protein (GFP) was used as a non-specific control. The adenoviral shuttle vector pAd-CMV and adenoviral gene expression vector pAdEasy-1 were homologously recombined in Escherichia coli strain BJ5183. The recombined plasmid Ad-RKIP was then propagated in HEK293 cells. The recombinant adenoviruses were harvested and the titers were detected using the p24 ELISA kit (Cell Biolabs, USA) before use.

Phagocytosis assay

BV2 microglia were plated at a density of 1×10^4 cells/well in a 96-well plate and switched to serum-free media 24 h. Then, microglia were transduced with Ad-RKIP or Ad-GFP for 24 h. Phagocytosis was measured by exposing the cultures to fluorescently-labeled E. coli particles (Invitrogen, Carlsbad, CA, USA) for 2 h. Cells were incubated with trypan blue and rinsed with PBS to remove non-internalized particles prior to measuring fluorescence at 480 nm excitation and 520 nm emission on a Fluoroskan multi-well plate reader.

ELISA

The production of TNF-α and IL-1β in the culture supernatants was measured by ELISA according to the manufacturer's instructions (BD PharMingen, San Diego, CA, USA).

Intracellular Reactive Oxygen Species Measurement

Intracellular accumulation of ROS was detected using dichlorodihydro-fluorescein diacetate (H_2DCF-DA; Sigma-Aldrich) as described previously with modifications (16). In brief, microglial cells were stimulated with erythrocyte lysis for 16 h and stained with 50 µM H_2DCF-DA in PBS buffer for 1 h at 37°C. DCF fluorescence intensity was measured at a 485 nm excitation and a 535 nm emission on a fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA).

Cell Viability Assay

Cell viability was assessed by MTT (3-[4,5-dimethylthiazol-2-yl]- 2,5-diphenyltetrazolium bromide) assay. Neuro2a cells were incubated with conditioned media from erythrocyte lysis-treated BV2 cells transfected with Ad-RKIP or Ad-GFP. After 24 h of incubation, MTT was added to the cells at a final concentration of 0.6 mg/ml and the plates were incubated for 4 h at 37°C. The reaction was terminated by adding 200 µl/well dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA). Absorbance at 570 nm was measured using a Bio−Rad microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Apoptosis Assay

The cell apoptosis was evaluated by using the Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (Invitrogen, Carlsbad, CA, USA). In brief, BV-2 cells were transduced with Ad-RKIP or Ad-GFP for 24 h, cells were then treated with PBS or erythrocyte lysis for 48 h. Neuron was treated with medium from above mentioned microglia. After 24 h of incubation, cells were harvested and washed with PBS. After washing, the cells were resuspended in 500 µl Annexin V binding buffer, followed by the addition of 5 µl Annexin V-FITC and 5 µl PI. The samples were incubated for 15 min at 37°C in the dark. Then, 300 µl of binding buffer was added to each tube. The percentage of cells undergoing apoptosis and necrosis was quantified using a FACSCalibur cytometer (BD Biosciences, Mountain View, CA, USA).

Statistical Analysis

Results are expressed as mean ± SD from at least three independent experiments. Statistical analysis was performed using Student's t-tests and one-way analysis of variance. A difference was considered statistically significant at P<0.05.

Results

The Expression of RKIP in Microglia Induced by Erythrocyte Lysis

First, we detected the expression of RKIP in erythrocytes lysate-treated BV2 cells at the indicated time points. We observed that erythrocyte lysate treatment significantly inhibited RKIP mRNA expression after 3 h (Figure 1A). To confirm the changes in RKIP expression at the protein level, we performed western blot analysis in erythrocyte lysate-treated BV2 microglia. Erythrocyte lysate treatment also dramatically inhibited RKIP protein expression in a time-dependent manner (Figure 1B).
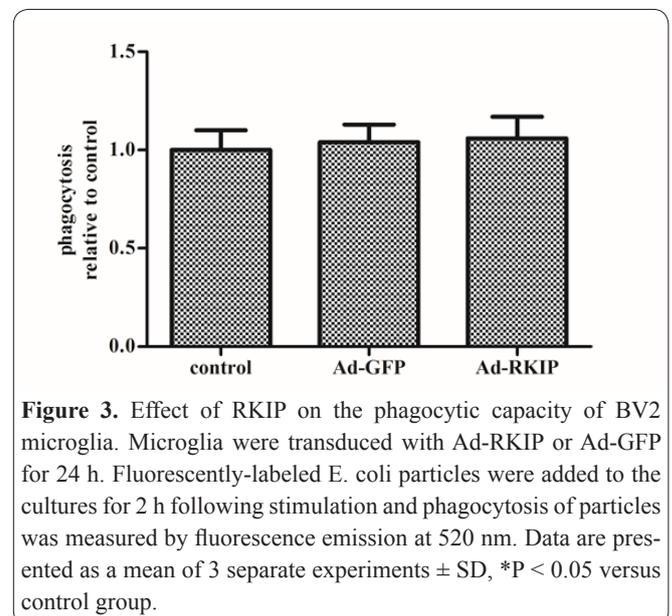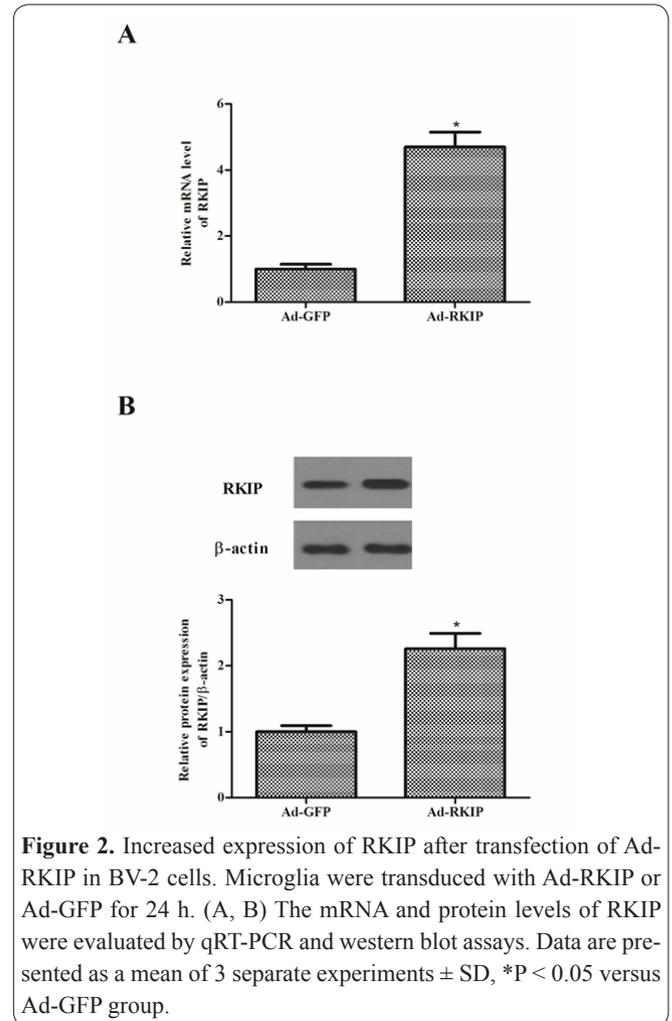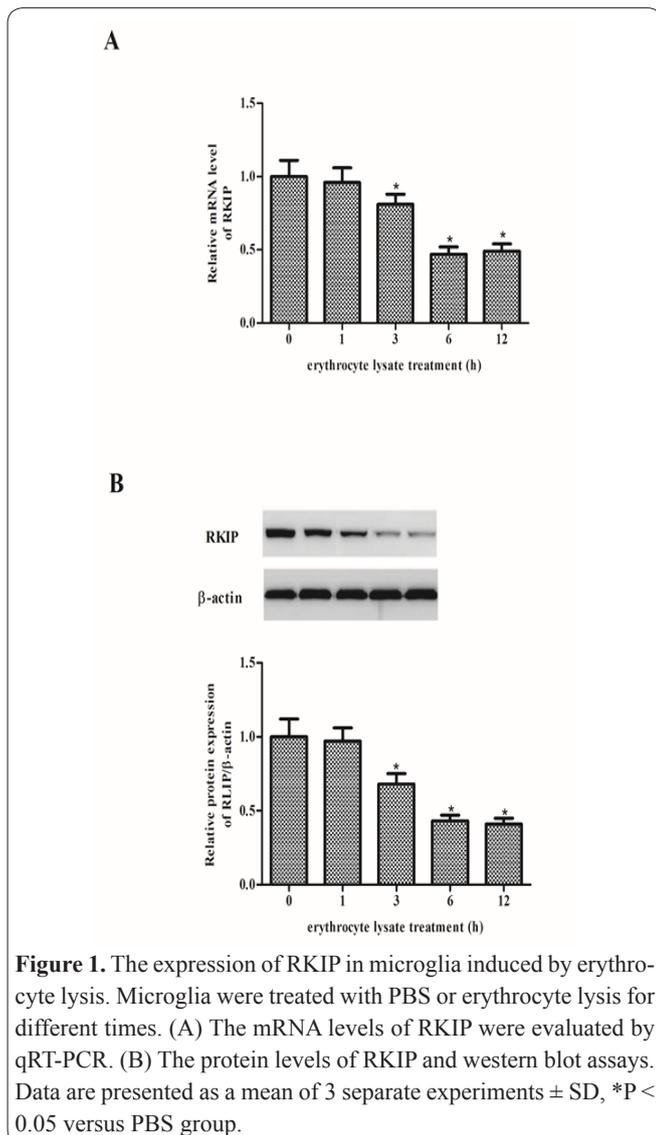
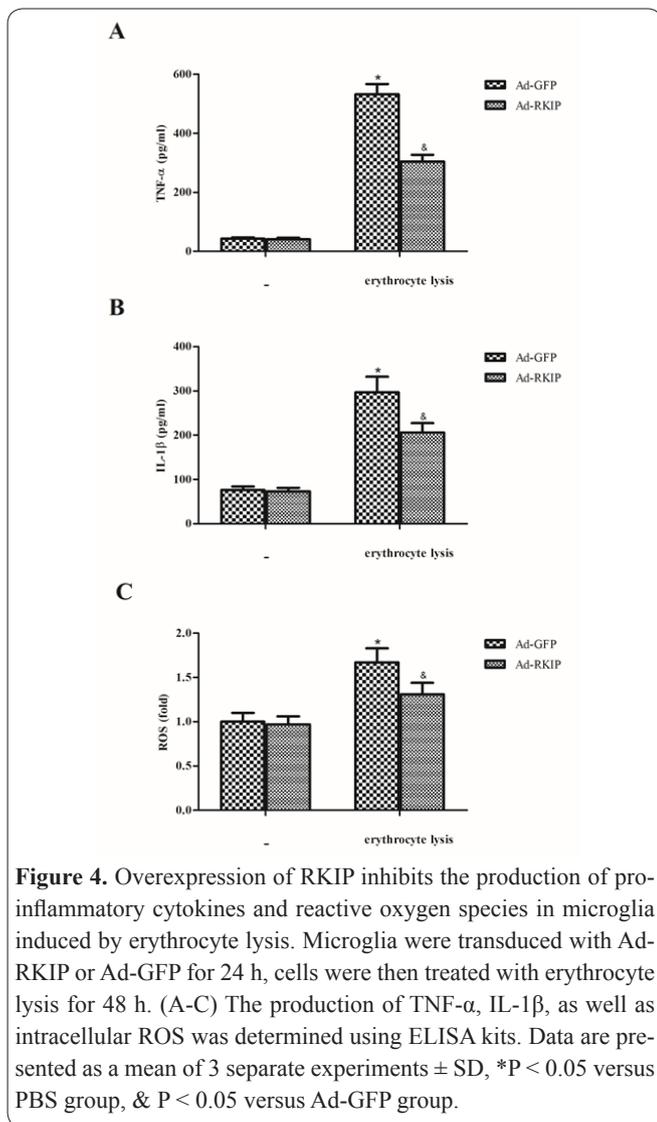### Effect of RKIP on the Phagocytic Capacity of BV2 Microglia

To investigate the role of RKIP in activated microglia, the RKIP expression vector was introduced into BV2 cells by transient transfection. We found that the expression of RKIP was upregulated in Ad-RKIP-trans-

fected BV-2 cells, as compared with the Ad-GFP-transfected cells (Figure 2), suggested that the transduction was successful. Then, we investigated the effect of RKIP on the phagocytic capacity of BV2 microglia. We found that RKIP had no effect on the phagocytic activity (Figure 3).

Overexpression of RKIP Inhibits the Production of Pro-Inflammatory Cytokines and Reactive Oxygen Species in Microglia Induced by Erythrocyte Lysis

Next, we evaluated the effect of RKIP on the pro-

Figure 2. Increased expression of RKIP after transfection of Ad-RKIP in BV-2 cells. Microglia were transduced with Ad-RKIP or Ad-GFP for 24 h. (A, B) The mRNA and protein levels of RKIP were evaluated by qRT-PCR and western blot assays. Data are presented as a mean of 3 separate experiments ± SD, *P < 0.05 versus Ad-GFP group.

Figure 3. Effect of RKIP on the phagocytic capacity of BV2 microglia. Microglia were transduced with Ad-RKIP or Ad-GFP for 24 h. Fluorescently-labeled E. coli particles were added to the cultures for 2 h following stimulation and phagocytosis of particles was measured by fluorescence emission at 520 nm. Data are presented as a mean of 3 separate experiments ± SD, *P < 0.05 versus control group.

Figure 1. The expression of RKIP in microglia induced by erythrocyte lysis. Microglia were treated with PBS or erythrocyte lysis for different times. (A) The mRNA levels of RKIP were evaluated by qRT-PCR. (B) The protein levels of RKIP and western blot assays. Data are presented as a mean of 3 separate experiments ± SD, *P < 0.05 versus PBS group.

Figure 4. Overexpression of RKIP inhibits the production of pro-inflammatory cytokines and reactive oxygen species in microglia induced by erythrocyte lysis. Microglia were transduced with Ad-RKIP or Ad-GFP for 24 h, cells were then treated with erythrocyte lysis for 48 h. (A-C) The production of TNF-α, IL-1β, as well as intracellular ROS was determined using ELISA kits. Data are presented as a mean of 3 separate experiments ± SD, *P < 0.05 versus PBS group, & P < 0.05 versus Ad-GFP group.

duction of pro-inflammatory cytokines and ROS in erythrocytes lysate-treated BV2 microglia. The results demonstrated that overexpression of RKIP suppressed erythrocyte lysate-induced secretion of TNF-α, IL-1β, as well as intracellular ROS production in BV-2 cells (Figure 4).
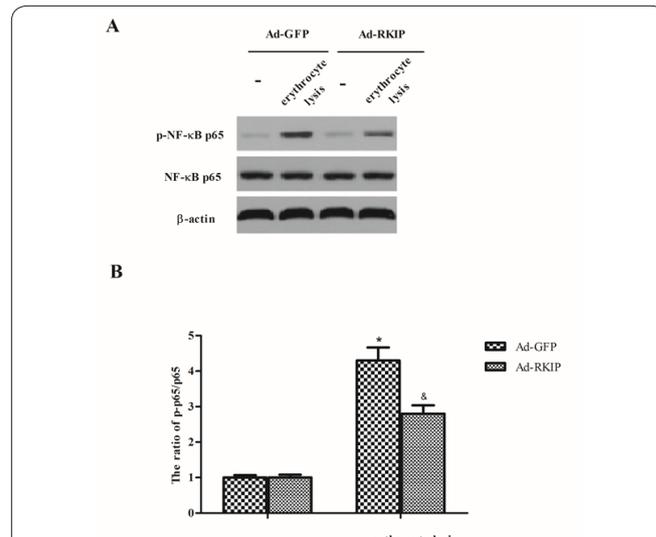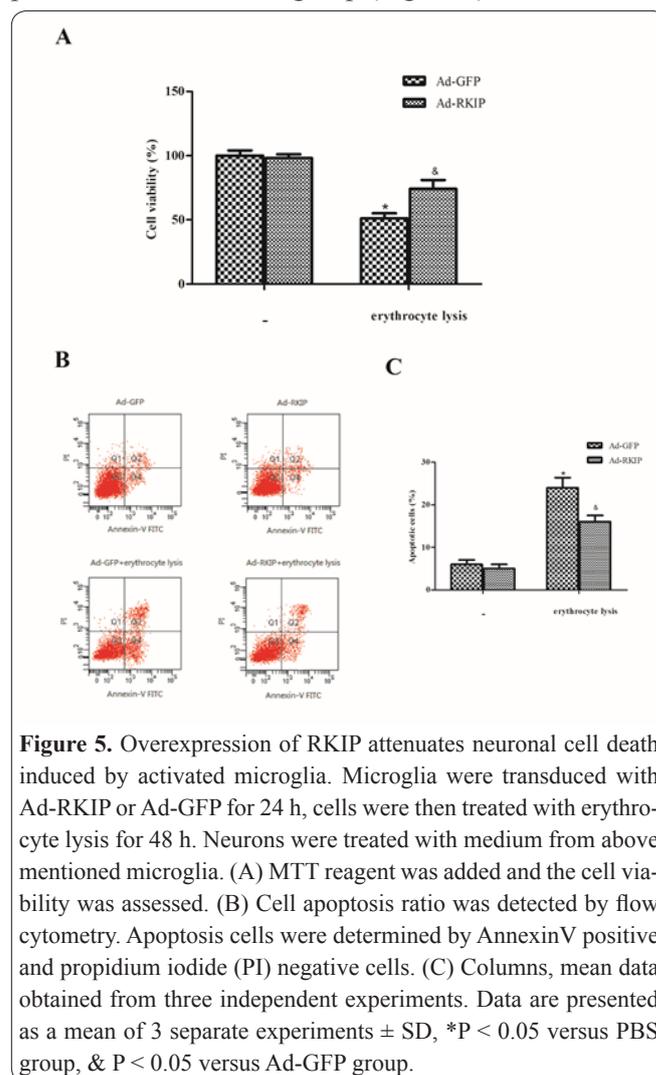
Overexpression of RKIP attenuates neuronal cell death induced by activated microglia

We examined whether RKIP affects the viability of neuronal cells by modulating microglial activation. As shown in Figure 5A and 5B, when neurons were treated with conditioned medium from erythrocyte lysis treated microglia, the cell viability decreased and apoptosis ratio increased. However, overexpression of RKIP increased the cell viability and decreased the apoptosis ratio in erythrocytes lysate-treated neurons.

Overexpression of RKIP Attenuates Microglia Inflammation through Inhibiting the NF-κB Signaling Pathway

The activation of the NF-κB is known to result in the production of inflammatory cytokines, which is activated in ICH. In addition, RKIP could negatively regulate NF-κB through binding of upstream activators of NF-κB. So, we examined the effects of RKIP on NF-κB p65 phosphorylation by western blot analysis. The results revealed that erythrocyte lysis significantly up-regu-

lated NF-κB p65 phosphorylation, however, Ad-RKIP treatment attenuated NF-κB p65 phosphorylation compared with the Ad-GFP group (Figure 6).

Figure 5. Overexpression of RKIP attenuates neuronal cell death induced by activated microglia. Microglia were transduced with Ad-RKIP or Ad-GFP for 24 h, cells were then treated with erythrocyte lysis for 48 h. Neurons were treated with medium from above mentioned microglia. (A) MTT reagent was added and the cell viability was assessed. (B) Cell apoptosis ratio was detected by flow cytometry. Apoptosis cells were determined by AnnexinV positive and propidium iodide (PI) negative cells. (C) Columns, mean data obtained from three independent experiments. Data are presented as a mean of 3 separate experiments ± SD, *P < 0.05 versus PBS group, & P < 0.05 versus Ad-GFP group.

Figure 6. Overexpression of RKIP attenuates microglia inflammation through inhibiting the NF-κB signaling pathway. Microglia were transduced with Ad-RKIP or Ad-GFP for 24 h, cells were then treated with erythrocyte lysis for 30 min, and were subjected to immunoblot analysis using antibodies against the p-NF-κB p65 and NF-κB p65. (B) Quantification of western blot data. A, The expression of p-NF-κB p65 and NF-κB p65 was measured by Western blot. Data are presented as a mean of 3 separate experiments ± SD, *P < 0.05 versus PBS group, &P < 0.05 versus Ad-GFP group.

Discussion

In the present study, we found that the expression of RKIP was significantly decreased by erythrocyte lysate treatment in BV2 microglia. Overexpression of RKIP inhibited the production of pro-inflammatory molecules. In addition, overexpression of RKIP attenuated neuronal cell death induced by activated microglia. Moreover, RKIP suppressed the activation of NF-κB signaling pathway in erythrocytes lysate-treated BV2 cells.

Previous studies have reported the role of RKIP in the central neuron system. Wen *et al.* reported that cyclin-dependent kinase 5 (CDK5)-mediated phosphorylation and autophagy of RKIP are involved in the over-activation of the extracellular signal-related kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway, leading to S-phase reentry and neuronal loss in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine hydrochloride-induced and transgenic Parkinson's disease (PD) models (17). Hellmann *et al.* reported that RKIP(+) cells showed accelerated neurite outgrowth, formation of elaborated neuronal networks and increased neuronal marker expression in retinoic acid (RA)-induced neuroblastoma cells (8). In the present study, we found that the expression of RKIP was significantly decreased by erythrocyte lysate treatment in BV2 microglia. All these data suggest that RKIP could produce protective effects on ICH.

Inflammation is an important host defense response to brain injury after ICH. The activation of microglia following ICH releases inflammatory cytokines, as well as amplify the inflammatory response in an autocrine or paracrine manner (18, 19). Moreover, in response to certain environmental toxins and endogenous proteins, microglia can enter an over-activated state and release reactive oxygen species (ROS) that cause neuronal death (4). In the present study, we found that overexpression of RKIP inhibited the production of pro-inflammatory cytokines, the cell viability of Neuro2a was markedly improved by Ad-RKIP compared with that in the Ad-GFP group. These results suggest that the neuro-protective effects of RKIP were due to the reduced secretion of pro-inflammatory/neurotoxic mediators from RKIP-overexpressed microglia.

NF-κB is pivotal in the transactivation of promoters for genes involved in inflammation, immune responses (20, 21). NF-κB can be activated by a wide array of factors such as thrombin, TNF-α, IL-1, oxidative stress, and growth factors (22). Mounting evidence indicates that NF-κB was a critical regulator of cell fate and function in the nervous system (22-24). In the progression of ICH, NF-κB activation promotes the release of series of inflammatory substances, such as TNF-α, IL-1β, induced nitric oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), and so on (25). In addition, previous investigations have shown that NF-κB activation was closely related with cell death and had an important function in secondary brain damage after ICH in patients (26, 27). So, understanding the signaling mechanisms underlying ICH-induced NF-κB activation may facilitate identification of therapeutic targets. In the present study, we found that erythrocyte lysis significantly up-regulated p65 phosphorylation, however, Ad-RKIP treatment attenuated p65 phosphorylation compa-

red with the Ad-GFP group. These results suggest that overexpression of RKIP may suppress NF-κB signaling pathway, reducing the production of NO, intracellular ROS, TNF-α and IL-1β in erythrocytes lysate-treated BV2 cells.

In conclusion, our results suggest that overexpression of RKIP attenuated microglia inflammation through inhibiting the NF-κB signaling pathway in erythrocytes lysate-treated BV2 cells. The present study provides preliminary evidence that RKIP may be used as an effective molecular target for the treatment of ICH.

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