Original Article

Downregulation of Aquaporin 4 Expression through Extracellular Signal-regulated Kinases1/2 Activation in Cultured Astrocytes Following Scratch-injury*

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Abstract

Objective To investigate the role of extracellular signal-regulated kinase1/2 (ERK1/2) pathway in the regulation of aquaporin 4 (AQP4) expression in cultured astrocytes after scratch-injury.

Methods The scratch-injury model was produced in cultured astrocytes of rat by a 10-μL plastic pipette tip. The morphological changes of astrocytes and lactate dehydrogenase (LDH) leakages were observed to assess the degree of scratch-injury. AQP4 expression was detected by immunofluorescence staining and Western blot, and phosphorylated-ERK1/2 (p-ERK1/2) expression was determined by Western blot. To explore the effect of ERK1/2 pathway on AQP4 expression in scratch-injured astrocytes, 10 μmol/L U0126 (ERK1/2 inhibitor) was incubated in the medium at 30 min before the scratch-injury in some groups.

Results Increases in LDH leakage were observed at 1, 12, and 24 h after scratch-injury, and AQP4 expression was reduced simultaneously. Decrease in AQP4 expression was associated with a significant increase in ERK1/2 activation. Furthermore, pretreatment with U0126 blocked both ERK1/2 activation and decrease in AQP4 expression induced by scratch-injury.

Conclusion These results indicate that ERK1/2 pathway down-regulates AQP4 expression in scratch-injured astrocytes, and ERK1/2 pathway might be a novel therapeutic target in reversing the effects of astrocytes that contribute to traumatic brain edema.

Key words: Astrocytes; Aquaporin 4; Scratch-injury; Extracellular signal-regulated kinases1/2

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INTRODUCTION

Traumatic brain injury (TBI) is a leading cause of death and disability in both youth and adults. Brain edema is a common and serious complication of TBI that accounts for approximately 50% of the deaths of TBI patients[1]. Due to progressive increases in both extracellular and intracellular brain water contents, brain edema leads to intracranial hypertension, a reduction in cerebral blood flow, brain herniation, and brainstem compression. Thus, brain edema has critical effects on morbidity and mortality following TBI and remains a crucial therapeutic target for the treatment of TBI. However, for many years, the available treatments for brain edema in TBI patients have remained symptomatic and rather non-specific. This situation has changed little due to our incomplete understanding of the mechanisms underlying brain edema.

The discovery of aquaporins (AQPs) established novel targets for the investigation of water movement in the brain. AQPs are a transmembrane protein family and selectively allow water transport through the plasma membrane[2]. To date, there are at least 13 known AQP subtypes (AQP0-12) that have been identified in mammals[3]. Among these, aquaporin 4 (AQP4) is the major water channel protein in the brain and is primarily distributed in the perivascular and subpial astrocytic end-feet[4]. AQP4 plays vital roles in cerebral water balance, astrocyte migration, neural signal transduction and ion homeostasis[5]. Previous studies have suggested that AQP4 is involved in TBI-induced brain edema[6]. However, the underlying mechanisms of the regulation of AQP4 expression induced by TBI remain elusive.

Extracellular signal-regulated kinase1/2 (ERK1/2) plays critical roles in the control of the expressions of many genes that are involved in cell proliferation and apoptosis[7], memory formation, aging, and neurodegeneration[8]. Ohsumi et al. reported that the phosphorylation of ERK1/2 (p-ERK1/2) is increased in rat astrocytes 6 h following TBI[9]. To our knowledge, there is no previous study examining the ERK1/2 pathway that regulates AQP4 expression in astrocytes after TBI. A previous investigation found that the activation of ERK1/2 pathways up-regulates AQP4 after ischemic damage in astrocytes[10]. Therefore, we hypothesized that ERK1/2 pathway mediate AQP4 expression in astrocytes following TBI.

AQP4 facilitates water movement into and out of the brain and plays an important role in brain edema, but the existing studies on the changes in AQP4 expression following TBI *in vivo* are contradictory. Our previous study using a rat impact-acceleration head injury model demonstrated that AQP4 expression was increased at 1 h post-injury in the injured regions[11]. A few of reports have also described increases in AQP4 expression following TBI[12-13]. However, other studies have indicated that AQP4 expression is decreased following TBI[14-15]. The reasons for these discrepancies of the changes of AQP4 expression were due to that we could not distinguish between primary and secondary response of astrocyte to TBI *in vivo*. So we intended to use an *in vitro* scratch-injury model in astrocyte to investigate the direct effect of injury on AQP4 expression and determine the effect of ERK1/2 activation on the change of AQP4 expression induced by injury.

MATERIALS AND METHODS

Astrocyte Culture

One-day-old Wistar rats were used in this experiment. All rats were provided by the Institute of Laboratory Animal Science affiliated with the Chinese Academy of Medical Sciences [Certificate No, SCSK (Beijing) 2005-0013]. The animal protocol was approved by the Animal Care and Use Committee of Beijing Neurosurgical Institute and was consistent with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Astrocyte cultures were prepared according to the method described by McCarthy et al.[16] with minor modifications[17]. Wistar rats were anesthetized with ether, and then dipped into 75% alcohol to sterilize. Cerebral cortex was taken from skulls, and meninges were carefully removed off. The cerebral tissues were cut into small pieces and dissociated into single cells by gentle pipetting. After filtering, the isolated cells were maintained in modified eagle medium (MEM, Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco). All cultures were incubated with 5% CO_2 and 95% air at 37 °C, and the culture medium was changed every 3-4 d. The purities of the cultured astrocytes were confirmed by immunofluorescence staining for glial fibrillary acidic protein (GFAP), a marker of astrocytes. For getting more astrocytes, the subculture was carried out when the cultured astrocytes were confluent on the tenth day of

primary culture, and the secondary cultured astrocytes were used in all experiments.

Scratch-injury Model in Cultured Astrocytes and U0126 Pretreatment

The scratch-injury model in cultured astrocytes was induced as described by Yu et al.[18]. The culture medium was changed one hour before scratch-injury, and then the cultured astrocytes in 6-well plates were scratched with a 10 μL pipette tip 7 times in the same direction followed by 7 scratches that were perpendicular to the direct of the first set of scratches. An interval of 0.5 cm was included between each parallel scratch. Immediately after scratch-injury, the medium was changed to fresh MEM containing 10% FBS to remove the detached cells. The astrocytes without scratch-injury were used as controls. The astrocyte morphology, lactate dehydrogenase (LDH) leakages, AQP4 expression and ERK1/2 activation were observed at 1, 12, and 24 h after scratch-injury.

For determining the role of ERK1/2 activation in AQP4 expression after scratch-injury in astrocytes, the ERK1/2 kinase inhibitor U0126 (Biomol) was added into the culture medium 30 min before scratch-injury. U0126 was dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium at a final concentration of 10 μmol/L. The astrocytes treated with DMSO (0.2%) were used as controls. AQP4 expression was detected by immunofluorescence staining and Western blot, and phosphorylated-ERK1/2 (p-ERK1/2) expression was determined by Western blot at 12 h after scratch-injury.

Immunofluorescence Staining

Astrocytes were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. Next, the astrocytes were incubated with polyclonal rabbit antibody against GFAP (DAKO, rabbit anti-rat, 1:50) or AQP4 (Abcam, rabbit anti-rat, 1:30) diluted in PBS overnight at 4 °C. After three 3 min washes in PBS, the astrocytes were incubated with FITC–conjugated goat anti-rabbit IgG (Jackson, 1:200) for 3 h at room temperature. Negative controls were created by the omission of the primary antibody. Images were acquired using an inverted Leica FW4000 fluorescence microscope.

Determination of Lactate Dehydrogenase Leakage

To assess the damage to cultured astrocytes following scratch-injury, we examined the lactate dehydrogenase (LDH) leakages into the medium using a LDH assay kit according to the manufacturer's instructions. The cells were digested with 0.2 mmol/L NaOH, and samples were taken for protein determination by the Bradford procedure[19]. The LDH leakages are expressed as LDH/protein (U/mg). All experiments were replicated three times with three samples for each condition in each experiment.

Western Blot

The AQP4 expression and ERK activation of astrocytes following scratch-injury were determined by Western blot as described in our previous publication[20]. The astrocytes were added to 0.25% trypsin for 8 min at room temperature, and then were collected through the cell scraper. The harvested astrocytes were lysed in cell lysis buffer on ice for 30 min, and protein concentrations were determined by the Bradford method. Protein samples (90 μg for AQP4 or 30 μg for p-ERK1/2 and ERK1/2) were denatured at 95 °C for 5 min followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels. The gels were blotted onto polyvinylidene fluoride (PVDF) membranes at 1 mA/cm^2 for 40 min. Non-specific binding sites were blocked with 5% nonfat milk in Tris-saline buffer (TBS) overnight at 4 °C. The PVDF membranes were incubated with polyclonal antibodies against AQP4 (1:400) and monoclonal antibodies against p-ERK1/2 and ERK1/2 (Cell Signaling Technology, rabbit anti-rat, 1:1000) for 2 h at room temperature. Subsequently, the PVDF membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Technology, 1:1000) at room temperature for 1 h. The specific bands were detected using an enhanced chemiluminescence reagent kit (Beijing CNS bioservices) according to the manufacturer's instructions. β-actin (Sigma, mouse anti-rat, 1:5000) was used as a loading control. The bands were imaged with the BioSpectrum 400 (UVP) and analyzed using the Quantity One software (Bio-Rad). All results are based on five independent samples that were repeated three times. AQP4 protein expression levels were normalized to β-actin. The p-ERK levels were normalized to total ERK.

Statistical Analysis

SPSS 11.5 for Windows was used for statistical analysis. All data are expressed as the means±SD.

One-way ANOVA analyses followed by least significant difference test (LSD) were used for the statistical analysis of the LDH leakeges, AQP4 and p-ERK1/2 expression. A *P*-value<0.05 was considered statistically significant.

RESULTS

Morphology Changes of Astrocytes and Increased LDH Leakages Following Scratch-injury in Cultured Astrocytes

Immunofluorescence staining revealed that over 95% of cells were GFAP-positive (Figure 1A), and the monolayer cultures of astrocytes were presented as uniform shapes under the phase contrast microscopic. One hour after scratch-injury, the edges of the scratches were lined with irregular-shaped cells (Figure 1B). Then the astrocytic processes at the edges of the scratches extended into the cell-free area by 12 h (Figure 1C), and were further elongated by 24 h after injury with astrocytes occasionally present in the injured core (Figure 1D). Compared with control, LDH leakages from the scratch-injured astrocytes were significantly increased at 1, 12, and 24 h after injury (*P*<0.05; Figure 1E). These observations showed that scratch-injury in cultured astrocytes induced changes in astrocyte morphologies and increases in LDH leakage due to the loss of cell membrane integrity.

Decrease in AQP4 Expression Following Scratch-injury in Cultured Astrocytes

The expression of AQP4 in cultured astrocytes was determined by immunofluorescence staining and Western blot. Immunofluorescence staining for AQP4 revealed that AQP4 reactivity was decreased in the areas surrounding the scratch-injury at 1, 12, and 24 h after injury (Figure 2A to 2D). Consistently, significant reductions in the AQP4 expression of cultured astrocytes were observed at 1, 12, and 24 h after scratch-injury by Western blot analysis (*P*<0.05; Figure 2E). These results demonstrated that AQP4 expression was down-regulated after scratch-injury in cultured astrocytes.

Activation of ERK1/2 Pathway Following Scratch-injury in Cultured Astrocytes

We determined the ERK1/2 activation in scratch-injured astrocytes with Western blot. ERK1/2 activation was indicated by the changes of p-ERK1/2 expression. Significant increases in p-ERK1/2 expression were observed at 1, 12, and 24 h after scratch-injury in cultured astrocyte (*P*<0.05). Pretreatment with the ERK1/2 inhibitor U0126 blocked the increase in p-ERK1/2 expression at 12 h after scratch-injury (*P*<0.05; Figure 3). These analyses indicated that p-ERK1/2 expression was up-regulated following scratch-injury in astrocytes, and ERK1/2 inhibitor could block the ERK1/2 activation

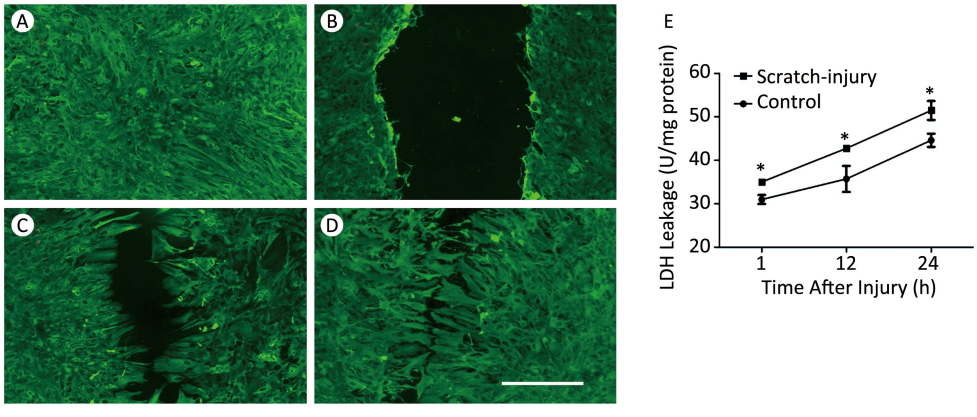

Figure 1. Morphological changes and increases of lactate dehydrogenase (LDH) leakage in cultured astrocytes following scratch-injury. (A) A representative immunofluorescence microscopy image depicts the glial fibrillary acidic protein (GFAP) staining (green) of astrocyte culture. Representative immunofluorescence microscopies of GFAP staining at 1 (B), 12 (C), and 24 h (D) after scratch-injury in cultured astrocytes. (E) Increases in LDH leakages in the scratch-injured astrocytes at 1, 12, and 24 h after injury. The data are presented as the mean±SD (*n*=9 for each time point), $^{*}P$<0.05 compared with the control group. Scale bars: 200 μm.

induced by scratch-injury.

Blockage of Decreased AQP4 Expression Following Scratch-injury by Pretreatment with U0126

Immunofluorescence staining revealed that DMSO did not influence the AQP4 reactivity at 12 h after scratch-injury in comparison with the control group (Figure 4A to 4C). But pretreatment with U0126 blocked the decreased AQP4 reactivity induced by scratch-injury (Figure 4D). Western blot analysis confirmed the finding that U0126 restrained the decrease of AQP4 level following scratch-injury in cultured astrocyte (P<0.05; Figure 4E). These data suggested that the activation of ERK1/2 pathway directly down-regulated AQP4 expression in scratch-injured astrocytes.

DISCUSSION

With an *in vitro* astrocyte scratch-injury model, we demonstrated that the scratch-injury directly decreased the AQP4 expression in cultured astrocytes, which can be regulated by activation of ERK1/2 pathway. (i) The scratch-injury leaded to the changes of astrocyte morphology, increases of LDH leakages and decreases of AQP4 expression; (ii) ERK1/2 pathway was activated following scratch-injury, as determined by Western blot; (iii) Pretreatment with ERK1/2 inhibitor U0126 blocked the ERK1/2 activation and decreases of AQP4 expression induced by scratch-injury. Thus, the activation of ERK1/2 pathway can down-regulate AQP4 expression following scratch-injury in cultured astrocytes, which might lead to the development of novel therapeutic target for brain edema after TBI.

The scratch-injury model used in this study mimics the injuries that are most frequently experienced by astrocytes *in vivo* after mechanical trauma[18]. Chen et al. reported that morphology changes and increased LDH release are observable after cortical contusion injury[21]. Consistent with their results, we also observed changes in astrocytic morphology and LDH leakage following scratch-injury, which confirmed that the astrocytes were subjected to direct damage in the scratch-injury model. Moreover, we observed that the expression of AQP4 was markedly decreased after scratch-injury, which suggested the decrease of AQP4 expression in astrocyte was the primary response of astrocyte to mechanical injury. Nito and Yamamoto's studies have also revealed that the expression of AQP4 is significantly decreased after oxygen-glucose deprivation injury and hypoxia in astrocytes[22-23]. Accordingly, we speculate that the decrease in AQP4 expression in astrocytes *in vitro* might be the primary response to injuries, while increase in AQP4 expression *in vivo* after TBI observed by our and other previous study might be a secondary response. A growing body of research suggests that alterations in AQP4 expression affect the formation and resolution of traumatic brain edema, and the down-regulation AQP4 expression after TBI can promote the development of brain edema. Therefore, intervention of AQP4 expression in astrocytes after injury is very important to the treatments for brain edema in TBI patients.

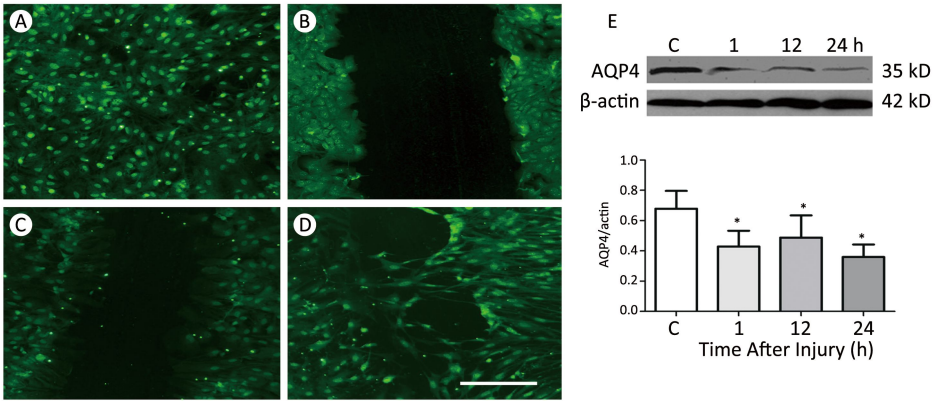

Figure 2. Decreased aquaporin 4 (AQP4) expression following scratch-injury in cultured astrocytes. A representative immunofluorescence microscopy image depicts AQP4 staining (green) before (A) and at 1 (B), 12 (C), and 24 h (D) after scratch-injury. Reductions in AQP4 staining were observed in the scratch-injured astrocytes. (E) Representative and quantitative analysis of the Western blot of AQP4 and β-actin indicated that AQP4 expression significantly decreased at 1, 12, and 24 h after scratch-injury in cultured astrocyte. β-actin was used as a loading control. The data are presented as the mean±SD (n=5 for each time point), $^{*}P$<0.05 compared with the control group. Scale bars: 200 μm.

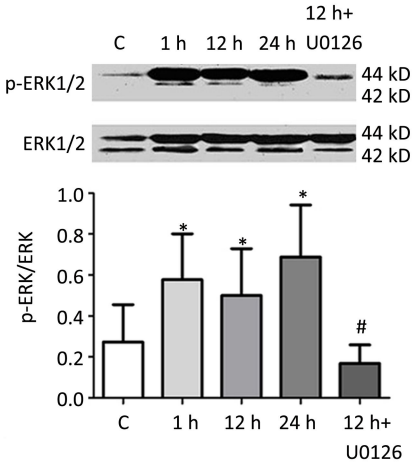

Figure 3. Inhibition of phosphorylated-extracellular signal-regulated kinase 1/2 (p-ERK1/2) expression in scratch-injured astrocytes by pretreatment with ERK1/2 kinase inhibitor (U0126). Significant increases in p-ERK1/2 were detected at 1, 12, and 24 h after scratch-injury in cultured astrocytes, and pretreatment with U0126, ERK1/2 kinase inhibitor, significantly inhibited the activation of ERK1/2 in scratch-injured astrocytes as evidenced by the reduction of p-ERK1/2 at 12 h after scratch-injury in astrocytes. The data are presented as the ratios of p-ERK/ERK (mean±SD, n=5 for each time point), $^{*}P$<0.05 compared with the control group, $^{\#}P$<0.05 compared with the 12 h after scratch-injury.

Given the important roles of AQP4 in TBI, understanding the mechanisms that underlie the expression of AQP4 after TBI may shed light on the treatment of brain edema. It has been indicated that AQP4 is regulated by intracellular signaling pathways in response to extracellular stimuli[24]. Previous studies showed that mitogen-activated protein kinases can regulate different cellular programs by relaying extracellular signals to intracellular responses[7], and mainly includes ERK1/2, p38 MAPK and JNK signaling pathway. Increases in p-ERK1/2 have been found in astrocytes following TBI in rats[9]. In the present study, we found that scratch-injured astrocytes increased the activation of ERK1/2 and decreased the expression of AQP4. Pretreatment with U0126, an ERK1/2 inhibitor, blocked both the ERK1/2 activation and the decrease in AQP4 expression induced by scratch-injury. These results demonstrated that the activation of the ERK1/2 pathway decreased the expression of AQP4 in scratch-injured astrocytes. It would be very likely that treatment with ERK1/2 inhibitor U0126 may benefit neurological outcomes by relieving brain edema after TBI, which suggest the possible therapeutic targets along the ERK1/2-AQP4 pathway for TBI patients with brain edema. It has also been illustrated that AQP4 expression was suppressed by an inhibitor of p38 MAPK in hyperosmotic condition[25-26]. Further studies are needed to explore if other signaling

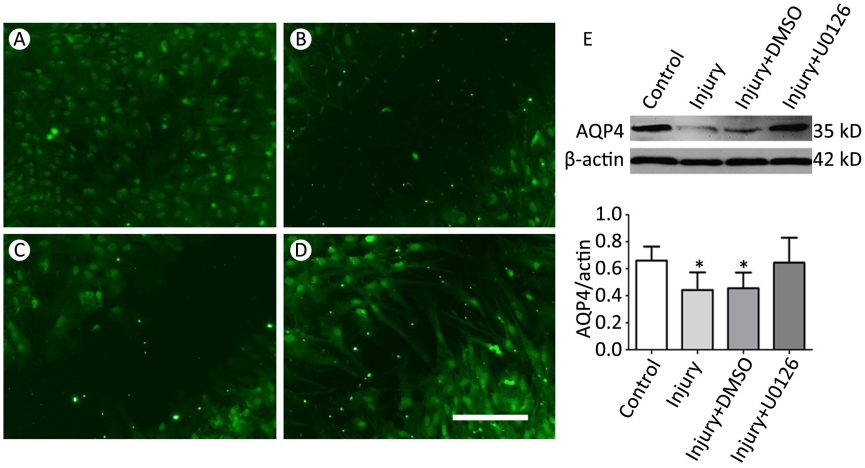

Figure 4. Blockage of decreased AQP4 expression following scratch-injury by pretreatment with U0126. Representative immunofluorescence microscopy images depict AQP4 staining (green) in intact (A) and scratch-injured astrocytes (B) that received treatments of DMSO vehicle (C) or U0126 (D). Decreases in AQP4 staining were observed in the cultured astrocytes at 12 h after scratch-injury with or without DMSO. Increases in AQP4 staining were found in the scratch-injured astrocytes that had received pretreatment with U0126. (E) Representative Western blot and quantitative analysis of AQP4 and β-actin indicated a significant decrease in AQP4 expression after scratch-injury with or without pretreatment with DMSO. This decrease was abolished by U0126. β-actin was used as loading control. The data are presented as the mean±SD (n=5 for each group), $^{*}P$<0.05 compared with the control group. Scale bars: 200 μm.

pathways including p38MAPK and JNK signaling pathway involved in regulation of AQP4 expression after scratch-injury.

In conclusion, we found ERK1/2 pathway directly down-regulated AQP4 expression in scratch-injured astrocytes. The regulation of AQP4 expression by the activation of ERK1/2 phosphorylation in astrocytes may lead to the development of novel therapeutic agents in reversing the effects of astrocytes that contribute to brain edema induced by TBI. Further research on the effect of ERK1/2 pathways on the regulation of AQP4 expression induced by TBI *in vivo* will be required to explore the potential of ERK1/2 pathway as a therapeutic target for brain edema induced by brain damage.

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AUTHOR CONTRIBUTIONS

YUAN Fang designed research, analyzed the results, wrote the manuscript and got a grant from the National Natural Science Foundation of China. SHI Zhong Fang performed the scratch-injury model in cultured astrocytes and U0126 treatment, immunofluorescence staining and wrote the manuscript. ZHAO Wei Jiang performed the Western blot. XU Li Xin performed the astrocyte culture. DONG Li Ping performed the determination of LDH leakage. YANG Shao Hua performed the analysis of results, revised the manuscript and got a grant from the National Natural Science Foundation of China.

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