Triptolide promotes nerve repair after cerebral ischemia reperfusion injury by regulating the NogoA/NgR/ROCK pathway

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Abstract

Activation of the NogoA/NgR/ROCK pathway limits nerve repair after brain ischemia-reperfusion (I/R) injury. Triptolide displays anti-inflammatory, anti-oxidant, and immunosuppressive effects and is derived from the traditional Chinese medicine Tripterygium wilfordii Hook F. This agent can also penetrate the blood-brain barrier, where it has a neuroprotective effect and ameliorates cerebral I/R injury via an as yet unknown mechanism(s). Here, an animal model of middle cerebral artery occlusion and reperfusion (MCAO/R) was employed to assess triptolide's therapeutic impact on brain I/R injury and the possible mechanism of action. The results indicate that triptolide treatment can decrease cerebral infarction and nerve injury after cerebral I/R injury. Importantly, in vivo and in vitro experiments revealed that treatment with triptolide decreased NogoA, NgR, p75NTR and ROCK2 expression, and upregulated the expression of GAP43 and PSD-95, thus suggesting improved synaptic function. These results indicate that triptolide can promote nerve repair following brain I/R injury by inhibiting NogoA/NgR/ROCK signalling.

Key words: triptolide, cerebral ischemia/reperfusion, NogoA/NgR/ROCK signalling pathway, neuroregeneration.

Introduction

The incidence rate of stroke increases year by year, with approximately 80% of these events being ischemic stroke [6]. With the expansion of therapeutic time windows, the principal treatment approach at present is to quickly restore the blood supply to the ischemic area [18]. However, ischemia-reperfusion (I/R) injury sometimes occurs, which can then further induce neuronal damage. It has proven difficult to recover neuronal structure and function after injury as the inhibitory microenvironment at the site of injured neurons can impede neurological repair after brain I/R injury.

Neurite outgrowth inhibitor A (NogoA) is an important component of this inhibitory microenvironment [25]. Physiologically, NogoA is involved in the development of oligodendrocytes and neurons and their maturation, as well as myelination, regulation of dendritic spine morphology, and synaptic plasticity [22,23]. NogoA is bound by Nogo-66 receptor (NgR) to form a complex with p75 neurotrophin receptor (p75NTR) and BK channel regulator (LINGO1) and activate RhoA/Rho kinase (ROCK) signalling, thereby inhibiting axonal regeneration and functional recovery after brain injury [7,14]. Inhibition of this NogoA/NgR/ROCK signalling axis can promote axonal regeneration in an animal model of cerebral I/R injury [12,26]. Fasudil is a ROCK inhibitor that contributes to the recovery of neural function and improvement of the blood-brain barrier (BBB) in an experimental model for stroke [2]. These findings suggest that inhibiting the NogoA/NgR/ROCK pathway may contribute to recovery from ischemic brain injury.

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Triptolide is an active component in *Tripterygium wilfordii* Hook F from Chinese medicinal preparations. This compound is known to have anti-inflammatory, anti-oxidant, and immunosuppressive properties [1]. In addition, triptolide has a small molecular weight and high lipophilicity, thus allowing it to penetrate the BBB and exert a protective effect on the nervous system [30]. Studies have shown that triptolide can reduce brain I/R injury through limiting inflammatory and oxidative damage, as well as by inhibiting the activation of apoptosis [13,21]. However, the molecular mechanisms by which triptolide can reduce cerebral I/R injury are not yet understood. Here, we used rat models of middle cerebral artery occlusion (MCAO) and SH-SY5Y cell oxygen glucose deprivation/reoxygenation (OGD/R) to evaluate the effects of triptolide on brain I/R injury and shed light on the possible mechanism of action.

Material and methods

Animal

Sprague-Dawley rats (males, 280 ±20 g) were purchased from a commercial supplier. All experiments received animal ethics approval (No. 2021025, Shanxi Datong University).

Animals were divided equally into three groups of 15 rats per group as follows: sham operated, I/R, and triptolide (TP) treatment. Triptolide was purchased from Chengdu Desite Biotechnology Co., China. It was injected intraperitoneally (0.2 mg/kg) at the start of reperfusion in animals from the TP group [17]. The same amount of DMSO vehicle was injected intraperitoneally into animals from the sham and I/R groups.

Model for brain I/R

A brain I/R injury model was developed as described in a previous report [19]. Following anesthetization with sodium pentobarbital, the right common, external and internal carotid arteries (CCA, ECA and ICA, respectively) were separated. A cut was made at the right CCA and a monofilament coated with a silicon tip inserted into the ICA until some resistance was felt. The monofilament was removed following 2 h of ischemia, followed by reperfusion for 24 h.

Neurological function scores

Neurological deficits were evaluated 24 h following I/R injury as previously described [19]. Points were awarded as follows: 0 – no deficit, 1 – rat failed to extend left forepaw, 2 – rat circles to left side, 3 – rat falls to left side, 4 – rat unable to walk.

Infarct volume

After evaluation of neurological function, the volume of cerebral infarct was estimated with triphenyl tetrazolium chloride (TTC) staining (n = 5 per group). Briefly, brain tissue was sliced and incubated for 15 min at 37°C with a 2% solution of TTC. Next, areas of red staining for the normal brain and white staining for the infarct brain were measured.

Nissl staining

After evaluation of neurological function, rats (5 from each group) were anesthetized and then perfused using saline and 4% paraformaldehyde. Frozen sections of brain tissue were obtained and Nissl staining performed as recommended by the manufacturer (Beyotime Biotechnology, China). Subsequently, neuronal death was analysed by light microscopy.

Cell culture and establishment of the OGD/R model

SH-SY5Y cells were grown in a 5% CO_2 humidified atmosphere at 37°C in DMEM that contained 10% foetal bovine serum (Gibco), 100 U/ml penicillin and 100 µg/ml streptomycin. For the OGD procedure, SH-SY5Y cells were grown in glucose-free DMEM for 4 h at 37°C in an incubator containing 94% N_2, 5% CO_2 and 1% O_2. The medium was then replaced with complete DMEM and the cells maintained at 37°C in 5% CO_2 during 24 h to reoxygenate.

Four groups of cells were prepared: normal control (control group), model (OGD/R group), triptolide intervention (TP group), and ROCK inhibitor fasudil intervention (Fasudil group). 1 nmol/LTP (Lemeitian Medicine, China) was added to the TP group after replacing complete growth medium with glucose-free DMEM. Similarly, 15 µg/ml fasudil (Tianjin Chase Sun, China) was added to the Fasudil group after placing the cells in glucose-free DMEM.

Cell viability assay

Cell Counting Kit-8 (CCK8) assay (Beyotime Biotechnology, China) was performed to evaluate cell viability. Briefly, SH-SY5Y cells grown in 96-well plates were exposed to OGD/R or to treatment with TP. After this, CCK8 solution (20 µl) was added and the incubation time was 2 h at 37°C. Following measurement of absorbance at 450 nm, the cell activity was calculated.

Immunofluorescence microscopy

For immunohistochemistry, brain tissue sections or SH-SY5Y cells grown in coverslips were blocked for 1 h using 0.3% Triton X-100 in 1% bovine serum albumin and phosphate buffered saline. They were then incubated overnight at 4°C with primary antibodies against MAP2 (Abcam), NeuN (Abcam), NogoA (Cell Signaling Technology [CST]), NgR (Abcam), ROCK2 (CST) and GAP43 (ABclonal). The following day, specimens were incubated for 2 h with Alexa Fluor 488- or 594-conjugated secondary antibody (Abcam) at room temperature. A confocal laser scanning microscope was used to obtain images, and staining was quantitatively analysed using software from Image-Pro Plus.

Western blot experiments

RIPA buffer was used to extract protein from rat ischemic brain tissue (n = 5 per group) and SH-SY5Y cells. Proteins (25 µg) underwent electrophoresis by 10% SDS-PAGE and transfer to nitrocellulose membrane. Blocking was performed at room temperature for 2 h with TBST containing 5% non-fat milk. Subsequent incubation with primary antibodies to NogoA (CST), NgR (Abcam), p75NTR (CST), ROCK2 (CST), PSD-95 (CST), GAP43 (ABclonal) and GAPDH (CST) was conducted overnight at 4°C. Membranes were then washed and incubated with HRP-conjugated secondary antibody for 2 h at room temperature. Greyscale images were viewed by chemiluminescence and evaluated with Image Lab Software (Bio-Rad, Hercules, CA, USA).

Statistical analyses

GraphPad Prism 5.0 software was used to perform statistical analysis. Results are presented as the mean ±SEM. Shapiro-Wilk test was used for normality test. Data from each group was normally distributed and showed homogeneity of variance. ANOVA followed by Tukey's post-hoc test was performed to compare results between different groups. Results were considered as being statistically significant when $p < 0.05$.

Results

Triptolide attenuates neurological deficits in rats with brain I/R and decreases the volume of infarction

The I/R group rats exhibited significant levels of neurological deficit, and hence their neurological scores were higher than those observed in sham animals ($p < 0.001$). Triptolide treatment significantly reduced neurological scores ($p < 0.001$) (Fig. 1A).

Staining with TTC revealed no infarcted tissue in the sham group, but a significantly larger infarct volume in the I/R rats ($p < 0.001$). Infarct volume in the TP group was smaller than that observed in the I/R group ($p < 0.001$) (Fig. 1B).

Triptolide attenuates neuronal injury in rats with brain I/R

Nissl staining indicated that the morphology of neurons remained intact and appropriately arranged in sham
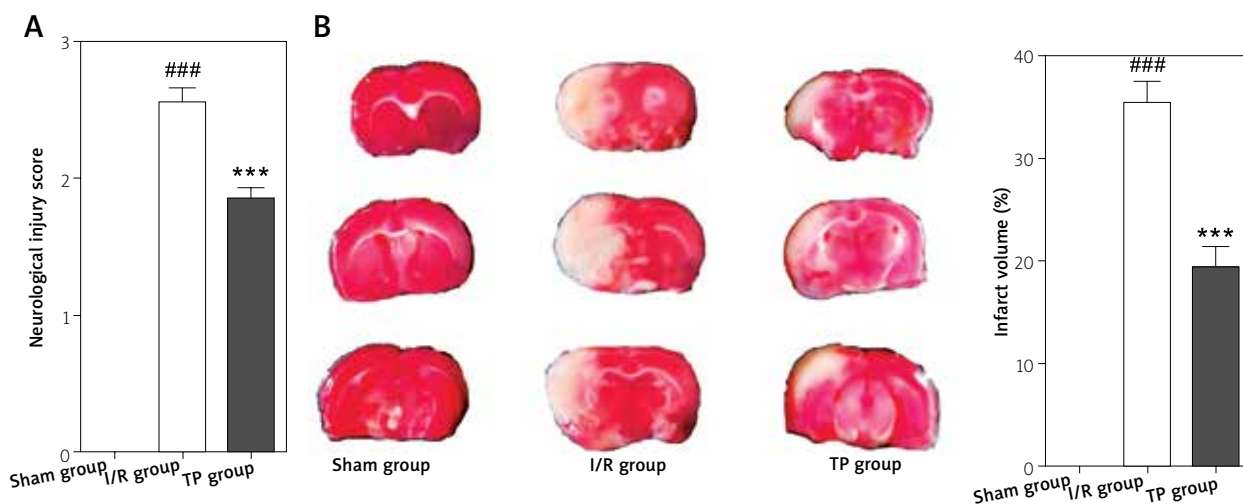

Fig. 1. Triptolide protects rats against brain damage after I/R. **A**) Scores of neurological functions at 24 hours after I/R, n = 15. **B**) Infarct area at 24 hours after I/R. n = 5. $^{\#\#\#}p < 0.001$ vs. the sham group, $^{***}p < 0.001$ vs. the I/R group.

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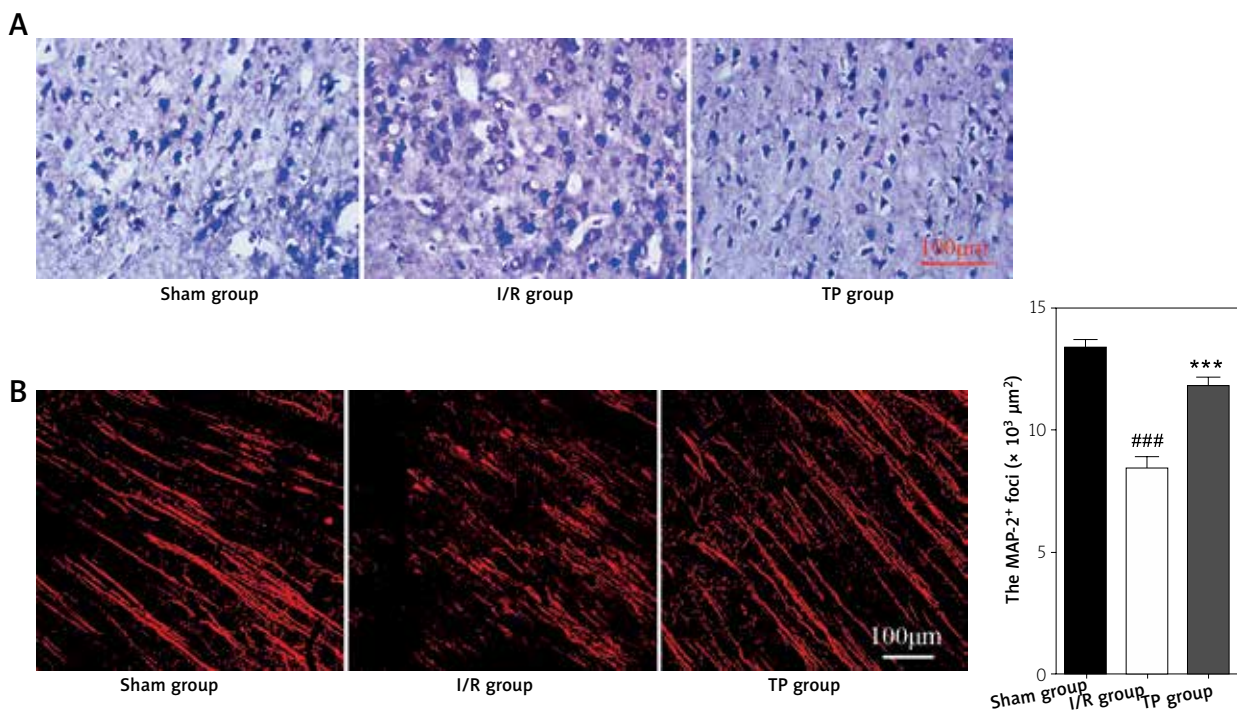

Fig. 2. Triptolide attenuates neuronal injury after brain I/R injury. **A)** Nissl stain ing. **B)** Representative confocal images of MAP2 immunofluorescence staining, $n = 5$. ###$p < 0.001$ vs. the sham group, ***$p < 0.001$ vs. the I/R group.

rats, and the Nissl body was deeply stained. In contrast, I/R rats showed many vacuolated spaces and Nissl bodies were significantly reduced. In the TP group, neuron morphology was improved and there were significantly more Nissl bodies compared to I/R rats (Fig. 2A).

MAP2 is a marker of dendrites and axons [3]. Immunofluorescence staining showed that neuronal processes in I/R rats were incomplete and that MAP2 expression was lower than that observed in sham rats. Neuronal morphology in the TP group showed improvement over that seen in I/R rats. Specifically, the length of processes was increased, the structure was relatively complete, and MAP2 expression was increased (Fig. 2B).

Effect of triptolide on PSD-95 and GAP43 expression

Post-synaptic density-95 (PSD-95) and growth-associated protein-43 (GAP43) play central roles in synaptic plasticity and axonal regeneration [4,20]. To investigate whether triptolide improves synaptic function, we evaluated the expression of PSD-95 and GAP43. Compared to the sham group, PSD-95 expression in I/R rats was lower, while GAP43 was increased ($p < 0.001$). Moreover, the expression of both PSD-95 and GAP43 improved in response to treatment with triptolide ($p < 0.05$) (Fig. 3).

Triptolide inhibits NogoA/NgR/ROCK signalling in rats with brain I/R

The molecular mechanism by which triptolide improves brain I/R injury was studied by examining the effects of triptolide on NogoA/NgR/ROCK signalling. Double immunofluorescence staining showed that expression levels for neuronal NogoA, NgR and ROCK2 were all higher in brain tissue from I/R rats compared to sham operated rats ($p < 0.001$). However, neuronal expression levels of NogoA, NgR and ROCK2 were all significantly lower in the triptolide vs. the I/R group ($p < 0.001$) (Fig. 4A-C). Western blot experiments revealed similar findings. Specifically, the expression levels for NogoA, NgR and ROCK2 proteins were all higher in the I/R group vs. the sham group ($p < 0.001$). However, the increase was sharply attenuated by treatment with triptolide ($p < 0.001$) (Fig. 4D).

Triptolide increases the viability of SH-SY5Y cells following OGD/R injury

An OGD/R model using cultured SH-SY5Y cells was developed to assess the neuroprotective effect of triptolide *in vitro* and the possible mechanism. CCK8 assay showed that OGD/R reduced the cell viability vs.
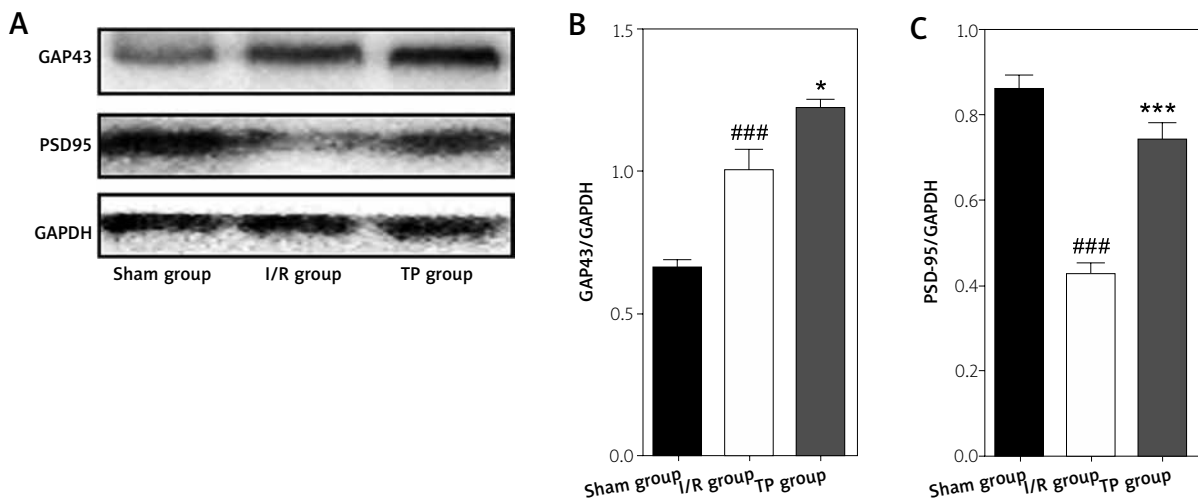
Fig. 3. Triptolide improves synaptic plasticity after brain I/R injury. The protein expression and quantitative analysis of GAP43 and PSD-95, $n = 5$. $^{###}p < 0.001$ vs. Sham group, $^{*}p < 0.05$ vs. I/R group, $^{***}p < 0.001$ vs. I/R group
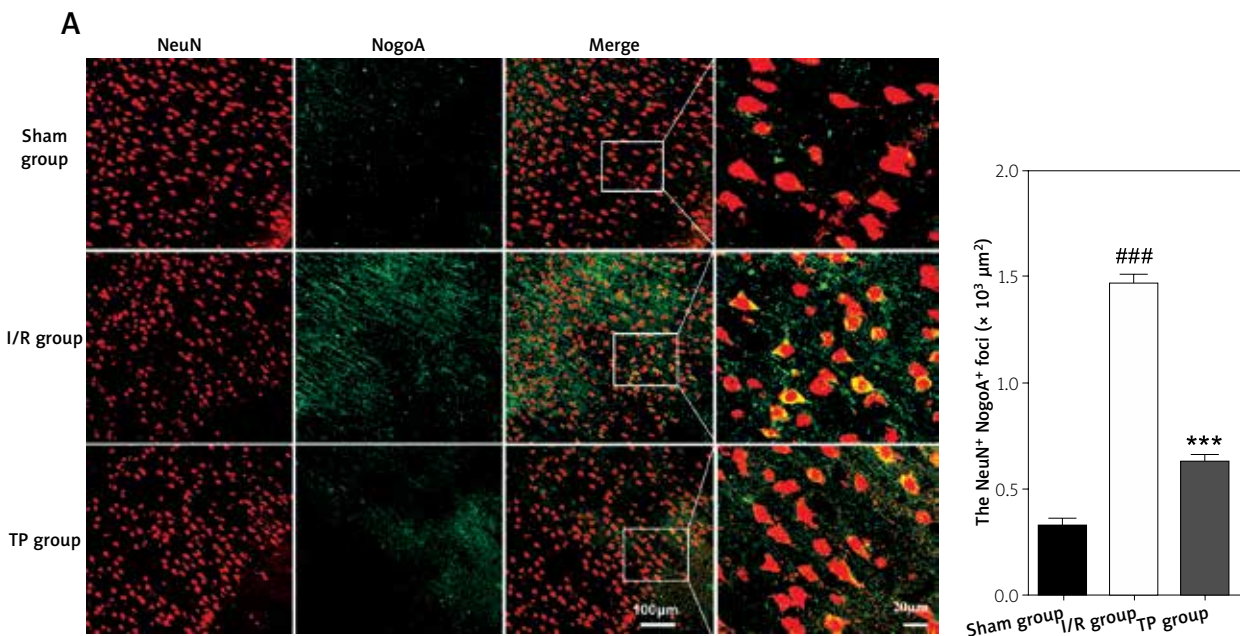

Fig. 4. Triptolide inhibited the activation of NogoA/NgR/ROCK pathway after brain I/R injury. **A**) Immunofluorescence image of NogoA (green) and NeuN (red).

the controls ($p < 0.001$). Treatment with triptolide and fasudil both increased OGD/R cell viability ($p < 0.01$). No significant difference in cell viability was observed between triptolide and fasudil treated groups (Fig. 5).

Effect of triptolide on GAP43 expression following OGD/R injury

GAP43 expression in OGD/R-treated cells was lower than that observed in controls ($p < 0.001$) (Fig. 6). Of

note, both triptolide and fasudil significantly increased GAP43 expression vs. the OGD/R group ($p < 0.001$).

The effect of triptolide on expression of NogoA, NgR and ROCK2 after OGD/R injury

Both immunofluorescence and western blot methods showed that NogoA, NgR and ROCK2 expression in OGD/R-treated cells was higher than that observed in
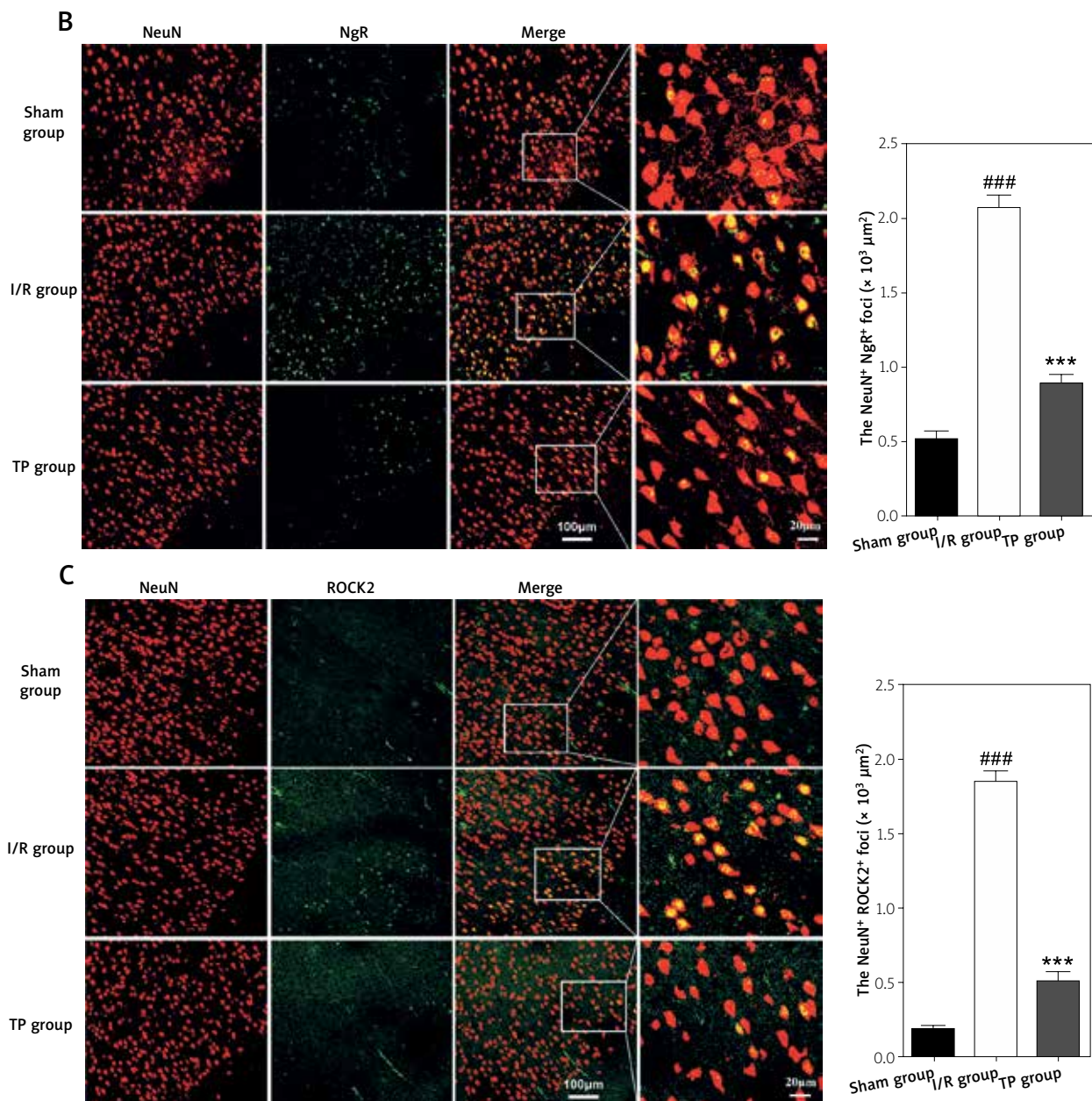
Fig. 4. Cont. **B**) Immunofluorescence image of NgR (green) and NeuN (red). **C**) Immunofluorescence image of ROCK2 (green) and NeuN (red).

controls ($p < 0.001$). However, both triptolide and fasudil significantly reduced NogoA, NgR and ROCK2 expression vs. the OGD/R group ($p < 0.001$). Moreover, no significant difference in the expression level of these proteins was observed between triptolide and fasudil groups (Fig. 7).

Discussion

Several studies have shown that triptolide is able to improve cerebral I/R injury. Triptolide was reported to achieve this by attenuating the nuclear factor-kappa B (NF-κB) and p53-upregulated modulator of apoptosis (PUMA) signalling pathways [28]. Li *et al.* [17] found that triptolide can decrease apoptosis in focal cerebral ischemia rat brain models. Their study further showed that triptolide can inhibit oxidative stress after brain I/R injury, with the likely mechanism involving regulation of Wnt/β-catenin signalling [21]. Triptolide has also been shown to reduce neurological deficit scores and brain infarct volumes, as well as reducing OGD-induced
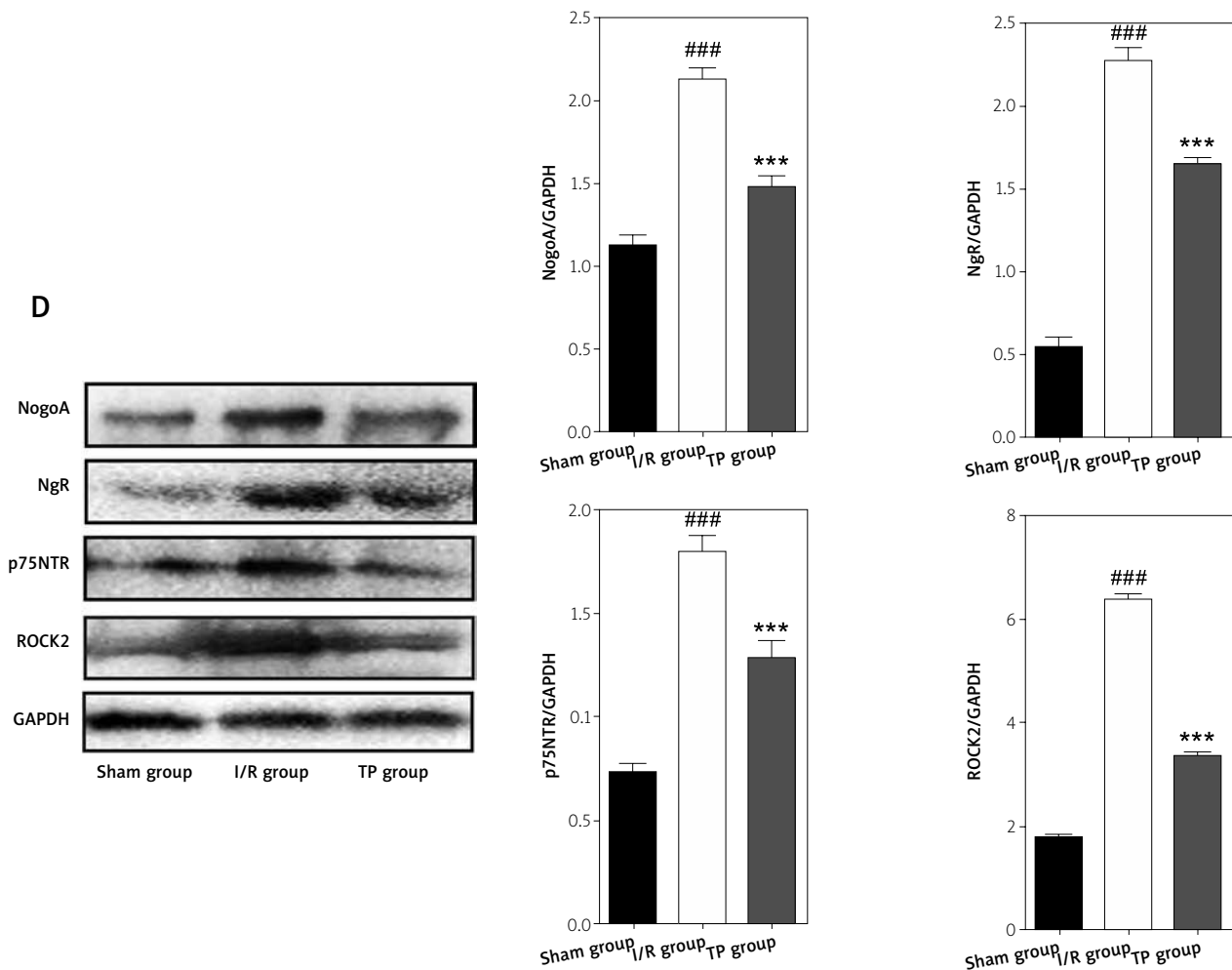
Fig. 4. Cont. **D**) The protein expression and quantitative analysis of NogoA, NgR, p75NTR and ROCK2, *n* = 5.
###*p* < 0.001 vs. the Sham group, ***p* < 0.001 vs. I/R the group.

cytotoxicity and apoptosis [10]. The present study further confirms that treatment with triptolide reduces the volume of brain infarct and improves neurological deficit scores, while also improving neuronal morphology. In addition, we observed that triptolide reduced OGD/R-induced cytotoxicity. Hence, the findings from our *in vivo* and *in vitro* experiments suggest that triptolide exerts protective effects following brain I/R injury.

GAP43 is a neuron-specific protein that is considered to be an internal determinant of axon plasticity and an important marker for axon regeneration [8]. It is involved in events such as axonal outgrowth, myelin repair, and synaptic plasticity [29]. Moreover, an increase in GAP43 expression is thought to be a key mechanism in the recovery from cerebral ischemia [16]. PSD-95 is central to the establishment and maintenance of postsynaptic density in excitatory neurons, a process that is closely related to neuronal function and survival. Increased expression of PSD-95 promotes synaptic plasticity and is an effective target for promoting neuroprotection in response to stroke [24]. In the current work, GAP43 expression in ischemic brain clearly increased and that of PSD-95 decreased following brain I/R. Furthermore, the expression of GAP43 and PSD-95 were both significantly increased by triptolide, suggesting it increases neuronal plasticity by regulating their expression.

Following ischemia injury to the brain, dysregulated synaptic regeneration is affected by many molecular mechanisms. NogoA has a critical key role through its inhibition of axon regeneration and nerve repair. Moreover, NogoA can activate RhoA/ROCK signalling by binding to the receptor complex formed by NgR, p75 NTR and LINGO-1. This, in turn, inhibits axon growth and nerve regeneration and repair [7,14]. The expression of NogoA in rats with global cerebral ischemia was
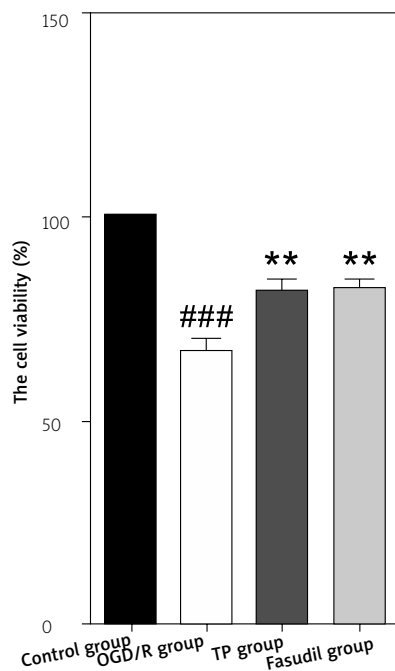
Fig. 5. Triptolide protected SH-SY5Y cells against OGD/R-induced cell injury. Cell viability measured by CCK8 assay, $n = 5$. $^{###}p < 0.001$ vs. the control group, $^{**}p < 0.001$ vs. the OGD/R group.

found to be significantly elevated at 6 hours after injury, and to persist for 7 days [31]. Eslamboli *et al.* [5] reported that NogoA expression continued to rise for 2 months after cerebral ischemia in marmoset monkeys. The expression of NogoA was also found to be elevated in the brain tissue of this animal model following cerebral I/R injury [11,27]. Therefore, NogoA is widely thought to be implicated in the underlying pathology of ischemic stroke. Anti-NogoA immunotherapy may improve nerve regeneration and neural plasticity after central nervous system injury, as well as improving the prognosis in animal models of stroke [15].

The ROCK inhibitor fasudil has been found to stimulate axonal regeneration using an animal model of cerebral I/R injury [9], suggesting that inhibition of the NogoA/NgR/ROCK signalling axis may contribute to recovery. NogoA, NgR, p75NTR, p-ROCK2 and ROCK2 expression levels were found in the present study to be significantly increased in animals with brain I/R injury. This finding is consistent with results from previous studies. In addition, we found that triptolide reduced NogoA, NgR, p75NTR, p-ROCK2 and ROCK2 expression, suggesting that its neuroprotective effects may involve inhibition of the NogoA/NgR//ROCK2 pathway.

Our studies using OGD/R-stimulated SH-SY5Y cells also confirmed the impact that triptolide has on brain I/R injury. The present *in vitro* studies confirmed that
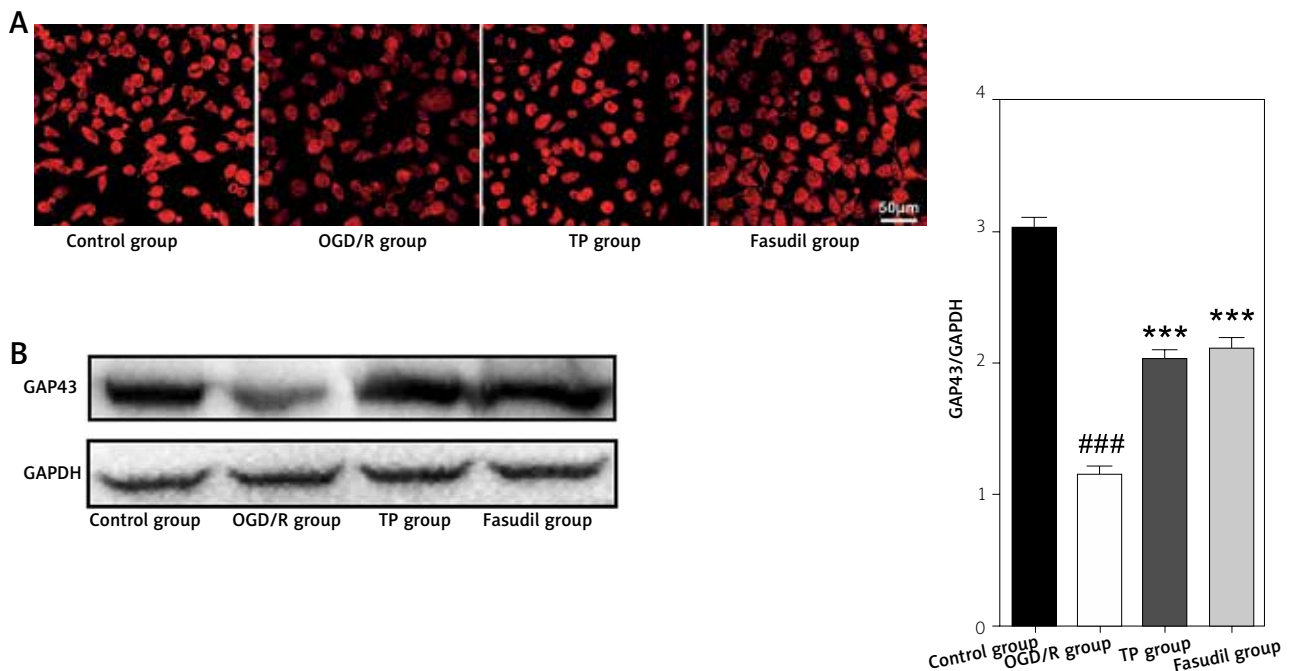

Fig. 6. Triptolide restored the expression of GAP43 in OGD/R-induced SH-SY5Y cells. **A)** Representative images of GAP43 immunofluorescence staining. **B)** The protein expression and quantitative analysis of GAP43, $n = 3$. $^{###}p < 0.001$ vs. the control group, $^{***}p < 0.001$ vs. the OGD/R group.
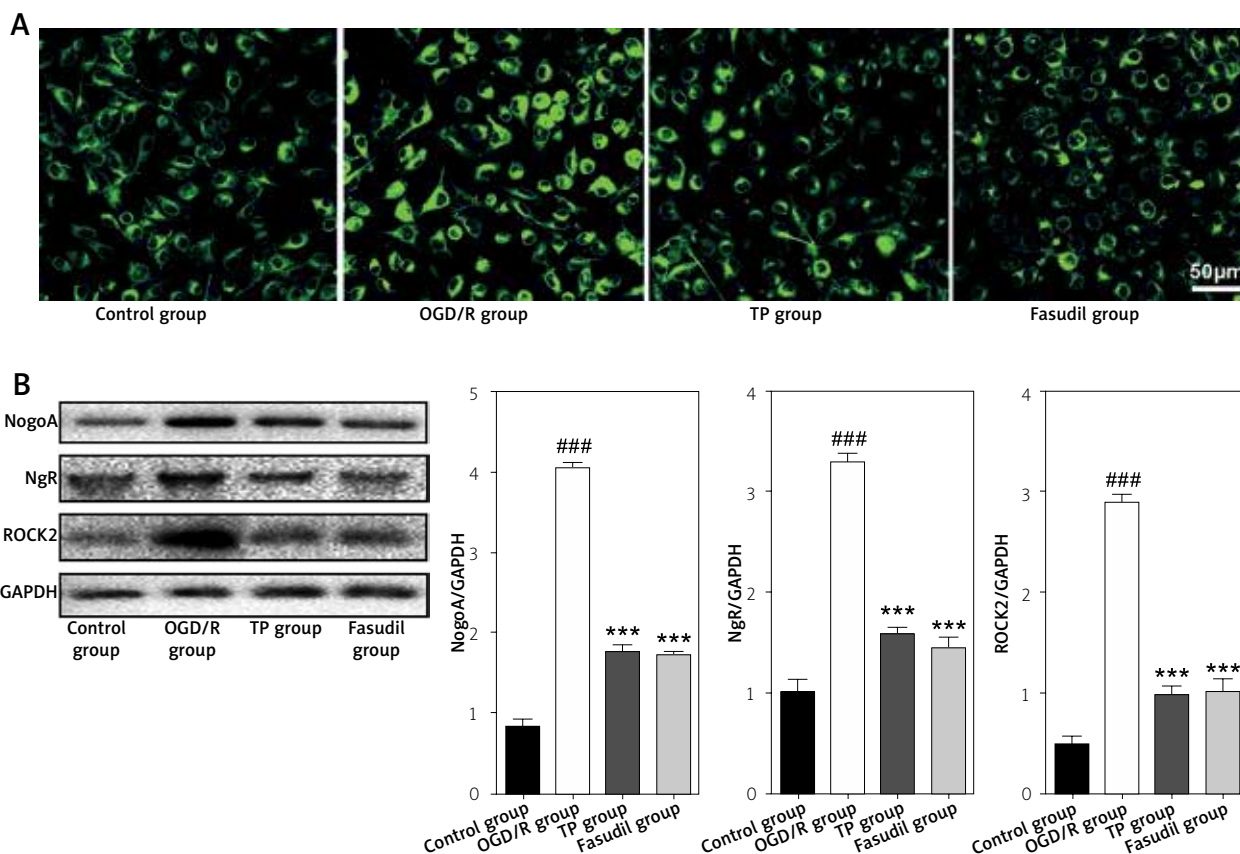
Fig. 7. Triptolide inhibited the expression of NogoA, NgR and ROCK2 in OGD/R-induced SH-SY5Y cells. **A)** Representative images of NogoA immunofluorescence staining. **B)** The protein expression and quantitative analysis of NogoA, NgR and ROCK2, $n = 3$. ###$p < 0.001$ vs. the control group, ***$p < 0.001$ vs. the OGD/R group.

both triptolide and the ROCK inhibitor fasudil increased SH-SY5Y cell activity following OGD/R, while increasing GAP43 and PSD-95 expression. In contrast, the levels of NogoA, NgR and ROCK2 expression were downregulated in both the triptolide- and fasudil-treated groups. No significant differences were observed between triptolide and fasudil intervention groups regarding NogoA/NgR/ROCK expression. These results indicate the neuroprotective effect of triptolide may be due to inhibition of NogoA/NgR/ROCK signalling.

In conclusion, our study found that triptolide reduces I/R–induced brain damage and maintains synaptic function through its inhibition of NogoA/NgR/ROCK signalling. These findings indicate that triptolide could be a promising new treatment for stroke victims.

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Disclosure

The authors report no conflict of interest

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