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Original Article

Toll-like receptor 3 agonist Poly I:C protects against simulated cerebral ischemia *in vitro* and *in vivo*

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Aim: To examine the neuroprotective effects of the Toll-like receptor 3 (TLR3) agonist Poly I:C in acute ischemic models *in vitro* and *in vivo*.
Methods: Primary astrocyte cultures subjected to oxygen-glucose deprivation (OGD) were used as an *in vitro* simulated ischemic model. Poly I:C was administrated 2 h before OGD. Cell toxicity was measured using MTT assay and LDH leakage assay. The levels of TNFα, IL-6 and interferon-β (IFNβ) in the media were measured using ELISA. Toll/interleukin receptor domain-containing adaptor-inducing IFNβ (TRIF) protein levels were detected using Western blot analysis. A mouse middle cerebral artery occlusion (MCAO) model was u sed for *in vivo* study. The animals were administered Poly I:C (0.3 mg/kg, im) 2 h before MCAO, and examined with neurological deficit scoring and TTC staining. The levels of TNFα and IL-6 in ischemic brain were measured using ELISA.
Results: Pretreatment with Poly I:C (10 and 20 µg/mL) markedly attenuated OGD-induced astrocyte injury, and significantly raised the cell viability and reduced the LDH leakage. Poly I:C significantly upregulated TRIF expression accompanied by increased downstream IFNβ production. Moreover, Poly I:C significantly suppressed the pro-inflammatory cytokines TNFα and IL-6 production. In mice subjected to MCAO, administration of Poly I:C significantly attenuated the neurological deficits, reduced infarction volume, and suppressed the increased levels of TNFα and IL-6 in the ischemic striatum and cortex.
Conclusion: Poly I:C pretreatment exerts neuroprotective and anti-inflammatory effects in the simulated cerebral ischemia models, and the neuroprotection is at least in part due to the activation of the TLR3-TRIF pathway.

Keywords: Toll-like receptor; Poly I:C; stroke; astrocyte; oxygen-glucose deprivation; middle cerebral artery occlusion; inflammation; TNFα; IL-6; interferon-β; Toll/interleukin receptor domain-containing adaptor-inducing interferon β (TRIF)

Introduction

Cerebral ischemic preconditioning (IPC) refers to a transient, sublethal ischemia which results in tolerance to a subsequent lethal cerebral ischemia. IPC is believed to trigger the most effective form of intrinsic neuroprotective mechanism[1, 2]. A better understanding of this endogenous neuroprotective mechanism could help in the development of prophylactic strategies in high-risk populations for pathological cerebrovascular events and neurosurgical procedures. Many attempts have been made to explore the mechanisms underlying this intrinsic neuroprotection of ischemic preconditioning. Post-ischemic inflammation is a significant factor that increases

damage associated with ischemic brain injury[3, 4]. Many studies have shown that the prevention of the inflammatory response might be a contributing mechanism by which IPC induces protection against brain ischemia[5, 6]. Bowen *et al* showed that prior IPC, a 10 min middle cerebral artery occlusion (MCAO), decreases the infarct volume and neurological deficits caused by focal ischemia (60 min MCAO 72 h after PC). IPC has also been shown to reduce the post-ischemia increase in the expression of many pro-inflammatory genes and prevent neutrophil and macrophage infiltration in the ipsilateral cortex of rats subjected to focal ischemia[5, 6].

Recent evidence indicates that Toll-like receptors (TLRs) are involved in the preconditioning-induced inflammatory prevention and ischemic tolerance[7, 8]. TLRs are innate immunity receptors that are expressed in a wide range of cell types, including in the central nervous system (CNS). Ten currently

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known TLR family members signal through two main pathways: a myeloid differentiation factor 88- (MyD88-) dependent pathway and a Toll/interleukin receptor domain-containing adaptor-inducing interferon β (TRIF)-dependent pathway. The MyD88-dependent pathway acts via nuclear factor κB (NF-κB) to induce pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα). The TRIF-dependent pathway acts via type I interferon to increase expression of anti-inflammatory molecules such as interferon β (IFNβ).

Of the TLRs, only TLR4 utilizes both of these pathways, and TLR3 signals exclusively through the MyD88-independent pathway. In contrast to the detrimental role of TLRs in response to ischemia, stimulation of TLR4 with low dose systemic administration of lipopolysaccharide (LPS) prior to ischemia provides robust neuroprotection accompanied by attenuated inflammatory response in the animal brain[9, 10]. Mice lacking TLR4 show less IPC-induced neuroprotection than wild-type mice[11]. Studies have suggested pretreatment with LPS or IPC cause cells to switch their transcriptional response by enhancing the TRIF-induced anti-inflammatory cytokine and suppressing the NF-κB induced pro-inflammatory cytokine[7, 8, 12]. Interestingly, LPS stimulation of macrophages has been shown to upregulate TLR3 expression[13], suggesting that LPS preconditioning may upregulate TLR3 to further enhance the TRIF pathway signaling. Because TLR3 signals exclusively through TRIF pathway, TLR3 stimulation may contribute to the finely controlled shift in the balance of pro-inflammatory and anti-inflammatory cytokines and represent an endogenous neuroprotective mechanism. However, whether TLR3 regulates the inflammatory response in the brain during cerebral ischemia and exerts cytoprotective effects against ischemia remains unexplored.

We have previously shown that TLR3 ligand polyinosinic-polycytidylic acid (Poly I:C) preconditioning can protect rat mixed cortical neuronal-glial cell cultures against oxygen-glucose deprivation (OGD)-induced injury and inhibited OGD-induced inflammatory cytokine IL-6 release into the medium[14]. In the present study, an *in vivo* model of focal cerebral ischemia and an *in vitro* model of cultured astrocytes subjected to OGD injury were used to further verify the neuroprotection of Poly I:C. The protective effects of Poly I:C were also investigated to determine whether this neuroprotection is related to Poly I:C's regulation of the inflammatory response during the ischemic period.

Materials and methods
Drugs and reagents
Poly I:C was obtained from Guangdong BangMin Pharmaceutical Co, Ltd (Jiangmen, China) and dissolved in saline. For *in vitro* experiments, Poly I:C was diluted with culture medium before being added to cell cultures. Poly-D-lysine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma Chemical Co (St Louis, MO, USA). A lactate dehydrogenase (LDH) assay kit was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Rat IFN-β, IL-6, and TNF-α ELISA kits were pur-

chased from R&D Systems (Minneapolis, MN, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Bio-Rad Laboratories Inc (Hercules, CA, USA). Antibody against TRIF was supplied by ABCAM (Cambridge, UK) and antibody against β-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other analytical grade chemicals were from commercial sources.

For primary astrocyte cultures, 1-d-old Sprague-Dawley rats were supplied by the Animal Center of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China). Kun-Ming strain mice at an age of 8 to 10 weeks and weighing 20 to 22 g were also supplied by the Animal Center. All animal experiments were carried out in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Astrocyte cell culture
Astrocytes were isolated from 1-d-old Sprague-Dawley rats. Briefly, brain hemispheres of newborn rats were removed from the skulls aseptically, and the meninges were carefully removed from the hemispheres in cold D-Hanks solution. The tissues were minced and incubated in 0.125% trypsin at 37 °C for 8 min. Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham's (DMEM/F-12) medium containing 20% fetal bovine serum (FBS) was added to terminate digestion. The suspension was filtered through stainless steel (200 mesh, hole width 95 μm). The filtrate was twice centrifuged at $300 \times g$ for 10 min. The precipitation was resuspended in DMEM/F-12 medium containing 20% FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL). The concentration of cells in suspension was adjusted to 1×10^6 cells/mL and plated in 25-cm² flasks. Cultures were incubated in DMEM/F-12 containing 20% FBS at 37 °C in 95% air and 5% CO_2 with 95% relative humidity (CO_2-Incubator, SHELLAB, USA). The total volume of culture medium was changed twice a week. The cells were cultured for two weeks until they reached confluence. On the 14th day *in vitro* (DIV), contaminated microglia and oligodendrocytes were removed by shaking at 200 rounds per minute with an orbital shaker for 5 h. After 5 d, shaking was repeated at 200 rounds per minute with an orbital shaker for 5 h. Under these conditions, microglial cells were almost completely detached from the layer of astrocytes. Astrocytes remaining in the flask were harvested with 0.125% trypsin. The suspension was centrifuged at $300 \times g$ for 10 min. The concentration of cells in precipitation was adjusted to $1-2 \times 10^5$ cells/mL with culture medium containing 20% FBS. Cells were plated to achieve a confluent monolayer on plastic 96-well culture plates and 35-mm (diameter) plastic dishes (Costar, Vitaris, Baar, France) that were previously coated with poly-D-lysine (100 μg/mL). To identify astrocytes, cultures were analyzed by immunochemical staining for glial fibrillary acidic protein (GFAP) (Sigma, USA) and counterstained using DAPI. Analyses of the cultures showed that 95%–98% of the cells were GFAP-positive, and 2%–5% cells were indeterminate types. All experiments were performed on 22-day old cultures.

Treatment of astrocyte cultures

At 22 DIV, a portion of the astrocytes were exposed to Poly I:C (10 and 20 μg/mL) for 24 h in serum-free DMEM, and a separate portion was cultured in serum-free DMEM for 24 h without Poly I:C treatment. Then, the culture medium was removed and replaced with pre-warmed glucose-free Earle's balanced salt solution, which was pre-incubated with 95% N_2 and 5% CO_2 for 30 min to remove the oxygen in the medium. The cultures were further incubated for 12 h in an airtight box that was continuously filled with 95% N_2 and 5% CO_2 to induce an oxygen-glucose deprivation (OGD) condition as described by Liu *et al*[15]. Cultures treated with Earle's balanced salt solution containing 10 mmol/L glucose in CO_2-incubator throughout were assigned to the control group.

Cell viability assay

The cell viability of the astrocytes was evaluated using the MTT conversion method[16]. The cells' ability to convert MTT to an insoluble purple formazan indicates mitochondrial integrity and activity, which in turn indicates cell viability. Briefly, after exposure to 12 h OGD, MTT was added to the cultures at a final concentration of 0.5 mg/mL and incubated for 4 h at 37 °C. The blue reaction product formazan was dissolved by 100 μL DMSO. The absorbance value at 570 nm was read using a microplate reader. Each experimental condition was performed in triplicate.

LDH release assay

Cell injury was quantitatively assessed using a cytotoxicity detection kit that measures cytosolic LDH release into the medium during OGD exposure. Absorbance at 492 nm was read on a microplate reader, and LDH values were normalized to the mean maximal LDH value in sister cultures continuously exposed to 0.1% Triton X-100, which causes near-complete glial cell death (100%). The results are expressed as a percentage of the maximal LDH level. Each experimental condition was performed in triplicate.

IFNβ, IL-6, and TNFα levels analysis

Secreted IFNβ, IL-6, and TNFα levels in the culture supernatants and in the ischemic brain were determined with commercially available ELISA kits according to the manufacturer's instructions. Absorbance at 450 nm was read on a microplate reader.

Protein extraction and Western blot analysis for TRIF

Astrocyte cultures were washed with ice-cold phosphate-buffered saline (PBS) and the proteins were extracted with 80 μL of lysis buffer: 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 0.5% Igepal, 0.1% SDS, 10 μg/mL phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL pepstatin and 10 μg/mL of heat-activated sodium orthovanadate. After 30 min on ice, the cell lysates were centrifuged at 12 000×*g* for 15 min at 4 °C and the supernatants were harvested. The protein concentrations in the samples were determined according to the Bradford method with serum albumin as a standard.

Equal amounts of the protein samples were loaded per lane and electrophoresed in 12% dodecylsulfate-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, and the membranes were incubated overnight at 4 °C with rabbit anti-TRIF polyclonal antibody (1:600 dilution) and goat polyclonal β-actin antibody (1:500 dilution). The membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies diluted at 1:5000 for 1 h at room temperature. The positive bands were revealed using enhanced chemiluminescence detection reagents and autoradiography film. The optical densities of the bands were scanned and quantified with ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). β-Actin served as an internal control.

Induction of focal cerebral ischemia and reperfusion in mice

Transient focal ischemia was produced by intraluminal MCAO with a nylon filament, as we have previously described[17]. All animal experiments were carried out in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. After performing a midline neck incision, the left common carotid artery, external carotid artery and internal carotid artery were carefully separated. The proximal left common carotid artery and the external carotid artery were ligated. A 6–0 nylon monofilament (Ethicon) with a heat-blunted tip was introduced through a small arteriotomy of the common carotid artery into the distal internal carotid artery and was advanced 8–9 mm distal to the origin of the middle cerebral artery (MCA) until the MCA was occluded. The suture was withdrawn from the carotid artery under anesthesia 2 h after insertion to enable reperfusion. Then, the wound was closed. Mice were maintained in an air-conditioned room at 25 °C during the reperfusion period of 22 h. Mice that failed to extend the contralateral forelimb were verified as adequate occlusion and selected for further measurements. Poly I:C (0.3 mg/kg in saline, im) was administered 24 h before MCAO. In sham-operated mice, the arteries were separated without occlusion. In each group, 8 animals were used to determine infarct volume. An additional 8 animals were used to determine IL-6 and TNFα levels in the brain by ELISA.

Evaluation of neurological deficit score and determination of infarct size

Neurological deficits of each mouse were evaluated at 23 h after MCAO by the Zea-Longa method: 0=normal spontaneous movement; 1=failure to extend the contralateral forelimb; 2=circling to affected side; 3=partial paralysis on affected side; 4=no spontaneous motor activity. Mice were then killed with an overdose of pentobarbital at 24 h after MCAO. The brains were immediately removed and frozen at -20 °C for 20 min and sectioned into five coronal slices. The brain slices were incubated in 2% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC) at 37 °C for 15 min, followed by 4% paraformaldehyde overnight. The brain slices were photographed and the area of ischemic damage was measured by an image analysis

system. The total infarct volume was calculated by integration of the infarct areas in sequential 1-mm-thick brain sections.

Statistical analysis

All results were expressed as the mean±SD. The significance of the differences among different groups was determined using SPSS (Statistical Package for the Social Sciences) for Windows (version 18.0) with one-way ANOVA. Ranked data of neurological deficit were analyzed by the nonparametric Kruskal-Wallis test. Differences were considered significant when $P<0.05$.

Results

Protective effects of Poly I:C on cultured astrocytes against OGD-induced injury

In cultured rat astrocytes, 95%–98% of the astrocytes were identified (Figure 1A). OGD induced significant cell injury as indicated by morphological observation. Cultured rat astrocytes exposed to OGD for 12 h demonstrated a marked decrease in the cell number and branches of the cell bodies, and the remaining cells were round and small. However, the cells appeared to be much healthier in the groups pretreated with 10 µg/mL and 20 µg/mL Poly I:C (Figure 1B). This observation indicates some degree of protection by Poly I:C against OGD-induced injury in cultured astrocytes. The cytoprotection by Poly I:C was further evaluated by MTT assay and LDH leakage.

The 12 h OGD exposure of astrocytes markedly decreased OD value *vs* the normal group as determined by the MTT assay (0.46±0.04, 0.93±0.17 in OD value, respectively, $P<0.05$) (Figure 2A). However, pretreatment with Poly I:C significantly attenuated OGD-induced cell toxicity. Pretreatment with 10 µg/mL and 20 µg/mL Poly I:C prior to exposure to 12 h OGD markedly raised astrocyte viability to 0.59±0.04 and 0.64±0.07 in OD value, respectively $P<0.05$).
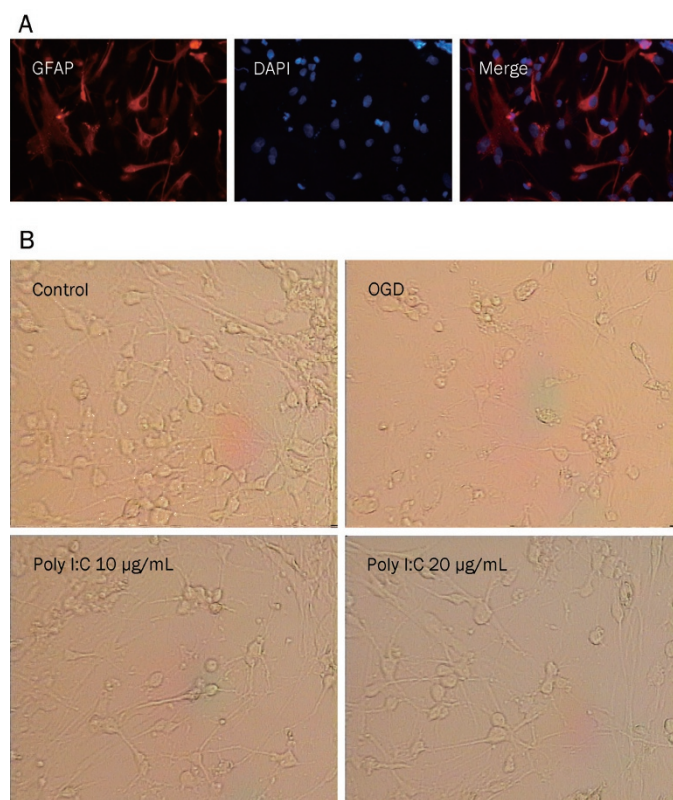

Figure 1. Microphotographs of cultured astrocytes. (A) 95%–98% GFAP-positive cells are shown in the cultures. Anti-GFAP antibody was used to identify cultured rat astrocytes (red), in which nuclei were counterstained using DAPI (blue). The microphotographs were taken at the 22nd DIV under a fluorescent microscope (Magnification, 400×). (B) Effects of Poly I:C against OGD-induced injury in cultured astrocytes. Astrocyte cultures treated with 10 µg/mL and 20 µg/mL Poly I:C (added 24 h before OGD) or untreated (OGD alone). The microphotographs were taken with a conventional optical microscope (Magnification, 200×) 12 h after exposure to OGD.
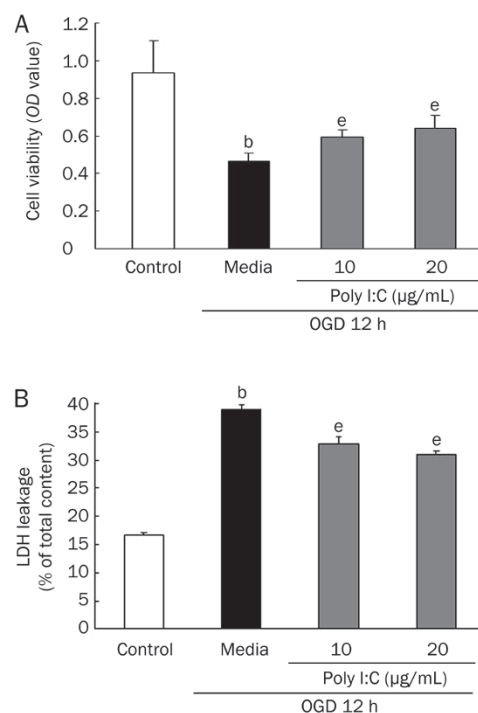

Figure 2. MTT assay (A) and LDH release (B) in astrocyte cells under simulated ischemia. Astrocyte cultures treated with 10 µg/mL and 20 µg/mL Poly I:C (added 24 h before OGD) or untreated (OGD alone). Each independent experiment was carried out in triplicate. Values are expressed as the mean±SD. [b]$P<0.05$ compared with control group, [e]$P<0.05$ compared with cells exposed to OGD alone.

LDH leakage, another indicator of cell toxicity, was evaluated to further investigate the protective effect of Poly I:C. As shown in Figure 2B, LDH leakage increased to 39.0%±0.9% compared with the control group after the cells were exposed to 12 h OGD, double the level of the control group ($P<0.05$). Pretreatment with 10 µg/mL and 20 µg/mL Poly I:C markedly attenuated OGD-induced cell death, reducing LDH leakage to 32.9%±1.2% and 31.0%±0.9%, respectively ($P<0.05$).

Effects of Poly I:C on IFNβ, TNFα, and IL-6 release in astrocyte cell cultures exposed to simulated ischemia *in vitro*

Exposure of astrocytes to simulated ischemia for 12 h resulted in notably increased IFNβ release. 10 µg/mL Poly I:C did not increase IFNβ levels in comparison with ischemic cultures. However, 20 µg/mL Poly I:C pretreatment significantly increased IFNβ levels after 12 h exposure to ischemia ($P<0.05$) (Figure 3A). *In vitro* exposure of astrocytes to 12 h of OGD also markedly increased TNFα and IL-6 levels, indicating that ischemic conditions stimulated astrocytes *in vitro* to produce pro-inflammatory cytokines. A lower Poly I:C concentration (10 µg/mL) did not decrease TNFα level in comparison with ischemic cultures ($P>0.05$). However, 20 µg/mL Poly I:C pre-

treatment notably decreased TNFα levels after 12 h exposure to ischemia ($P<0.05$) (Figure 3C). Pretreatment with 10 µg/mL and 20 µg/mL Poly I:C both attenuated IL-6 release induced by ischemia *in vitro* ($P<0.05$) (Figure 3B).

Poly I:C preconditioning upregulates TRIF protein expression in astrocyte cell cultures exposed to simulated ischemia *in vitro*

TLR3 is unique among the TLRs because its signaling occurs solely via recruitment of the protein TRIF. TRIF signaling occurs via the interferon-regulated factor-3 (IRF3) complex and induces the anti-viral IFNβ via the TRIF-IRF3 axis. We examined the expression of TRIF protein in astrocyte cultures by Western blot analysis. We found that TRIF levels were reduced in astrocyte cultures exposed to simulated ischemia compared with the control group. However, TRIF levels were upregulated in Poly I:C-pretreated cultures at both concentrations ($P<0.05$) (Figure 4).
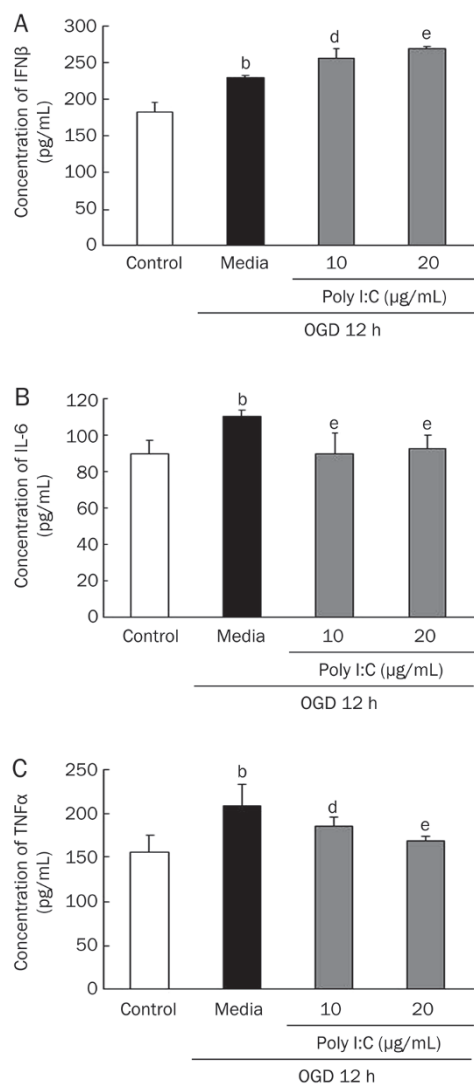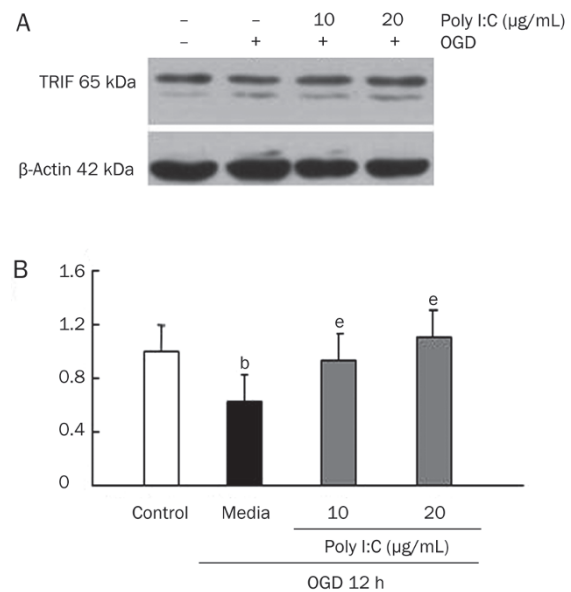

Figure 3. Anti-inflammatory effects of Poly I:C in astrocyte cells subjected to 12 h OGD. (A) The effects of Poly I:C on IFNβ levels in culture medium are shown. (B) The effects of Poly I:C on IL-6 levels in culture medium are shown. (C) The effects of Poly I:C on TNFα levels in culture medium are shown. Each independent experiment was carried out in triplicate. Values are expressed as the mean±SD. [b]$P<0.05$ *vs* control group, [d]$P>0.05$, [e]$P<0.05$ *vs* cells exposed to OGD alone.

Figure 4. Western blot analysis of the modulatory effects of Poly I:C on TRIF protein expression in astrocytes subjected to 12 h OGD. Astrocyte cultures were pretreated with 10 µg/mL and 20 µg/mL Poly I:C and then exposed to OGD for 12 h. At the end of the treatment, cells were harvested for Western blot analysis with β-actin as a protein loading control. (A) representative Western blot; (B) statistical results. Each independent experiment was carried out in triplicate. Values are expressed as the mean±SD. [b]$P<0.05$ *vs* control group; [e]$P<0.05$ *vs* OGD alone.

Neuroprotective and anti-inflammatory effects of Poly I:C on mice subjected to transient MCAO

Because the present experiments in cultured astrocytes suggested anti-inflammatory activities of Poly I:C under ischemic conditioning, further *in vivo* evaluation of this compound in the MCAO model was performed. A 2 h transient MCAO in mice resulted in an infarct that included almost 50% of the

ipsilateral brain and caused obvious neurological dysfunction. However, the total infarct volume was greatly reduced by the administration of Poly I:C compared with that in the untreated mice (*P*<0.05) (Figure 5). Similarly, neurological deficit after MCAO was ameliorated in Poly I:C-treated animals (*P*<0.05) (Figure 5). In addition, inflammatory responses were investigated in ipsilateral and contralateral brains. The 2 h transient MCAO in mice caused increased TNFα and IL-6 levels in the ischemic brain compared with those in the sham group, whereas 0.3 mg/kg Poly I:C pretreatment markedly reduced TNFα and IL-6 levels in the ischemic brain (*P*<0.05) (Figure 6). The expression of these pro-inflammatory cytokines remained the same in the contralateral brain (data not shown).

Discussion

Astrocytes are the most abundant cells in the CNS and perform many functions, including maintenance of the blood–brain barrier, supply of nutrients to neurons, modulation of synaptic transmission, and regulation of the inflammatory response to brain injury. Neurons may undergo damage that is more severe when astrocytes are injured by ischemia[18, 19]. TLR3 is expressed throughout the CNS and is most prominent in astrocytes[8]. Unique among the TLRs, TLR3 signals exclusively through the TRIF-dependent pathway and is purported to participate in a comprehensive neuroprotective action. It has been reported that the activation of TRIF reduces neuronal death in *in vitro* stroke models. Mice lacking TRIF/IRF3 were not protected by LPS preconditioning in an *in vivo* model[7].

Our previous studies showed that TLR3 ligand Poly I:C preconditioning protects mixed cortical cultures against OGD-induced injury and inhibits OGD-induced IL-6 release[14]. In the present study, we further examined the neuroprotective potential of Poly I:C in cultured rat astrocytes in a simulated ischemia model, which may contribute to clarifying our understanding of drug mechanisms that protect the brain against ischemic injury[20–22]. Our study showed that the TLR3 agonist Poly I:C can reduce ischemic damage to astrocytes in a simulated ischemic condition, as indicated by cellular mor-
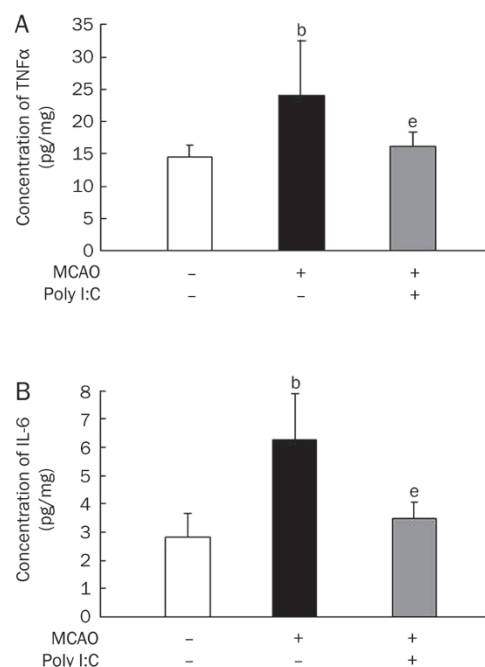

Figure 6. Anti-inflammatory effects of Poly I:C (0.3 mg/kg) in mice subjected to MCAO. (A) Poly I:C reduced TNFα levels in the ischemic striatum and cortex. (B) Poly I:C reduced IL-6 levels in the ischemic striatum and cortex. TNFα and IL-6 levels in the contralateral brain between groups showed no notable difference (data not shown). Values are expressed as the mean±SD, *n*=8. [b]*P*<0.05 *vs* control group, [e]*P*<0.05 *vs* MCAO group.

phology, mitochondrial function and LDH leakage. Bsibsi *et al*[23] reported that Poly I:C-conditioned medium improves neuronal survival in organotypic human brain slice cultures, and freshly added Poly I:C to control medium promoted neuronal survival equally well. This finding suggests that in the context of such slices, local astrocytes can be activated by Poly I:C to produce neuroprotective mediators in culture. Poly I:C has also shown neuroprotective effects under ischemic condi-
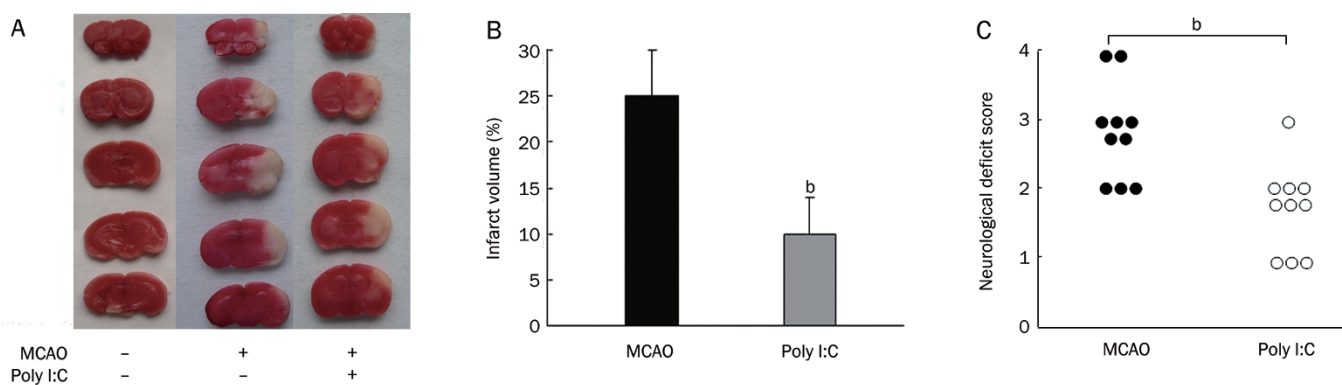

Figure 5. Poly I:C (0.3 mg/kg) reduced cerebral infarct size and improved neurological deficit in mice subjected to MCAO. Poly I:C was administered intramuscularly 24 h before MCAO. (A) Five consecutive TTC-stained coronal brain slices are arranged in cranial to caudal order. (B) Statistical results of infarct volumes in groups are shown. Values are expressed as the mean±SD, *n*=8. [b]*P*<0.05 *vs* MCAO group. (C) Results of neurological deficit score in groups are shown, and the data were analyzed by the nonparametric Kruskal-Wallis test. *n*=10. [b]*P*<0.05 *vs* MCAO group.

tions. Marsh *et al*[7] found that acute Poly I:C *in vitro* treatment in mouse mixed cortical cultures exposed to OGD markedly reduced OGD-mediated cell death. Our findings in the present study are consistent with those of Marsh *et al*.

In several models of brain ischemia, systemic administration of the TLR4 ligand LPS induces tolerance to injury[24–26] that is most likely through induction of IFNβ and interferon-stimulated genes through the TLR4 adapter molecule TRIF[7]. Poly I:C-induced tolerance to ischemic damage is similar to the phenomenon of LPS-induced tolerance to ischemic injury. IFNβ is an important downstream chemical product of TLR3 and TLR4 via TRIF. It has been shown that direct administration of IFNβ reduced ischemic brain damage in both rat and rabbit models of ischemic stroke[27-29]. The protective effects of IFNβ are associated with both preventing neutrophil infiltration and attenuating blood-brain barrier damage[28]. IFNβ has already been approved for human use as a treatment for the chronic inflammatory disorder multiple sclerosis. In contrast to IFNβ, TNFα, and IL-6 are important downstream products of the TLR-MyD88 pathway. TNFα and IL-6 are critically important in mediating leukocyte infiltration in tissues via the initial induction of leukocyte adhesion molecules such as vascular cell adhesion molecule-1, intercellular cell adhesion molecule-1 and E-selection on endothelial cells[30, 31]. Because TLR3 activation exclusively signals through the TRIF pathway, signaling via the TRIF-dependent pathway might serve as a neuroprotective mechanism associated with Poly I:C preconditioning in ischemia. To explore the neuroprotective mechanism related to Poly I:C, we examined IFNβ, TNFα, and IL-6 levels in the cultured medium of ischemic astrocytes. It was demonstrated that preconditioning with the TLR3 ligand Poly I:C enhanced IFNβ levels in the culture medium from ischemic astrocytes and inhibited TNFα and IL-6 production from the ischemic astrocytes. Similar inhibitory effects of Poly I:C on TNFα and IL-6 levels in ischemic mouse brains were observed *in vivo*. The results of Western blot analysis further showed that Poly I:C preconditioning markedly enhanced TRIF protein expression in the ischemic astrocyte cultures. These findings suggest that Poly I:C preconditioning may activate TLR3 in astrocytes and signal through TRIF to induce its downstream component IFNβ production. Reduced TNFα and IL-6 production is purported to be a result of disrupted signaling through the TLR-MyD88 pathway. Poly I:C preconditioning, similar to LPS, may inhibit the pro-inflammatory MyD88 pathway by upregulating inflammatory pathway inhibitors in the brain; alternatively, the activation of the TLR3-TRIF pathway before a stroke can directly inhibit the activation of TLR-MyD88 pathway in the setting of ischemia.

In conclusion, the protective potential of TLR3 ligand Poly I:C has been investigated in brain ischemia models *in vivo* and *in vitro*. The underlying protective mechanisms for Poly I:C preconditioning may be partly related to its activation of the TRIF–IFNβ pathway, which results in reduced pro-inflammatory cytokine production and enhanced anti-inflammatory cytokine IFNβ production from the ischemic astrocytes. This is the first demonstration of the neuroprotective mechanism of Poly I:C and its modulation of the TRIF-dependent signaling pathway. Activation of TLR3-TRIF by drug preconditioning might be a useful and novel anti-inflammatory strategy to protect the brain from ischemic damage.

Author contribution
Dr Qing LU and Dr Wei ZHU designed the study and wrote the manuscript; Dr Xu-lin XU helped write the manuscript, Lin-na PAN conducted the research and analyzed the data; Cai LI helped with portions of the research; Prof Lian-jun GUO helped design the study and revised the paper.

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