Poly(I:C) preconditioning ameliorates cognitive dysfunction after cerebral I/R injury by inhibiting TRAF6 signaling

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Abstract
Objective: Toll-like receptor (TLR) activation plays an important role in cerebral ischemia-reperfusion injury. In addition, increasing evidence suggests that TLRs may affect cognitive behavior through TLR-mediated signaling. Here, we explored the protective effects of TLR3 on cognitive dysfunction after ischemia in the context of poly(I:C) preconditioning.

Materials and Methods: Mice (n=84) were randomly divided into the sham group, AAV (vector) group, middle cerebral artery occlusion (MCAO) model group, poly(I:C) (pre) + MCAO model group, and AAV (TRAF6) + poly(I:C) (pre) + MCAO model group. The mice were injected i.p. with poly(I:C) (1.25 mg/g) 24 h prior to cerebral ischemia. Then, neurological scores were assessed, and the infarct volume was measured after cerebral ischemia-reperfusion. We evaluated the poly(I:C) preconditioning-induced attenuation of neuronal damage using Nissl and TUNEL staining. We assessed the poly(I:C) preconditioning-mediated inhibition of I/R-induced glial activation, inflammatory factor levels and TRAF6 expression. We also assessed whether TRAF6 affects poly(I:C) preconditioning to improve cognitive dysfunction and neuroprotection.

Results: The results showed that compared with those of the sham group and AAV (vector) group, the functional neurological scores and focal infarct volume of the MCAO group and poly(I:C) preconditioning group were significantly increased. The results also showed that compared with those of the MCAO group, the functional neurological scores and focal infarct volume of the poly(I:C) preconditioning group were significantly reduced. Our results indicated that poly(I:C) preconditioning significantly attenuated neuronal apoptosis and cell loss. Poly(I:C) preconditioning also inhibited I/R-induced glial cell activation and reduced NF-κB, TNF-α and IL-β levels. Our findings showed that poly(I:C) preconditioning affected cognitive dysfunction following cerebral I/R. Here, we observed that poly(I:C) preconditioning affected the expression and distribution of TRAF6 following cerebral I/R. TRAF6 overexpression abolished poly(I:C)-induced neuroprotection and worsened cognitive dysfunction in cerebral I/R injury.

Significance: Our findings suggested that poly(I:C) preconditioning ameliorates cognitive dysfunction after cerebral I/R injury by inhibiting TRAF6 signaling, which is a potential therapeutic target for the
treatment of cognitive dysfunction after stroke.

Introduction

Stroke is the third leading cause of death and the leading cause of long-term disability in the United States[1]; however, in China, stroke has become the leading cause of both death and disability, the latter of which is often accompanied by cognitive dysfunction [2]. Ischemic stroke caused by cerebral ischemia/reperfusion (I/R) injury accounts for ~83% of all stroke cases [1]. At present, IV r-tPA thrombolysis is an effective treatment for acute cerebral I/R injury, but the narrow time window and complications involving intracerebral hemorrhage limit its clinical application [3]. Moreover, many survivors suffer from severe disability, including cognitive impairment. Therefore, it is of great importance to find other ways to alleviate the cognitive impairment induced by ischemic stroke. Recent studies have suggested that Toll-like receptor (TLR)-mediated innate immune and inflammatory responses contribute to cerebral I/R injury [4, 5]. TLR-mediated signaling pathways predominately activate NF-κB, which is a critical transcription factor that regulates the expression of genes involved in innate and inflammatory responses [6]. Consequently, TLRs may be important targets for the development of new treatment approaches for cerebral I/R injury [7]. Toll-like receptor 3 is located in intracellular endosomes and recognizes double-stranded RNA (dsRNA), resulting in the induction of antiviral immune responses, and polyinosinic-polycytidylic acid [poly(I:C)], a synthetic analogue of dsRNA, stimulates TLR3-mediated responses[8]. Our previous research has shown that the administration of poly(I:C) after focal cerebral ischemia can decrease the brain infarct volume and reduce neurological deficit scores (NDSs) through the downregulation of TLR4 signaling and has therapeutic effects against cerebral ischemic I/R[9]. In addition, some studies have demonstrated that preconditioning mice with poly(I:C) attenuates neurological deficits and reduces the infarct volume following cerebral I/R injury [10-12]. These studies showed that TLR3 activation and TLR3 preconditioning plays an important role in cerebral ischemia-reperfusion injury.

Over the past few years, cognitive changes following brain infarction have been demonstrated in stroke patients and animals. The mechanism of poststroke cognitive impairment remains uncertain [13]. As of yet, there is no effective treatment for cognitive impairment following stroke. Recently,
increasing evidence has shown that TLRs might also impact cognitive processes in brain injury [14]. A recent study showed that TLR4 and TLR2 may be involved in cognitive dysfunction associated with intracerebral hemorrhage and Alzheimer's disease [15, 16]. It was recently shown that TLR3 has broad effects on cognitive dysfunction and that the activation of TLR3 by specific TLR agonists of poly(I:C) diminishes working memory performance. Meanwhile, poly(I:C) preconditioning provides neuroprotection in focal cerebral ischemia [9], so we wondered whether poly(I:C) preconditioning can ameliorate cognitive dysfunction associated with focal cerebral I/R injury.

In recent years, tumor necrosis factor receptor-associated factor (TRAF) family members have received much interest from the research community; these receptor-associated factors are adaptor proteins that are important for the assembly of receptor-associated signaling networks that link upstream receptors to downstream effector molecules[17]. Among them, tumor necrosis factor receptor-associated factor 6 (TRAF6) is an important multifunctional intracellular adaptin of the tumor necrosis factor superfamily and Toll/IL-1 receptor (TIR) superfamily. Recent studies have shown that TRAF6 plays important roles in regulating host immunity, embryonic development, tissue homeostasis, and neurodegenerative disease[18, 19]. Our previous study revealed that TRAF6 polyubiquitination may participate in secondary inflammatory injury in a mouse model of intracerebral hemorrhage [20]. Meanwhile, TRAF6 expression has been reported to be upregulated in the peripheral blood of ischemic stroke patients, as well as in neurons from a rat model of cerebral I/R [21]. Moreover, as a ubiquitin E3 ligase, TRAF6 exacerbates cerebral I/R, and its ablation has been shown to decrease the infarct volume and neurological deficit scores by alleviating proinflammatory signaling after cerebral I/R[17]. These results suggest that TRAF6 participates in inflammatory injury after cerebral I/R and that TRAF6 is an important treatment target. In particular, the signaling components TLR3-TRAF6 comprise a pathway independent of the TLR3-mediated activation of NF-κB and MAP kinases[22]. Therefore, we speculate that poly(I:C) preconditioning might improve cognitive dysfunction after focal ischemic cerebral injury by reprogramming TRAF6 signaling.

In this study, we employed a mouse middle cerebral artery occlusion (MCAO) model to evaluate the effects of poly(I:C) preconditioning on cognitive dysfunction and the related mechanisms. The results
suggested that poly(I:C) preconditioning ameliorates cognitive dysfunction associated with cerebral I/R injury by inhibiting TRAF6 signaling. Poly(I:C) is a promising new drug candidate for the treatment of cognitive impairment induced by cerebral ischemia.

Materials And Methods

Experimental animals

Male C57BL/6J mice (aged 10-12 weeks with an initial body weight of 20 ± 2 g) were purchased from the Third Military Medical University Animal Center. The mice were housed at a temperature of 25 ± 2 °C and a relative humidity of 70% ± 5% under natural light/dark conditions and allowed free access to food and water. The animal experiments were performed in strict accordance with the ethical guidelines of the Third Military Medical University Animal Studies Committee and the National Institutes of Health Guide (NRC 2011) for the Care and Use of Laboratory Animals.

MCAO model

To establish the middle cerebral artery occlusion (MCAO) model[9], the mice were anesthetized with isoflurane (3% for induction and 1.5% for maintenance). After a ventral neck incision was made, the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were carefully exposed. Microvascular aneurysm clips were applied to the right CCA and the ICA. A-coated 6-0 filament (RWD, Shenzhen, China) was introduced into the arteriotomy hole, fed distally into the ICA, and advanced to a predetermined distance 8 mm from the carotid bifurcation toward the MCA. After 60 min of MCAO, the filament was withdrawn, the collar suture was tightened, and the skin incision was closed. Sham mice underwent neck dissection and coagulation of the ECA but not MCA occlusion. Laser Doppler flowmetry (PeriFlux system 5000; Perimed, Stockholm, Sweden) was used as a quality control; success was determined based on an 80% decrease in regional CBF in the mice after MCAO. Dead animals and failed MCAO model mice were excluded from the experiments.

Western blot assay

Total protein samples from the brain tissues of mice were lysed using cold RIPA buffer containing 1 mM phenylmethylsulfonylfluoride according to the manufacturer’s protocol (Beyotime Technologic Inc., China). The protein content was determined using a bicinchoninic acid protein assay kit.
(Beyotime Technologic Inc., China). Equal amounts of protein lysates (30 μg in each lane) were loaded onto sodium dodecyl sulfate (SDS)-polyacrylamide gels (8-12%), electrophoretically separated and transferred onto PVDF membranes (Millipore, USA). After blocking nonspecific binding sites with 5% dry milk for 1 h in TTBS at 37 °C, the membranes were individually incubated with primary antibodies against TRAF6 (Bioss Technologic Inc., China) and β-actin at 4 °C overnight. Then, the membranes were further incubated with a corresponding horseradish peroxidase (HRP)-conjugated secondary antibody at a 1:500 dilution for 2 h at room temperature. Protein bands were visualized with enhanced chemiluminescence (ECL) reagents (Pierce; Thermo Fisher Scientific Inc., Waltham, MA, USA). β-Actin was used as an internal reference for relative quantification. The densitometric values were analyzed with ImageJ 1.43u (National Institutes of Health, USA) and normalized to β-actin as an internal control (IOD of target protein versus IOD of GAPDH).

**Quantitative real-time PCR assay**

Total RNA samples were obtained from brain tissues using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s protocol. cDNA was synthesized using a SuperScript cDNA Synthesis Reagent Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using Maxima SYBR Green/ROX qPCR Master Mix according to the manufacturer’s instructions (Thermo Fisher Scientific) on an ABI-StepOnePlus Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The cycle threshold (CT) values for each sample were normalized to those of GAPDH, and the fold differences were determined using the 2^-ΔΔCT method.

**Immunohistochemistry**

Immunostaining was performed as described previously[23]. Paraffin-embedded sections of brain tissues were dewaxed and rehydrated with xylene and graded alcohols. Endogenous peroxidase activity was blocked with 0.3% H2O2 in methanol for 5 min, and then the sections were microwaved in EDTA buffer solution (pH 8.0) for 20 min and cooled. After the sections were incubated with 5% BSA and 0.1% Triton X 100, they were incubated with a rabbit anti-TRAF6 antibody (Bioss Technologic Inc.,
China) at 4 °C overnight. Normal nonimmune rabbit serum was used as a negative control. After incubation with a secondary antibody at room temperature for 60 min, the slides were incubated with goat anti-rabbit immunoglobulin conjugated to peroxidase-labeled dextran polymer (EnVision+ System-HRP; Boster, China) for 20 min. Then, the sections were stained with 3,3-diaminobenzidine (DAB) and counterstained with hematoxylin. After dehydration and drying, the sections were mounted with neutral gum, covered with coverslips and observed under a microscope.

**Immunofluorescence staining**

The sections were treated with 5% goat serum and 0.1% Triton X-100 to block nonspecific binding. Then, the sections were incubated overnight at 4 °C with a rabbit anti-TRAF6 antibody (Bioss Technologic Inc., China) and an anti-GFAP antibody (mouse monoclonal, 1:600; CST, USA). After washing with PBS, the sections were incubated with a mixture of Alexa Fluor 647-conjugated donkey anti-rabbit IgG and Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:500; Life Technologies) for 30 min at 37 °C. To identify the nuclei of the cells, the sections were incubated with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. After staining, the sections were mounted with Ultramount (DAKO) and photographed with a confocal fluorescence microscope (TCS-TIV; Leica, Nussloch, Germany).

**Neurological score**

Animals were scored as previously described[24, 25]. The behavior of MCAO mice was assessed 24 h after cerebral ischemia. Animal behavior was recorded and subsequently analyzed by two observers who were blinded to the animal group designations. Neurological deficits were scored using the following scale: 0 points, escapes to supports; 1 point, hangs onto string with forepaws, hindpaw(s) and tail; 2 points, hangs onto string with forepaws and hindpaw(s); 3 points, hangs on with forepaws and moves laterally on string; 4 points, hangs on with forepaw(s); 5 points, falls off within 2 s. The mean score on the scale was used as the final score for each animal.

**TTC staining and assessment of cerebral infarct volume**

The cerebral infarct volume was determined as previously described and assessed using the TTC method[9, 26]. After MCAO, animals were sacrificed, and the brains were removed and frozen at -80 °C for 5 min. The brains were then quickly removed and sectioned coronally to obtain 7 sections. The
slices were incubated for 20 min at 37 °C in darkness with 2% triphenyl tetrazolium chloride (TTC) solution (Sigma-Aldrich, St. Louis, MO). After rinsing with PBS (0.1 M), the slices were fixed overnight in 4% PFA (4 °C) and photographed with a camera. Red areas indicated normal brain tissue, whereas pale white areas indicated the infarcted area. The infarct area was measured by using the following formula: percentage of infarct volume = 100 × (unstained volume (mm³)/edema index)/total volume (mm³).

**Nissl staining**

Nissl staining was performed as previously described[27]. Paraffin-embedded sections of brain tissues were dewaxed and rehydrated with xylene and graded alcohols. The sections were incubated in 0.5% cresyl violet solution (Beyotime Technologic Inc., China) for 20 min. After washing with distilled water, the sections were hydrated in serial alcohol solutions (70%, 80%, 95%, 100%) and xylene. The sections were mounted with neutral gum, covered with coverslips and observed under a microscope. Quantitative analysis of the ratios of viable neurons was performed using Image-Pro Plus 5.0 image processing software (Media Cybernetics, Rockville, MD).

**TUNEL staining**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed using a TUNEL kit (Roche, Mannheim, Germany) according to the manufacturer’s protocol as previously described.[27] After the sections were deparaffinized and rehydrated, antigen retrieval was performed, and endogenous peroxidase activity was blocked with methanol solution containing 0.3% H₂O₂ for 5 min. Then, the brain slides were incubated with proteinase K for 30 min at 37 °C and TUNEL reaction mixture for 1 h at 37 °C. After three washes with PBS, the sections were treated with 50 ml converter-POD for 30 min at 37 °C. Three or more random fields were used to assess cell death and calculate the apoptotic index. The apoptosis level was expressed as the percentage of TUNEL-positive cells relative to total cells.

**Water maze test**

To assess spatial learning and memory, the mice were subjected to the Morris water maze test.
starting from day 14 after MCAO/reperfusion[28]. Briefly, the Morris water maze apparatus consisted of a circular tank containing water in a room with salient visual cues. The mice were trained to find a hidden platform in the water maze (0.8 m in diameter) for 5 consecutive days. Each mouse underwent five trials (with randomly assigned starting positions) per day to locate the platform, and the intertrial interval was 10 s. For each trial, the mouse started from one of the four quadrants facing the wall of the pool; the trial ended when the animal climbed onto the platform, which was submerged 2 cm underneath the water in the middle of one of the quadrants of the water tank. If a mouse did not locate the platform within 60 s, it was guided to the platform. The swimming path and the percentage of time spent in the target quadrant were recorded by a video camera. The mean latency was recorded for each, and the spatial test was performed on the fifth day to evaluate the memory retention of each animal.

**Lentiviral vector transfection**

In vivo lentiviral vector transfection was performed as described previously. Mice were injected with a TRAF6-overexpressing lentivirus vector or a TRAF6-negative lentivirus vector. The mice were anesthetized with isoflurane (3% for induction and 1.5% for maintenance), their heads were shaved, and they were placed in a stereotaxic frame. A midline incision was made, and burr holes were made using a high-speed dental drill. Next, 1-2 μl of the TRAF6-overexpressing lentivirus vector or TRAF6-negative lentivirus vector was injected into the brain over a 5-10 min period. Virus administration was performed with a 10-μl Hamilton syringe (Hamilton Company, USA), which was controlled by a microinfusion pump (RWD, Shenzhen, China). The location of the lentivirus infusion was determined by immunohistochemistry.

**Statistical analysis**

All assays were performed in triplicate. The data are shown as the mean ± SD. To compare mean values between two groups, independent Student’s t-test was used. To compare mean values among more than two groups, two-way ANOVA was used. Fisher’s exact test was used to compare the tumor metastasis rate between two groups.

**Results**
1. Poly(I:C) preconditioning significantly attenuated focal cerebral I/R injury

To evaluate the effect of poly(I:C) preconditioning on focal cerebral I/R injury, the mice were injected i.p. with poly(I:C) (1.25 mg/g) 24 h prior to cerebral ischemia. Then, neurological scores were assess, and the infarct volume was measured after cerebral ischemia-reperfusion. First, we assessed the effect of poly(I:C) preconditioning on functional neurological scores. The results showed that compared with those of the sham group and AAV (vector) group, the functional neurological scores of the MCAO group and poly(I:C) preconditioning group were significantly increased (P<0.001, Fig. 1A). The results also showed that compared with those of the MCAO group, the functional neurological scores of the poly(I:C) preconditioning group were significantly reduced (P<0.001, Fig. 1A). Moreover, we assessed the effect of poly(I:C) preconditioning on the focal infarct volume. The results showed that compared with that of the sham group and AAV (vector) group, the focal infarct volume of the MCAO group and poly(I:C) preconditioning group was significantly increased (P<0.001, Fig. 1B, C). The results also showed that compared with that of the MCAO group, the focal infarct volume of the poly(I:C) preconditioning group was significantly reduced (P<0.001, Fig. 1B, C). These data indicated that poly(I:C) preconditioning significantly attenuated focal cerebral I/R injury.

2. Poly(I:C) preconditioning attenuated neuronal damage

Many study observations have provided a foundation for investigations of brain injury induced by ischemia and reperfusion, as well as morphological evidence that most structural damage occurs during reperfusion[29]. We evaluated the effect of poly(I:C) on neuronal damage following cerebral I/R using Nissl staining and TUNEL staining. We first evaluated the effect of poly(I:C) preconditioning on neuronal cell loss with Nissl staining. Our results showed that compared with that in the sham group and AAV (vector) group, the number of neuronal cells in the MCAO group and poly(I:C) preconditioning group was significantly decreased (P<0.001, Fig. 2A-E, K). The results also showed that compared with that in the MCAO group, the number of neuronal cells in the poly(I:C) preconditioning group was significantly increased (P<0.001, Fig. 2 A-E, K). We next evaluated the effect of poly(I:C) preconditioning on neuronal apoptosis with TUNEL staining. Our results showed that compared with that in the sham group and AAV (vector) group, the number of TUNEL+ cells in the MCAO group and
poly(I:C) preconditioning group was significantly increased (P<0.001, Fig. 2F-J, L). The results also showed that compared with that in the MCAO group, the number of TUNEL+ cells in the poly(I:C) preconditioning group was significantly decreased (P<0.001, Fig. 2 F-J, L). Our results indicated that poly(I:C) preconditioning significantly attenuated neuronal apoptosis and neuronal cell loss.

3. Poly(I:C) preconditioning inhibited I/R-induced glial cell activation and reduced NF-κB, TNF-α and IL-β levels

We evaluated the effect of poly(I:C) preconditioning on microglia and astrocytes following cerebral I/R. Our results showed that compared with that in the sham group and AAV (vector) group, astrocyte activation in the MCAO group and poly(I:C) preconditioning group was significantly increased (P<0.001, Fig. 3A-E, K). The results also showed that compared with that in the MCAO group, astrocyte activation in the poly(I:C) preconditioning group was significantly decreased (P<0.001, Fig. 3 A-E, K). Our results also showed that compared with that in the sham group and AAV (vector) group, microglial activation in the MCAO group and poly(I:C) preconditioning group was significantly increased (P<0.001, Fig. 3F-J, L). The results also showed that compared with that in the MCAO group, microglial activation in the poly(I:C) preconditioning group was significantly decreased (P<0.001, Fig. 3F-J, L). Our results indicated that poly(I:C) preconditioning significantly attenuated glial activation.

We further evaluated the levels of TNF-α, IL-1β, iNOS and IFN-β after poly(I:C) administration. The PCR results showed that NF-κB expression was markedly decreased in the poly(I:C) group compared with the MCAO group. ELSIA results showed that IL-1β, TNF-α, iNOS and IFN-β levels were significantly reduced in the poly(I:C) group. These results indicated that poly(I:C) preconditioning attenuated inflammatory injury after focal cerebral ischemia (P<0.001, Fig. 4).

4. Poly(I:C) preconditioning induced neuroprotection involving TRAF6

TRAF6 is critical for the production of inflammatory cytokines in various TLR-mediated signaling pathways, and TRAF6 is essential for poly(I:C)-dependent cytokine production by promoting the TLR3 adaptor protein TRIF-assembled signaling complex[30]. TRAF6 has indispensable roles in the regulation of host immunity, embryonic development, tissue homeostasis, and neurodegenerative diseases (Zucchelli et al., 2011; Xie, 2013). TRAF6 expression has been reported to be upregulated in
the peripheral blood of ischemic stroke patients, as well as in neurons in a rat model of cerebral I/R. Recently, we and others revealed the functions of TRAF6 in ischemic stroke[17]. Here, we observed the effect of poly(I:C) preconditioning on TRAF6 following cerebral I/R. The Western blot results showed that poly(I:C) administration decreased TRAF6 levels compared with those in the MCAO group (P<0.001, Fig. 5A). Immunohistochemistry showed that TRAF6 expression in the poly(I:C) preconditioning group was significantly decreased compared with that in the sham group and AAV (vector) group (P<0.001, Fig. 5B-F). Compared with that in the MCAO group, TRAF6 expression in the poly(I:C) preconditioning group was significantly decreased (P<0.001, Fig. 5B-F). Our data also showed that TRAF6 was mainly expressed in neurons and astrocytes. Our immunofluorescence staining further indicated that compared with that in the MCAO group, TRAF6 expression in the poly(I:C) preconditioning group was significantly decreased in astrocytes (P<0.001, Fig. 5G-N).

5. Poly(I:C) preconditioning improved cognitive dysfunction
The most common cognitive deficits after stroke are aphasia and hemispatial neglect[31]. We evaluated the effect of poly(I:C) preconditioning on cognitive dysfunction following cerebral I/R. The results showed that compared with that of the sham group and AAV (vector) group, the cognitive dysfunction of the MCAO group and poly(I:C) preconditioning group was significantly increased (P<0.001, Fig. 6A-D). Our findings showed that poly(I:C) preconditioning improved cognitive dysfunction following cerebral I/R.

6. TRAF6 overexpression abolished poly(I:C)-induced neuroprotection and worsened cognitive dysfunction in cerebral I/R injury.
We know that Poly(I:C) preconditioning affects the expression of TRAF6. We next observed that TRAF6 affected poly(I:C)-induced neuroprotection and improved cognitive dysfunction in cerebral I/R injury. Our data showed that functional neurological scores were significantly decreased in the poly(I:C)+ TRAF6 overexpression group compared with the poly(I:C) group (P<0.001, Fig. 1A). Our data also showed that the cerebral infarct volume was significantly increased in the poly(I:C)+ TRAF6 overexpression group compared with the poly(I:C) group (P<0.001, Fig. 1B-C). Moreover, our data showed that cognitive dysfunction was significantly decreased in the poly(I:C)+ TRAF6 overexpression
group compared with the poly(I:C) group (P<0.001, Fig. 5G-N). These results further demonstrated the protective effect of poly(I:C) preconditioning induced through the inhibition of TRAF6 signaling.

Discussion

In this study, we employed a mouse model of MCAO to evaluate the effects of poly(I:C) preconditioning on cognitive dysfunction after cerebral I/R injury and the related mechanisms. We first found that poly(I:C) preconditioning ameliorated cognitive dysfunction; furthermore, poly(I:C) preconditioning decreased neuronal damage and inhibited the activation of microglia and astrocytes and TRAF6 signaling. In contrast, TRAF6 overexpression abolished poly(I:C)-induced neuroprotection in cerebral I/R injury. The results suggested that poly(I:C) preconditioning ameliorates cognitive dysfunction associated with cerebral I/R injury by inhibiting TRAF6 signaling. Poly(I:C) is a promising new drug candidate for the treatment of cognitive impairment induced by cerebral ischemia.

Recent studies have indicated that TLR-mediated signaling plays an important role in cerebral I/R injury[4, 32]. TLR4 deficiency[5] or TLR2 modulation[33] protects the brain from I/R injury. However, TLR3 deficiency does not induce a neuroprotective effect against cerebral I/R [34].

Meanwhile, ischemic tolerance induced by preconditioning has attracted increasing attention. Preconditioning is a procedure by which a noxious stimulus near but below the threshold of damage is applied to the tissue. Shortly after preconditioning or after a delay, the organ develops resistance to or tolerance of the same stimulus or even different noxious stimuli at a level beyond the threshold of damage. Preconditioning thereby protects against subsequent injury[35]. Recent studies have found that a small dose of a TLR ligand before cerebral ischemia results in tolerance of subsequent cerebral ischemic damage. It has been reported that preconditioning with the ligands TLR2, 4, 7, and 9 induces TLRs to generate ischemic tolerance and reduces cerebral ischemic damage[36-39]. Poly(I:C) administration 24-72 h prior to cerebral ischemia significantly reduces infarction volume and decreases NDS[10-12]. Moreover, our previous study showed that the administration of poly(I:C) 3 h after cerebral ischemia also reduces cerebral I/R injury[9]. However, whether poly(I:C) preconditioning can ameliorate cognitive dysfunction still needs to be further investigated. Our study is the first to demonstrate that poly(I:C) preconditioning not only reduces the cerebral infarct volume but also
ameliorates cognitive dysfunction. This study provides new strategies for treating cognitive dysfunction after cerebral ischemia.

The inflammatory response and apoptosis are both critical to determining cell fate in the secondary response to focal ischemia[40]. Astrocytes and microglia are the most abundant cell types in the mammalian central nervous system (CNS) and provide innate immunity in the CNS. They are essential for maintaining a homeostatic environment in the CNS and for supporting normal neuronal function[41]. Microglia and astrocytes are activated after cerebral ischemia, and this activation contributes to secondary inflammation injury and the deterioration of the NDS[42]. In contrast, inhibiting cerebral inflammatory cell activation and decreasing inflammatory factor levels can alleviate cerebral ischemia reperfusion injury[43]. Consistent with these findings, we observed that poly(I:C) preconditioning inhibited the activation of microglia and astrocytes, increased the number of Nissl-stained cells and decreased the number of TUNEL+ cells, suggesting that poly(I:C) decreases neuronal apoptosis by inhibiting the inflammatory response after cerebral ischemia.

Tumor necrosis factor receptor-associated factor 6 (TRAF6) is a member of the TRAF family and an important multifunctional intracellular adaptin of the tumor necrosis factor and Toll/IL-1 receptor (TIR) superfamilies. Recent studies have shown that TRAF6 plays important roles in the regulation of host immunity, embryonic development, tissue homeostasis, and neurodegenerative disease[18, 19]. A recent study showed that TRAF6 expression is induced in neurons after I/R injury. In a rat model of traumatic brain injury, TRAF6 levels are significantly improved 7 days after injury, and TRAF6 is coexpressed with NeuN and GFAP based on immunofluorescence[44]. In SAH model rats compared with sham rats, the expression levels of TRAF6 increase gradually and peak 24 h after SAH. Moreover, the application of a TRAF6 overexpression plasmid and gene silencing by siRNA can increase or decrease the expression of TRAF6 and severely exacerbate or relieve EBI after SAH[45]. Another study showed that TRAF6 is upregulated in spinal cord astrocytes in the late phase after nerve injury, which causes the maintenance of neuropathic pain by integrating TNF-α and IL-1β signaling in astrocytes[46]. Importantly, TRAF6 overexpression in neurons leads to exacerbated cerebral damage, whereas the loss of TRAF6 expression is neuroprotective through the ubiquitination and activation of
Rac1[17]. Our study showed that TRAF6 overexpression increases the cerebral infarct volume and worsened cognitive dysfunction after cerebral I/R injury. The above studies suggest that TRAF6 is an important interventional target for central nervous system diseases, including ischemic stroke. The mechanisms of ischemic preconditioning and TLR ligand exposure have been thoroughly explored. Susan L. Stevens et al. showed that exposure to TLR ligands and brief ischemia induces genomic changes in the brain characteristic of a TLR pathway-mediated response. Interestingly, preconditioning with brief ischemia, TLR4 and TLR9 results in a reprogrammed response to stroke injury that involves a shared subset of 13 genes not evident in the genomic profile of brains subjected to stroke without prior preconditioning, which includes IRF3 and IRF7[47]. Similar to the above result, which showed reprogramming of the response to stroke injury, our results showed that poly(I:C) preconditioning decreased TRAF6 levels after cerebral I/R instead of increasing them. Moreover, TRAF6 overexpression abolished the protective effect of poly(I:C) preconditioning on cerebral I/R. Our study also showed that poly(I:C) preconditioning decreased NF-κB expression and reduced the levels of TNF-α and IL-1β after cerebral I/R, suggesting that poly(I:C) preconditioning ameliorates cognitive dysfunction after cerebral I/R by inhibiting TRAF6 signaling to reduce inflammatory reactions, as TRAF6 functions as an adaptor, positively regulating NF-κB, activating the NF-κB pathway and inducing the production of proinflammatory cytokines[48]. Therefore, our results further showed that TLRs are implicated in the pathogenesis of cognitive impairment induced by inflammation and are involved in TRAF6 signaling.

Conclusion
We are the first to show that poly(I:C) preconditioning ameliorates cognitive dysfunction. Furthermore, poly(I:C) preconditioning decreases neuronal damage, inhibits the activation of microglia and astrocytes and TRAF6 signaling and reduces the inflammatory reaction after cerebral I/R injury. These results suggest that TRAF6 is a new interventional target and that poly(I:C) is a promising drug candidate for cognitive dysfunction associated with cerebral I/R injury.

Abbreviations

**TRAF6** Tumor necrosis factor receptor-associated factor 6
TLR: Toll-like receptor
I/R: ischemia/reperfusion (I/R)
poly(I:C): polyinosinic-polycytidylic acid
NDS: neurological deficit scores
MCAO: middle cerebral artery occlusion
CCA: right common carotid artery
ECA: external carotid artery
ICA: internal carotid artery

Declarations

Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Contributions
PFW, WBZ and FXW conceived and designed the study. PFW, WBZ and XHJ performed the experiments and collected materials and the data. ZGL and FXW wrote the manuscript, with substantial contribution by all other authors. The authors read and approved the final manuscript.

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Ethics declarations
Ethics approval and consent to participate

The animal experiments were performed in strict accordance with the ethical guidelines of the Third Military Medical University Animal Studies Committee and the National Institutes of Health Guide (NRC 2011) for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Poly(I:C) reduced ischemic cerebral I/R injury. Poly(I:C) or vehicle was injected i.p. into mice 1 h before cerebral ischemia. Mice were subjected to cerebral ischemia (60 min) followed by reperfusion (24 h). (A) Neurological deficit scores (5 was a perfect score, and 0 indicated death due to cerebral I/R injury; n = 10). (B) TTC staining images of the sham group, AAV (vector) group, MCAO model group, poly(I:C) (pre) + MCAO model group, and AAV(TRAF6) + poly(I:C) (pre)+ MCAO model group. (C) Quantification of the infarct volume (n = 9). ***P < 0.01 vs. the sham group or AAV (vector) group; ####P < 0.01 vs. the MCAO model group; $$$P < 0.01 vs. the MCAO model group.
Alterations in neurons in ischemic cerebral I/R injury treated with poly(I:C) preconditioning. (A-E) Images of Nissl staining of dead neurons. (F-J) Images of TUNEL staining of apoptotic neurons. The number of dead neurons detected by Nissl staining (K) and the number of apoptotic neurons detected by TUNEL staining (L) in the sham group, AAV (vector) group, MCAO model group, poly(I:C) (pre) + MCAO model group, and AAV(TRAF6) + poly(I:C) (pre) + MCAO model group. ***P < 0.01 vs. the sham group or AAV (vector) group; ####P < 0.01 vs. the MCAO model group. Scale bars = 40 μm (400×).
Figure 3

Activation of astrocytes and microglia in ischemic cerebral I/R injury treated with poly(I:C) preconditioning. (A-E) Images of GFAP staining of astrocytes. (F-J) Images of IBA-1 staining of microglia. The state of astrocytes detected by GFAP staining (K) and the number of microglia detected by IBA-1 staining (L) in the sham group, AAV (vector) group, MCAO model group, poly(I:C) (Pre) + MCAO model group, and AAV(TRAF6) + poly(I:C) (pre) + MCAO model group. ***P < 0.01 vs. the sham group or AAV (vector) group; ###P < 0.01 vs. the MCAO model group. Scale bars = 40 μm (400×).
Cytokine levels in poly(I:C)-preconditioning mice subjected to ischemic cerebral I/R injury.

Cytokine levels in poly(I:C)-preconditioned mice subjected to ischemic cerebral I/R injury and MCAO model mice were determined by qualitative RT-PCR. Twenty-four hours after ischemic cerebral I/R injury, the cytokine levels of IL-1β, TNF-α, iNOS and IFN-β were significantly increased in mice subjected to ischemic cerebral I/R injury compared with sham mice (**P < 0.01, #P < 0.05), and the cytokine levels of IL-1β, TNF-α, iNOS and IFN-β were significantly decreased in mice subjected to ischemic cerebral I/R injury compared with MCAO mice (**P < 0.01, #P < 0.05).
Figure 5

Effect of poly(I:C) preconditioning on TRAF6 expression in mice subjected to ischemic cerebral I/R injury. A) Representative bands and densitometry quantification of TRAF6 expression in poly(I:C)-preconditioned mice subjected to ischemic cerebral I/R injury. B-E) Representative images of TRAF6 immunostaining in poly(I:C)-preconditioned mice subjected to ischemic cerebral I/R injury. Scale bars =?. F) Comparison of the number of GFAP-positive astrocytes showing TRAF expression between MCAO mice and poly(I:C)-preconditioned mice.
subjected to ischemic cerebral I/R injury. G-J) Representative photomicrographs showing cells double-labeled for GFAP (H, green) and TRAF6 (J, red) and the colocalization of GFAP and TRAF6 in MCAO mice. K-N) Representative photomicrographs showing cells double-labeled for GFAP (L, green) and TRAF6 (M, red) and the colocalization of GFAP and TRAF6 in poly(I:C)-preconditioned mice subjected to ischemic cerebral I/R injury. ***P < 0.01 vs. the sham group or AAV (vector) group; ###P < 0.01 vs. the MCAO model group. Scale bars = 40 μm (400×).
Effect of poly(I:C) preconditioning on cognitive dysfunction in mice subjected to ischemic cerebral I/R injury. A-E) Representative travel path of poly(I:C)-preconditioned mice subjected to ischemic cerebral I/R injury in the Morris water maze. F) Escape latency to reach the platform of the poly(I:C)-preconditioned mice subjected to ischemic cerebral I/R injury. G) The time spent in the target quadrant by poly(I:C)-preconditioned mice subjected to ischemic cerebral I/R injury. n = 8 per group, ***P < 0.01 vs. the PBS-treated group; #P < 0.01 vs. the KA 3 h and 24 h groups. ***P < 0.01 vs. the sham group or AAV (vector) group; ###P < 0.01 vs. the MCAO model group.