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ORIGINAL PAPER

Knockdown of TRIM32 Protects Hippocampal Neurons from Oxygen–Glucose Deprivation-Induced Injury

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Abstract

Tripartite motif 32 (TRIM32) is a member of TRIM family that plays a potential role in neural regeneration. However, the biological function of TRIM32 in cerebral ischemia reperfusion injury has not been investigated. In the present study, we evaluated the expression level of TRIM32 in hippocampal neurons following oxygen–glucose deprivation/reperfusion (OGD/R). The results showed that TRIM32 expression was significantly elevated in hippocampal neurons subjected to OGD/R as compared to the neurons cultured in the normoxia condition. To further evaluate the role of TRIM32, hippocampal neurons were transfected with TRIM32 small interfering RNA (si-TRIM32) to knock down TRIM32. We found that knockdown of TRIM32 improved cell viability of OGD/R-stimulated hippocampal neurons. Generation of reactive oxygen species was decreased, while contents of superoxide dismutase and glutathione peroxidase were increased after si-TRIM32 transfection. Knockdown of TRIM32 suppressed cell apoptosis, as proved by the increased bcl-2 expression along with decreased bax expression and caspase-3 activity. We also found that TRIM32 knockdown enhanced OGD/R-induced activation of Nrf2 signaling pathway in hippocampal neurons. Furthermore, siRNA-Nrf2 was transfected to knock down Nrf2. SiRNA-Nrf2 transfection reversed the protective effects of TRIM32 knockdown on neurons. These data suggested that knockdown of TRIM32 protected hippocampal neurons from OGD/R-induced oxidative injury through activating Nrf2 signaling pathway.

Keywords Tripartite totif 32 (TRIM32) · Cerebral ischemia reperfusion (I/R) injury · Hippocampal neurons · Oxygen–glucose deprivation/reperfusion (OGD/R) · Oxidative stress · Nrf2 signaling pathway

Introduction

Stroke is a medical condition that remains one of the leading causes of morbidity and mortality worldwide [1]. There are two main types of stroke, ischemic and hemorrhagic [2]. It has been surveyed that more than 80% of strokes are ischemic in which obstruction of one or more cerebral

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arteries result in a consequence of a critical reduction of regional cerebral blood flow [1]. Cerebral ischemia leads to severe oxygen and glucose deprivation, resulting in depletion of ATP production [3]. Neurons have higher energy demand when compared to other brain cells, thus, lack of blood supply disturbs cellular homeostasis, culminating in neuronal death [3]. Besides, reperfusion causes overproduction of reactive oxidative species (ROS), which may lead to oxidative damage, cellular death and neuronal loss [4, 5]. Therefore, cerebral ischemia reperfusion (I/R) is the main mechanism of stroke. Alleviating I/R-induced neurons injury may be meaningful for the preventing stroke.

Tripartite motif (TRIM) family, also known as RING B-box coiled-coil (RBCC), is a group of proteins that are associated with a variety of physiological processes including cell proliferation, pluripotency, DNA repair, transcription, and signal transduction [6]. Alterations in TRIM proteins are thought to be involved in various pathological conditions, including cancer, inflammatory and innate immune responses, and I/R injury [6]. TRIM72 (MG53), a member of TRIM protein family, participates in cardio protection of cardiac ischemic, preconditioning, and postconditioning via activating the PI3K/Akt/GSK3β and ERK1/2 signaling pathways [7, 8]. Activation of nuclear factor-erythroid 2 related factor 2 (Nrf2) signaling attenuates the generation of ROS of neurons, showing a promising neuroprotective effect against cerebral I/R injury [9]. TRIM8 knockdown significantly improved nuclear factor-erythroid 2 related factor 2 (Nrf2) and heme oxygenase-1 (HO-1) expressions in lung of LPS-treated mice [10]. TRIM32 has been shown to play a role in progression of neurological diseases. TRIM32 affects axonal regeneration and the recovery of motor function following spinal cord injury through regulating proliferation of glial cells [11]. However, the role of TRIM32 in cerebral I/R injury has not been fully evaluated. Therefore, the objective of this study was to investigate the effect of TRIM32 on cerebral I/R injury and explore whether the Nrf2 signaling is involved in TRIM32-mediated neuroprotective effect.

Materials and Methods

Primary Hippocampal Neurons Isolation and Culture

Ten pregnant Wistar rats were obtained from Experimental Animal Center of Beijing Institute of Radiation Medicine (Beijing, China). The rats were anaesthetized by isoflurane and sacrificed by decapitation. The hippocampus samples were dissected from the embryos, then the primary hippocampal neurons were prepared as described previously [12]. Briefly, the hippocampus samples were cut and digested with 0.25% trypsin–EDTA (Gibco Laboratories, Grand Island, NY, USA) at 37 °C for 20 min. The cells were suspended with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (Gibco), 10% horse serum (Gibco), 1% L-glutamine (Invitrogen, Carlsbad, CA, USA) and 1% penicillin–streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA). Then the cells were plated on poly-D-lysine-coated dishes at a density of 5×10^5/ml. After 24 h, the plating medium was replaced with Neurobasal/B27 medium. To inhibit the growth of glial cells, 3 µg/ml cytarabine (Sigma) was added. Then, half of the medium was changed every 2 days. After 2 weeks, cells were collected for further experiments. All protocols were approved by the Institutional Animal Care and Use Committee of Xi'an Medical University (Xi'an, China).

Cell Transfection and Oxygen–Glucose Deprivation/Reperfusion (OGD/R) Model

TRIM32 small interfering RNA (si-TRIM32), siRNA-Nrf2 and negative control siRNA (si-NC) were constructed by Gene Pharma (Shanghai, China). For in vitro transfection, hippocampal neurons were plated at 1×10^6 cells per well in a six-well cell culture plate for 24 h. Then, Lipofectamine 2000 (Invitrogen) was added into the medium of cells and supplemented with 5 nmol/l siRNA solution. After 24 h, the transfection efficiencies were evaluated using western blot.

Hippocampal neurons were cultured in DMEM without glucose and maintained in a humidified chamber with 1% O_2/94% N_2/5% CO_2 at 37 °C for 3 h. After that, cells were incubated with DMEM containing 4.5 g/ml glucose in normoxic conditions for 24 h at 37 °C.

Cell Viability Assay

Hippocampal neurons were cultured in 96-well plates at a density of 5000 cells/well. After different treatments, cell viability was measured using the Cell Counting Kit-8 (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a spectrophotometer (Bio-Tek, Winooski, VT, USA).

Assessment of Reactive Oxygen Species (ROS) Level

ROS generation was assayed quantitatively by using oxidant-sensitive probe DCFH-DA (Sigma). Briefly, hippocampal neurons were seeded in 6-well plates (1×10^5 cells/well) and then subjected to different treatments. Then the cells were loaded with 10 µM DCFH-DA for 30 min in the dark, after that, the fluorescence intensity was measured by flow cytometry.

Antioxidant Enzymes Content Measurement

Cell culture supernatant of hippocampal neurons was collected after indicated treatments. The contents of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) as described in the manufacturer's instructions.

Caspase-3 Activity

Caspase 3 activity was detected with Caspase 3 activity assay kit (Beyotime) per the manufacturer's instructions. Briefly, hippocampal neurons were lysed and incubated with the substrate Ac-DEVD-pNA (2 mM) at 37 °C for 1 h. Then the absorbance values were read at 405 nm.

Analysis of TRIM32 Expression

Total RNA was extracted from hippocampal neurons using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using a commercial kit (TaKaRa Bio, Shiga, Japan) to synthesize the cDNA. Then the RT-PCR amplifications were performed using the SYBR Green Master Mix (Roche, Mannheim, Germany) on an ABI 7500 Cycler (Applied Biosystem, Foster, CA, USA). TRIM32, forward 5′-CGGAGCATGGAAGTCACAG-3′ and reverse 5′-ACCACAGCCAGGAAACCC-3′; β-actin, forward 5′-TCACTATTGGCAACGAGCGGTTC-3′ and reverse 5′-GCACTGTGTTGGCATAGAGGTCTT-3′.

Western Blotting

Hippocampal neurons were lysed in radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime) supplemented with protease inhibitor (Roche, Basel, Switzerland). Nuclear fractions were extracted using a protein extraction kit (Beyotime Biotechnology). After measurement of the protein concentration using bicinchoninic acid (BCA) assay (Beyotime), equal amounts of protein (50 μg) were loaded onto 10% SDS-PAGE gels and then transferred to nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature, and then probed with anti-TRIM32 (1:1500), anti-bax (1:2500), anti-bcl-2 (1:3000), anti-Nrf2 (1:1000), anti-Lamin B2 (1:2500), anti-β-actin antibody (1:2000; Abcam, Cambridge, MA, USA) at 4 °C overnight. Then, the membranes were incubated with the horseradish peroxidase (HRP)-labeled secondary antibody (Abcam) for 1 h at room temperature. The blots were processed using an enhanced chemiluminescent detection kit (Millipore). The signals were quantified by Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

The data are shown as the means ± SD. SPSS 18.0 (SPSS, Chicago, IL, USA) was used for the statistical analysis A one-way analyses of variance (ANOVA) followed by Dunnett's post hoc test or student's t-test was utilized for the comparisions. $*p < 0.05$.

Results

The Expression of TRIM32 was Significantly Increased in OGD/R-Induced Neurons

First, the expression of TRIM32 in neurons was measured using qRT-PCR and western blot. As shown in Fig. 1a, the expression of TRIM32 at the mRNA level was significantly increased (5.47 fold) in hippocampal neurons subjected to OGD/R when compared to the neurons cultured in the normoxia condition. Consistently, western blot analysis showed that the protein expression of TRIM32 was also increased (3.21 fold) in OGD/R-stimulated hippocampal neurons (Fig. 1b).

Knockdown of TRIM32 Ameliorated OGD/R-Induced Neurons Injury

In order to study the roles of TRIM32 in OGD/R-induced neurons injury, TRIM32 was knocked down by transfection with si-TRIM32 in hippocampal neurons. Western blot analysis results showed that si-TRIM32 significantly reduced TRIM32 protein levels to 25.2% of the control (Fig. 2a). It was found that cell viability hippocampal neurons were obviously decreased to ~37.2% after OGD/R stimulation. On the contrary, cell viability was markedly increased to ~67.3% in the si-TRIM32 transfected neurons (Fig. 2b).

Knockdown of TRIM32 Ameliorated OGD/R-Induced Oxidative Stress and Cell Apoptosis in Hippocampal Neurons

Then we investigated the effects of si-TRIM32 on OGD/R-induced oxidative stress in hippocampal neurons. ROS level was markedly increased to 346.3% in hippocampal neurons subjected to OGD/R compared with control (Fig. 3a). Besides, the activities of SOD and GPx were significantly decreased to 45.1% and 33.4%, respectively, in OGD/R-induced hippocampal neurons (Fig. 3b, c). However, knockdown of TRIM32 ameliorated increased ROS generation (214.2%) and decreased SOD and GPx activities (67.6% and 69.2%) in OGD/R-induced hippocampal neurons (Fig. 3a, c).

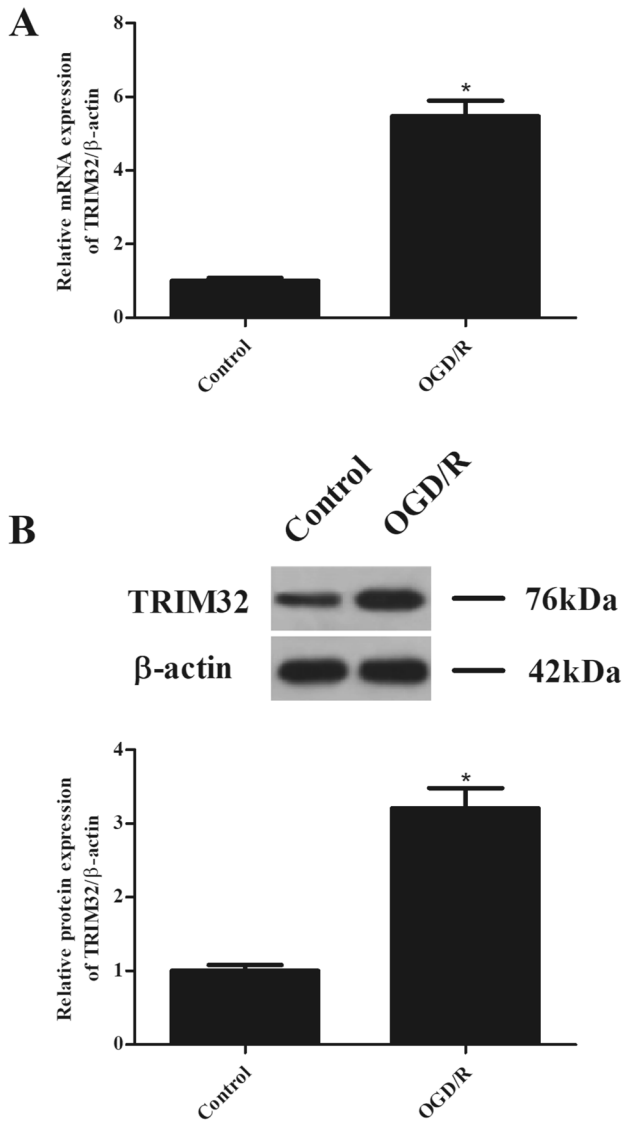

Fig. 1 TRIM32 expression was significantly upregulated in neurons subjected to OGD/R stimulation. The primary hippocampal neurons were subjected to OGD/R, then the expression of TRIM32 was measured using qRT-PCR (**a**) and western blot (**b**). n = 4; *$p < 0.05$

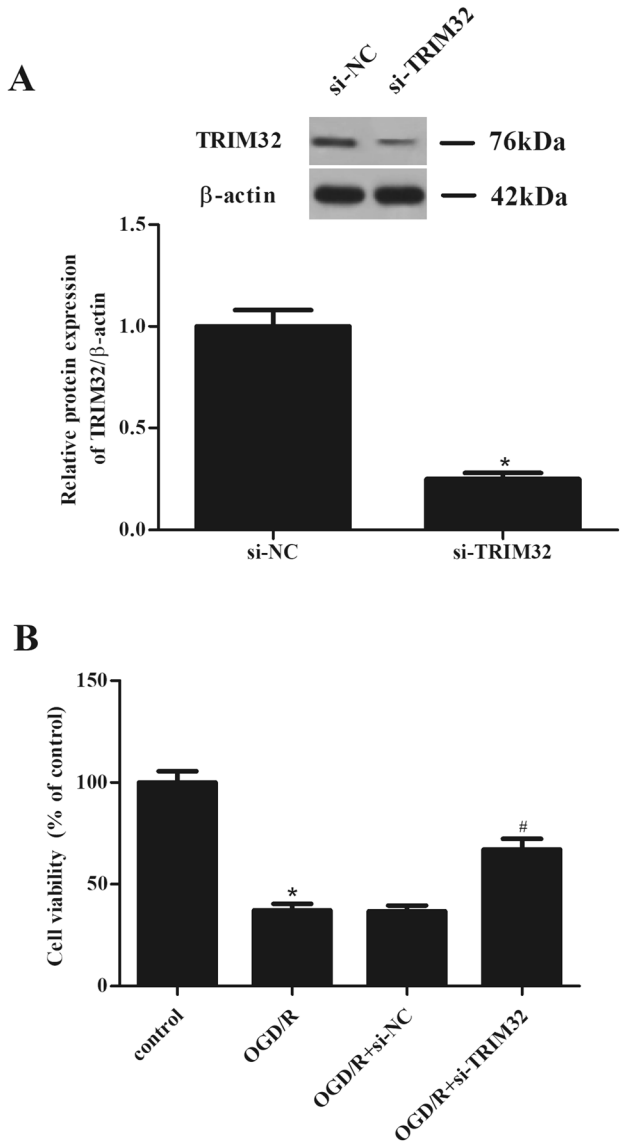

Fig. 2 TRIM32 silencing attenuated OGD/R-induced neurons injury. Hippocampal neurons were transfected with si-TRIM32 or si-NC for 24 h and then subjected to 24 h OGD/R treatment. **a** Western blot analysis was performed to evaluate TRIM32 expression. *$p < 0.05$ vs. si-NC group. **b** CCK-8 assay was carried out to measure cell viability. n = 5; *$p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. OGD/R group

To further explore the role of TRIM32 in OGD/R-induced cell apoptosis, expression levels of bax and bcl-2, as well as caspase-3 activity were measured. As shown in Fig. 3d, bax expression was significantly increased (3.76 fold), while bcl-2 expression was dramatically decreased (0.26 fold) after OGD/R treatment. However, the changes in expression levels of bax and bcl-2 caused by OGD/R were mitigated by knockdown of TRIM32. Furthermore, we observed that caspase-3 activity was significantly increased (3.64 fold) in hippocampal neurons subjected to OGD/R; however, caspase-3 activity was markedly decreased to 2.23-fold in the si-TRIM32 transfected neurons (Fig. 3f).

Knockdown of TRIM32 Induced the Activation of Nrf2 Signaling Pathway in Hippocampal Neurons

Previous studies have demonstrated that Nrf2 signaling pathway plays crucial roles in I/R injury [13, 14]. Therefore, we evaluated whether Nrf2 signaling pathway was involved in the effects of TRIM32. Figure 4a displayed that OGD/R-induced increased expression level of Nrf2 was markedly enhanced by TRIM32 knockdown. Knockdown of Nrf2 using si-Nrf2 transfection significantly decreased the expression of Nrf2 in the nucleus (Fig. 4b). Moreover,

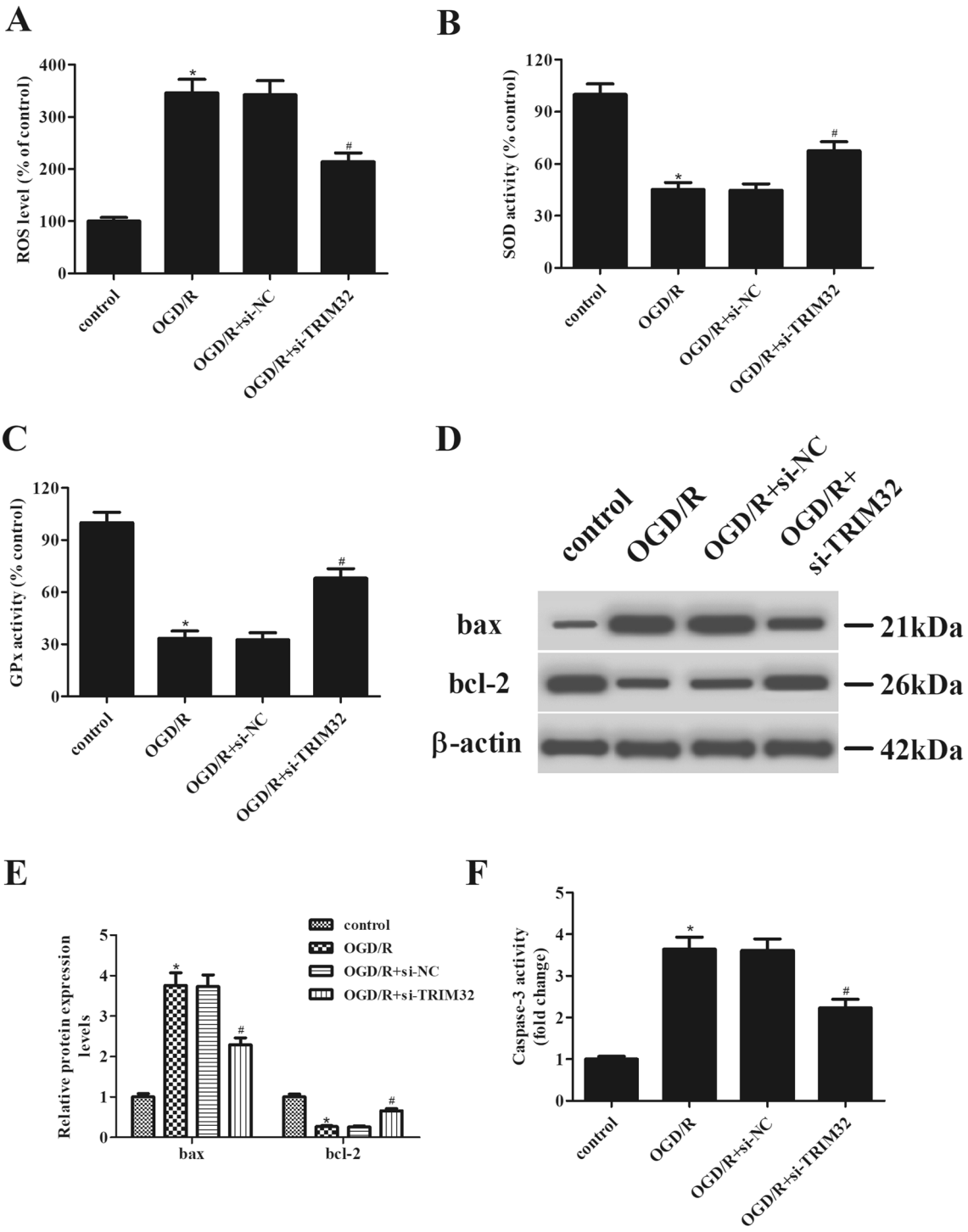

Fig. 3 TRIM32 silencing ameliorates OGD/R-induced oxidative damage and cell apoptosis in neurons. Hippocampal neurons were transfected with si-TRIM32 and then treated with or without OGD/R stimulation. ROS generation (**a**), activities of SOD (**b**) and GPx (**c**) were measured, respectively. **d** Western blot analysis was performed to evaluate expression levels of bax and bcl-2. **e** Quantification analysis of bax and bcl-2. **f** Caspase-3 activity of neurons was determined. n = 4; $^{*}p < 0.05$ vs. control group, $^{\#}p < 0.05$ vs. OGD/R group

knockdown of Nrf2 greatly abolished the protective effect of TRIM32 knockdown on cell viability (45.2%; Fig. 4c). Moreover, si-Nrf2 resulted in significant increase in ROS generation (287.4%) along with decrease in the activities of SOD and GPx (51.3% and 45.2%, respectively; Fig. 4d–f). The decreased caspase-3 activity in si-TRIM32 transfected

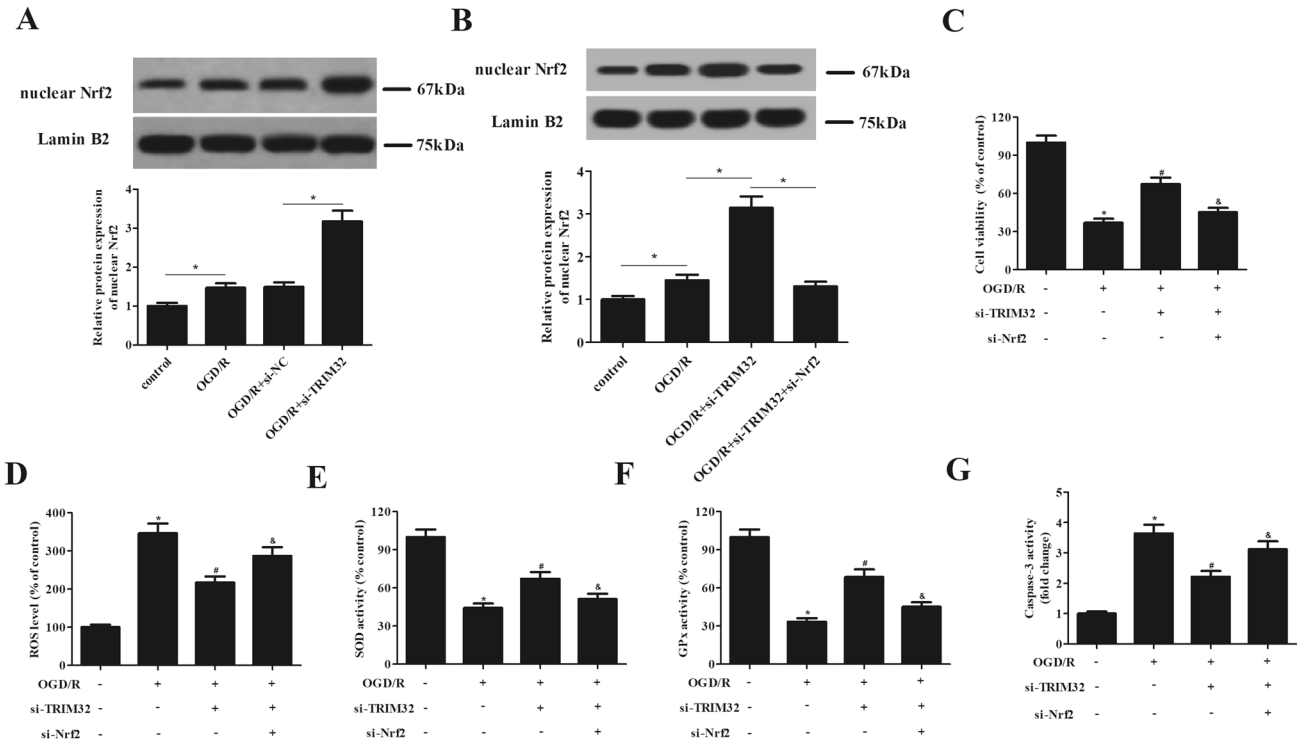

Fig. 4 Effect of TRIM32 knockdown was mediated by the activation of Nrf2 signaling pathway in neurons. **a** After different treatments, expression level of Nrf2 was measured using western blot. Hippocampal neurons were co-transfected with si-TRIM32 and si-Nrf2 for 24 h before OGD/R stimulation. **b** Nuclear expression of Nrf2 was detected using western blot. $^{*}p<0.05$. **c** Cell viability after si-Nrf2 transfection. **d**–**f** ROS generation and SOD and GPx levels after si-Nrf2 transfection. **g** Caspase-3 activity after si-Nrf2 transfection. n = 3; $^{*}p<0.05$ vs. control group, $^{\#}p<0.05$ vs. OGD/R group, $^{\&}p<0.05$ vs. OGD/R+si-TRIM32 group

neurons were alleviated by si-Nrf2 transfection (3.11 fold; Fig. 4g).

Discussion

TRIM32 has been found to play essential roles in various cellular processes, such as cell differentiation, proliferation, apoptosis, antiviral response, and oncogenesis [15–17]. There are multiple lines of evidence indicating that TRIM32 is important for maintenance of the physiological function of nervous system [18–21]. TRIM32 has been observed to be dysregulated in many neurological diseases, including Alzheimer's disease and Parkinson's disease [18–20]. TRIM32 is expressed in neural progenitor cells in the developing brain and exerts important functions in neuronal differentiation [21]. TRIM32 is barely detected in glial cells in the normal uninjured spinal cord, while is highly upregulated in astrocytes and microglia of the injured spinal cord [11]. After spinal cord injury (SCI), TRIM32$^{-/-}$ mice (deficiency of TRIM32) exhibit increased numbers of astrocytes and microglia, accompanied by enhanced proliferation of astrocytes and microglia as well as increased secretion of interleukin (IL)-1 and IL-10. Furthermore, the axonal regeneration is impaired, and motor recovery is delayed in the spinal cord of TRIM32$^{-/-}$ mice following SCI [11]. Moreover, expression of TRIM32 is upregulated in the sciatic nerve upon injury, implying a potential role of TRIM32 in neural regeneration [22]. In the present study, TRIM32 expression was significantly increased after OGD/R stimulation in hippocampal neurons. The neuro-regulatory role of TRIM32 may be disparate in different cell types and context.

It is well known that ischemia can result in tissue injury and organ dysfunction [23]. A profound and lengthy period of tissue hypoxia may lead to the consequent depletion of cellular ATP. Early restoration of cerebral blood flow is crucial for sustaining neuronal viability. Nevertheless, reperfusion is believed to promote delayed secondary brain injury as the freshly arriving oxygen will serve as a substrate for excessive ROS, and excessive ROS production is the main cause of brain damage during cerebral I/R injury [24–26]. The balance between the rate of generation of ROS and the tissue's ability to scavenge ROS is broken during I/R injury. The excess ROS generation may cause activation of oxidative signals, which leads to a cascade

of I/R injury [26]. The present study addressed the role of TRIM32 in OGD/R-induced oxidative damage in hippocampal neurons. The results showed that knockdown of TRIM32 suppressed ROS generation, and elevated antioxidative defense system as proved by the increased levels of SOD and GPx. Moreover, knockdown of TRIM32 improved cell viability and inhibited cell apoptosis in OGD/R-induced hippocampal neurons.

Nrf2 signaling has emerged as one of the most important cellular defense and survival pathway against oxidative stress and toxicants [27]. Nrf2 is a transcription factor that is an important part of antioxidative defense system. Nrf2 is kept in the cytoplasm by Kelch like-ECH-associated protein 1 (KEAP1) under normal conditions [28]. However, oxidative stress disrupts the complex and activates Nrf2. It has been demonstrated that activation of Nrf2 results in the induction of many cytoprotective proteins. That is because the activated Nrf2 translocates into the nucleus and binds to the antioxidant response element (ARE) in the upstream promoter region of many antioxidative genes. Therefore, Nrf2 regulates the expression of multiple antioxidant enzymes, such as heme oxygenase-1 (HO-1), SOD, catalase (CAT), NAD(P)H quinone oxidoreductase-1 (NQO-1), and γ-glutamylcysteine synthase (γ-GCLC), thereby regulating protecting against oxidative damage triggered by injury and inflammation [29]. In recent years, several drugs that stimulate the Nrf2 signaling pathway are being studied for treatment of oxidative stress related diseases. In the current study, we found that knockdown of TRIM32 induced the activation of Nrf2 signaling pathway in OGD/R-induced hippocampal neurons. Furthermore, siRNA-Nrf2 reversed the protective effects of TRIM32 knockdown on neurons. These findings suggested that knockdown of TRIM32 exerted its protective effects on OGD/R-induced oxidative injury in hippocampal neurons through activating Nrf2 signaling pathway.

In conclusion, our study indicated that knockdown of TRIM32 protected hippocampal neurons from OGD/R-induced oxidative injury. Knockdown of TRIM32 attenuated oxidative stress, apoptosis and improved endogenous antioxidant defenses in OGD/R-induced hippocampal neurons. The protective effects were attributed to the activation of Nrf2 signaling pathway.

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Compliance with Ethical Standards

Conflicts of interest The authors have no competing interests to disclose.

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