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Research Article

Ginsenoside Rg1 attenuates cerebral ischemia-reperfusion injury due to inhibition of NOX2-mediated calcium homeostasis dysregulation in mice

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ABSTRACT

Background: The incidence of ischemic cerebrovascular disease is increasing in recent years and has been one of the leading causes of neurological dysfunction and death. Ginsenoside Rg1 has been found to protect against neuronal damage in many neurodegenerative diseases. However, the effect and mechanism by which Rg1 protects against cerebral ischemia-reperfusion injury (CIRI) are not fully understood. Here, we report the neuroprotective effects of Rg1 treatment on CIRI and its possible mechanisms in mice.

Methods: A bilateral common carotid artery ligation was used to establish a chronic CIRI model in mice. HT22 cells were treated with Rg1 after OGD/R to study its effect on $[Ca^{2+}]_i$. The open-field test and pole-climbing experiment were used to detect behavioral injury. The laser speckle blood flowmeter was used to measure brain blood flow. The Nissl and H&E staining were used to examine the neuronal damage. The Western blotting was used to examine MAP2, PSD95, Tau, p-Tau, NOX2, PLC, p-PLC, CN, NFAT1, and NLRP1 expression. Calcium imaging was used to test the level of $[Ca^{2+}]_i$.

Results: Rg1 treatment significantly improved cerebral blood flow, locomotion, and limb coordination, reduced ROS production, increased MAP2 and PSD95 expression, and decreased p-Tau, NOX2, p-PLC, CN, NFAT1, and NLRP1 expression. Calcium imaging results showed that Rg1 could inhibit calcium overload and resist the imbalance of calcium homeostasis after OGD/R in HT22 cells.

Conclusion: Rg1 plays a neuroprotective role in attenuating CIRI by inhibiting oxidative stress, calcium overload, and neuroinflammation.

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Abbreviations: ANOVA, One-way analysis of variance; ASC, Apoptosis-associated speck-like protein containing a CARD; caspase-1, Cysteinylnl aspartate-specific protease-1; CBF, cerebral blood flow; CIRI, cerebral ischemia/reperfusion injury; CN, Calcineurin; DHE, Dihydroethidium; FBS, fetal bovine serum; I/R, ischemia reperfusion; IL-1 β , Interleukin-1 β ; IPP, Image-Pro Plus; MAP2, microtubule-associated protein 2; NFAT1, nuclear factor of activated T-cells 1; NLRP1, Nucleotide-binding oligomerization domain like receptor protein 1; NMDA, N-methyl-D-aspartate; NOD, nucleotide-binding oligomerization domain; NOX, Nicotinamide adenine dinucleotide phosphate oxidase; OFT, Open field test; OGD/R, oxygen glucose deprivation/reperfusion; PCT, Pole-climbing test; PIP2, phosphatidylinositol biphosphate; PLC, phospholipase C; PSD95, postsynaptic density protein 95; Rg1, Ginsenoside Rg1; ROS, reactive oxygen species; SCIL, Spinal cord ischemia-reperfusion injury; TBST, Tris buffered saline Tween.

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1. Introduction

Strokes can be divided into hemorrhagic stroke and ischemic stroke, with the latter accounting for about 80 % of all cases [1]. The most frequent reason of ischemic stroke is a sudden thrombus formation leading to vascular occlusion, which blocks blood flow and interrupts the glucose and oxygen required to maintain brain metabolism [2,3]. The restoration of cerebral blood flow after stroke can rescue ischemia neuronal damage, but it can also cause a cascade of neurological impairments known as cerebral ischemia/reperfusion injury (CIRI) [1,4]. The mechanisms leading to CIRI are complex and multifactorial, including disturbances in energy metabolism, free radical damage, inflammatory response, and induction of apoptosis [5]. Currently, there are still no clinically effective drugs for treatment of CIRI [6], and it is essential to study the mechanisms and neuroprotective agents for CIRI.

It is now widely accepted that the overproduction of reactive oxygen species (ROS) has a critical role in the development of CIRI. During the process of cerebral ischemia and reperfusion, excessive ROS accumulation can induce DNA damage, lipid peroxidation, and protein oxidation, thus leading to cellular integrity and dysfunction along with reversible or permanent neurological deficit [7–10]. NADPH oxidase (NOX) is regarded as a major source of intracellular ROS production [11]. Among them, NOX2 and NOX4 are reported to be the central sources of ROS within 8–16 h after stroke [12]. It has been reported that knockdown of the NOX2 gene and specific inhibitors of NOX2 significantly reduce oxidative stress injury in mice with cerebral ischemic [13]. Calcium overload is another key mechanism leading to persistent neuronal damages after cerebral I/R [14]. In the prophase of cerebral ischemia, transient glucose and oxygen deprivation trigger continuous stimulation to the N-methyl-D-aspartate (NMDA) receptor, which leads to activation of related Ca^{2+} channels and Ca^{2+} influx into the cells, which in turn causes neuronal injury and even death [15,16]. ROS has been reported to affect the influx of Ca^{2+} into cells and the storage of Ca^{2+} [17]. However, it is still unknown whether NOX2 involves in cerebral I/R-induced Ca^{2+} homeostasis disorder. Additionally, inflammation after ischemic stroke is another important factor leading to neuronal dysfunction [18,19]. The inflammasome complexes are the families of nucleotide-binding oligomerization domain (NOD)-like receptor (NLR), including NLRP1, AIM2, NLRP3, and NLRC4 [20]. Growing studies reported that NLRP1 inflammasome participated in aging-related cognitive dysfunction in many animal models [20,21]. A recent study suggested that mitochondrial ROS and Ca^{2+} influx involved in the activation of NLRP3 inflammasome in endothelial cells [22]. Therefore, we speculated that NOX2-mediated ROS accumulation might induce Ca^{2+} homeostasis disorder, which further increased NLRP1 inflammasome expression in neurons, eventually resulting in CIRI.

Ginsenoside Rg1 (Rg1) is one of the major active substances of ginseng, and growing evidence has suggested that Rg1 has a variety of pharmacological effects such as regulating cell proliferation, differentiation, and regeneration, as well as anti-apoptotic, antioxidant, and anti-inflammatory effects [23]. In our previous study, Rg1 was shown to have a significant protective effect on H_2O_2 -induced neuronal damage via inhibiting the NOX2 in hippocampal neurons [24]. Our recent study also showed that Rg1 treatment significantly attenuated neuronal injury through inhibiting NOX2 and NLRP1 inflammasome in SAMP8 mice [25]. In the present study, we investigated the protective effect and mechanism of Rg1 treatment on CIRI in mice. We found that Rg1 treatment had potential to improve CIRI by inhibiting NOX2-mediated Ca^{2+} overload and resulting in down-regulation of NLRP1 inflammasome in mice.

2. Materials and methods

2.1. Chemicals and reagents

Rg1 (>98 %) was purchased from Chengdu Desite Biotechnology Co., China. The tempol and apocynin were obtained from Merck Millipore.

2.2. Animals

The male 3-month-old ICR mice (30–40 g) were obtained from the Laboratory Animal Center of Anhui Medical University and raised in a light/dark cycle for 12 h, humidity and temperature (22–24 °C) control room. The animals were then randomized into 6 groups (n = 14): sham operation group (Sham); cerebral I/R group (model); apocynin (50 mg/kg) group, tempol (50 mg/kg) group, and Rg1 (5, 10 mg/kg) groups. The drugs were administered by gavage (0.1 ml/10 g) before cerebral I/R for 5 days and after cerebral I/R for 14 days. The sham and model groups were given distilled water by gavage (0.1 ml/10 g). All tests were endorsed by the Research facility Creature Morals Committee of Anhui Medical University.

2.3. Cells culture and treatment

HT22 cells were cultured in the high-sugar DMEM medium in supplementation with 10 % FBS solution in 5 % CO_2 at 37 °C. When the cells reached about 70 % in the high-sugar serum medium, they were replaced with the sugar-free medium and incubated in a triple-gas incubator ($\text{N}_2:\text{O}_2:\text{CO}_2 = 94:1:5$) for 6 h. Then the cells were supplanted with the high-sugar serum-free medium and reoxygenated for 24 h in a normoxic incubator by adding the corresponding drugs in each group. Experimental groups: control group, OGD/R group, tempol (50 μM) group, apocynin (50 μM) group and Rg1 (5, 10 μM) group.

2.4. Bilateral common carotid artery ligation and reperfusion and Laser speckle blood flowmeter measurement

The animals were anesthetized by using pentobarbital sodium (0.35 %, 0.1 ml/10 g) by intraperitoneal injection. The mice were fixed supine and both common carotid arteries were exposed and isolated. Insert threads under the common carotid artery and place the fishing line parallel to the common carotid artery, and ligate the bilateral common carotid arteries together with the fishing line to prevent the blood for 1 h. Then pull out the fishing lines and cut the threads for reperfusion.

For laser speckle blood flowmeter measurement, the mice (n = 5) were firstly fixed prone. The skin of the head is cut about 1 cm, the subcutaneous fascia is separated and the skull is exposed to detect the brain blood flow. Then the mice were performed the cerebral I/R. Blood flow is detected by using the PeriCam Perfusion Speckle Imager System (Perimed, Järfälla, Sweden) in each mouse before cerebral ischemia, after cerebral ischemia 40 min, and after reperfusion 20 min. The average blood flow of the three detection points was calculated to indicate the changes in blood flow.

2.5. Open field test (OFT)

The OFT was employed to detect the activities of mice after the cerebral I/R 14 days. Briefly, the mice (n = 8) were put in the middle of the box to adapt to the environment for 2 min, then the movements of the mice were recorded by the behavioral tracking system (ANY-maze software, Stoeling Company) for 3 min. The total moving distance (TMD, m), the mean moving speed (MMS, m/s),

and the line crossing and standing up times were recorded respectively to indicate the motor and exploratory behavior.

2.6. Pole-climbing test (PCT)

The PCT was carried out after the cerebral I/R 14 days. A cork ball of 3 cm in diameter was fixed on the top of the stick. The mice ($n = 8$) were placed head up upon the ball, then the time that the mice turned around on the ball and the time that the mice climbed the whole pole were recorded. All mice need to be trained three times to familiarize themselves with the whole process before testing.

2.7. Hypology

The mice ($n = 4$) were performed cardiac perfusion with saline and 4 % paraformaldehyde. The brain tissue was removed and placed in 4 % paraformaldehyde for 24 h. The brain tissue was embedded in paraffin and sliced into 5 μm sections for H&E staining and Nissl staining. The neuronal morphology and the changes of Nissl bodies in the cortex, hippocampal CA1 and CA3 were observed with a microscope (Olympus IX72, Japan). For the Nissl staining, the density from three random fields ($400 \times$) in each area of cortex, hippocampus CA1 and CA3 was measured by using the Image-Pro Plus (IPP) 6.0 to evaluate the changes of Nissl bodies in neurons.

2.8. DHE fluorescence staining

Dihydroethidium (DHE), a superoxide fluorescent probe reagent, is commonly used to label the ROS in living cells. The mice ($n = 3$) were injected from the tail vein with DHE working solution (0.33 g/L, 0.1 ml/10 g) after I/R 24 h. Thirty minutes later, the brain tissues were taken out and frozen at -20°C in OCT (Sakura, Torrance, USA). The brain tissue was sectioned into 10 μm slices with a freezing microtome (Leica CM3050, Germany) at -20°C . Then the slices were stained with Hoechst 33,258 for 2 min. The fluorescence microscope was used to photograph the DHE and Hoechst. The red fluorescence density was measured using IPP 6.0 from three random fields in each area of cortex, hippocampus CA1, and CA3 to indicate ROS production.

2.9. Western blotting

The brain tissues ($n = 3$) of the cortex and hippocampus or HT22 cells were adopted to extract the total proteins by using RIPA lysate (containing 1 % protease and phosphatase inhibitors). Then protein was separated with SDS-PAGE gel (10%) electrophoresis and further transferred to the PVDF membrane (Millipore, Bedford, USA). Then the membrane was sealed in the closed buffer for 1 h and further treated with primary antibodies overnight at 4°C (Supplementary Table S1). Then the membrane was followed by secondary antibodies for 1 h at 25°C . After three washes in TBST, the results were visualized by using the Imaging System (Chemidoc MP, Bio-Rad, Britain). Protein band density was measured using Image J and normalized to β -actin. Then the relative density was used to indicate the expression of the target protein.

2.10. Calcium imaging

The cells were seeded in a 35 mm dish and performed OGD/R and drug treatment. After 24 h of reoxygenation, replaced with fluorescent dye solution (DMEM: F-127: Fura-2 AM = 500: 1: 1) for 20 min, and then washed 3 times with artificial extracellular solution. Fluorescence of Fura-2 AM was measured at 340 nm (F340)

and 380 nm (F380) under alternating excitation per second using an Olympus Digital Calcium Imaging System (IX73, DG-4PLUS/OF30, Japan). Images were acquired for 300s to obtain baseline Ca^{2+} levels with extracellular Ca^{2+} (1 mM). The extracellular solution was then replaced by adding BAPTA (1 mM, MedChemExpress, USA) or CaCl_2 (Ca^{2+} , 2 mM) and the ratio (F340/F380) was recorded. The Δ ratio (F340/F380) was calculated to indicate the change of $[\text{Ca}^{2+}]_i$ levels before and after treatment with BAPTA or high calcium solution. The experiments were performed at least three independent experiments.

2.11. Data analysis

All data are showed as mean \pm standard deviation. Statistical differences were analyzed by using GraphPad Prism 6. And one-way ANOVA was employed to compare differences among groups, the Tukey's test was used to compare the differences between groups. $P < 0.05$ was regarded as statistical significance.

3. Results

3.1. Rg1 treatment improves spontaneous activity and motor coordination in cerebral I/R mice

The OFT results indicated that the TMD (m), the MMS (m/s), and the lines crossing and the standing up times were significantly diminished after cerebral I/R. The PCT results indicated that the turning around time and the pole-climbing time were increased after cerebral I/R, suggesting that cerebral I/R affects the brain function that regulates the spontaneous activity and motor coordination, which mimics the process by which humans are unable to walk independently after stroke (Fig. 1A–F). However, these behavioral phenotypes can be effectively improved by apocynin, tempol, and Rg1 (5, 10 mg/kg) treatment (Fig. 1A–F). And compared with Rg1 (5 mg/kg) group, Rg1 (10 mg/kg) treatment had more behavioral improvement effect, but there was no significant difference. These data proposed that Rg1 treatment could improve mobility and motor coordination in cerebral I/R mice by approximately twofold compared to the model group.

3.2. Rg1 treatment increases cerebral blood flow (CBF) in cerebral I/R mice

We detected the changes in CBF in mice. The results indicated that the CBF was markedly reduced after ischemia 40 min and reperfusion 20 min after cerebral I/R (Fig. 2A–B). While treatment with tempol, apocynin, and Rg1 (5, 10 mg/kg) significantly improved the CBF (Fig. 2A–B). Among them, Rg1 (10 mg/kg) improved CBF better and increased by about 97 % compared with the model group during ischemia and reperfusion.

3.3. Rg1 treatment ameliorates neuropathological damages in the cortex and hippocampus in cerebral I/R mice

We further detect the pathological morphological changes in the regions of cortical, hippocampus in cerebral I/R mice. H&E staining showed that there were obvious pathological damages in the regions of the cortex and hippocampus after cerebral I/R, especially in the cortex region (Fig. 3 A). A large number of cells showed unclear outlines and nuclear pyknosis. While treatment with apocynin, tempol, and Rg1 (5, 10 mg/kg) could significantly alleviate pathological injury described above (Fig. 3 A). The results of Nissl staining were consistent with the aforementioned results. Compared with the model group, Rg1 (10 mg/kg) increased the density of Nissl staining about 120 % in the cortical and

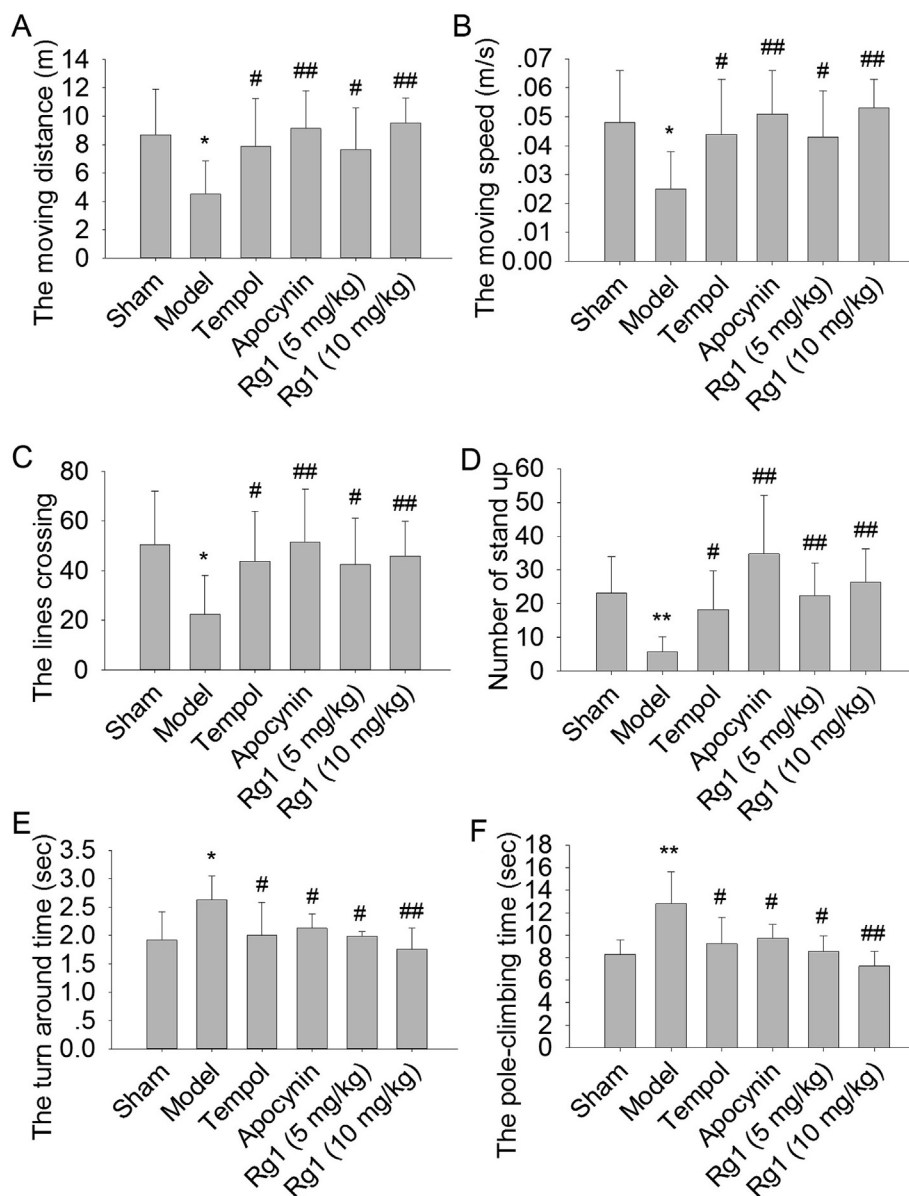


Fig. 1. Impacts of Rg1 on spontaneous movement and motor coordination in cerebral I/R mice. (A) The moving distance (m); (B) The moving speed (m/s); (C) The lines crossing; (D) The number of standing up; (E) The turning around time (s); (F) The pole-climbing time (s). Data are shown as mean ± SD, n = 8. *P < 0.05, **P < 0.01 vs. control group, #P < 0.05, ##P < 0.01 vs. model group.

hippocampal CA3 region, and the effect of apocynin was better in the hippocampal CA1 region, increasing the density of Nissl staining by about 78 % (Supplementary Fig. S1 A-D). These results suggested that Rg1 treatment effectively alleviates neuronal damage induced by cerebral I/R.

3.4. Rg1 treatment increases expressions of MAP2 and PSD95 and decreases p-Tau in the cerebral I/R mice

We further measured the expressions of microtubule-associated protein 2 (MAP2) and postsynaptic density protein 95 (PSD95). The results indicated that the expressions of MAP2 and PSD95 were dramatically reduced and the expression of p-Tau was elevated after cerebral I/R. While tempol, apocynin, and Rg1 (5, 10 mg/kg) administration could reverse the above changes (Fig. 3B–E). The data indicated that Rg1 treatment could attenuate neuronal

microtubule structural damage and inhibit phosphorylation of Tau after cerebral I/R in mice.

3.5. Rg1 treatment decreases ROS production and NOX2-related proteins in the cortex and hippocampus in cerebral I/R mice

We then examined the ROS accumulation after cerebral I/R by using DHE. The results demonstrated that the ROS accumulation in the hippocampus and cortex regions was markedly increased after cerebral I/R. However, administration with apocynin, tempol, and Rg1 (5, 10 mg/kg) markedly decreased ROS accumulation after cerebral I/R, especially in Rg1 (10 mg/kg) group (Fig. 4A–D). Meanwhile, we also detected the expressions of NOX2-associated proteins in brain tissue. The results indicated that the levels of p22phox, p47phox, and NOX2 were markedly aggrandized in the model group (Fig. 4E–G). Nevertheless, Rg1 (5, 10 mg/kg) and

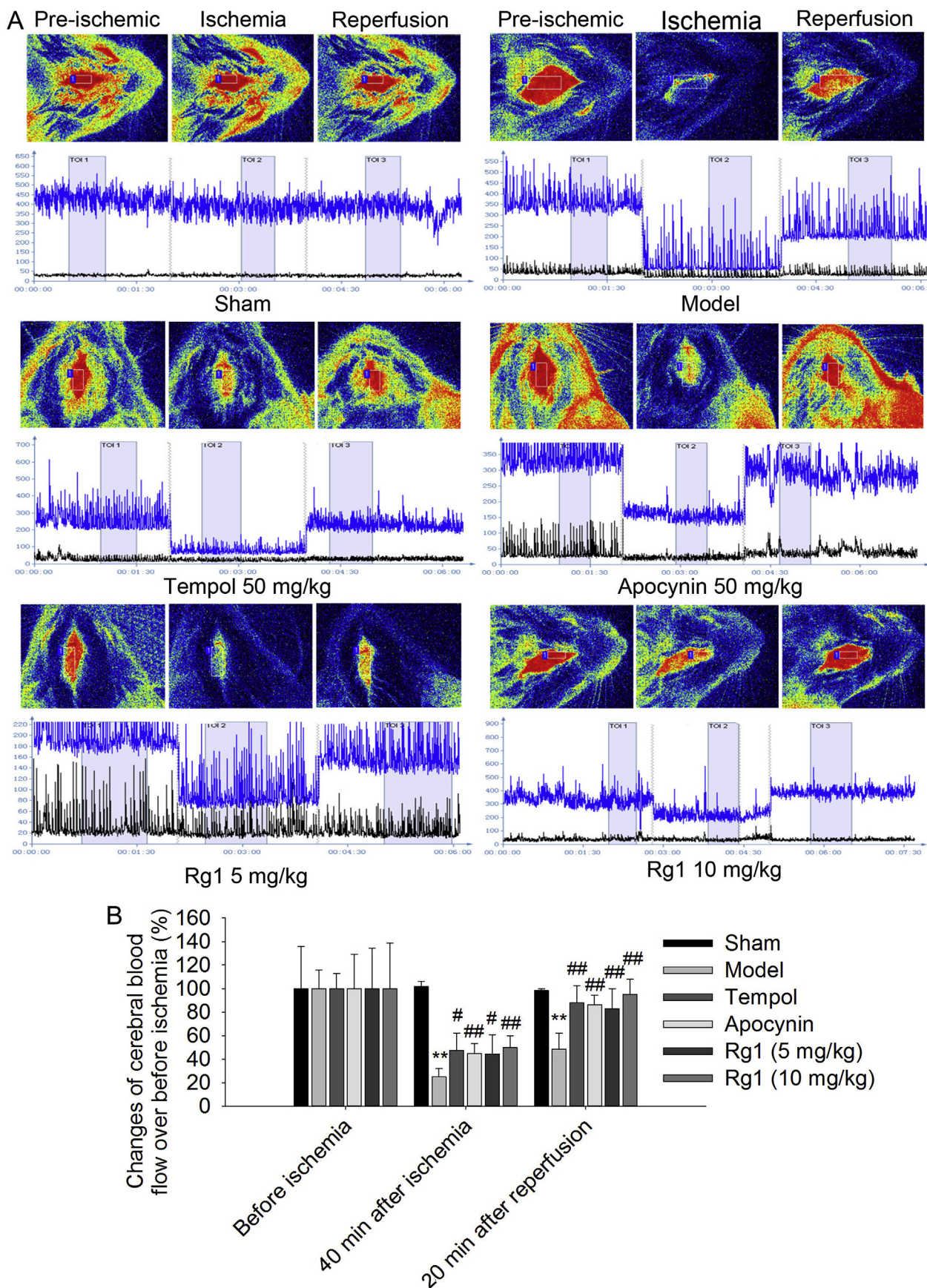


Fig. 2. Impacts of Rg1 on cerebral bloodstream in cerebral I/R mice. (A) Laser speckle contrast imaging. (B) The examination of relative changes of cerebral blood flow over before ischemia. Data are shown as mean ± SD, n = 5. ***P* < 0.01 vs. control group; **P* < 0.05, ###*P* < 0.01 vs. model group.

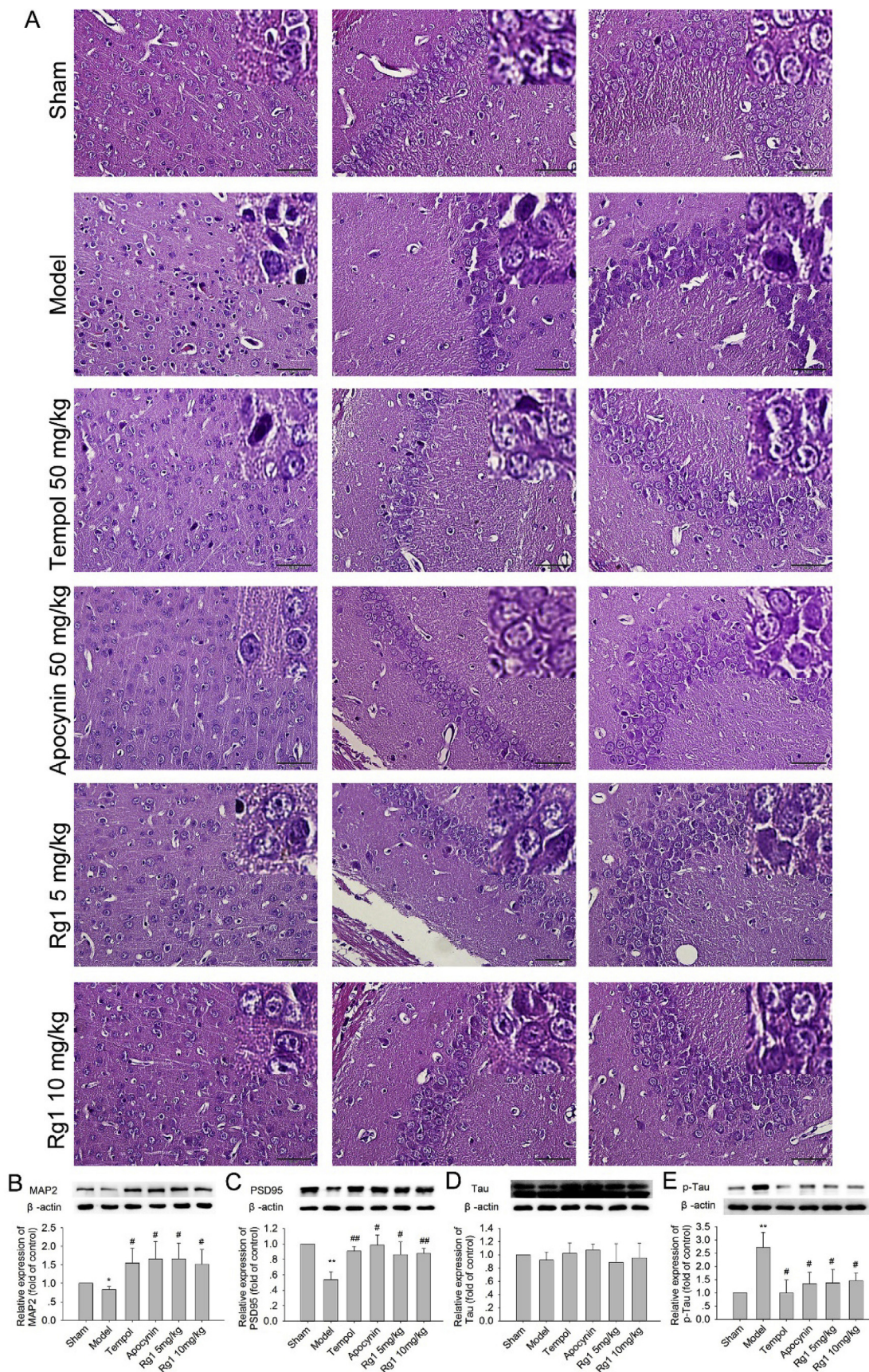


Fig. 3. Impacts of Rg1 on neuropathological variation and the expressions of MAP2, PSD95, TAU, and p-TAU in cerebral I/R mice. (A) Changes of pathological morphology (n = 4). (B–E) The relative expressions of MAP2, PSD95, TAU, and p-TAU (n = 3). Data are shown as mean ± SD, *P < 0.05, **P < 0.01 vs. control group; #P < 0.05, ##P < 0.01 vs. model group.

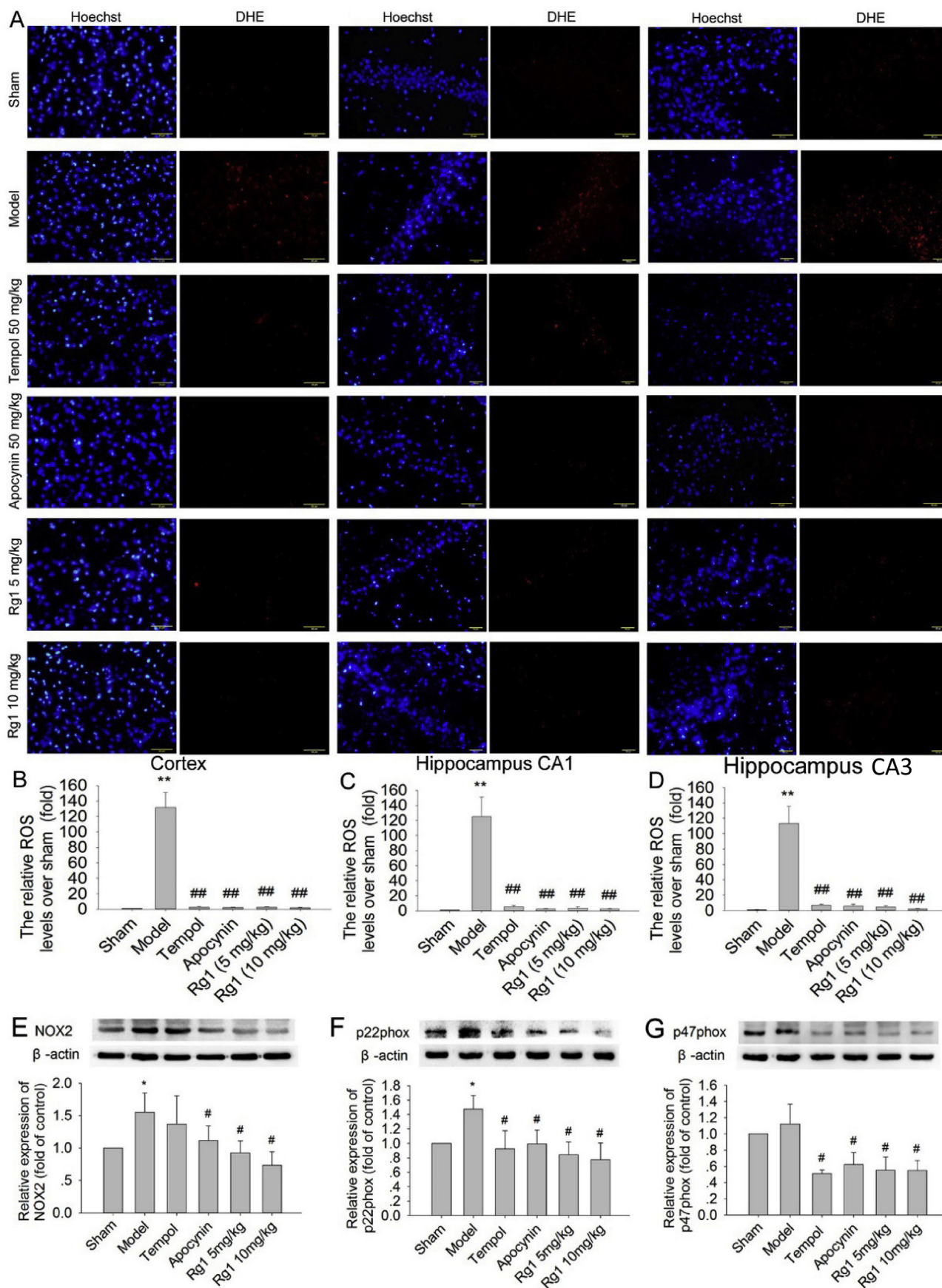


Fig. 4. Impacts of Rg1 treatment for ROS production and the expressions of NOX2-related protein in cerebral I/R mice. (A) The ROS accumulation (n = 4). (B–D) Analysis of the relative ROS levels over control. (E–G) The relative expressions of NOX2-related protein (n = 3). Data are shown as mean ± SD, *P < 0.05, **P < 0.01 vs. control group; #P < 0.05, ##P < 0.01 vs. model group.

apocynin administration could observably down-regulate the expressions of p22phox, p47phox, and NOX2 (Fig. 4E–G). Tempol treatment showed no significant difference in NOX2 expression, but signally down-regulated the levels of p22phox and p47phox (Fig. 4E–G). These data suggested that Rg1 could alleviate CIRI by inhibiting NOX2-mediated oxidative stress in mice.

3.6. Rg1 treatment ameliorates the calcium overload and inhibits the PLC–CN–NFAT1 signaling in vivo and in vitro

We further investigate the impact of Rg1 on the regulation of the PLC–CN–NFAT1 signaling in cerebral I/R mice and OGD/R HT22 cells. The data showed that the expressions of p-PLC, CN, and NFAT1 were markedly increased after cerebral I/R in vivo (Fig. 5A–D) and in vitro (Fig. 5I–N). While apocynin, tempol, and Rg1 (5, 10 mg/kg) administration markedly decreased the levels of p-PLC, CN, and NFAT1 in vivo (Fig. 5A–D) and in vitro (Fig. 5I–N). These results indicated that Rg1 administration might ameliorate CIRI by inhibiting the PLC–CN–NFAT1 signaling pathway.

We further performed calcium imaging to detect the impact of Rg1 on $[Ca^{2+}]_i$ in OGD/R-induced HT22 cells. The results showed that the HT22 cells underwent significant calcium overload after OGD/R, with an increase of $[Ca^{2+}]_i$ (F340/F380 ratio), an imbalance of calcium homeostasis (Δ RatioF340/F380) after treatment with BAPTA and $CaCl_2$ compared to before treated (Fig. 5E–H). While compared with the OGD/R group, treatment with apocynin, tempol, and Rg1 markedly decreased the levels of $[Ca^{2+}]_i$ and the Δ RatioF340/F380 after treatment with BAPTA and $CaCl_2$ (Fig. 5E–H), especially in Rg1 (10 mg/kg) group, suggesting that Rg1 could inhibit calcium overload and resist the calcium homeostasis disorder induced by OGD/R in HT22 cells.

3.7. Rg1 treatment inhibits NLRP1 inflammasome in cerebral I/R mice

We then detected the expressions of NLRP1-associated proteins in brain tissue. The results indicated that the NLRP1, caspase-1, ASC, and IL-1 β expressions were markedly increased after cerebral I/R. While tempol and apocynin could decrease the expressions of NLRP1, caspase-1, and IL-1 β in brain tissue, but had no impact on ASC expression. However, Rg1 (5, 10 mg/kg) administration markedly decreased the expressions of the NLRP1-associated proteins, especially in Rg1 (10 mg/kg) group (supplementary Fig. S2 A–D). These data indicated that Rg1 treatment could inhibit NLRP1 inflammasome in brain tissue of cerebral I/R mice.

4. Discussion

The incidence of ischemic cerebrovascular disease is increasing yearly and has been one of the leading causes of neurological dysfunction and death. It has been reported that excessive ROS accumulate after cerebral I/R [26], and neuronal cells undergo calcium overload [15], which upregulates inflammasomes through multiple pathways, resulting in neuroinflammation and neuronal damage [27]. However, the specific mechanism of CIRI remains to be elucidated. Ginseng is a commonly utilized herbal medication for stroke [28], and ginsenoside Rg1 has a significant defensive impact on the central nervous system [29]. This study was committed to explore the impact and mechanism of Rg1 on CIRI in mice. The results indicated that ROS oxidative stress, calcium homeostasis disorder, and neuroinflammation are closely correlated with CIRI in mice. And Rg1 treatment markedly improved the motor and coordinative abilities and decreased the expressions of NOX2, p-PLC, CN, NFAT1, and NLRP1 in cerebral I/R mice, and diminished the levels of $[Ca^{2+}]_i$ in HT22 cells. These results

suggested that Rg1 administration might be a beneficial strategy for protecting against CIRI.

Cerebral ischemia and hypoxia are critical causes of CIRI. The bilateral common carotid arteries ligation is often used to establish a mouse model of CIRI caused by cerebral blood flow hypoperfusion. The increase of cerebral blood flow can significantly reduce the volume of cerebral infarction and improve brain dysfunction after cerebral I/R [30]. In the present study, we found that Rg1 pretreatment could effectively increase CBF after cerebral ischemia and reperfusion. It was reported that cerebral I/R could induce neuronal damage in the cortex and hippocampus, such as nuclear consolidation, fission, and eosinophilic degeneration, as well as the decrease of Nissl bodies in neurons, resulting in a decrease of motor and coordination ability [31]. In this paper, we found that Rg1 significantly improve motor and coordination abilities and attenuated the neuronal damage in the regions of the cortex and hippocampus, which confirmed the therapeutic role of Rg1 in CIRI. To further confirm the protective effects of Rg1 on CIRI, we detected the expressions of MAP2, PSD95, and p-Tau in cerebral I/R mice. MAP2, as a cytoskeletal and neuronal marker protein, plays pivotal roles in maintaining neuronal function [32,33]. PSD95 is a core scaffolding protein in the dense region of the glutamatergic postsynaptic membrane, which plays critical roles in learning, memory, and synaptic plasticity [34,35]. Tau is one of the most abundant microtubule-associated proteins and its hyperphosphorylation is thought to be one of the important reasons for neuronal functional impairment in Alzheimer's patients [36]. Our study showed that the expressions of MAP2 and PSD95 were decreased and the level of p-Tau was markedly elevated after CIRI in mice, while these changes could be reversed by Rg1 treatment. These data suggested that Rg1 could attenuate neuronal damage induced by cerebral I/R.

Multiple pathophysiological mechanisms are involved in CIRI, including oxidative stress, calcium overload, neuroinflammation, and energy metabolism disorder et al. [37–39]. In particular, oxidative stress has been reported to play critical roles in the pathogenesis of CIRI [40]. The NOX family includes Duox 1/2 and NOX1-5, which can transport the electrons passing the biological membranes to produce ROS in cells. NOX2 is an isoform widely distributed in the brain, especially in neurons. NOX2 consists of four cytoplasmic subunits (p47phox, gp67phox, gp40phox and Rac) and two membrane subunits (gp91phox and gp22phox), with gp91phox referred to as NOX2 [41]. Increasing evidence indicated that NOX2 overexpression was associated with aggravation of ischemic injury in many models of stroke [41,42]. In the present study, we found that Rg1 treatment notably reversed the overexpression of ROS, NOX2, p22phox and p47phox induced by cerebral I/R. The data suggested that NOX2 plays a crucial role in CIRI and Rg1 might effectively attenuate CIRI by inhibiting the activation of NOX2.

Calcium ion, an important second messenger in intracellular, participates in regulations of many physiological and pathological processes, such as cell apoptosis and inflammation. Imbalance of $[Ca^{2+}]_i$ homeostasis involves in many neurodegenerative diseases. It was reported that excessive mitochondrial ROS could induce a sustained increase of $[Ca^{2+}]_i$. And antioxidants could prevent the increase of $[Ca^{2+}]_i$ and attenuated NMDA-induced neuronal damage [43]. Moreover, oxidative stress is also reported to lead to an increase of $[Ca^{2+}]_i$ released from the endoplasmic reticulum (ER) [44]. However, it is still unclear whether NOX2 activation participates in $[Ca^{2+}]_i$ homeostasis disorder in CIRI. It has been reported that NOX2 activation involves in lipopolysaccharide (LPS)-induced cardiomyocytes $[Ca^{2+}]_i$ homeostasis dysregulation by increasing mitochondrial ROS, and inhibition of NOX2 significantly preserves $[Ca^{2+}]_i$ homeostasis and alleviates cardiomyopathy [45].

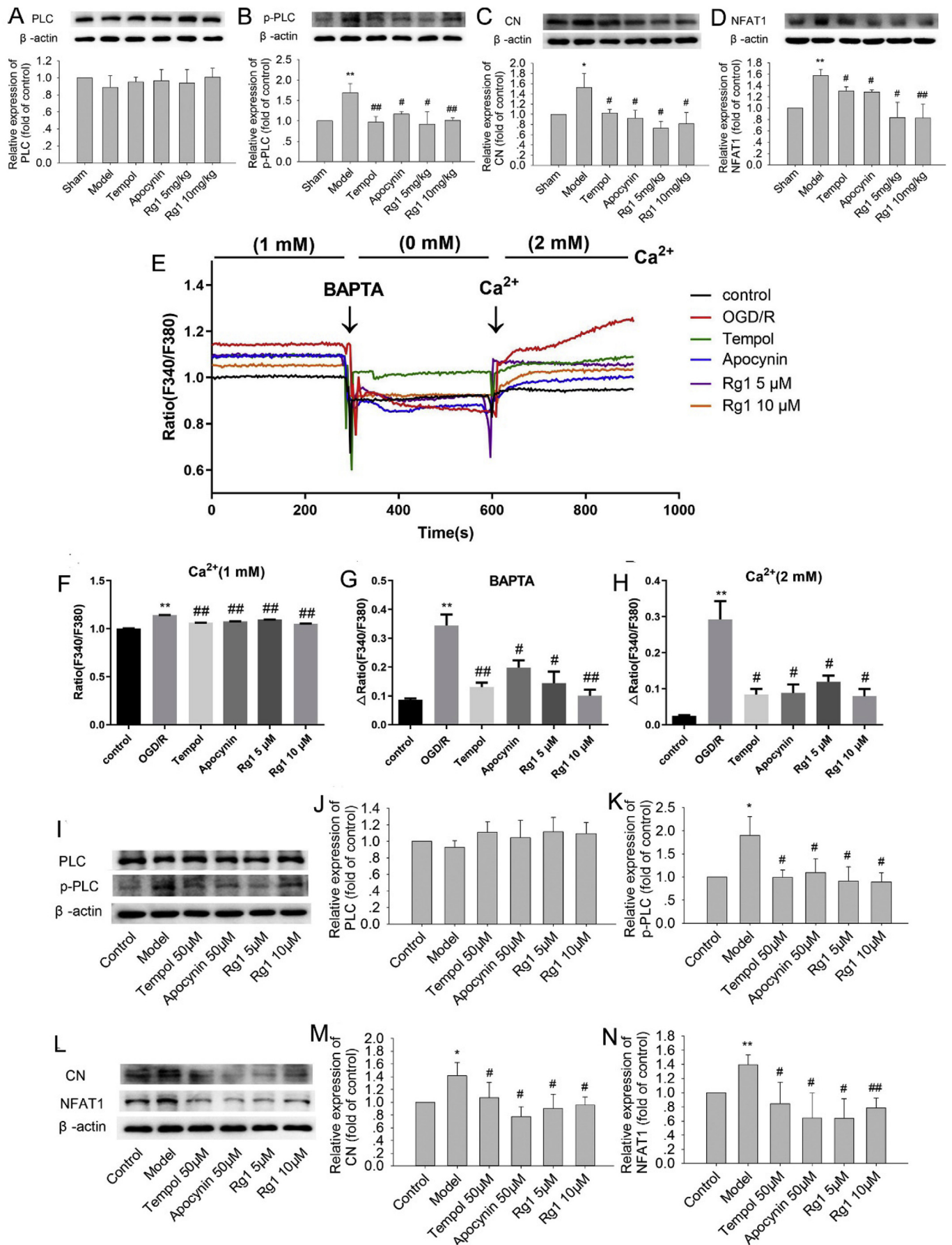


Fig. 5. Impacts of Rg1 on $[Ca^{2+}]_i$ in OGD/R HT22 cells and the expressions of PLC, p-PLC, CN, and NFAT1 in vivo and vitro. (A–D) The relative expressions of PLC, p-PLC, CN, and NFAT1 in cerebral I/R mice. (E) The Ratio (F340/F380) responds to BAPTA and $CaCl_2$ (2 mM) in OGD/R HT22 cells. (F) The Ratio (F340/F380) of basal $[Ca^{2+}]_i$ (0–300s). (G) The Δ Ratio (F340/F380) after BAPTA (300–600s). (H) The Δ Ratio (F340/F380) after $CaCl_2$ (600–900s). (I–K) The bands and relative expressions of PLC and p-PLC in HT22 cells. (L–N) The bands and relative expressions of CN and NFAT1 in HT22 cells. Data are shown as mean \pm SD, $n = 3$. * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. model group.

Additionally, Vitamin E and Trolox, potent ROS scavengers, was reported to inhibit intracellular Ca^{2+} signaling by suppressing phospholipase C (PLC) activity [46]. Activation of PLC can catalyze the phosphatidyl-inositol bisphosphate (PIP2) to DAG and IP3. IP3 can act with IP3R to promote a rapid release of Ca^{2+} from the ER into the cytoplasm, which further induces Ca^{2+} influx [47]. In this study, we found that the expressions of NOX, ROS and p-PLC were significantly increased in brain after cerebral I/R, and the level of $[\text{Ca}^{2+}]_i$ in OGD/R HT22 cells was significantly increased. While Rg1 treatment significantly decreased the levels of NOX, ROS and p-PLC in vivo and preserved $[\text{Ca}^{2+}]_i$ homeostasis in vitro. Therefore, we speculated that Rg1 administration might improve CIRI by reducing NOX2-mediated ROS production, which inhibits PLC to reduce $[\text{Ca}^{2+}]_i$ and stabilize calcium homeostasis.

Calcineurin (CN), a calcium-dependent protein phosphatase, has been reported to induce neuronal death after brain ischemia through dephosphorylation of nuclear factor of activated T-cells (NFAT) [48]. NFAT1 is an important isoform widely expressed in the brain. Upon activation, NFAT1 translocate into the nucleus to regulate its target gene expressions, such as NLRP1 and IL-1 β [49]. It has also been reported that cognitive dysfunction in Alzheimer's disease is correlated with selective alterations in CN/NFAT signaling [50]. In the study, we also found that the levels of CN and NFAT1 were significantly increased after cerebral I/R, and Rg1 administration dramatically decreased the expressions of CN and NFAT1 in vivo and in vitro. The data suggested that calcium overload might activate NLRP1 inflammasome by CN-NFAT1 signaling, and Rg1 administration might improve CIRI by inhibiting calcium-mediated CN-NFAT1 pathway.

Neuroinflammation plays pivotal role in CIRI. The entire process of I/R is accompanied by inflammatory response [51]. It has been reported that, in spinal cord ischemia-reperfusion injury (SCII), the restoring blood supply after spinal cord ischemia leads to further damage by inflammatory factor release, cellular edema, and neuronal apoptosis [52,53]. Similar to SCII, CIRI also involves the release of inflammatory factors. It has been reported that NLRP1 and NLRP3 inflammasomes play important roles in neuronal damages and behavioral dysfunctions after cerebral I/R [54]. A recent study suggested that CD21, a kind of phthalide derivative, might protect against CIRI by inhibiting NLRP3 inflammasome activation in a model of global cerebral ischemia of mice [55]. In addition, studies have found that CaCl_2 treatment can upregulate NLRP3 at the transcription level by activating NFAT1 signaling in HNSCC cell lines [56]. Our previous study indicated that Rg1 treatment could delay neuronal senescence through inhibiting NOX2 and NLRP1 in neurons [25]. In the present study, we found that Rg1 treatment significantly reversed the cerebral I/R-induced increase of IL-1 β , caspase-1, ASC and NLRP1. These findings suggested that Rg1 might attenuate neuroinflammation in CIRI by inhibiting NOX2-mediated ROS accumulation and calcium-mediated CN-NFAT1 activation in neurons after cerebral I/R.

To confirm our hypothesis, we further explored the effect of apocynin and tempol on CIRI in vivo and in vitro. Apocynin, an inhibitor of NOX, has been reported to protect against neuronal damage and improve learning and memory impairments by inhibiting NOX [57]. Tempol, often used as a superoxide dismutase mimic, has been reported to ameliorate several neurodegenerative diseases [58]. Our results also indicated that apocynin and tempol treatment significantly improved motor ability and neuronal damage elevated the levels of PSD95 and MAP2, reduced the level of p-Tau in cerebral I/R mice. Meanwhile, we found that apocynin and tempol treatment could reduce the levels of IL-1 β and ROS, and decreased the expressions of NLRP1 and NOX2 in cerebral I/R mice. Further research indicated that apocynin and tempol treatment attenuated calcium overload in OGD/R HT22 cells and reduced the

levels of p-PLC, CN, and NFAT1 in vivo and in vitro. The data suggested that NOX2 is closely involved in calcium-mediated CN-NFAT1 and NLRP1 inflammasome activation in CIRI. Moreover, in most indicators, tempol, apocynin, and Rg1 treatment had similar protective effect on CIRI in mice, while the results also indicated that Rg1 (10 mg/kg) had a better protective effect on CIRI.

Overall, Rg1 could effectively ameliorate I/R-induced motor dysfunction and neuronal damage in mice. The mechanism might contribute to the inhibition of ROS-related calcium overload and PLC–CN–NFAT1 signaling, which further down-regulates NLRP1 inflammasome. Given that Rg1 has a profound effect on CIRI, further study is warranted to investigate the effect and mechanism of Rg1 treatment on CIRI.

Declaration of competing interest

The authors declare that there are no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jgr.2021.08.001>.

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