



# TRPC6 Attenuates Cortical Astrocytic Apoptosis and Inflammation in Cerebral Ischemic/Reperfusion Injury

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Transient receptor potential canonical 6 (TRPC6) channel is an important non-selective cation channel with a variety of physiological roles in the central nervous system. Evidence has shown that TRPC6 is involved in the process of experimental stroke; however, the underlying mechanisms remain unclear. In the present study, the role of astrocytic TRPC6 was investigated in an oxygen–glucose deprivation cell model and middle cerebral artery occlusion (MCAO) mouse model of stroke. HYP9 (a selective TRPC6 agonist) and SKF96365 (SKF; a TRPC antagonist) were used to clarify the exact functions of TRPC6 in astrocytes after ischemic stroke. TRPC6 was significantly downregulated during ischemia/reperfusion (IR) injury in cultured astrocytes and in cortices of MCAO mice. Application of HYP9 *in vivo* alleviated the brain infarct lesion, astrocytes population, apoptosis, and interleukin-6 (IL-6) and IL-1 $\beta$  release in mouse cortices after ischemia. HYP9 dose-dependently inhibited the downregulation of TRPC6 and reduced astrocytic apoptosis, cytotoxicity and inflammatory responses in IR insult, whereas SKF aggravated the damage *in vitro*. In addition, modulation of TRPC6 channel diminished IR-induced Ca<sup>2+</sup> entry in astrocytes. Furthermore, decreased Ca<sup>2+</sup> entry due to TRPC6 contributed to reducing nuclear factor kappa light chain enhancer of activated B cells (NF- $\kappa$ B) nuclear translocation and phosphorylation. Overexpression of astrocytic TRPC6 also attenuated apoptosis, cytotoxicity, inflammatory responses, and NF- $\kappa$ B phosphorylation in modeled ischemia in astrocytes. The results of the present study indicate that the TRPC6 channel can act as a potential target to reduce both inflammatory responses and apoptosis in astrocytes during IR injury, subsequently attenuating ischemic brain damage. In addition, we provide a novel view of stroke therapy by targeting the astrocytic TRPC6 channel.

**Keywords:** apoptosis, astrocytes, Ca<sup>2+</sup>, inflammation, ischemic stroke, NF- $\kappa$ B, TRPC6

## INTRODUCTION

The transient receptor potential (TRP) channels are cation-permeable membrane proteins with common structural features of six transmembrane segments. Based on the homology of amino acid sequences, the TRP superfamily can be subdivided into seven subfamilies: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPN (*Drosophila* NOMPC), TRPP (polycystin), and TRPML (mucolipin) (Venkatchalam and Montell, 2007). Among the channels, the TRPC channel, which is most closely related to *Drosophila* TRPs, is widely distributed in different tissues

and governs the fate and functions of various cell types (Curcic et al., 2019). TRPCs are broadly expressed in brain, lung, heart, kidney, liver, spleen, and other organs in mammalian animals like human, mouse, rat, and rabbit (Montell, 2001; Venkatachalam and Montell, 2007). In terms of similarity to amino acid sequences and function, TRPC members can be further classified into four subgroups: TRPC1, TRPC2, TRPC4/5, and TRPC3/6/7 (Wang et al., 2020a).

The TRPC6 channel is emerging as an important target for the control of  $\text{Ca}^{2+}$  currents in a wide range of disorders, including immune-mediated diseases, pulmonary arterial hypertension, atherosclerosis, and central nervous system (CNS)-related diseases, such as autism spectrum disorders, glioma, depression, traumatic brain injuries, seizure, Alzheimer's disease, and ischemic stroke (Hamid and Newman, 2009; Ding et al., 2010; Du et al., 2010; Griesi-Oliveira et al., 2015; Kim and Kang, 2015; Zhang et al., 2015, 2016; Pochwat et al., 2018; Ramirez et al., 2018; Chen et al., 2019). TRPC6 is abundantly expressed in various anatomical regions of the CNS, such as the cerebellum, hippocampus, middle frontal gyrus, and cortex (Riccio et al., 2002; Du et al., 2010). As a regulator of  $\text{Ca}^{2+}$  influx, TRPC6 is involved in neuronal survival, synapse formation, neuronal nerve-growth-cone guidance, and sensory transduction (Li et al., 2005; Jia et al., 2007; Zhou et al., 2008; Quick et al., 2012). Dysfunction of the TRPC6 channel may trigger a series of downstream events and neurobiological disorders.

Ischemic stroke is a life-threatening condition caused by a vascular embolism due to cardiac events, artery-to-artery embolism, or *in-situ* small artery disease (Hankey, 2017). Several underlying mechanisms, including excitotoxicity, ionic imbalance, oxidative and nitrative stress, inflammation, and apoptosis, are involved in the pathophysiological process of cerebral ischemia (Khoshnam et al., 2017).  $\text{Ca}^{2+}$  overload has a critical role and initiates the ischemic cascade during brain ischemia/reperfusion (IR) injury. Increasing evidence indicates an important role of TRPC6 in cerebral IR injury (Liu et al., 2020). TRPC6 is identified on cortical neurons and astrocytes, and is downregulated in neurons after brain ischemic injury (Du et al., 2010; Guo et al., 2017; Qu et al., 2017; Shirakawa et al., 2017). Notably, maintaining the TRPC6 protein level in neurons improves neuronal survival and behavioral performance, thus alleviating ischemic brain damage (Du et al., 2010; Guo et al., 2017). However, the roles of the TRPC6 channel in mouse cortical astrocytes following IR injury have not been evaluated.

In the present study, the specific effects of astrocytic TRPC6 on ischemic stroke were investigated. TRPC6 protein expression in primary mouse astrocytes was downregulated following IR injury. Inhibition of TRPC6 downregulation via HYP9 or TRPC6 overexpression protected astrocytes and the brain against IR insults. These results indicate that the TRPC6 channel contributes to neuroprotection in cerebral ischemia by promoting astrocyte survival. Furthermore, this study provides therapeutic evidence for the treatment of ischemic stroke by targeting the TRPC6 channel.

## MATERIALS AND METHODS

### Animals

C57BL/6J male mice 8–10 weeks of age were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). All animal experiments were approved by the Animal Care Committee of the First Affiliated Hospital at Zhejiang University. Mice were housed in polypropylene cages and maintained at  $25 \pm 1^\circ\text{C}$  under 12 h light/12 h dark cycles with free access to rodent chow and water. Mice were randomly allocated to each group before any treatment. Proper anesthetic procedures were used to ensure that the mice did not suffer unnecessarily during or after the experimental procedure. A total of 82 mice were used in this study (including 10 mice that died).

### *In vivo* Model of Focal Cerebral Ischemia

Focal cerebral ischemia was induced by middle cerebral artery occlusion (MCAO). Animals were anesthetized with 1% sodium pentobarbital (75–100 mg/kg, intraperitoneal injection); body temperature was maintained at  $37 \pm 0.5^\circ\text{C}$  during the operation using a heating pad. Transient MCAO was generally performed as previously reported (Lin et al., 2013a). Briefly, the left common carotid artery was exposed to separate the internal carotid artery (ICA) and the external carotid artery (ECA). Next, a 6-0 monofilament nylon suture (RWD Life Science Co., LTD, Shenzhen, China) with a rounded tip was inserted through the exposed left ICA and advanced into the middle cerebral artery. After 1 h of occlusion, the filament was gently withdrawn to allow reperfusion. At 24 h after reperfusion, animals were sacrificed and the brain tissues were obtained for future assays.

### Intracerebroventricular Injection

Mouse intracerebroventricular injection was performed using a stereotaxic instrument (RWD Life Science Co., Ltd., Shenzhen, China) with a micro-syringe pump under anesthesia. Drugs or vehicle (5  $\mu\text{L}$  in total) were slowly injected (1  $\mu\text{L}/\text{min}$ ) into the left ventricle at a depth of 2.5 mm below the brain surface, 1.0 mm lateral and 0.5 mm posterior to the bregma. After injection, animals were allowed to recover from anesthesia under a heating pad.

### Cell Culture

As previously reported (Shen et al., 2016), primary astrocytes from cerebral cortices of 0–1-day-old post-natal C57BL/6 mice were isolated under sterile conditions. Astrocytes were grown in culture medium [Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin]. Dissociated cortical cells were seeded onto poly-D-lysine (PDL, 10  $\mu\text{g}/\text{mL}$ ; # P7405; Sigma-Aldrich St. Louis, MO, USA)-coated T-75 flasks (Costar; Corning Inc., Corning, NY, USA) at a density of three cortices per flask and incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  in a humidified incubator. The medium was changed every other day. After 8–10 days, confluent cultures were shaken (250 rpm at  $37^\circ\text{C}$ ) for 12 h to reduce microglial contamination. Then, purified astrocytes were cultured in medium supplemented with 20  $\mu\text{M}$  cytosine-1- $\beta$ -D-arabinofuranosid (Sigma-Aldrich) for the next 2–3 days. The remaining attached cells were digested with 0.25% trypsin

(#25200056; Gibco, Grand Island, NY, USA) and then reseeded at a density of  $0.03\text{--}0.05 \times 10^6/\text{cm}^2$  in PDL-coated six-well plates at  $37^\circ\text{C}$  in an incubator with  $5\% \text{CO}_2$ . After 5–7 days when the astrocytes reached 90–95% confluence, cells were prepared for subsequent experiments. More than 95% of cells in culture were astrocytes (results not shown).

## Oxygen-Glucose Deprivation and Re-oxygenation (OGD/R)

OGD/R experiments were established to mimic the *in vitro* condition of IR injury. The cultures were incubated for 1–4 h in DMEM medium without glucose in a humidified incubator chamber at  $37^\circ\text{C}$  with  $95\% \text{N}_2$  and  $5\% \text{CO}_2$ . Subsequently, the astrocytes were returned to the original culture conditions and then incubated under normoxic conditions for the next 24 h.

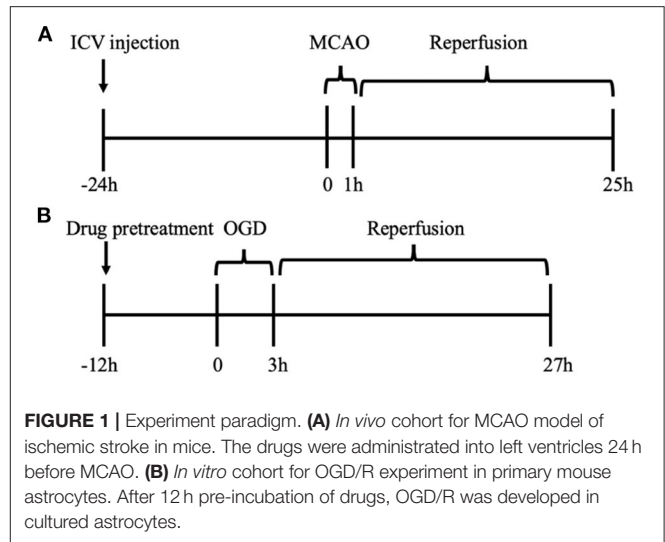
## Drugs and Experimental Groups

The TRPC6-specific agonist HYP9 (Leuner et al., 2010) (#H9791; Sigma-Aldrich), dissolved in dimethyl sulfoxide (DMSO; the final maximum DMSO concentration was  $<0.05\%$ ), was used to determine the role of TRPC6 in astrocytes following IR injury. To verify the optimum concentration, 0, 1, 5, 10, 15, 20, or  $30 \mu\text{M}$  HYP9 was preincubated *in vitro*. SKF96365 (SKF; #ab120280; Abcam, Cambridge, MA, USA) was originally recognized as a major inhibitor of TRPC channels (Singh et al., 2010). In the present study, SKF (dissolved in deionized water) at 0, 0.05, 0.5, 5, 10, 20, 30, or  $40 \mu\text{M}$  concentration was used to treat primary mouse astrocytes. The *in vitro* groups consisted of eight subgroups: (1) control group (CON + Naive); (2) control combined with vehicle (DMSO) group (CON + Vehicle); (3) control combined with HYP9 ( $15 \mu\text{M}$ ) group (CON + HYP9); (4) control combined with SKF ( $30 \mu\text{M}$ ) group (CON + SKF); (5) OGD/R group (OGD + Naive); (6) OGD/R combined with vehicle (DMSO) group (OGD + Vehicle); (7) OGD/R combined with HYP9 ( $15 \mu\text{M}$ ) group (OGD + HYP9); (8) OGD/R combined with SKF ( $30 \mu\text{M}$ ) group (OGD + SKF).

Animals were randomly assigned into eight groups before any procedure as follows: (1) sham operation group (sham + Naive) ( $n = 9$ ); (2) sham operation combined with vehicle (DMSO) group (sham + Vehicle) ( $n = 3$ ); (3) sham operation combined with HYP9 ( $5 \mu\text{g}$ ) group (sham + HYP9) ( $n = 3$ ); (4) sham operation combined with SKF ( $20 \mu\text{g}$ ) group (sham + SKF) ( $n = 3$ ); (5) MCAO operation group (MCAO + Naive) ( $n = 18$ ); (6) MCAO operation combined with DMSO group (MCAO + Vehicle) ( $n = 12$ ); (7) MCAO operation combined with HYP9 ( $5 \mu\text{g}$ ) group (MCAO + HYP9) ( $n = 12$ ); (8) MCAO operation combined with SKF ( $20 \mu\text{g}$ ) group (MCAO + SKF) ( $n = 12$ ). **Figure 1** shows the experiment paradigm.

## Lentivirus Infection

Lentivirus vectors were chemically synthesized by Obio Technology (Shanghai) Corp., Ltd. To overexpress astrocytic TRPC6, we infected primary astrocytes with lentiviruses carrying FLAG-tagged full-length mCherry-WT-TRPC6 (WT-TRPC6) or mCherry-WT-TRPC6-null (Vehicle1). Besides, to knock down TRPC6 in primary astrocytes, lentiviruses



carrying mCherry-shRNA-TRPC6 (sh-TRPC6) or mCherry-shRNA-TRPC6-null (Vehicle2) were used to infect primary cortical astrocytes. Targeted sequences for TRPC6 and Vehicle2 siRNAs are: GCTTGCCAACATTGAGAAA and TTCTCCGAACGTGTCACGT, respectively. Astrocytes at 70% confluence were infected with lentiviruses according to the vendor's protocol. After 72–96 h lentiviral infection, astrocytes were used for subsequent experiments. The efficiency of infections was detected by western bolts.

## TTC Staining

Animals were sacrificed at 24 h after reperfusion. The infarct volume was tested based on 2,3,5-triphenyltetrazolium chloride (TTC) staining. The brains were sectioned into 2-mm coronal slices using a brain matrix. The brain slices were incubated in 2% TTC solution staining for 20 min at  $37^\circ\text{C}$  and then fixed in 4% paraformaldehyde (PFA). The ischemic volume was quantified using ImageJ software (version 2.0). The infarct percentage was shown as the ratio of the ischemic area to the entire slice area.

## Analysis of Cell Viability and Lactate Dehydrogenase (LDH) Release

An LDH release quantification kit (#11644793001; Sigma-Aldrich) was used to evaluate cell viability. The cell-free supernatant was obtained by centrifugation at  $\sim 250 \times g$  for 10 min. Subsequently, the supernatant was incubated in working solutions in the dark for 1 h at room temperature ( $\sim 22^\circ\text{C}$ ). The absorbance of the samples was measured at 490 nm using a microplate reader (SpectraMax i3x; Molecular Devices, Sunnyvale, CA, USA). To determine the cytotoxicity percentage, all groups were compared with the control groups.

## Flow Cytometry

The flow cytometry measurement of astrocyte apoptosis and  $\text{Ca}^{2+}$  concentration was performed using a BD flow cytometer (BD Biosciences, San Jose, CA, USA). Astrocytes were digested

with trypsin. The harvested cells were washed with phosphate-buffered saline (PBS) twice before incubating in buffer containing 5  $\mu$ L of fluorescein isothiocyanate-annexin V and 5  $\mu$ L of propidium iodide or 0.5  $\mu$ g/ml DAPI for 15 min at room temperature in the dark. Subsequently, the cells were detected and analyzed.

The  $\text{Ca}^{2+}$  concentration was also measured using the BD flow cytometer. Cortical astrocytes were loaded with 2  $\mu$ M Fluo-4 AM (#F14217; Invitrogen, Carlsbad, CA, USA) in culture medium for 1 h at 37°C in an incubator with 5%  $\text{CO}_2$ . Next, the medium was changed to Hank's balanced salt solution (#14025092; Gibco) and the astrocytes were incubated for another 30 min at 37°C. Then, the astrocytes were collected and analyzed using flow cytometry.

## Western Blot

Western blot analysis was performed as previously described with slight modifications (Li et al., 2020). Samples (20–40  $\mu$ g) were separated on 12% gels and transferred onto 0.45- $\mu$ m polyvinylidene difluoride membranes (#IPVH00010; Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk for 1 h and then incubated with the following primary antibodies at 4°C overnight: TRPC6 (1:1,000; #21403; SAB Biotherapeutics, Sioux Falls, SD, USA), caspase-3 (1:1,000; #ab214430; Abcam, Cambridge, MA, USA), NF- $\kappa$ B (1:1,000; #6956S; Cell Signaling Technology), phospho-NF- $\kappa$ B (1:1,000; #3033S, Cell Signaling Technology), IL-1 $\beta$  (1:2,000; #ab9722; Abcam), Flag (1:1,000; #F1804, Sigma-Aldrich),  $\beta$ -actin (1:2,000; #60008-1-Ig; Proteintech, Rosemont, IL, USA). Then, the membranes were incubated for 1 h with secondary antibodies (1:8,000; #SA00001-1 and #SA00001-2; Proteintech). Protein-specific signals were visualized using the Bio-Rad ChemiDoc™ MP imaging system (Hercules, CA, USA).

## Immunofluorescence

Brain tissues were cut into 20  $\mu$ m slices using a cryostat (#CM1950; Leica, Wetzlar, Germany). Tissues were blocked in 5% goat serum containing 0.3% Triton X-100 for 1 h at 37°C and then incubated with the following primary antibodies overnight at 4°C: GFAP (1:400; #80788S and #3670S; Cell Signaling Technology), TRPC6 (1:100; #ab62461; Abcam). Next, the slices were washed with PBS and incubated with secondary fluorescent antibodies (1:100; #SA00013-1, SA00013-2, SA00013-3, or SA00013-4; Proteintech) for 1 h at room temperature. In addition, 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; 1  $\mu$ g/mL; #4083; Cell Signaling Technology) staining was used to visualize the nuclei. Images were acquired using a confocal laser-scanning microscope (Nikon A1 Ti, 600 $\times$  magnification; Leica TCS SP8, 400 $\times$  magnification).

Astrocytes plated on glass coverslips 12 mm in diameter were collected and fixed in 4% PFA for 15 min at room temperature. Subsequently, the fixed cells were permeabilized with 0.1% Triton X-100 for 15 min at room temperature followed by blocking in 10% goat serum at 37°C for 30 min. Before incubation with secondary fluorescent antibodies, the astrocytes were labeled with the following primary antibodies: GFAP (1:400; #80788S and #3670S; Cell Signaling Technology), NF- $\kappa$ B (1:100; #6956S; Cell Signaling Technology). Next, DAPI (1  $\mu$ g/mL) was used to stain

nuclei. Specific fluorescent signals were tested using a confocal microscope (Leica TCS SP8, 630 $\times$  or 1,890 $\times$  magnification).

## Enzyme-Linked Immunosorbent Assay

The release of interleukin-6 (IL-6) and IL-1 $\beta$  from cortices and cultured astrocytes was quantified using enzyme-linked immunosorbent assay (IL-6, #70-EK206HS; IL-1 $\beta$ , #70-EK201BHS-96; MULTI SCIENCES, Hangzhou, China). The experiments were performed according to the manufacturer's instructions. The final absorbance was detected at 450 nm and the background correction was set at 570 nm on a microplate reader (SpectraMax i3x; Molecular Devices).

## Statistical Analyses

Data are presented as the means  $\pm$  standard error of the mean (SEM) of at least three independent analyses. SPSS ver. 25.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA) were used for statistical analyses. One-way analysis of variance with Tukey's test for *post-hoc* analysis were performed. *P*-value <0.05 was considered statistically significant.

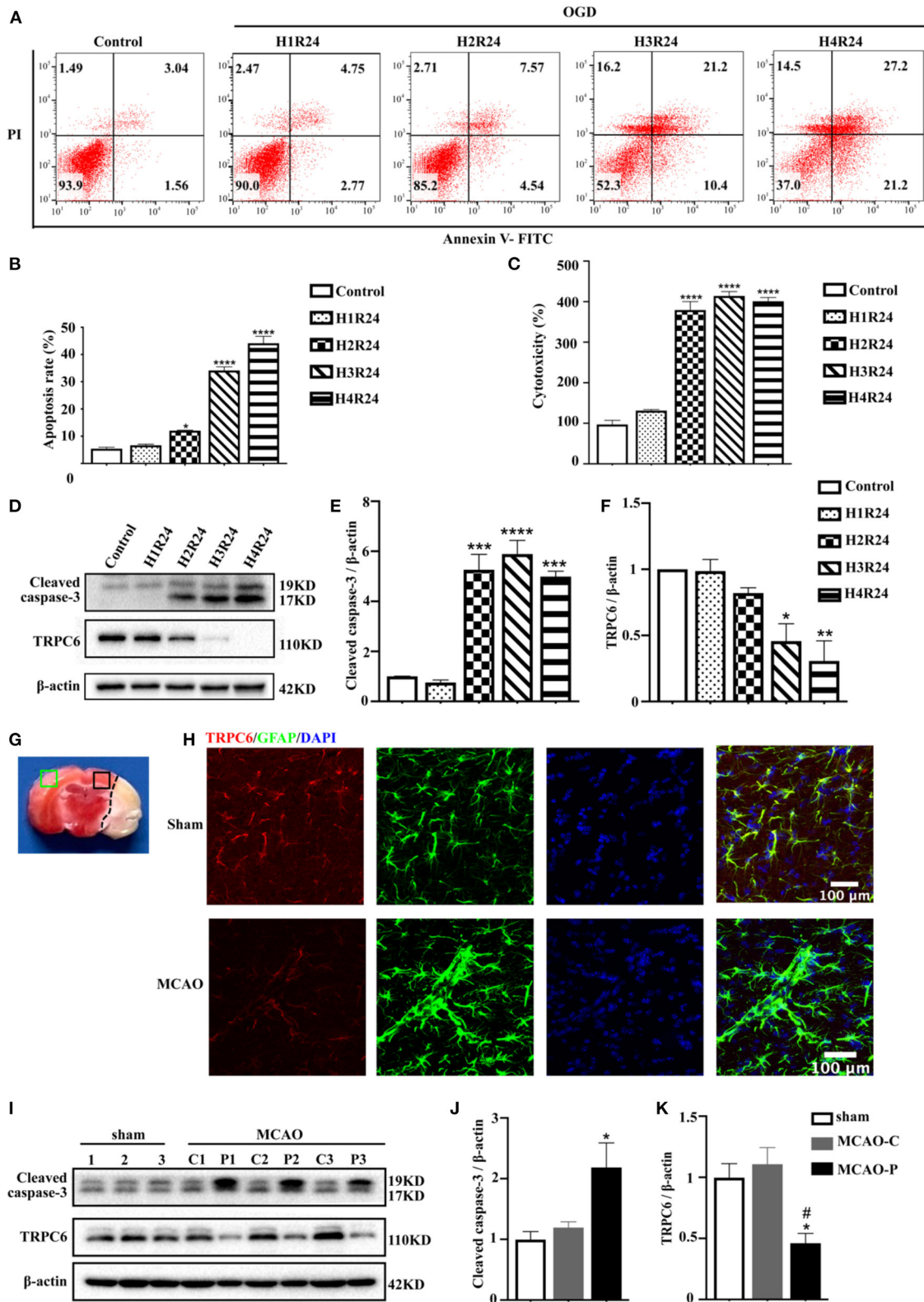
## RESULTS

### TRPC6 in Mouse Cortical Astrocytes Is Downregulated After Ischemia

In several studies, TRPC6 in neurons reportedly declines after cerebral ischemia, and maintaining the expression of neuronal TRPC6 protein contributes to neuronal survival and reduced cerebral ischemic insult (Du et al., 2010; Lin et al., 2013a; Guo et al., 2017). However, the role of the astrocytic TRPC6 channel after stroke remains unclear.

Astrocytes have provided important insights into ischemic stroke (Liu and Chopp, 2016). To confirm the vital functions of the astrocytic TRPC6 channel in cerebral ischemia, we established an *in vitro* model of stroke in primary mouse astrocytes using an OGD/R experiment. Astrocytes were exposed to 1–4 h of OGD followed by 24 h reperfusion. The apoptosis rate was elevated and cell viability was decreased in astrocytes following hypoxia injury (Figures 2A–C). The TRPC6 protein levels in astrocytes after OGD/R were significantly downregulated (Figures 2D,F). Conversely, the expression of the apoptosis-specific biomarker, cleaved caspase-3, was gradually increased after stroke compared with the control groups (Figures 2D,E). As shown in Figures 2A–D, 3 h OGD and 24 h reperfusion triggered appropriate hypoxic damage in astrocytes; thus, the subsequent *in vitro* OGD/R experiments were performed under this condition.

The *in vivo* stroke model is mimicked by MCAO in mice. Immunofluorescence signals showed that astrocytic TRPC6 was downregulated in the ischemic penumbra (Figures 2G,H). In parallel with the *in vitro* results, the TRPC6 protein level in peri-infarct areas was decreased compared with that in contralateral cortices (Figures 2I,K). In addition, after ischemic stroke, the cleaved caspase-3 expression in the ipsilateral hemisphere was upregulated compared with the contralateral hemisphere (Figures 2I,J). Furthermore, immunofluorescence



**FIGURE 2 |** TRPC6 in the cortical astrocytes is downregulated in ischemia. **(A)** Cultured astrocytes were exposed or not to OGD/R, and their apoptosis and death was detected by FITC/PI flow cytometry analysis. The percentage of apoptosis in scatter plots was presented as the proportion of apoptotic cells vs. total cell

(Continued)

**FIGURE 2** | population (H1R24: 1 h OGD followed by 24 h reperfusion; H2R24: 2 h OGD followed by 24 h reperfusion; H3R24: 3 h OGD followed by 24 h reperfusion; H4R24: 4 h OGD followed by 24 h reperfusion). **(B)** Bar chart showed the statistical results of apoptosis rate measured by flow cytometry in each group in three independent experiments. Data were shown as mean  $\pm$  SEM ( $n = 3$ ). **(C)** LDH assay was used to measure the cytotoxicity of astrocytes in control and OGD groups. Data were shown as mean  $\pm$  SEM ( $n = 4$ ). **(D)** The protein level of TRPC6 and cleaved caspase-3 in astrocytes were tested by western blot analysis.  $\beta$ -actin was used as a loading control. **(E,F)** Quantifications of cleaved caspase-3 and TRPC6 protein levels shown in **(D)**. Data were shown as mean  $\pm$  SEM ( $n = 3$ ). **(G)** Brain slices with TTC staining. Green rectangle: contralateral cortex; Black rectangle: peri-infarct cortex. **(H)** Representative images of the cortex after sham or MCAO operation (peri-infarct cortex) double-stained with the TRPC6 (Red) and GFAP (Green) antibodies (Scar bar: 100  $\mu$ m; 600 $\times$  magnification). **(I)** Mice were exposed to sham operation or MCAO procedure ( $n = 3$ ). The expressions of TRPC6 and cleaved caspase-3 in sham group (left hemisphere) and in contralateral (C) or peri-infarct (P) cortex were detected by western blotting.  $\beta$ -actin was used as a loading control. The number "1," "2," "3" represents cortices in each group were extracted from three different mice. **(J,K)** Quantifications of cleaved caspase-3 and TRPC6 protein levels shown in **(I)**. Data were shown as mean  $\pm$  SEM ( $n = 3$ ). MCAO-C, contralateral cortices in MCAO mice; MCAO-P, peri-infarct cortices in MCAO mice. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  (all comparisons to control group or sham group). # $p < 0.05$  (comparisons to MCAO-C group).

signals showed that astrocytic TRPC6 was downregulated in the ischemic penumbra (Figure 2H).

These results indicate that IR injury-induced changes in TRPC6 channel protein levels may have important functions in brain ischemia.

### Reducing IR-Mediated TRPC6 Downregulation With HYP9 Alleviates Ischemic Insults in the Mouse Cortex

Consequently, to determine the role of TRPC6 in an *in vivo* model of ischemic stroke, intraventricular administration of HYP9 or SKF was performed in mice. MCAO resulted in increased infarct size and pro-inflammatory cytokine release compared with the sham groups (Figure 3). Pretreatment with HYP9 alleviated cortical injury, reduced infarct volume (Figures 3A,B), astrocytic population (Figure 3C), cleaved caspase-3 expression (Figures 3D,E), and inhibited pro-inflammatory cytokine (IL-6 and IL-1 $\beta$ ) generation after MCAO (Figures 3G,H). However, the effects of SKF on an animal model of stroke regarding infarction volume, astrocytic population, cleaved caspase-3 protein level, and cytokine generation were not significantly different. In conclusion, the results show that application of the TRPC6 agonist HYP9 can alleviate IR-induced cerebral injury in an animal model of stroke.

### Dose-Dependent HYP9 Inhibition of TRPC6 Downregulation in Astrocytes Promotes Astrocyte Survival During IR Injury

HYP9 is a specific TRPC6 channel activator (Leuner et al., 2010). Treatment with HYP9 (0, 1, 5, 10, 15, 20, or 30  $\mu$ M) was used to verify whether TRPC6 channel degradation is responsible for IR-induced astrocyte insults. Astrocytes were pre-incubated with different concentrations of HYP9 for 12 h in a CO<sub>2</sub> chamber and then exposed to normal or OGD/R conditions. Application of 15  $\mu$ M HYP9 contributed to a significant decrease in OGD/R-induced astrocyte apoptosis and cytotoxicity (Figures 4A–C). Western blot results showed that HYP9 (15  $\mu$ M) suppressed the downregulation of TRPC6 and decreased cleaved caspase-3 protein levels in primary mouse astrocytes in the OGD group (Figures 4D–F). These results indicate that maintaining the protein level of the TRPC6 channel in astrocytes with HYP9 diminishes OGD/R-induced astrocyte apoptosis and improves cell vitality.

### TRPC Antagonist SKF Aggravates Astrocyte Damage Dose-Dependently in the OGD/R Experiment

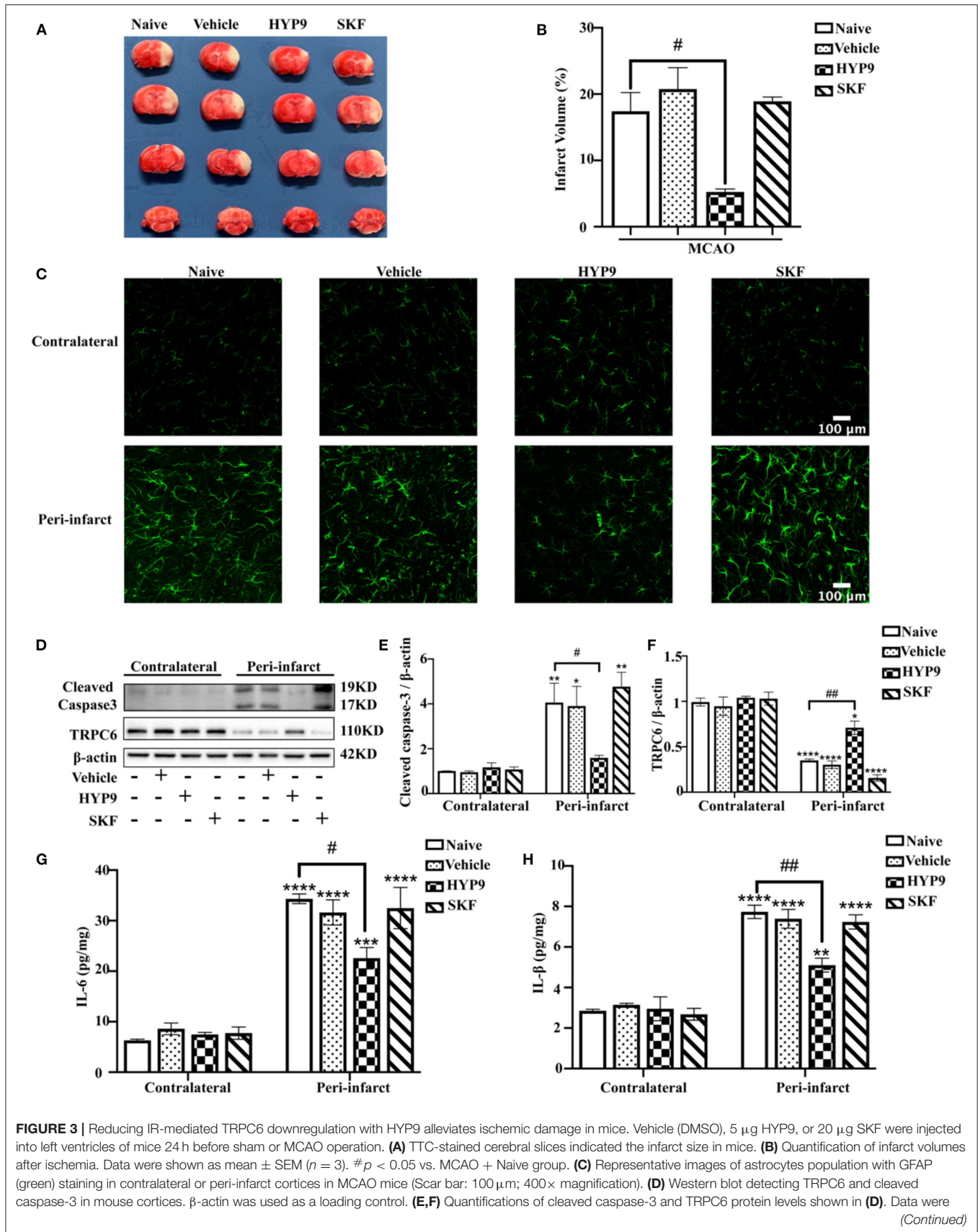
Next, whether blockage of the TRPC6 channel by SKF results in increased astrocyte damage after OGD/R was investigated. Cultured astrocytes were pretreated with 0, 0.05, 0.5, 5, 10, 20, 30, or 40  $\mu$ M SKF for 12 h before the OGD/R experiment. As shown in Figures 5A,B, the apoptosis of astrocytes was markedly increased in the OGD group supplemented with 30 or 40  $\mu$ M SKF compared with the OGD group without SKF pretreatment. In addition, the effects of SKF on astrocyte cytotoxicity in the control and OGD groups were investigated using the LDH assay. Notably, IR injury-mediated astrocyte cytotoxicity was elevated after pre-incubation with 20, 30, or 40  $\mu$ M SKF in OGD groups (Figure 5C). In addition to the increased cleaved caspase-3 expression, the OGD/R-induced degradation of TRPC6 protein was not rescued after treatment with SKF (Figures 5D–F). Taken together, pretreatment of cultured astrocytes with 30 or 40  $\mu$ M SKF aggravates apoptosis and cytotoxicity in OGD groups.

### TRPC6 Protects Astrocytes Against IR Injury in Modeled Ischemia

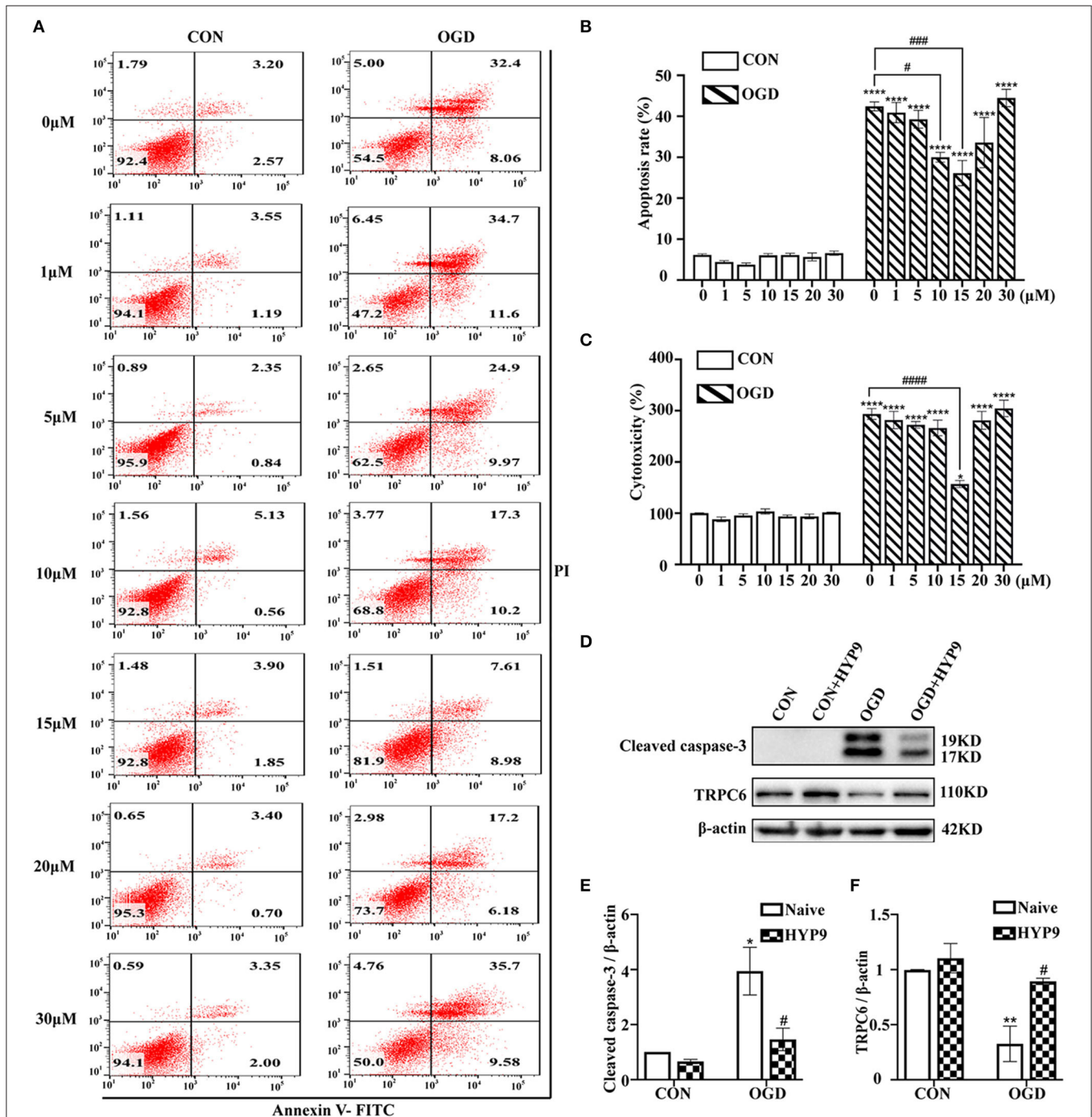
To further clarify the protective role of astrocytic TRPC6 during IR injury, we then infected primary cortical astrocytes with lentivirus vectors to overexpress (WT-TRPC6) or knock down (sh-TRPC6) TRPC6. The efficiency of lentiviral infections was shown in Figure 6D. OGD/R notably decreased the protein levels of TRPC6 compared with that in control groups in astrocytes (Figure 6D). Overexpression of TRPC6 strikingly reduced OGD/R-induced astrocyte apoptosis (Figures 6A,B), cytotoxicity (Figure 6C), and cleaved caspase-3 protein levels (Figures 6D,E). However, knocking down TRPC6 via sh-TRPC6 did not exacerbate IR injury-mediated apoptosis, cytotoxicity or cleaved caspase-3 protein levels in astrocytes (Figure 6). In general, TRPC6 attenuates astrocytes IR injury in modeled ischemia *in vitro*.

### Maintaining the Protein Level of the TRPC6 Channel in Astrocytes Alleviates Astrocytic Inflammatory Responses

Astrocytic inflammatory response also has a critical effect on regulating the progress and prognosis of ischemic stroke (Cekanaviciute and Buckwalter, 2016; Deng et al., 2018). In

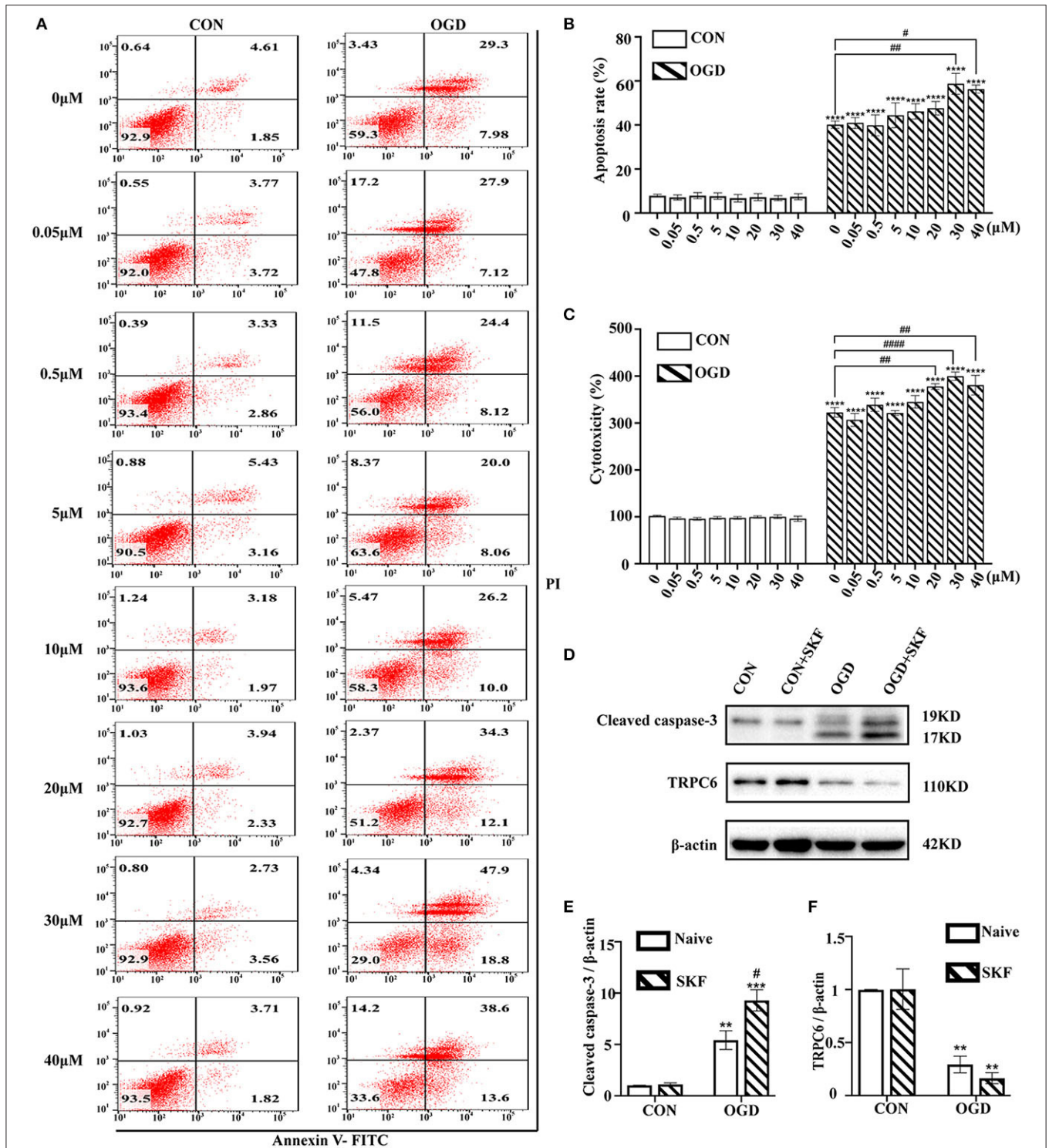


**FIGURE 3** | shown as mean  $\pm$  SEM ( $n = 3$ ). **(G,H)** The release of IL-6 and IL-1 $\beta$  was determined by ELISA assay. Data were shown as mean  $\pm$  SEM ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  vs. MCAO + Naïve group in contralateral cortices; # $p < 0.05$ , ## $p < 0.01$  vs. MCAO + Naïve group in peri-infarct area.

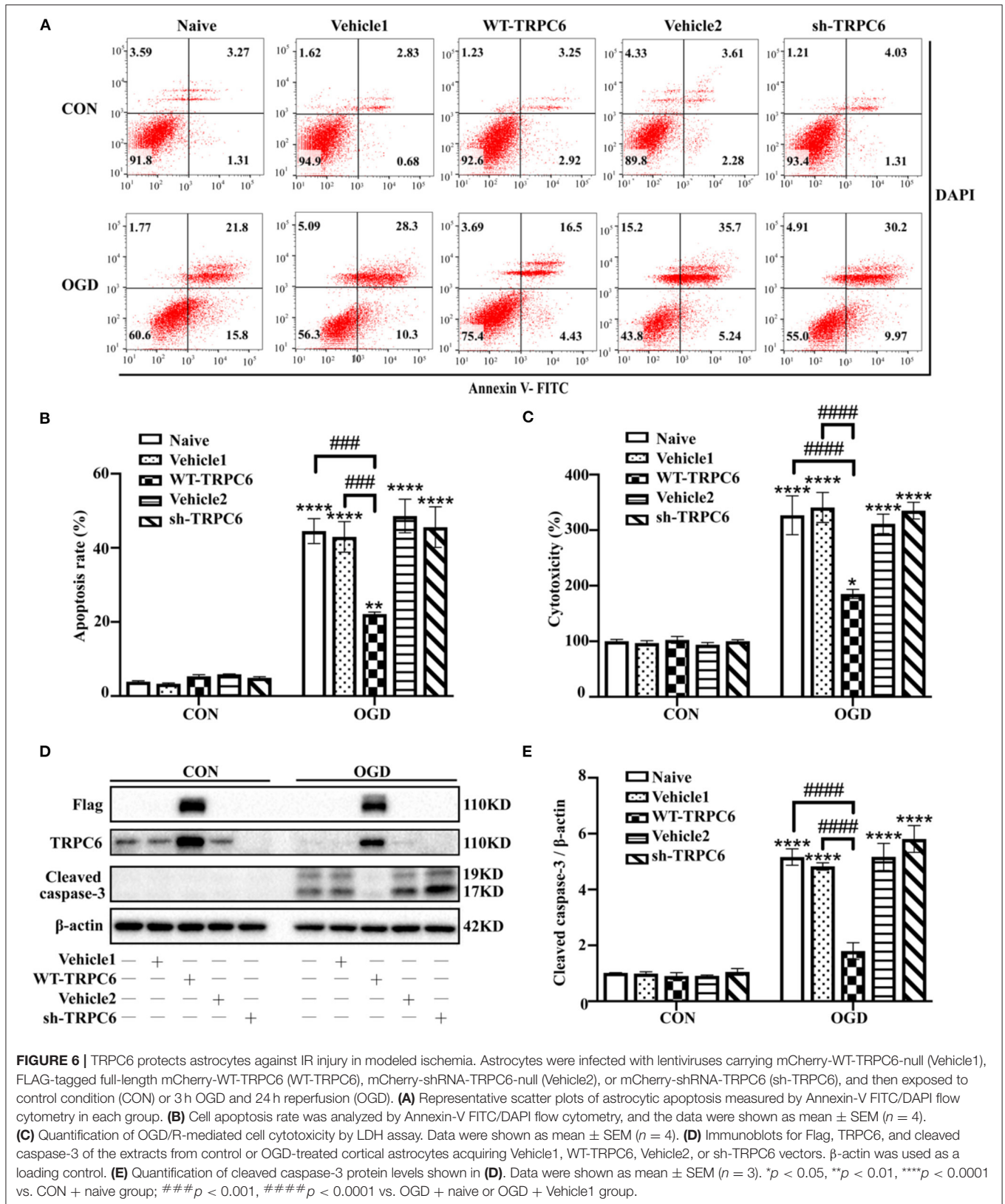


**FIGURE 4** | Inhibition of TRPC6 downregulation by HYP9 protects astrocytes from ischemic damage. Astrocytes were pre-incubated with 0, 1, 5, 10, 15, 20, and 30  $\mu$ M HYP9 for 12 h, and then subjected to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). **(A)** Representative scatter plots of astrocytic apoptosis measured by Annexin-V FITC/PI flow cytometry in each group. **(B)** Cell apoptosis rate was analyzed by Annexin-V FITC/PI flow cytometry, and the data were shown as mean  $\pm$  SEM ( $n = 4$ ). **(C)** Quantification of OGD/R-mediated cell cytotoxicity by LDH assay. Data were shown as mean  $\pm$  SEM ( $n = 4$ ). **(D)** Immunoblots for TRPC6 and cleaved caspase-3 of the extracts from CON or OGD-treated cortical astrocytes acquiring 15  $\mu$ M HYP9 or not.  $\beta$ -actin was used as a loading control. **(E,F)** Quantifications of cleaved caspase-3 and TRPC6 protein levels shown in **(D)**. Data were shown as mean  $\pm$  SEM ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$  vs. CON (HYP9 = 0  $\mu$ M) group; # $p < 0.05$ , ### $p < 0.001$ , #### $p < 0.0001$  vs. OGD (HYP9 = 0  $\mu$ M) group.





**FIGURE 5 |** TRPC6 antagonist SKF aggravates OGD/R induced astrocytes insults. Astrocytes were pre-incubated with 0, 0.05, 0.5, 5, 10, 20, 30, and 40 μM SKF for 12 h, and then subjected to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). **(A)** Representative scatter plots of astrocytic apoptosis measured by Annexin-V FITC/PI flow cytometry in each group. **(B)** Cell apoptosis rate was analyzed by Annexin-V FITC/PI flow cytometry, and the data were shown as mean ± SEM (*n* = 4). **(C)** Quantification of OGD/R-mediated cell cytotoxicity by LDH assay. Data were shown as mean ± SEM (*n* = 4). **(D)** Immunoblots for TRPC6 and cleaved caspase-3 of the extracts from CON or OGD-treated cortical astrocytes acquiring 30 μM SKF or not. β-actin was used as a loading control. **(E,F)** Quantifications of cleaved caspase-3 and TRPC6 protein levels shown in **(D)**. Data were shown as mean ± SEM (*n* = 3). \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001 vs. CON (SKF = 0 μM) group; #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.0001 vs. OGD (SKF = 0 μM) group.



addition to cell apoptosis and vitality, the expression of the pro-inflammatory cytokines IL-6 and IL-1 $\beta$  were measured to confirm the effects of HYP9 and SKF on ischemic astrocytic inflammation. The IL-6 and IL-1 $\beta$  protein levels were significantly increased in the OGD groups compared with the control groups. Notably, HYP9 and WT-TRPC6 reduced the elevated level of IL-6 and IL-1 $\beta$  in astrocytes in the OGD groups (Figures 7A–F).

The data show that inhibition of TRPC6 downregulation with HYP9 or WT-TRPC6 reduces inflammatory responses in astrocytes after OGD/R.

### Inhibiting TRPC6 Downregulation Suppresses Intracellular Ca<sup>2+</sup> Overload in Primary Astrocytes

The above reported results indicate the TRPC6 channel is closely associated with apoptosis, vitality, and inflammatory response in cultured cortical astrocytes exposed to IR damage. As a Ca<sup>2+</sup> channel, TRPC6 modulates the concentration of intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) (Wang et al., 2020a). To understand the potential mechanism of TRPC6 channel against cerebral ischemic insults in astrocytes, the effects of HYP9 and SKF on [Ca<sup>2+</sup>]<sub>i</sub> in astrocytes was measured. Consistent with previous research (Li et al., 2015; Rakers and Petzold, 2017), OGD/R dramatically increased astrocytic [Ca<sup>2+</sup>]<sub>i</sub> compared with control conditions (Figures 8A,B). Notably, application of HYP9 statistically reduced the elevated [Ca<sup>2+</sup>]<sub>i</sub> in the OGD group (Figures 8A,B). In addition, SKF raised [Ca<sup>2+</sup>]<sub>i</sub> in ischemic astrocytes (Figures 8A,B). These results indicate that astrocytic TRPC6 channel triggers a variety of protective cellular responses by inhibiting brain ischemia-induced Ca<sup>2+</sup> overload.

### TRPC6 Diminishes NF- $\kappa$ B Nuclear Translocation and Phosphorylation

NF- $\kappa$ B phosphorylation and nuclear translocation are considered vital pathways that mediate the inflammatory cascade, including cytokine generation (Kopitar-Jerala, 2015; Shih et al., 2015). Therefore, the influence of HYP9 and SKF on NF- $\kappa$ B nuclear translocation and phosphorylation in primary mouse astrocytes exposed to ischemic damage was investigated. Immunofluorescent signals showed the nuclear distribution of NF- $\kappa$ B was increased after OGD/R (Figure 9). Notably, the TRPC6-specific activator HYP9 attenuated IR injury-induced NF- $\kappa$ B nuclear translocation (Figure 9). Furthermore, HYP9 and WT-TRPC6 inhibited the phosphorylation of NF- $\kappa$ B in astrocytes subjected to OGD/R (Figures 10A–G). Collectively, the findings indicate that NF- $\kappa$ B phosphorylation and nuclear translocation may be pivotal downstream pathways that contribute to the protective effects of TRPC6 in astrocytes after stroke.

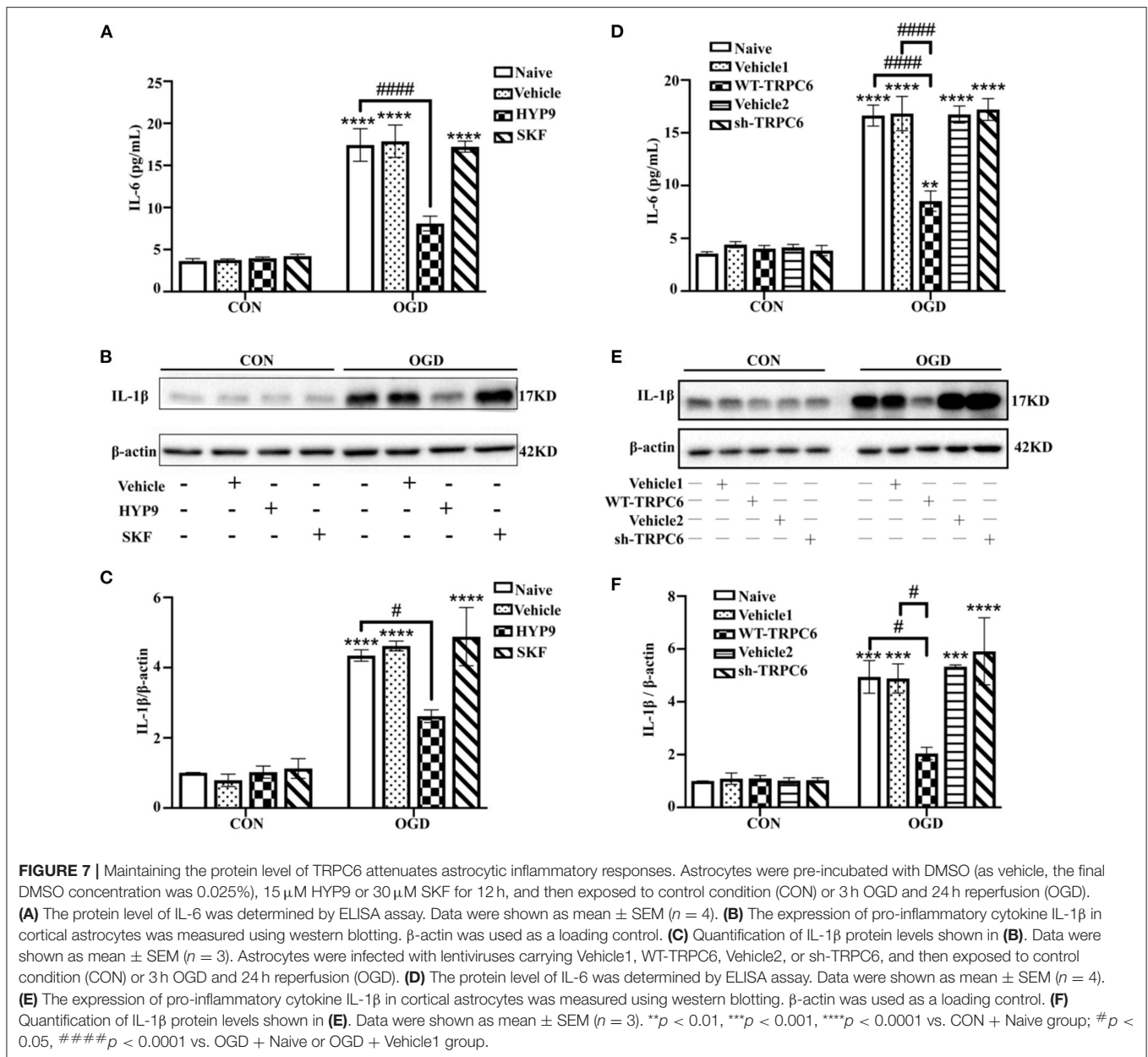
## DISCUSSION

In cerebral ischemia, several clinical trials have failed due to narrow therapeutic window, adverse effects, and individual differences (Petrovic-Djergovic et al., 2016). A breakthrough in stroke therapeutic strategies is considered urgent. In the

present study, significant downregulation of TRPC6 protein was observed in both cultured astrocytes and cerebral cortices after IR injury. *In vivo* results confirmed that the TRPC6 channel-specific activator HYP9 attenuated the infarct volume, astrocytes population, apoptosis, and the generation of pro-inflammatory cytokines (IL-6 and IL-1 $\beta$ ) caused by MCAO. However, significant effects of SKF on infarct lesion or cortical IL-6 and IL-1 $\beta$  release in MCAO mice were not observed. In addition, HYP9 was shown to attenuate apoptosis rate, improve cell vitality, and reduce the generation of IL-6 and IL-1 $\beta$  in cultured astrocytes subjected to OGD/R. Furthermore, the TRPC channel antagonist SKF aggravated astrocytic apoptosis and cytotoxicity in OGD groups. In addition, inhibition of the TRPC6 channel downregulation with HYP9 suppressed the increase in [Ca<sup>2+</sup>]<sub>i</sub> in ischemic astrocytes. The IR-mediated increased NF- $\kappa$ B phosphorylation and nuclear translocation were inhibited by HYP9 pretreatment in primary mouse astrocytes. In parallel, overexpression of TRPC6 also decreased IR injury-induced astrocytic apoptosis, cytotoxicity, inflammatory responses and NF- $\kappa$ B phosphorylation in modeled ischemia *in vitro*.

Previously, neuron-specific-related mechanisms are primarily considered in the pathogenesis of brain ischemia. As knowledge increased, astrocytes and other CNS cells are shown to be closely involved in ischemic stroke (Pekny et al., 2016; Werner et al., 2020). Astrocytes interact with neurons and participate in structural support, neuronal metabolism, synaptogenesis, synaptic transmission, axonal remodeling, and neurogenesis (Halassa and Haydon, 2010; Liu and Chopp, 2016). IR injury-activated astrocytes initiate the CNS inflammatory response, which in turn exacerbates brain insults during ischemic stroke (Cekanaviciute and Buckwalter, 2016). Thus, maintaining the normal function of astrocytes likely promotes post-ischemic neurological recovery. Therapeutic targeting of astrocytes is expected to be a future area of research regarding treatment and prognosis of brain ischemia (Neuhaus et al., 2017).

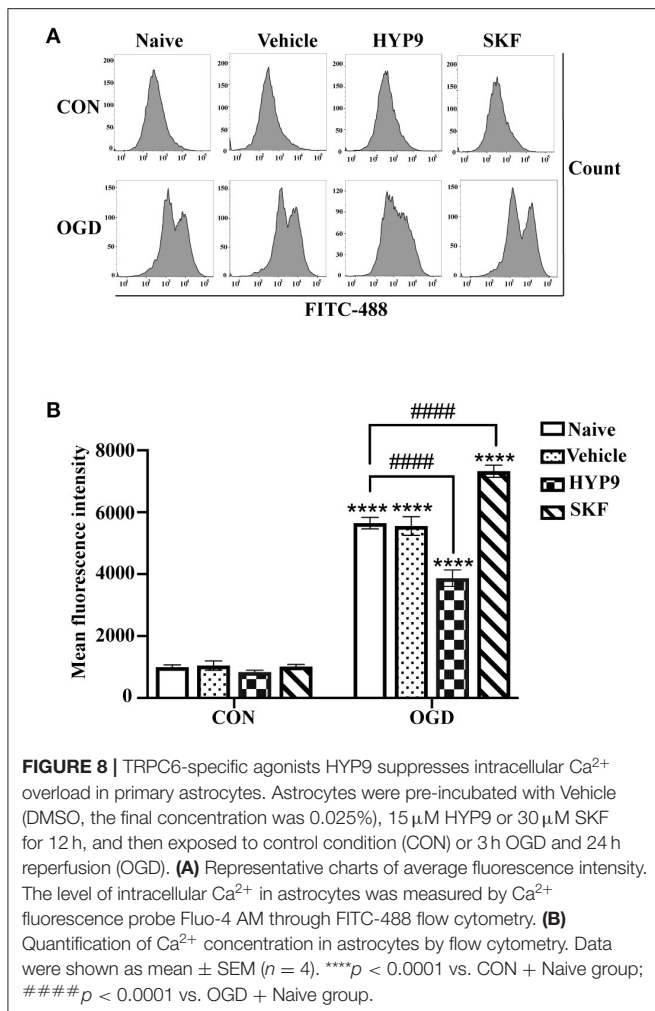
Evidence shows that TRPC6 is a crucial regulator of inflammatory cascades, especially in pulmonary inflammation (Chen et al., 2020; Ortiz-Muñoz et al., 2020). TRPC6 contributes to platelet activation, leukocyte transendothelial migration, lung vascular barrier disruption, and airway inflammation (Tauseef et al., 2012; Weber et al., 2015; Chen et al., 2020; Ortiz-Muñoz et al., 2020). In addition, activation of the TRPC6 channel promotes apoptosis in neonatal glomerular mesangial cells and renal tubular epithelial cells under different injury conditions (Soni and Adebisi, 2016; Hou et al., 2018). TRPC6 inhibits N-methyl-D-aspartate (NMDA)-mediated Ca<sup>2+</sup> elevation and reduces neuronal ischemic excitotoxicity (Li et al., 2012). Furthermore, inhibition of TRPC6 degradation in neurons through cAMP-response element binding protein (CREB) pathway alleviates ischemic cerebral insults (Du et al., 2010). (-)-Epigallocatechin-3-gallate, resveratrol, and neuroprotectin D1 contribute to neuroprotective effect on brain IR damage through TRPC6/CREB pathways (Lin et al., 2013b; Yao et al., 2013, 2014). In particular, Hyperforin, a selective TRPC6 agonist, alleviates cerebral IR injury by suppressing the degradation of TRPC6 and increasing phosphorylated CREB in Ca<sup>2+</sup>/calmodulin-dependent kinase IV (CaMKIV) signaling pathway (Lin et al., 2013a). Collectively, these



evidences suggest the vital role of TRPC6/CREB pathway in ischemic stroke. Studies have shown that TRPC6 channel is expressed in microglia and involved in microglial activation and neuroinflammatory response in different diseases including hypertension, epilepsy, Alzheimer's disease, infectious diseases and neuropathic pain (Toth et al., 2013; Lee et al., 2014; Liu et al., 2017; Shinjyo et al., 2020; Wang et al., 2020b). However, the relevance of the TRPC6 channel in IR injury-induced astrocytic apoptosis and inflammation remains unclear. In addition, other downstream mechanisms beyond TRPC6/CREB pathways are worth exploring.

Hyperforin, a main compound of St. John's wort, can activate the TRPC6 channel and induce changes in  $[Ca^{2+}]_i$  currents (Leuner et al., 2007); however, hyperforin is chemically

unstable when subjected to oxygen and light. HYP9 is a stable synthetic phloroglucinol derivative of hyperforin that maintains the biological function of hyperforin in activating the TRPC6 channel (Leuner et al., 2010). Consequently, HYP9 was used in the present study to explore its function in the TRPC6 channel and the associated downstream cellular mechanisms in ischemic stroke. Inflammatory responses and apoptosis in ischemic astrocytes were inhibited by maintaining the protein level of the TRPC6 channel *via* HYP9. Overexpression of TRPC6 also reduced IR injury-induced astrocytic apoptosis, cytotoxicity, and inflammatory responses in experimental stroke *in vitro*. Meanwhile, SKF is a TRPC channel blocker (Song et al., 2014; Jing et al., 2016). SKF also affects other channels belonging to the TRPC channel subfamily. Evidence shows that TRPC1, TRPC3,



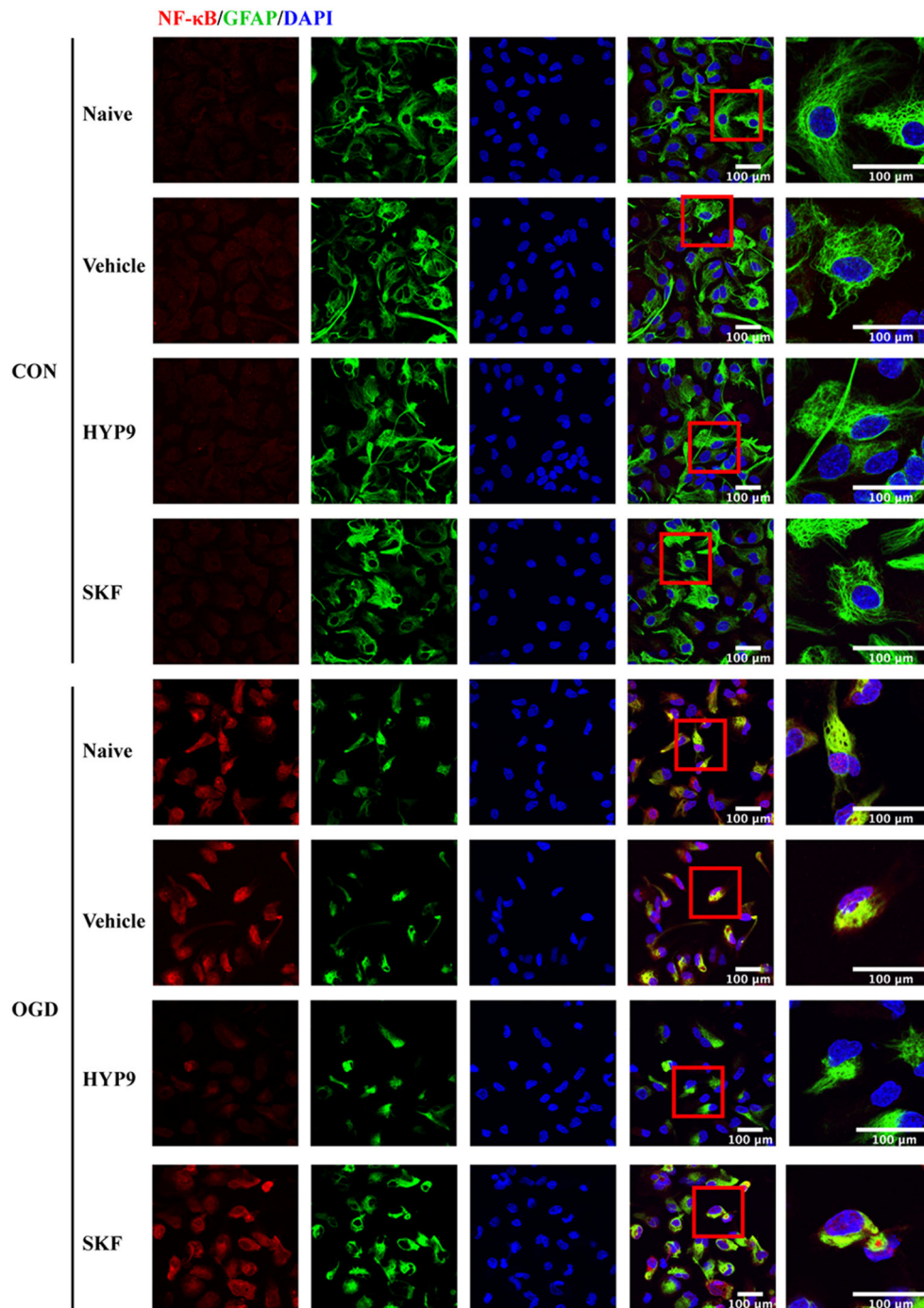
and TRPC7 are involved in cerebral ischemic stroke (Chen et al., 2017; Xu et al., 2018). In the present study, SKF aggravated astrocyte injury after ischemia *in vitro*. However, significant changes in infarct volume, cleaved caspase-3 expression, and cytokine release were not observed in the MCAO model after SKF application. The inconsistent results between cell and animal models of stroke may be due to the complex effects of SKF on cortical non-astrocytic cells. Noticeably, knocking down TRPC6 via sh-TRPC6 shown no significant effects on apoptosis, cytotoxicity, and inflammatory responses in primary astrocytes after OGD/R. Since TRPC6 is strikingly downregulated in astrocytes exposed to IR insults, knocking down of TRPC6 did not significantly aggravate astrocytic damage in OGD group. The dissimilar effects of SKF and sh-TRPC6 on ischemic astrocytes might be related to the side-effect of SKF on other TRPCs (TRPC3/7). Overall, further *in vivo* studies using astrocytic TRPC6-, siRNA-, or shRNA-targeted transgenic mice are needed to fully clarify the role of the astrocytic TRPC6 channel in stroke.

Activation of the cerebral inflammatory cascade has important roles in acute brain IR insults. The inflammatory response induced by IR injury directly affect the progress of cerebral

ischemia (Petrovic-Djergovic et al., 2016; Ramiro et al., 2018). Notably, astrocytes regulate the neuroinflammatory response by affecting the generation and release of diverse cytokines in ischemic stroke (Sofroniew, 2014). Astrocytes generate several pro-inflammatory cytokines and chemokines, including IL-6, IL-1β, tumor necrosis factor-α (TNF-α), C-C motif chemokine ligand (CCL)-2, CCL-5, CCL-7, C-X-C motif chemokine ligand (CXCL)-10, and vasoactive endothelial growth factor (John et al., 2005; Hamby et al., 2012; Sofroniew, 2015). Astrocyte-derived IL-6 and IL-1β are essential for secondary inflammatory damage in brain ischemia (John et al., 2005; Diaz-Cañestro et al., 2019). The pro-inflammatory nuclear transcription factor NF-κB belongs to the detrimental astrocytic signaling pathways (Colombo and Farina, 2016). NF-κB is involved in astrocyte inflammation and apoptosis (Su et al., 2019; Bai et al., 2020). Compelling evidence has shown that inhibition of NF-κB nuclear translocation or phosphorylation alleviates IR-mediated cytokine production and brain inflammation (Colombo and Farina, 2016; Liu et al., 2019; Xie et al., 2019; Bai et al., 2020). NF-κB activation is tightly modulated by various typical upstream signaling molecules, which include toll-like receptors, TNF receptors, IL-1 receptors, CAMKII, and stromal interaction molecule or Orai-mediated Ca<sup>2+</sup> signals (Verstrepen et al., 2008; Berry et al., 2018; Pires et al., 2018; Ye et al., 2019).

A crucial relationship exists between Ca<sup>2+</sup> and NF-κB signaling pathways (Wei et al., 2019). As a vital second messenger, Ca<sup>2+</sup> governs a variety of functions causing cellular pathological and physiological changes. Modulation of the Ca<sup>2+</sup> current has attracted interest as a promising medical strategy for ischemic stroke (Kalogeris et al., 2016; Secondo et al., 2018). The TRPC6 channel, a Ca<sup>2+</sup> permeable cation channel, has been associated with Ca<sup>2+</sup> entry and brain ischemic pathogenesis (Liu et al., 2020). In the present study, TRPC6 was dramatically downregulated during IR injury. The TRPC6-specific agonist HYP9 rescued TRPC6 degradation in primary cultured astrocytes and reduced [Ca<sup>2+</sup>]<sub>i</sub>. Consistently, the TRPC channel antagonist SKF contributed to increased [Ca<sup>2+</sup>]<sub>i</sub>. How a Ca<sup>2+</sup> entry activator reduces and a Ca<sup>2+</sup> channel blocker increases the Ca<sup>2+</sup> influx in primary astrocytes is notable. TRPC6 has been shown to block the NMDA receptor (NMDAR)-triggered [Ca<sup>2+</sup>]<sub>i</sub> increase and neurotoxicity in neurons after brain IR damage (Li et al., 2012). Overexpression of TRPC6 inhibits [Ca<sup>2+</sup>]<sub>i</sub> overload, prevents neuronal death, lessen infarct size, and improves behavior performance after ischemia. Similarly, another research team suggests that the TRPC6 channel selectively suppresses the NMDAR-mediated current in hippocampal neurons, which is increased *via* TRPC6 knockdown or interference with SKF (Shen et al., 2013). In another study, SKF increases intracellular Ca<sup>2+</sup> by promoting the reverse mode of the Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (Song et al., 2014). Thus, we hypothesized that the underlying mechanism of TRPC6-induced [Ca<sup>2+</sup>]<sub>i</sub> decrease in astrocytes after ischemic insults may be associated with the complex interaction between the TRPC6 channel and other ion exchangers.

Although the present study showed a protective role of the TRPC6 channel in astrocytes after ischemia, various questions remain unanswered. The effects of the astrocytic TRPC6

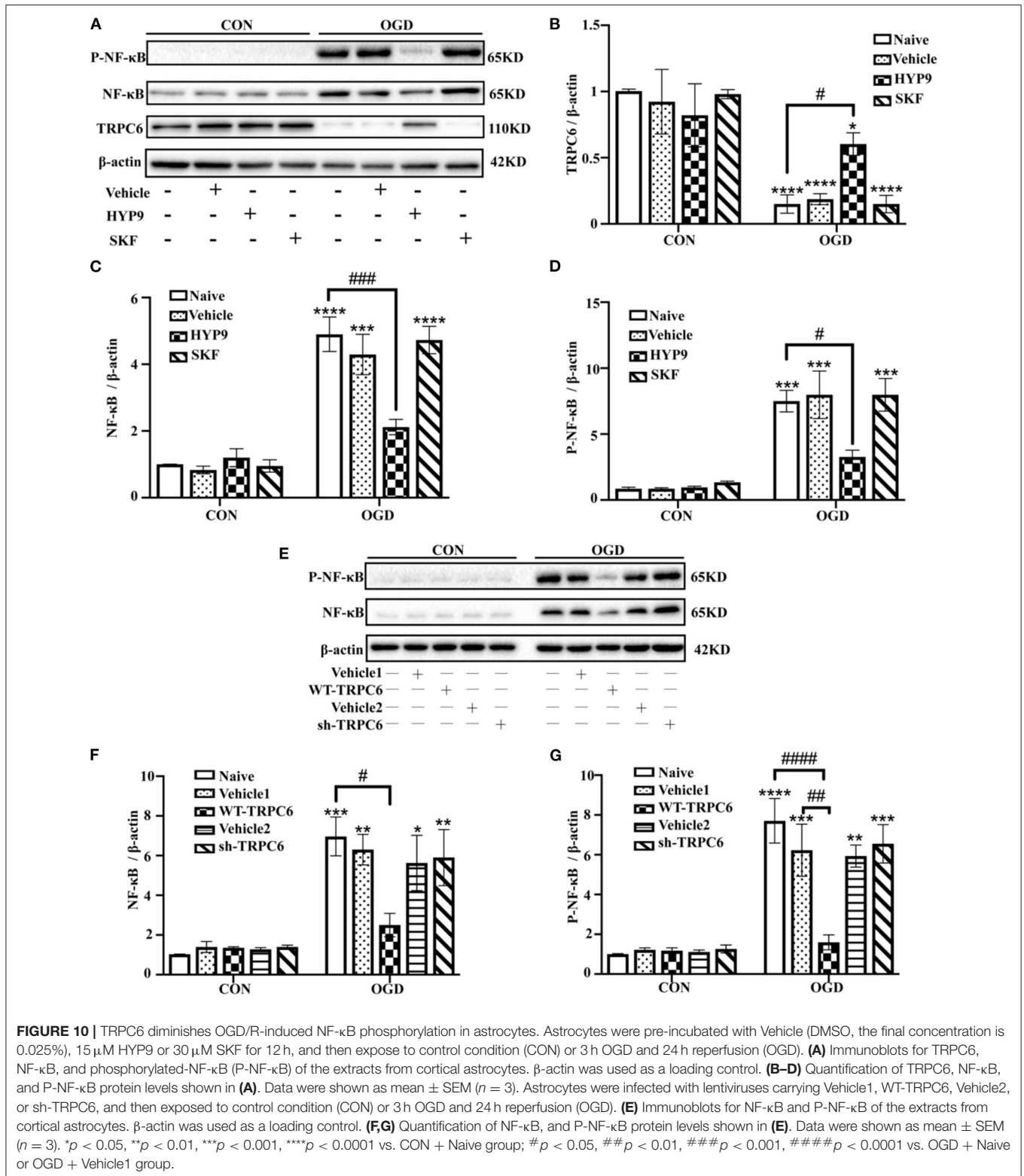


**FIGURE 9** | TRPC6 diminishes NF-κB nuclear translocation in astrocytes after ischemia. Astrocytes were pre-incubated with Vehicle (DMSO, the final concentration is 0.025%), 15 μM HYP9 or 30 μM SKF for 12 h, and then expose to control condition (CON) or 3 h OGD and 24 h reperfusion (OGD). Representative images of cortical astrocytes after OGD/R double-stained with the NF-κB (red) and GFAP (green) antibodies (Scale bar: 100 μm; 630× or 1,890× magnification).

channel on ischemic neurons will be addressed in our future work. Furthermore, the underlying mechanisms of TRPC6-triggered astrocytic  $Ca^{2+}$  changes in ischemic stroke warrant further investigations.

## CONCLUSION

The results of the present study indicate that HYP9 reduces the infarct size, apoptosis, and pro-inflammatory cytokine release in



a mouse model of ischemic stroke. Inhibition of TRPC6 channel downregulation *via* HYP9 or TRPC6 overexpression alleviates apoptosis and inflammatory response in astrocytes exposed to ischemic damage. Furthermore, the protective role of TRPC6 in

stroke is associated with  $Ca^{2+}$ /NF-κB-dependent pathways. The results of this study indicate that the astrocytic TRPC6 channel and TRPC6 agonist HYP9 might be a novel therapeutic approach to prevent ischemic stroke induced by brain injury.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The animal study was reviewed and approved by Animal Care Committee of the First Affiliated Hospital at Zhejiang University.

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## AUTHOR CONTRIBUTIONS

LL wrote the manuscript. LL, MC, and KL finished the experiment. SZ and XXio designed the general idea. All authors edited the drafts of the manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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