

TRPC3/6/7 Knockdown Protects the Brain from Cerebral Ischemia Injury via Astrocyte Apoptosis Inhibition and Effects on NF- κ B Translocation

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Abstract

Ischemia contributes significantly to morbidity and mortality associated with many common neurological diseases. Calcium overload is an important mechanism of cerebral ischemia and reperfusion (I/R) injury. Despite decades of intense research, an effective beneficial treatment of stroke remains limited; few therapeutic strategies exist to combat the consequences of cerebral ischemia. Traditionally, a “neurocentric” view has dominated research in this field. Evidence is now accumulating that glial cells, especially astrocytes, play an important role in the pathophysiology of cerebral ischemia. Here, we show that transient receptor potential (TRP)C3/6/7 knockout (KO) mice subjected to an I/R procedure demonstrate ameliorated brain injury (infarct size), compared to wild-type (WT) control animals. This is accompanied by reduction of NF- κ B phosphorylation and an increase in protein kinase B (AKT) phosphorylation in I/R-injured brain tissues in TRPC3/6/7 KO mice. Also, the expression of pro-apoptotic protein Bcl-2 associated X (Bax) is down-regulated and that of anti-apoptotic protein Bcl-2 is upregulated in TRPC3/6/7^{−/−} mice. Astrocytes isolated from TRPC3/6/7 KO mice and subjected to oxygen/glucose deprivation and subsequent reoxygenation (OGD-R, mimicking in vivo I/R injury) also exhibit enhanced Bcl-2 expression, reduced Bax expression,

enhanced AKT phosphorylation, and reduced NF- κ B phosphorylation. Furthermore, apoptotic rates of TRPC3/6/7 KO astrocytes cultured in OGD-R conditions were reduced significantly compared to WT control. These findings suggest TRPC3/6/7 channels play a detrimental role in brain I/R injury. Deletion of these channels can interfere with the activation of NF- κ B (pro-apoptotic), promote activation of AKT (anti-apoptotic), and ultimately, ameliorate brain damage via inhibition of astrocyte apoptosis after cerebral ischemia/reperfusion injury.

Keywords

Astrocyte Cerebral ischemia TRPC3/6/7 NF- κ B

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Introduction

Stroke is a central nervous system disease with the highest mortality and disability rates, which cause serious damage to human health. Stroke is mainly classified into hemorrhagic stroke and ischemic stroke. Over the past years, much effort has gone into prevention and treatment of stroke, such as thrombolysis, anti-coagulation, emergency intervention, and minimally invasive surgery, and greatly improved the prognosis of patients with stroke. But 75 % of the patients still suffer from varying degrees of sequelae. Recanalization that is promoted happens in about 30 % of ischemic stroke patients upon thrombolysis therapy. The progressive injury following stroke originates from complex pathologic mechanisms, including calcium overload, energy exhaustion, excitotoxicity, oxidative stress, inflammation, and apoptosis [1, 2, 3, 4, 5]. With respect to cell death, toxic intracellular accumulation of calcium plays an important role in the phenomenon of cerebral ischemia and reperfusion injury [6, 7, 8].

Astrocytes are the most abundant non-neuronal cell type in the central nervous system and constitute up to 50 % of total human brain cells. Neurons cannot survive in the brain without close interaction with astrocytes. Accordingly, astrocyte function can critically influence neuronal survival during ischemia and other brain insults [9, 10, 11, 12]. Several molecular pathways are involved in astrocyte apoptosis, such as Ca^{2+} overload, mitochondrial dysfunction, and oxidative stress. Thus, it is a very effective approach to protect the central nervous system in ischemia and reperfusion (I/R) injury by preventing astrocytes from cell apoptosis [13].

C-type of transient receptor potential (TRPC) channels belong to the TRP superfamily. The TRPC subfamily has seven members in mammals (TRPC1–7) which participate in store-operated calcium entry (SOCE) and/or receptor-operated calcium entry (ROCE) in various cell types, including astrocytes, and play a critical role in many cellular processes [14]. TRPC1–TRPC6 are expressed in cultured embryonic astrocytes [15, 16]. TRPC1 and TRPC3 likely contribute to SOCE in astrocytes, induced by endoplasmic reticulum depletion or metabotropic stimulation [17, 18]. TRPC6 is involved in ROCE in cultured embryonic cortical astrocytes [19]. The aim of this work was to explore the roles of TRPC3/6/7 subgroup in brain ischemic injury, specifically to examine the impact of the combined TRPC3/6/7 deletion on astrocyte survival in cerebral ischemia reperfusion injury.

Materials and Methods

Materials and Animals

Sources of reagents and antibodies included the following: Fura2-AM (Invitrogen), FBS (GIBCO), protease inhibitor cocktail (Roche), anti-p-NF- κ B (Ser-529) (Santa Cruz), anti-NF- κ B (Santa Cruz), anti-Bcl-2-associated X (Bax) (Cell Signaling), anti-Bcl2 (Cell Signaling), anti-cleaved caspase 3 (abcam), anti-phosphorylated protein kinase B (p-AKT) (Ser-473) (Cell Signaling), and anti-AKT (Cell Signaling), anti- β -actin (abcam).

TRPC3/6/7-deficient mice on a mixed C57BL/6J-129SvEv background were reconstituted from cryopreserved embryos. TRPC3/6/7 triple knockout (KO) parents were generated by crossing TRPC3 KO [20], TRPC6 KO [21], and TRPC7 KO [22] mice to the desired homozygosity at the Comparative Medicine Branch (CMB) of the National Institute of Environmental Health Sciences, North Carolina [20, 21]. Wild-type (WT) C57BL/6J/129SvEv mice also reconstituted from frozen embryos served as controls for the KO mice. Age-matched KO and WT controls were used for all studies. Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science). Animals were kept on a 12-h light-dark cycle in a temperature-controlled room with ad libitum access to food and water. All animal studies were approved by the Animal Care and Utilization Committee of Huazhong University of Science and Technology. Anesthetic procedures were used in full to ensure that animals do not suffer unduly during and after the experimental procedure. The genotype of TRPC3/6/7 triple KO was confirmed by PCR and RT-PCR as shown in Fig. 1a, b.

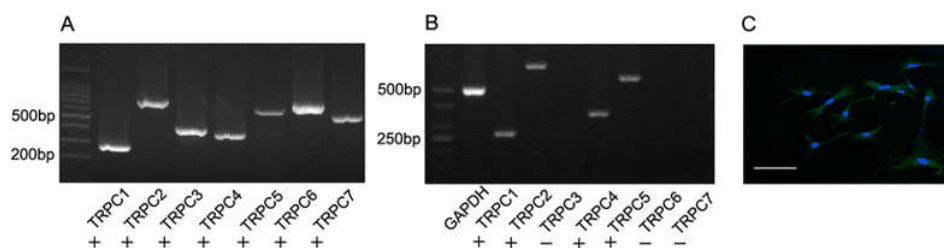


Fig. 1

TRPC3/6/7 gene knockout and positive control were confirmed by RT-PCR analysis of mRNA isolated from the mouse brain. **a** RT-PCR analysis for TRPC1–TRPC7 mRNA in WT mouse brain was used as a positive control. **b** RT-PCR analysis for TRPC1–TRPC7 mRNA in TRPC3/6/7 knockout mouse brain. **c** GFAP positive cells in primary cortical culture astrocytes. Astrocyte marker GFAP (*green*), DAPI (*blue*). *Scale bar c*: 50 μm

Surgical Procedures for Middle Cerebral Artery Occlusion

Focal cerebral ischemia was induced by occluding the middle cerebral artery (MCAO), as reported previously [23]. After animals were anesthetized with chloral hydrate, a nylon filament (10 mm total length and 0.22 ± 0.11 mm diameter) was inserted in the internal carotid artery. One hour later, the filament was removed to commence reperfusion for 24 h. Animals were distributed into groups as follows: WT sham group (control), WT model group, TRPC3/6/7^{-/-} sham group (control), and TRPC3/6/7^{-/-} model group. In the sham group, the external carotid artery was dissected, but the filament was not inserted.

Primary Astrocyte Culture

Primary astrocytes were isolated from the cerebral cortices of 3-day-old mice, as described previously [24]. Briefly, the dissociated cortical cells were suspended in DMEM-F12, containing 100 units/mL penicillin, 100 $\mu\text{g/mL}$ streptomycin, and 15 % fetal bovine serum. Cells were then placed into poly-L-lysine-coated culture flasks at a density 6×10^6 cells/cm². Astrocytes were obtained by the shaking method after 12–14 days of culture. Non-astrocytes, such as microglia and oligodendrocytes, were removed from the flasks. In these cultures, more than 98 % of the cells are positive for glial fibrillary acidic protein (GFAP), an astrocyte marker (Fig. 1c). Astrocytes in the flasks were dislodged with 0.25 % trypsin and plated in different culture dishes for further experiments.

The Oxygen and Glucose Deprivation Model

Astrocyte cultures were subjected to oxygen and glucose deprivation (OGD) followed by reoxygenation, to mimic the *in vitro* ischemic-like condition. For the OGD condition, the culture medium was replaced with glucose-free DMEM pretreated with 95 % N₂/5 % CO₂ atmosphere and cells were maintained in an hypoxic chamber (95 % N₂/5 % CO₂, 37 °C) for 12 h. Reoxygenation was achieved by placing OGD-treated cells in glucose-containing DMEM/F12 under normoxic condition for 4 h (OGD-reoxygenation (R)). Further *in vitro* analysis was performed with OGD-R-treated cell samples. Control astrocytes were maintained in a complete DMEM medium and incubated in a normoxic conditions throughout experiments (blank control). Astrocytes were assigned to four groups: WT control, WT OGD-R, TRPC3/6/7^{-/-} control, and TRPC3/6/7^{-/-} OGD-R.

Flow Cytometric Apoptosis Assay

Apoptosis rates were measured by flow cytometry using the AnnexinV-fluorescein isothiocyanate (FITC)/PI apoptosis detection kit (BioVision, USA), as described [25]. After treatment with or without OGD-R, cells were collected at a concentration of 1×10^5 cells/mL (total 8 mL for each), mixed with Annexin V-FITC and propidium iodide according to manufacturer's recommendation, and analyzed using a flow cytometer. Data were analyzed using the Cell Quest software (BD Biosciences, USA).

Modified Neurological Severity Score Tests

After 1 hour ischemic insult and 24 h reoxygenation, the neurological deficit scores of model animals were determined in a blinded manner according to the following graded scoring system: 0, no neurological deficit; 1, failure to extend left; 2, circling to the left side; 3, falling to the left side; and 4, did not walk independently and loss of consciousness [26].

2,3,5-Triphenyltetrazolium Chloride Staining

Mice were anesthetized with chloral hydrate and decapitated after 24 h of reperfusion. The brains were quickly removed and chilled at -20 °C for 10 min and then, five consecutive 1 mm-thick slices were cut with a metallic brain matrix. The brain slices were stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) at 37 °C for 15 min in dark and then fixed in 4 % paraformaldehyde for 1 h. The unstained area of the brain slice was defined as infarction, whereas normal tissue was stained as red; infarct volume ratio was measured and calculated as described [27].

Quantitative PCR

After reverse transcription of total RNA to cDNA using PrimeScript RT Reagent Kit (TaKaRa), real-time quantitative PCR was performed using the StepOne Real-Time PCR system (Applied Biosystems) in a final volume of 20 µL containing 0.5 µg of total RNA with Power SYBR Green PCR Master Mix (Applied Biosystem). Primer

pairs used for each gene are as follows: β -actin, 5'-CTGAGAGGGAAATCGTGCGT-3' and 5'-CCACAGGATTCCATACCCAAGA-3'; Bax, 5'-AGACAGGGGCCTTTTGTGCTAC-3' and 5'-AATTCGCCGGAGACACTCG-3'; and Bcl-2, 5'-CCGGGAGAACAGGGTATGATAA-3' and 5'-CCCACTCGTAGCCCCTCTG-3'. The temperature cycles were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The results were analyzed with Primer Express software (Applied Biosystem). The identity of the PCR product was confirmed by automated determination of the melting temperature of the PCR products. The results for each gene were normalized relative to β -actin messenger RNA (mRNA) levels measured in parallel in each sample.

Immunofluorescence

After fixation with 4 % paraformaldehyde, astrocytes cells were permeabilized with 0.1 % Triton-X and blocked with 3 % BSA in PBS for 30 min; then cells were incubated with anti-p65 and GFAP monoclonal antibodies overnight at 4 °C, washed in PBS for three times (10 min each time), and followed by incubation with goat anti-rabbit FITC. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) in PBS for 5 min. All slides were examined with an epifluorescence microscope (Olympus FV500).

Western Blot Analysis

Lysates from cultured astrocytes cells and brain tissues were prepared at 4 °C. Fifty micrograms of total proteins were resolved in SDS-PAGE and then transferred to a nitrocellulose membrane. Membranes were blocked with 5 % skim milk in TBS-T and incubated with the respective primary antibody (AKT, p-AKT, NF- κ B, p-NF- κ B, Bcl-2, Bax, β -actin) for 24 h at 4 °C. Then, membranes were detected with conjugated goat secondary antibody and signals were detected with an ECL kit.

Ca²⁺ Imaging

For measurement of [Ca²⁺]_i, astrocytes were cultured on polylysine-coated coverslips for 24 h and loaded with 2 μ M Fura2-AM by incubation for 35 min in a complete culture medium at 37 °C, then washed with Hepes buffered saline solution (HBSS) twice at room temperature. The HBSS contained (in mM) 140 NaCl, 5 KCl, 1MgCl₂, 2 CaCl₂, 10 glucose, and 10 HEPES (pH 7.4). [Ca²⁺]_i in individual cells was monitored with an Olympus IX51 inverted epifluorescence microscope and Slidebook software, using excitation wavelengths of 340 and 380 nm to detect Fura2/FuraCa fluorescence emission at 510 nm [28].

Statistical Analysis

Data are given as mean \pm standard deviation (S.D.). Student's *t* test was used to compare between two groups. Statistical differences were considered significant when $P < 0.05$.

Results

Decreased Infarct Volume and Neurological Deficits in TRPC3/6/7^{-/-} Mice

To decipher the function of the TRPC3/6/7 channels in cerebral I/R injury, we established the MCAO model with TRPC3/6/7 knockout mice and their wild-type control counterparts. Five consecutive TTC-stained coronal brain slices were arranged in cranial to caudal order (Fig. 2). The white brain area represents infarcted brain tissue. The TRPC3/6/7^{-/-} group had smaller infarct volumes compared to the WT group ($P < 0.01$, $n = 5$). Sham groups showed no obvious infarction. Also, the neurological deficits were assessed 24 h after I/R. The neurological scores in the TRPC3/6/7^{-/-} group were significantly reduced compared with those found in the WT group ($P < 0.05$, $n = 5$). Using this animal model system, we observed that (1) infarction volume (size) is reduced in TRPC3/6/7^{-/-} mice compared to that of WT mice and (2) motor behavior deficit is attenuated in TRPC3/6/7^{-/-} mice.

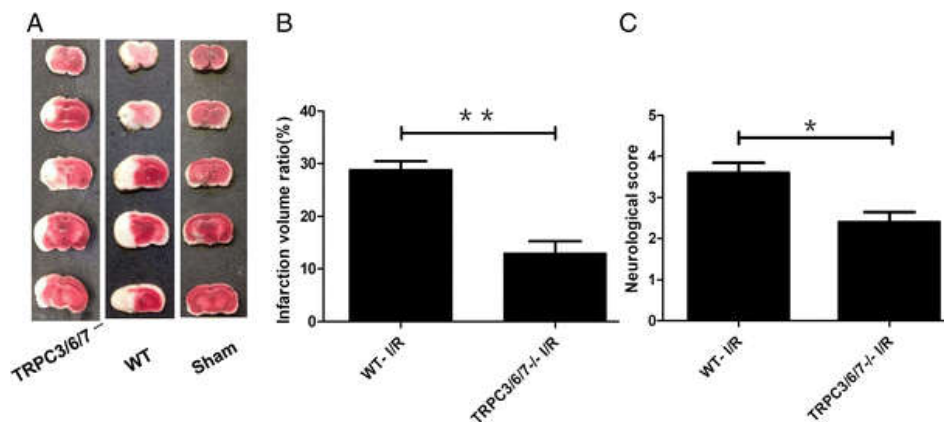


Fig.

Infarct volume is reduced and neurological deficits are ameliorated in TRPC3/6/7 KO mice. **a** TTC staining of the brain for each group. **b** The infarct volume was measured using the ImageJ analysis software. Values are expressed as mean \pm S.D. ($n = 5$); $P < 0.01$ between the WT group and

TRPC3/6/7^{-/-} group. **c** Evaluation of the neurological deficit was performed after occlusion of the MCA for 1 h followed by reperfusion for 24 h. The values are expressed as mean \pm S.D. ($n = 5$). $P < 0.05$ WT group vs TRPC3/6/7^{-/-} group

Apoptosis of Primary Cultured Astrocytes Is Diminished in TRPC3/6/7^{-/-} Mice

To test the impact of OGD-R on astrocyte apoptosis, astrocytes isolated from WT and TRPC3/6/7^{-/-} mice were subjected to the OGD-R procedure, followed by FACS analysis. Cells appeared in the right upper and right lower quadrants of a dot plot represent all apoptotic cells. Percentage of apoptotic cells in the WT/OGD-R group was 60.4 % (Fig. 3a). Compared to the WT/OGD-R group, astrocytes from the TRPC3/6/7^{-/-}/OGD-R group exhibit a much lower apoptotic rate (36.7 %, Fig. 3a, b). To confirm this observation, cleaved caspase-3, a pro-apoptotic protein, was profiled by Western blot. As shown in Fig. 3c, d, astrocytes from the TRPC3/6/7^{-/-}/OGD-R group demonstrated reduced cleaved caspase-3 expression, compared to that in the WT/OGD-R group.

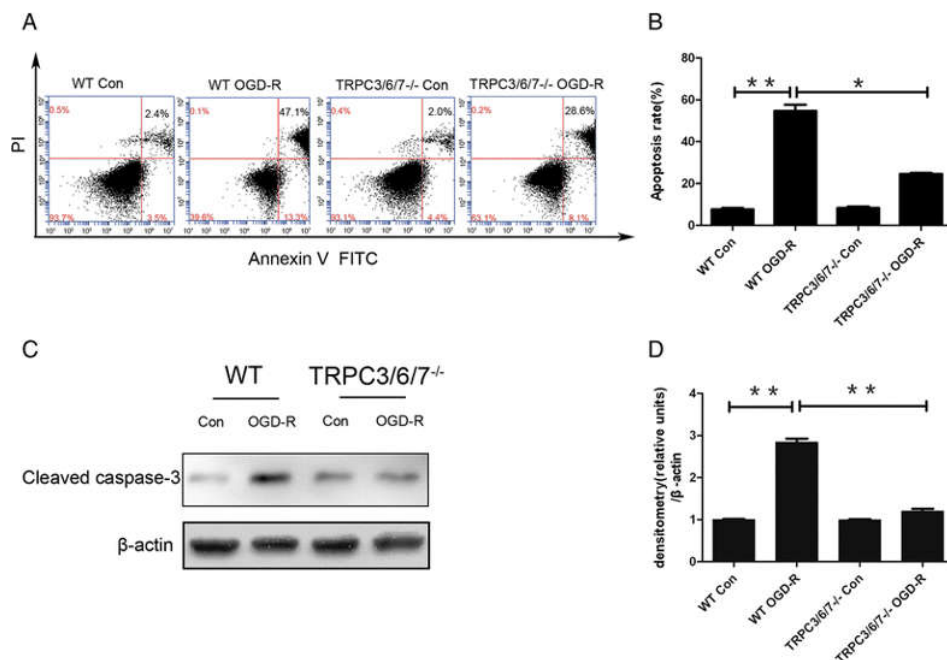


Fig.

Decreased cell susceptibility to OGD-R in TRPC3/6/7^{-/-} astrocytes. **a** WT and TRPC3/6/7^{-/-} cells were subjected or not to OGD-R, and their apoptotic status was analyzed by flow cytometry. **b** Data are shown as mean \pm S.D. ($n = 3$). $P = 0.01$, $P = 0.05$ by two-tailed Student's *t* test. **c** Western blot of cleaved caspase3 expression. **d** Densitometry of cleaved caspase3, expression normalized with respect to actin of blots such as shown in **c**. Data are presented as mean \pm S.D. ($n = 3$). $P = 0.01$ between TRPC3/6/7^{-/-} OGD-R and WT OGD-R by two-tailed Student's *t* test

Expression of Apoptosis-Related Proteins and mRNA Is Reduced by Loss of TRPC3/6/7 Channels both In Vivo and In Vitro

Bcl-2 and Bax belong to the Bcl-2 protein family; Bcl-2 promotes cell survival, whereas Bax accelerates apoptosis. In our in vitro study, Western blot analysis of astrocytes from the WT/OGD-R group showed that the expression level of pro-apoptotic Bax protein was significantly increased compared to the control group (Fig. 4e, f), but astrocytes from the TRPC3/6/7^{-/-}/OGD-R group exhibited significantly diminished Bax expression (Fig. 4e, f). In contrast, expression of the anti-apoptotic protein Bcl-2 was reduced in astrocytes from the WT/OGD-R group (Fig. 4e, f) but enhanced in astrocytes from the TRPC3/6/7^{-/-}/OGD-R group (Fig. 4e, f). mRNA levels of Bax and Bcl-2 in these groups of astrocytes showed expression profiles similar to the protein expression patterns (Fig. 4h). Statistical analysis of the Bcl-2/Bax ratio, an index of anti-apoptosis, revealed that the OGD-R induced a dramatic reduction in the Bcl-2/Bax ratio in WT astrocytes and that such a reduction was ameliorated in TRPC3/6/7 KO cells (Fig. 4g).

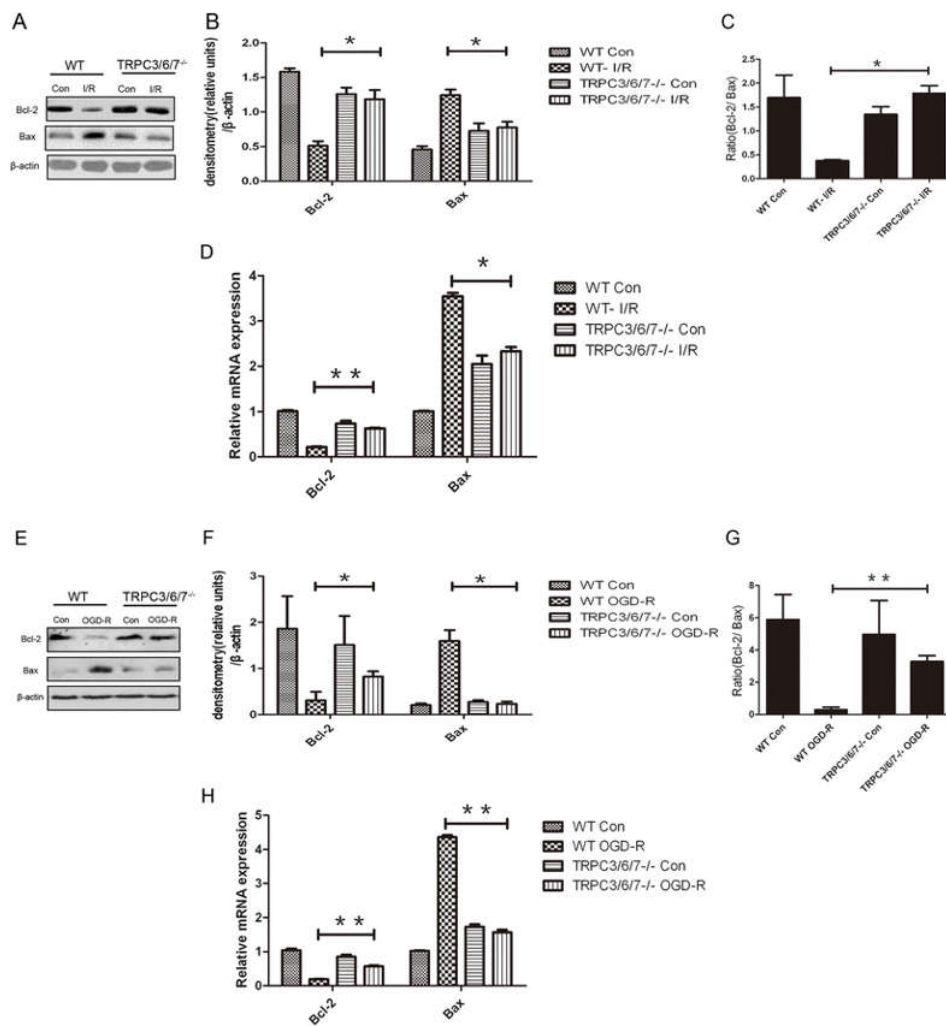


Fig.

Effect of TRPC3/6/7 ablation on MCAO and OGD-R induced changes in Bcl-2 and Bax expression. **a** Western blot of Bcl-2 and Bax expression. **b** Densitometry of Bax and Bcl-2, expression normalized with respect to actin of blots such as shown in **a**. Data are presented as mean \pm S.D. (n = 3). $P < 0.05$ between the TRPC3/6/7^{-/-} group and the WT group by two-tailed Student's *t* test. **c** Quantitation of Bcl-2/Bax data. $P < 0.05$, TRPC3/6/7^{-/-} I/R injury model

group compared to WT I/R injury model group. **d** Real-time PCR analysis of Bcl-2 and Bax relative mRNA expression. **e** Western blot of Bcl-2 and Bax expression. **f** Densitometry of Bax and Bcl-2, expression normalized with respect to actin of blots such as shown in **e**. Data are presented as means \pm S.D. ($n = 3$). $P < 0.05$ between TRPC3/6/7^{-/-} OGD-R and WT OGD-R by two-tailed Student's *t* test. **g** Quantitation of data as Bcl-2/Bax ratios. $P < 0.01$, TRPC3/6/7^{-/-} OGD-R compared to WT OGD-R. **h** Real-time PCR analysis of Bcl-2 and Bax relative mRNA expression

Western blot analysis of brain tissues from the MACO model demonstrated similar pattern changes of Bcl-2 and Bax. WT MCAO mice showed increased Bax expression and decreased Bcl-2 protein expression (protein in Fig. 4a, b; mRNA in Fig. 4d), whereas brain tissues from TRPC3/6/7^{-/-} MACO mice exhibited a clear increase in Bcl-2 and unnoticeable changes in Bax. Also, the ratio of Bcl-2/Bax was visibly increased in the TRPC3/6/7^{-/-} MACO mice, compared to WT MACO animals (Fig. 4c).

p-AKT Levels Are Increased and p-NF- κ B Levels Are Decreased in I/R-Subjected TRPC3/6/7^{-/-} Cells, both In Vivo and In Vitro

To determine the role of TRPC3/6/7 in AKT and NF- κ B signaling following OGD-R treatment in astrocytes, we analyzed the protein levels of p-AKT and p-NF- κ B. The OGD-R procedure increased NF- κ B phosphorylation and decreased AKT phosphorylation in WT astrocytes (Fig. 5c, d). The MACO surgery generates similar results in WT animals (Fig. 5a, b). But in TRPC3/6/7^{-/-} astrocytes, OGD-R induced marginal changes in NF- κ B phosphorylation and increased AKT phosphorylation. Similarly, MACO surgery also enhanced AKT phosphorylation in TRPC3/6/7^{-/-} mouse brain tissues and did little to NF- κ B phosphorylation (Fig. 5a, b).

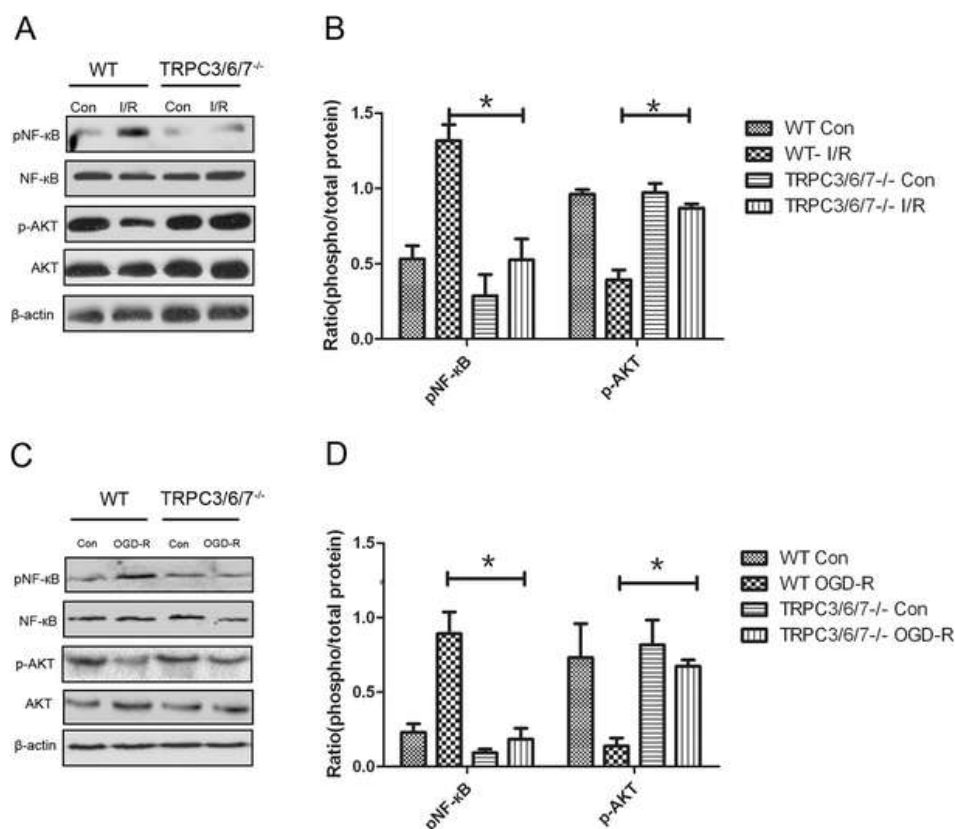


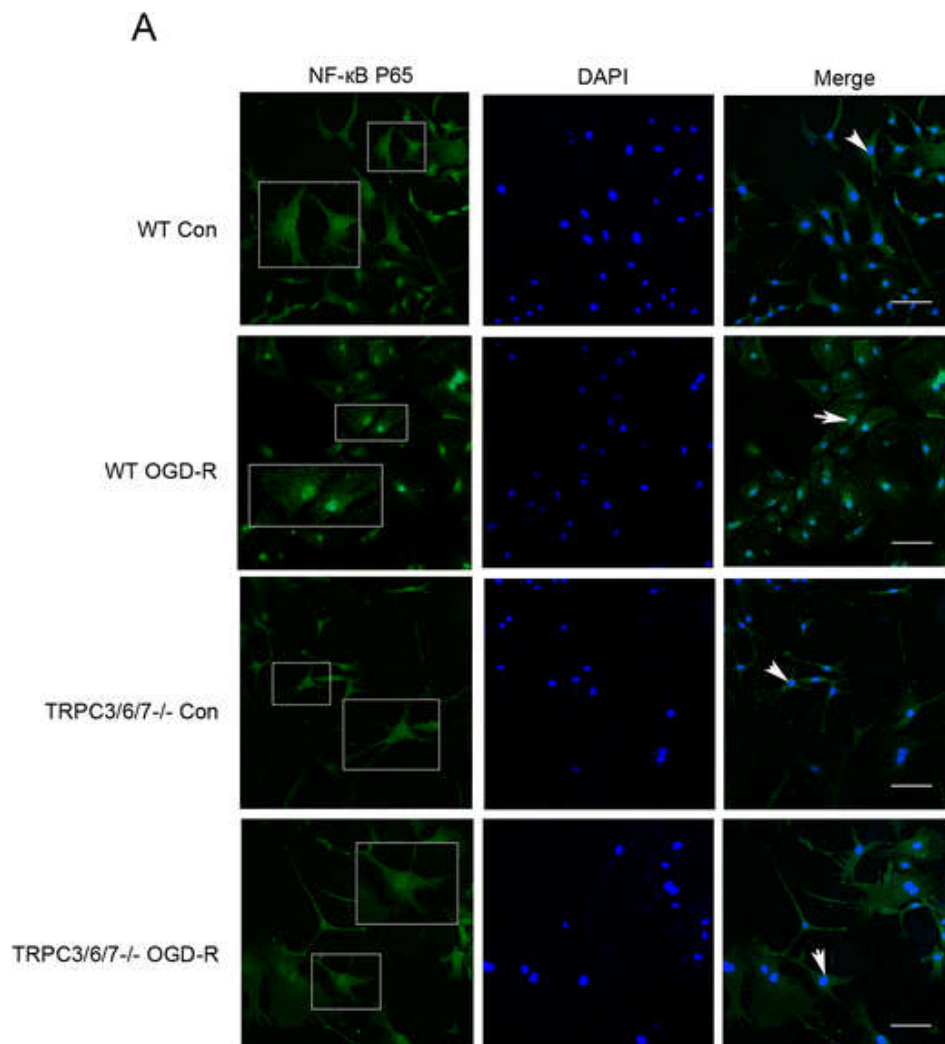
Fig.

Effect of TRPC3/6/7 on expression of p-NF-κB and p-AKT protein. **a** Western blot of p-NF-κB, p-AKT, total NF-κB, and total AKT, and of the corresponding β-actin, used as loading control. **b** Phosphoprotein levels as fraction of total. Data are presented as mean ± S.D. ($n = 3$). $P < 0.05$ between TRPC3/6/7^{-/-} I/R injury model and WT I/R injury model by two-tailed Student's *t* test. **c** Western blot of p-NF-κB, p-AKT, total NF-κB, and total AKT, and of the corresponding β-actin, used as loading control. **d** Phosphoprotein levels as fraction of total. Data are presented as

mean \pm S.D.($n = 3$). $P < 0.05$ between TRPC3/6/7^{-/-} OGD-R and WT OGD-R by two-tailed Student's *t* test

Loss of TRPC3/6/7 Attenuated NF- κ B Activation and Nuclear Translocation Induced by OGD-R

We investigated whether loss of TRPC3/6/7 could interfere with OGD-R-induced NF- κ B activation and nuclear translocation. Nuclear translocation of p65 was detected by immunofluorescence staining. OGD-R promoted massive nuclear translocation of p65, a subunit of NF- κ B in WT astrocytes (Fig. 6). But this translocation of p65 into nuclei was significantly compromised in TRPC3/6/7^{-/-} astrocytes upon OGD-R treatment (Fig. 6).



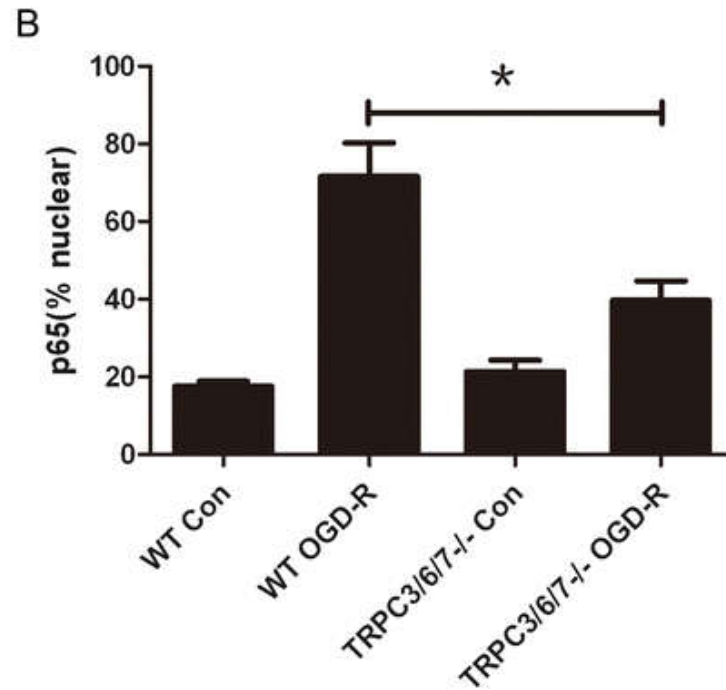


Fig.

OGD-R-induced alterations in cytoplasmic-nuclear distribution of the p65 subunit of NF- κ B in astrocytes. Cells were pretreated with vehicle or in OGD-R condition and analyzed by immunofluorescence microscopy to determine cells with p65 (*green fluorescence*) subunit present within their nuclei (labeled with DAPI, *blue fluorescence*). **a** OGD-R increased the trafficking of the p65 subunit of NF- κ B into the nucleus. The *arrowhead* indicated p65 subunit of NF- κ B in the nucleus. *Scale bar*: 50 μ m. **b** Quantification of the p65 subunit of NF- κ B in the nucleus, positive cells of total cells in each region.

The p65 subunit of NF- κ B in the nucleus decreased in TRPC3/6/7^{-/-} OGD-R compared to WT OGD-R, $P = 0.05$

TRPC3/6/7 Participates in Ca²⁺ Release and Ca²⁺ Influx in Astrocytes Induced by OGD-R

The effect of the OGD-R procedure on SOCE in WT and TRPC3/6/7^{-/-} astrocytes was examined using ratiometric fluorescence video microscopy of Fura2-loaded cells as described previously [29, 30, 31]. By comparing the second peaks of each trace, which represents the influx of free calcium into cells, we observed that the ER SERCA pump inhibitor thapsigargin induced a SOCE that is significantly lower in OGD-R-treated TRPC3/6/7 KO astrocytes than that in OGD-R-treated WT astrocytes ($P = 0.01$) (Fig. 7). This observation implies that TRPC3/6/7 channels participate in the OGD-R-induced calcium influx in astrocytes.

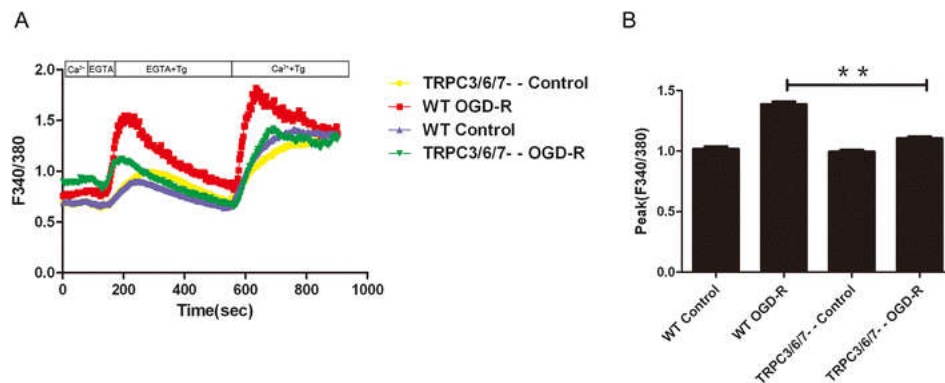


Fig.

OGD-R increased, and ablation of TRPC3/6/7 prevented the OGD-R-induced increase of Tg-induced SOCE in primary astrocytes. **a** Time course of Tg-induced intracellular Ca²⁺ changes (intracellular Ca²⁺ was monitored with the Fura2 Ca²⁺ sensitive fluorescent dye). **b** Peak 340/380 ratios $P = 0.01$, TRPC3/6/7 KO astrocytes compared to WT astrocytes

Discussion

Stroke triggers a complex cascade of pathophysiologic events in the brain that ultimately lead to cell death and infarction. Several molecular pathways are involved, such as calcium overload, oxidative stress, excitotoxicity, and apoptotic-like cell death. The abovementioned processes interact with each other and form a complex regulatory network. Calcium overload is an important mechanism of cerebral ischemia and reperfusion injury.

The past decade has been a period of rapid development in stroke research. Several different mechanisms have been implicated in ischemia-induced cell death, including excitotoxicity, oxidative stress, and inflammation, apoptosis, and cell death. In the process of cell death, intracellular accumulation of Ca^{2+} plays an important role. Past studies have provided ample of evidence indicating that glutamate receptors are the major trigger for Ca^{2+} influx in neurons following stroke. Recently, TRP channels have been shown to regulate Ca^{2+} homeostasis and to be involved in pathophysiology of stroke [6, 7, 8].

The mammalian TRP channel superfamily has approximately 30 members that are grouped into six subfamilies: TRPA, TRPC, TRPM, TRPML, TRPP, and TRPV. These channels are widely expressed in all cell types and tissues, They play a critical role in many cellular processes by changing cytosolic free Ca^{2+} concentrations [32]. TRPC channels are highly expressed in some regions of the brain. Within the seven members of the TRPC family (TRPC1–7), TRPC1 forms heteromers with TRPC4 or TRPC5. Similarly, TRPC3, TRPC6, and TRPC7 coassociate to form heteromers [33].

For decades, cerebral ischemia research was mainly focused on neuronal cells. It is a rather recent concept that astrocytes play significant roles in the demise of brain tissue after cerebral ischemia, in addition to protecting brain function and enhancing survival and regeneration under these conditions [9, 12]. Subtle and temporal regulation of astrocyte functions after stroke will undoubtedly impact the survival of neurons. TRPC1–TRPC6 are expressed in cultured embryonic astrocytes [15, 16]. The present study was designed to elucidate the role of the TRPC3/6/7 group of TRPC channels in brain ischemic injury, focusing on astrocytes in cerebral ischemia reperfusion injury.

To decipher the function of TRPC3/6/7 subfamily in cerebral ischemia/reperfusion injury in vivo, we established middle cerebral artery occlusion (MCAO)/reperfusion model with TRPC3/6/7 knockout mice and wild-type control mice. Using this animal model system, we found that (1) infarction volume (size) is reduced in TRPC3/6/7 KO mice compared to that in WT mice and (2) motor behavior deficit is ameliorated in TRPC3/6/7 KO mice. Western blot analysis revealed that MCAO followed by reperfusion enhances the phosphorylation status of NF- κ B and decreases that of AKT, increases Bax expression, and reduces Bcl-2 levels in cerebral cortex of WT

mice as seen 24 h after reperfusion. In contrast to WT mice, these changes were blunted in TRPC3/6/7^{-/-} mice ($P = 0.05$). The Bcl-2/Bax ratio was elevated 24 h after reperfusion in TRPC3/6/7 KO mice compared to WT mice.

To investigate whether the protection afforded by loss of TRPC3/6/7 cerebral ischemia injury is associated with parallel changes in astrocytes, we next studied primary astrocytes isolated from both KO and WT mice and subjected to OGD-R (mimicking the ischemia/reperfusion situation). Western blot analysis showed that the OGD-R protocol enhances the phosphorylation status of NF- κ B, increases Bax expression, and reduces Bcl-2 levels in astrocytes from WT mice. In contrast, the absence of TRPC3/6/7 markedly prevented the OGD-R-induced upregulation of phosphorylation status of NF- κ B, the increase in Bax levels, and the decrease in Bcl-2 levels ($P = 0.05$). Loss of TRPC3/6/7 prevented much of the OGD-R-induced apoptosis in astrocytes. These findings provide the evidence that loss of TRPC3/6/7 has neuroprotective activity on cerebral ischemia/reperfusion injury, and this effect may be attributable to an anti-apoptotic effect via inhibition of NF- κ B phosphorylation and promotion of AKT phosphorylation.

The TRPC family proteins can be responsible for SOCE and ROCE in a variety of cell types [34]. According to recent findings, Ca²⁺ entry through receptor-operated Ca²⁺ channels (ROCs) and store-operated Ca²⁺ channels (SOCs) contributes to shape cytoplasmic Ca²⁺ signals in astrocytes [15, 17, 35].

Astrocytes can remove glutamate from the synaptic cleft and thereby prevent Glu-induced excitotoxicity and associated elevations in intracellular Ca²⁺ due to activation of metabotropic glutamate receptors. These cells thus play an active, central role in brain physiology [36].

Although several members of the TRPC family of channels have been shown to play roles in astrocytic Ca²⁺ responses by acting as Ca²⁺ entry pathways across the plasma membrane [16, 19, 37, 38], there are only a few reports revealing the relationship between the physiological and pathophysiological functions of astrocytes and TRPC channel-mediated Ca²⁺ entry. Supporting a role for the TRPC channel in astrocyte-associated dysfunctions of the brain, the pro-inflammatory cytokine IL-1 can cause TRPC6-dependent changes in [Ca²⁺]_i in mouse astrocytes [18, 19]. Shirakawa [18] found that selective inhibition of TRPC3 attenuated thrombin-induced Ca²⁺ responses and consequent cellular responses in cultured cortical rat astrocytes.

In our studies, we examined SOCE in primary astrocytes isolated from both KO and WT mice. Using Fura2 to monitor intracellular calcium changes, we observed that Tg-induced SOCE in astrocytes subjected to OGD-R is significantly higher than in control astrocytes and that this increase is absent in TRPC3/6/7 KO astrocytes ($P = 0.01$). This adds to the list of finding that relates TRPC channels to astrocyte function.

Cerebral I/R triggers several molecular mechanisms including apoptosis and causes irreversible damages to cerebral tissues including astrocytes, and we hypothesized, TRPC3, C6, and/or C7 could participate in this pathological process. If so, ablation of

these channels should reduce the infarct size and cell apoptosis and decrease astrocyte apoptosis *in vitro*. This we found. We looked into the underlying mechanisms of TRPC3/6/7 ablation associated with astrocyte protection in cerebral I/R, which had not been studied previously. Astrocytes are the most numerous cell type in the higher mammalian nervous system. They play key roles in modulate synaptic activity and structural, trophic, and metabolic support to neurons. Accordingly, impairment of astrocyte functions during brain ischemia and other insults are likely to influence neuronal survival and plasticity [39]. AKT and Bcl-2 play vital roles in regulating the intracellular apoptotic signaling pathways. The PI3K/AKT signaling system is an upstream mediator of the Bcl-2 protein family. AKT phosphorylates Bad to suppress apoptosis, which then inactivates its inhibitory effects on the anti-apoptotic protein Bcl-2 and eventually inhibits the release of cytochrome c [40]. However, the level of p-AKT protein is decreased after cerebral I/R injury, implying a decrease of anti-apoptotic activity in the brain [41]. Indeed, enhancing phosphorylation of AKT and Bcl-2 expression or preventing downregulation of Bcl-2 is beneficial in cerebral I/R or stroke situations [42]. In the present study, increased tissue p-AKT and Bcl-2 in TRPC3/6/7 KO mice probably accounts for the improved neurological deficit scores and reduced infarction volume. Bcl-2 and p-AKT expression were also upregulated in TRPC3/6/7^{-/-} astrocytes upon OGD-R treatment *in vitro*. Our data imply that removal of TRPC3/6/7 attenuates astrocyte apoptosis probably by increasing the phosphorylation status of AKT and hence activating Bcl-2 expression.

NF-κB plays an important role in TRPC expression in many cell types. Tiruppathi showed that tumor necrosis factor (TNF) induces the NF-κB-dependent upregulation of TRPC1 in human umbilical vein endothelial cells (HUVECs) [43]. Thrombin-induced TRPC1 upregulation in HUVECs was due to the increase of NF-κB transcriptional activity by Ca²⁺ signaling in a feedforward manner and subsequent protein kinase C (PKC) activation [44]. TRPC3 is involved in astrogliosis after stroke. It has been shown that thrombin can initiate astrogliosis in cultured rat and human astrocytes and upregulate TRPC3 expression via nuclear factor-κB, extracellular signal-regulated protein kinase (ERK), protease-activated receptor 1 (PAR-1), and c-JunNH2-terminal kinase signaling pathways [18, 38]. We observed in this study that ischemia/reperfusion enhanced NF-κB phosphorylation and nuclear translocation in WT astrocytes, whereas TRPC3/6/7 deletion diminishes these NF-κB changes. Also these NF-κB changes are positively correlated with astrocyte apoptosis. Whether and how NF-κB activation regulates astrocyte apoptosis remains to be determined in the future studies.

Our results showed that disruption of TRPC3/6/7 subgroup of TRPC channels exerts a major protective effect against cerebral injury initiated by ischemia/reperfusion in mice, at least at the level of astrocytes during the cerebral ischemic process. In conclusion, astrocyte apoptosis was a major injury in cerebral I/R injury. Disruption of the TRPC3/6/7 could reduce the apoptosis of astrocytes in cerebral I/R injury. The recovered astrocytes provided helpful repairing conditions thus accelerating the recovery of insulted brain functions. Therefore, a TRPC-directed intervention offers great promise for recovery therapies of cerebral ischemia in patient after stroke.

Notes

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Compliance with Ethical Standards

Animals were treated in compliance with the Guide for the Care and Use of Laboratory Animals (National Academy of Science). All animal studies were approved by the Animal Care and Utilization Committee of Huazhong University of Science and Technology.

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