Original Paper

TRPC1 Deficiency Exacerbates Cerebral Ischemia/Reperfusion-Induced Neurological Injury by Potentiating Nox4-Derived Reactive Oxygen Species Generation

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Key Words

Ischemia/reperfusion injury • Neurological dysfunction • Reactive oxygen species • NADPH • Nox4 • TRPC1

Abstract

Background/Aims: Transient receptor potential cation channel 1 (TRPC1)-mediated the calcium (Ca^{2+}) influx plays an important role in several brain disorders. However, the function of TRPC1 in ischemia/reperfusion (I/R)-induced neurological injury is unclear. **Methods:** Wild-type or TRPC1 knockout mice underwent middle cerebral artery occlusion for 90 min followed by 24 h of reperfusion. In an *in vitro* study, neuronal cells were treated with oxygen–glucose deprivation and reoxygenation (OGD/R) to mimic I/R. The intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ was measured by Fura 2-AM under a microscope. Cerebral infarct volume was measured by triphenyltetrazolium chloride staining. Neurological function was examined by neurological severity score, Morris water maze test, rotarod test and string test. Oxidative parameters were detected by malondialdehyde, glutathione peroxidase, and superoxide dismutase commercially available kits. The protein expression levels of TRPC1, Nox4, p22phox, p47phox, and p67phox were analyzed by western blotting. **Results:** Brain tissues from cerebral I/R mice showed decreased TRPC1 expression. Similarly, TRPC1 expression was reduced in HT22 cells upon exposure to OGD/R treatment, followed by decreased Ca^{2+} influx. However, TRPC1 overexpression reversed the OGD/R-induced decrease in $[\text{Ca}^{2+}]_i$. TRPC1 knockout significantly exacerbated I/R-induced brain infarction, edema, neurological severity score, memory impairment, neurological deficits, and oxidative stress. In contrast, TRPC1 upregulation inhibited the increase in reactive oxygen species (ROS) generation induced by OGD/R. Analysis of key subunits of the Nox family and mitochondrial ROS revealed that the effects of TRPC1 downregulation on oxidative stress were associated with activation of Nox4-containing NADPH oxidase. TRPC1 interacted with Nox4 and facilitated Nox4 protein

degradation under OGD/R conditions. In addition, TRPC1 inhibition potentiated the OGD/R-induced translocation of p47phox and p67phox as well as the interaction between Nox4 and p47phox or p67phox, whereas TRPC1 overexpression had the opposite effects. **Conclusion:** TRPC1 deficiency potentiates ROS generation via Nox4-containing NADPH oxidase, which exacerbates cerebral I/R injury. TRPC1 may be a promising molecular target for the treatment of stroke.

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Introduction

Brain ischemic stroke is one of the leading causes of human disability and mortality worldwide [1]. An estimated 2.5 million people suffer from stroke in China per year, with 1 million people dying from stroke-related disability [2]. Blood flow restoration in the ischemic area is often used in the clinic to treat stroke patients [3]. Unfortunately, reperfusion itself often produces additional injuries in the ischemic brain, and effective treatments for ischemia and reperfusion (I/R) injury are lacking [2, 3]. The potential mechanisms of I/R are complicated and involve oxidative stress, calcium overload, inflammation, and neuronal apoptosis [4-6]. Oxidative stress is the major process that closely associated with neurological injury [6, 7]. Thus, inhibition of the increase in oxidative stress level would be an ideal therapeutics for I/R injury.

Reactive oxygen species (ROS)-induced neurological dysfunction is associated with an altered intracellular calcium (Ca^{2+}) concentration ($[\text{Ca}^{2+}]_i$) [8]. Cellular Ca^{2+} homeostasis mediated by store-operated Ca^{2+} (SOC) channels is implicated in the regulation of several neurological disorders [8-10]. The canonical transient receptor potential channel (TRPC) family is a type of non-selective cation channel responsible for Ca^{2+} influx [11]. Despite the controversial molecular identity of the SOC channels, TRPC members, mainly TRPC1, can be activated by store depletion and interact with the components of SOC channels, such as Stim1 [12, 13]. Among the seven TRPC subfamily members (TRPC1 through to TRPC7), TRPC1 is most widely expressed in many regions of the brain and plays a noteworthy role in central nervous system [8, 11, 14]. TRPC1 knockout mice show widespread loss of dopaminergic neuron, whereas TRPC1 overexpression has a neuroprotective effect on hippocampal neuron [14, 15]. Neurotoxins can initially inhibit the TRPC1-mediated Ca^{2+} influx, which in turn further augments the effects of neurotoxins [8]. These findings indicate the significance of TRPC1 in neurological function.

In this study, we found that TRPC1 expression is specifically decreased in cerebral I/R mice and in hippocampal neuronal cells under oxygen-glucose deprivation and reoxygenation (OGD/R) conditions. Overall, these findings demonstrate that TRPC1 deficiency increases Nox4-containing NADPH oxidase activity, leading to enhanced ROS generation and potentiating the I/R-induced neurological injury.

Materials and Methods

Materials and reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), streptomycin, penicillin, TRIzol reagent, PCR primers, Lipofectamin 2000 and Fura 2-AM were purchased from Invitrogen (Carlsbad, CA, USA). Adenovirus encoding mouse TRPC1 cDNA (Ad-TRPC1) or LacZ (Ad-LacZ) was obtained from RiboBio Co., Ltd (Guangzhou, China). Small interference RNA (siRNA) targeting mouse TRPC1, Nox1, Nox2, Nox3, Nox4 or Nox4, and negative siRNA were designed and constructed by FugenGen (Guangzhou, China). Thapsigargin (Tg), N-acetyl-L-cysteine (NAC), apocynin, triphenyltetrazolium chloride (TTC), 2',7'-dichlorofluorescein diacetate ($\text{H}_2\text{DCF-DA}$), dihydroethidium (DHE), MitoSOX Red, lucigenin, NADPH and cycloheximide (CHX) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Animal model

TRPC1 knockout mice and wild-type mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were carried out according to the institutional guidelines of the Care and Use of Laboratory Animals of Jilin University and were approved by the Institutional Animal Ethics Committee. Genotypes of wild-type, heterozygote, and knockout offspring were determined by reverse transcription polymerase chain reaction (RT-PCR) on tail DNA using the following primers: 5'-CCTCTTATTT CCCTTCTTAG ACCA-3' and 5'-ACCACATACA AAGCACAGAA AGG-3'. Wild-type mice had a band at 216 bp, heterozygote mice had bands at 216 bp and 278 bp, and knockout mice had a band at 278 bp. The middle cerebral artery occlusion (MCAO) model was established as previously described [16]. Briefly, mice were anesthetized with isoflurane and the right carotid artery was exposed by a skin incision. A surgical nylon filament coated with silicon hardener was inserted to the artery through the external carotid stump and advanced to a point approximately 12 mm to the carotid bifurcation. Mice with a greater than 90% decrease in blood flow after filament insertion were included in this study. After 90 min of MCAO, the filament was carefully removed to restore reperfusion for 24 h. The sham mice were received an identical procedure without the filament insertion.

Cell culture and treatment

The murine hippocampal neuronal cell line HT22 was obtained from the Cell Bank of the Chinese Academy of Medical Science (Shanghai, China) and cultured in DMEM containing 10% FBS, 100 µg/mL streptomycin and 100 U/mL penicillin in a humidified incubator with 5% CO₂ at 37°C. For oxygen-glucose deprivation and reoxygenation (OGD/R) induction, the cells were cultured in glucose-free DMEM in an anaerobic chamber with 94% N₂/5% CO₂ and 1% O₂ for 4 h at 37°C. Afterwards, the cells were cultured in completed medium under normoxic conditions for reoxygenation for 24 h at 37°C. siRNA transfection was performed 24 h before OGD/R induction using Lipofectamine 2000 according to the manufacturer's instructions. For adenoviral infection, HT22 cells were treated with Ad-TRPC1 or Ad-LacZ in DMEM containing 2% (v/v) FBS for 24 h and then subjected to OGD/R treatment. For pharmacological approaches, NAC (5 mmol/L) or apocynin (10 µmol/L) was added to the cells for 1 h before various treatments.

Intracellular Ca²⁺ concentration measurements

Cells were incubated with 2 µmol/L of Fura 2-AM in 2 mL of modified Krebs solution (in mmol/L: 119 NaCl, 2.5 KCl, 1 NaH₂PO₄, 1.3 MgCl₂, 20 HEPES, 11 glucose, 0.5 EGTA, pH 7.4) at 37°C for 30 min. Ca²⁺ stores were depleted with 1 µmol/L of thapsigargin followed by the addition of 2 mmol/L of Ca²⁺. Fluorescence was observed using a wide-field inverted microscope (IX81, Olympus, Tokyo, Japan) at 340 nm and 380 nm wavelengths [Ca²⁺]_i levels were analyzed with Olympus Cell[^]R software and expressed as the ratio of 340 nm to 380 nm.

Western blotting analysis

Brain tissues and HT22 cells were lysed with radioimmunoprecipitation assay buffer (RIPA buffer; Beyotime, Jiangsu, China) containing 1% protease and phosphatase inhibitors (Merck, Darmstadt, Germany). Cytoplasmic and membrane fractions were extracted using a Qproteome Cell Compartment Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Protein concentrations were quantified using a bicinchoninic acid kit (Bio-Rad, Hercules, CA, USA). Samples containing equal amounts of protein were separated by 8%–10% SDS-PAGE gels and then transferred onto nitrocellulose membranes (Millipore, Billerica, MA, USA). The membranes were blocked by 5% non-fat milk powder in TBST (in mmol/L, 10 Tris-HCl, 150 NaCl, 0.05% Tween-20, pH 7.6) and incubated with the following antibodies at 4°C overnight: TRPC1, Nox2, Na⁺/K⁺ ATPase (1:1000; Abcam, Cambridge, MA, USA), Stim1, Orai1 p22phox (1:2000; Alomone, Jerusalem, Israel), p47phox, p67phox, and GAPDH (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterward, the bands were visualized with HRP-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) followed by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ, USA). Image quantification was performed using ImageJ software (version 1.41, NIH, Maryland, MD, USA). For immunoprecipitation, protein lysates were incubated with A/G agarose beads (Santa Cruz Biotechnology) and the appropriate primary antibody at 4°C overnight. The amount of protein bound to the immunoprecipitated protein was determined by western blotting.

RT-PCR analysis

Total RNA from brain tissues and HT22 cells was isolated using TRIzol reagent following the manufacturer's instructions. RNA purity and concentration were determined by UV spectrometry (Qubit2.0, Invitrogen). One microgram of RNA was reverse-transcribed and amplified using the One Step RT-PCR Kit (Qiagen). The specific primer sequences for RT-PCR of gene expression were as follows:

TRPC1, 5'-GACGTCCATGCGACAGAAGA-3' and 5'-TGTGTACTGATAGCTGCCC-3';
TRPC2, 5'-CCTACCGCCTTCGAGTCATC-3' and 5'-TCCCAGGCATAGTCAGCTCT-3';
TRPC3, 5'-GGCACTGTACGCAGTGAGAT-3' and 5'-CCTGCTTCAGCGTTTGGATG-3';
TRPC4, 5'-AGGTACCCTGCCTACACCTT-3' and 5'-GCGTTGGCTGACTGTATTGT-3';
TRPC5, 5'-GGAGAGCCTTGTGACTTGCT-3' and 5'-CTGCTCATTAGGGGCAGGAC-3';
TRPC6, 5'-AATGGGGATGCAGAGACTCG-3' and 5'-AGGTCCAAAGCTTTCATTTGCTG-3';
TRPC7, 5'-ATAGCCGGCACTCCAAACAG-3' and 5'-GTCGGAGCGTTCACAACATA-3';
Nox1, 5'-TGGAACCTGCCTTTAGCTG-3' and 5'-GTGGGAAGCTTGGTCGTTCT-3';
Nox2, 5'-TGTGTGAATGCCAGAGTCGG-3' and 5'-CGCAGTCTGTCCACGTACAA-3';
Nox3, 5'-GAACGAGAGTGGGTCTTCG-3' and 5'-AAGGTGCGGACTGGATTGAG-3';
Nox4, 5'-CTGAACCTCAACTGCAGCCT-3' and 5'-CACCCGTCAGACCAGGAATG-3';
Nox5, 5'-CGATCAGTGAGGACTTGCGA-3' and 5'-GAGTAGGCGCCGAAATACCA-3';
GAPDH, 5'-GGGCACGAAGGCTCATCAT-3' and 5'-AGAAGGCTGGGGCTCATTTG-3'.

The amplified products were electrophoresed on 1% agarose gels and the bands were analyzed by ImageJ software.

Brain infarction measurement

Brain infarction was measured using TTC staining. After I/R surgery, the brains were removed and cut into 2-mm sections followed by staining with 2% TTC for 30 min at 37 °C. The brain sections were then fixed in 10% formaldehyde neutral buffer solution at 4 °C overnight. The unstained and red areas represented as infarct and normal regions, respectively. The infarct volume was assessed using ImageJ software and expressed as a percentage of the infarct volume compared with the contralateral hemisphere volume (considered 100%).

Brain water content

After 24 h of reperfusion, the brains were isolated and immediately weighed to determine wet weight. The brains were dried in an oven at 110°C for 24 h to obtain the dry weight. Brain water content was measured as (wet weight – dry weight)/wet weight × 100%.

Neurological severity score

Neurological dysfunction was evaluated after 24 h of reperfusion as previously described [17]. One point was assigned to each of 10 tasks that a mouse could not finish. Therefore, the higher the neurological severity score (NSS), the more severe the neurological deficit.

Morris water maze test

The memory deficits and spatial learning of mice were examined using the Morris water maze as previously described [18, 19]. The experimental apparatus (diameter = 120 cm, height = 50 cm) was divided into four quadrants, filled with water, and maintained at 25°C. A round platform (diameter = 9 cm) was placed 1 cm under the water surface at the midpoint of the fourth quadrant. Each mouse was released from four randomized starting points and allotted 90 s to find the location of the escape platform, which was kept constant. All mice were trained four times per day for 4 days. The test was performed after 24 h of reperfusion and four trials were averaged.

Rotarod test

The motor function of mice was evaluated using an automated Rotarod apparatus (Ugo Basile, Comerio, Italy) at a constant speed of 40 rpm. The average latency on the rod was recorded, and the maximum cutoff time was 180 s. Mice were trained before MCAO/reperfusion surgery for 4 days at a constant speed.

String test

Motor function was also assessed by string test as previously described [18]. Mice were placed on a string (diameter, 2 mm; length, 50 cm) between two vertical supports at a height of 37 cm. Scores were calculated according to the following system: 0, mouse falls off; 1, mouse hangs onto the string with two forepaws; 2, mouse hangs onto the string with both forepaws and attempts to climb onto the string; 3, mouse hangs onto the string with both forepaws and one hind paw; 4, mouse hangs onto the string with two forepaws and two paws; 5, mouse escapes. The string test was performed after 24 h of reperfusion and four trials were averaged.

ROS detection

ROS levels in brain tissues and HT22 cells were visualized by H₂DCF-DA, DHE, and MitoSOX Red staining. The cells and frozen brain sections (8 µm) were incubated with H₂DCF-DA (10 µmol/L), DHE (10 µmol/L), or MitoSOX Red (5 µmol/L) for 30 min at 37°C. Images were taken with a confocal laser-scanning microscope (FV1000, Olympus) at 488-nm excitation and 525-nm emission wavelengths for H₂DCF-DA or at 535-nm excitation and 610-nm emission wavelengths for DHE and MitoSOX Red. The fluorescence intensity was measured by ImageJ software.

Oxidative parameters detection

Brain tissues were homogenized in RIPA buffer on ice and then centrifuged at 5000 × *g* for 5 min at 4°C. The levels of malondialdehyde (MDA), glutathione peroxidase (GPx), and superoxide dismutase (SOD) in supernatant were determined using commercially available kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturers' protocols.

NADPH oxidase activity measurement

NADPH oxidase activity was measured by a lucigenin-enhanced chemiluminescence assay as described previously [20]. Briefly, brain tissues or HT22 cells were lysed in NADPH lysis buffer containing 1.0 mol/L K₂HPO₄, 0.1 mol/L EGTA, and 0.15 mol/L phosphate and protease inhibitor cocktail (pH 7.0). After centrifugation at 12000 × *g* for 5 min, the supernatant was collected and incubated with lucigenin (5 mmol/L) for 10 min at 37°C in the dark. The basal relative light units (RLU) of chemiluminescence were recorded using a luminometer (Promega, Madison, WI, USA). Afterward, NADPH (100 µmol/l) was immediately added to the suspension and the chemiluminescence was recorded every 15 s for 20 min as the experimental RLU. The NADPH oxidase activity was calculated as the mean RLU, which was normalized to the total protein concentration.

Immunofluorescent staining

To detect p47phox and p67phox translocation, HT22 cells were fixed and labeled with TRPC1 and p47phox or p67phox antibodies (1:100) overnight at 4°C, followed by incubation with the appropriate secondary antibodies (TRITC-labeled antibody for TRPC1 and FITC-labeled antibody for p47phox or p67phox, 1:200; Jackson Laboratory) for 1 h at room temperature. Fluorescent images were acquired using a Zeiss Axioplan2 fluorescence microscope.

Statistical analysis

All data were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed by SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) using two-tailed Student *t* test or one-way ANOVA, followed by the Bonferroni multiple comparison test. The regression analysis was determined by the Pearson correlation test. A *p* value less than 0.05 was considered as statistically significant.

Results

TRPC1 expression is decreased in the brain tissues of cerebral I/R mice

The protein expression of TRPC1 was significantly decreased in brains isolated from I/R mice compared with sham-operated mice (Fig. 1A). Consistently, the expression of TRPC1 in HT22 cells was reduced after reoxygenation in a time-dependent manner (Fig. 1B). Similarly, under I/R or OGD/R conditions, TRPC1 expression was also inhibited in the plasma membrane of brain tissues or HT22 cells (Fig. 1C and D). These findings were further supported by RT-PCR (Fig. 1E). However, no significant differences were observed in the mRNA expression of TRPC3, TRPC4 or TRPC7, and TRPC2 and TRPC6 were too faint to detect (Fig. 1E). Moreover, the expression of Stim1 and Orai1 were unchanged (Fig. 1F and G). Because TRPC1 is an important store-operated Ca^{2+} channel, we examined whether $[\text{Ca}^{2+}]_i$ is altered under OGD/R conditions *in vitro*. In the absence of extracellular Ca^{2+} , OGD/R treatment had no effect on thapsigargin-induced Ca^{2+} release from the endoplasmic reticulum store in HT22 cells. However, the Ca^{2+} influx caused by the addition of external Ca^{2+} (2 mmol/L) was markedly inhibited in OGD/R-treated cells in a time-dependent manner, which paralleled the OGD/R-induced decrease in TRPC1 expression (Fig. 1H–J).

To explore the possibility that the reduced TRPC1 expression was responsible for the decrease in $[\text{Ca}^{2+}]_i$, we overexpressed TRPC1 in HT22 cells. Upregulation of TRPC1 in HT22 was confirmed by western blotting (Fig. 1K). Ca^{2+} imaging showed that overexpression of TRPC1 significantly prevented the OGD/R-mediated inhibition of Ca^{2+} influx (Fig. 1L and M).

TRPC1 deletion exacerbates neurological dysfunction in cerebral I/R mice

Based on the decrease in TRPC1 expression and $[\text{Ca}^{2+}]_i$, we speculated that TRPC1 may be involved in the neurological deficit induced by I/R challenge. To verify this assumption, we established an *in vivo* MCAO/reperfusion model in wild-type and TRPC1 knockout mice. Mouse genotypes were determined by RT-PCR (Fig. 2A). This was further confirmed in brain tissues by western blotting analysis (Fig. 2B). The brain infarct area was analyzed by TTC staining after 24 h of reperfusion. There was no infarct area in wild-type or knockout mice after the sham operation (Fig. 2C). However, under I/R conditions, the TRPC1 deficiency markedly increased the total infarct size compared with wild-type mice. Quantitative analysis revealed that the infarct volume percentage was increased from 31.3% in wild-type mice to 48.5% in knockout mice (Fig. 2D). Moreover, brain water content was increased in both wild-type and knockout mice compared with the corresponding sham mice, but the increase was more noticeable in knockout mice (Fig. 2E).

We next examined the effects of TRPC1 deficiency on neurobehavioral outcomes. The NSS after MCAO/reperfusion surgery was markedly higher in knockout mice than in wild-type mice (Fig. 2F). The Morris water maze test showed that latency to escape was slightly increased in knockout mice compared with wild-type mice. After 24 h of reperfusion, knockout mice took significantly longer time to reach the platform (Fig. 2G). Additionally, we also performed rotarod and string tests to evaluate motor function. Knockout mice had a significantly shorter duration on the rotarod and string after 24 h of reperfusion compared with wild-type mice (Fig. 2H and I). These data indicate that TRPC1 deficiency aggravates cerebral I/R-induced neurological injury and deficit.

TRPC1 deficiency potentiates I/R-induced brain oxidative stress via NADPH oxidase

Increasing evidence indicates that oxidative stress is critical for I/R-induced neurological injury [21, 22]. Thus, we investigated whether TRPC1 could affect brain oxidative stress in I/R mice. ROS generation was determined by $\text{H}_2\text{DCF-DA}$ and DHE dyes. No significant differences were observed in ROS generation between wild-type and knockout mice after sham operation. After 24 h of reperfusion, the fluorescence was significantly increased, with the ROS generation increase more pronounced in knockout mice than in wild-type mice (Fig. 3A–D). Similarly, the level of MDA was significantly higher in I/R knockout mice in wild-

Fig. 1. OGD/R decreases TRPC1 expression and Ca²⁺ influx in HT22 cells. (A) Mouse cerebral ischemia/reperfusion (I/R) model was established as mentioned in method section. The total protein was isolated from brain tissues of the sham or I/R mice, and the expression of TRPC1 was determined by western blotting. ** $P<0.01$ vs. sham mice, $n=8$. (B) The cells were cultured in oxygen-glucose deprivation conditions for 4 h and then were placed in completed medium under normoxic conditions for reoxygenation (OGD/R) for 6, 12 or 24 h. The expression of TRPC1 expression in total protein was examined. (C and D) The content of TRPC1 in plasma membrane of brain tissues or HT22 cells treated as mentioned above. ** $P<0.01$ vs. control, $n=6$. (E) RT-PCR analysis of TRPC1-7 mRNA expression in brain tissues from sham or I/R mice. (F and G) Western blotting analysis of Stim1 (F) and Orai1 (G) expressions in HT22 cells. (H) The cells were loaded with Fura 2-AM probe and subjected to Ca²⁺ imaging experiment. (I) Quantification of intracellular calcium concentration [Ca²⁺]_i in the absence or presence of extracellular Ca²⁺, * $P<0.05$, ** $P<0.01$ vs. control, $n=5$. (J) The [Ca²⁺]_i was positively correlated with the TRPC1 expression. (K) Western blotting analysis of TRPC1 expression in HT22 cells treated with TRPC1 adenovirus (Ad-TRPC1) at different multiplicity of infection (MOI) for 24 h. ** $P<0.01$ vs. control, $n=6$. (L) The cells were treated with Ad-LacZ or Ad-TRPC1 (40 MOI) for 24 h before OGD/R treatment. Ca²⁺ imaging experiment was performed to measure ([Ca²⁺]_i). (M) Quantification of [Ca²⁺]_i, ** $P<0.01$ vs. control; ## $P<0.01$ vs. OGD/R, $n=6$.

Fig. 2. Deficiency of TRPC1 aggravates I/R-induced neurological injury. (A) Genotypes of wild-type (WT) and TRPC1 heterozygote and TRPC1 knockout (KO) offspring were determined by RT-PCR on tail DNA. (B) TRPC1 protein expression in brain tissues from wild-type, heterozygote and knockout mice was examined by western blotting. (C) Representative images of triphenyltetrazolium chloride-stained brain sections from wild-type or knockout mice showing the infarct area after 24 h of reperfusion. (D) Quantitative analysis of the total infarct volume. (E) Brain water content was measured. (F) Neurological severity score was recorded. (G) The learning ability and spatial memory of mice was examined by Morris water maze test. (H and I) The motor function of mice was assessed by rotarod (H) and string test (I). The duration on the rotarod and the scores of string grip were recorded. * $P < 0.05$, ** $P < 0.01$ vs. Sham WT; ## $P < 0.01$ vs. I/R WT, $n = 10$.

type mice (Fig. 3E). Moreover, endogenous antioxidant enzymes, such as GPx and SOD, were further decreased after TRPC1 knockout (Fig. 3F and G).

To explore how TRPC1 deficiency potentiates brain ROS generation, we measured both NADPH oxidase activity and mitochondrial ROS (mROS) generation. The I/R-induced increase in NADPH oxidase activity was more pronounced in knockout mice than in wild-type mice (Fig. 3H). However, the results of mROS generation using a highly selective indicator dye MitoSOX Red showed that TRPC1 deficiency had no effect on the I/R-induced increase in mROS generation (Fig. 3I and J). This excluded the possibility that TRPC1 deficiency accelerates oxidative stress by increasing mROS generation. Furthermore, in HT22 cells, OGD/R treatment also increased NADPH oxidase activity in a time-dependent manner (Fig. 3K). Interestingly, the OGD/R-induced decrease in $[Ca^{2+}]_i$ was negatively correlated with NADPH oxidase activity (Fig. 3L). The OGD/R-induced increase in ROS generation was markedly inhibited by TRPC1 overexpression but promoted by TRPC1 knockdown (Fig. 3M–O). As expected, the ROS scavenger NAC abolished the increase in ROS generation. More importantly, pharmacological inhibition of NADPH oxidase using apocynin largely abrogated the excessive ROS generation caused by TRPC1 inhibition (Fig. 3N and O). This supports the evidence indicating that TRPC1 specifically regulates the function of NADPH oxidase.

Nox4 is required for TRPC1 deficiency to increase ROS generation

The Nox family plays an important role in the regulation of NADPH activity [21]. To investigate which isoform(s) of Nox is responsible for TRPC1-mediated NADPH oxidase activity, we knocked down five different Nox isoforms—Nox1 to -5—in HT22 cells. RT-PCR showed that the respective endogenous expression of Nox was efficiently reduced after

Fig. 3. TRPC1 knockout potentiates NADPH oxidase-mediated ROS generation. (A) ROS generation in brain sections from wild-type and knockout mice with sham or I/R treatment was determined by H₂DCF-DA (10 μmol/L). (B) Quantitative analysis of DCF fluorescence intensity. **P<0.01 vs. Sham WT; ##P<0.01 vs. I/R WT, n=6. (C) Superoxide anion in brain sections was examined by dihydroethidium (DHE) staining under a confocal microscope. (D) Quantitative evaluation of DHE fluorescence intensity. **P<0.01 vs. Sham WT; ##P<0.01 vs. I/R WT, n=5. (E-H) The levels of MDA (E), GPx (F), SOD (G) and NADPH oxidase activity in brain homogenate were measured, respectively. **P<0.01 vs. Sham WT; ##P<0.01 vs. I/R WT, n=7. (I) Confocal microscopy was used to localize mitochondrial ROS generation using MitoSOX Red staining. (J) Quantitative evaluation of MitoSOX fluorescence intensity. **P<0.01 vs. Sham WT, n=5. (K) Quantitation analysis of NADPH oxidase activity in OGD/R-treated HT22 cells. **P<0.01 vs. control, n=5. (L) The correlation between [Ca²⁺]_i and NADPH oxidase activity was analyzed. (M) Western blotting analysis of TRPC1 expression in HT22 cells treated with different concentrations of TRPC1 siRNA (5, 10 or 20 nmol/L) for 24 h. **P<0.01 vs. control, n=6. (N) HT22 cells were pretreated with N-acetyl-L-cysteine (NAC, 5 mmol/L) or apocynin (10 μmol/L) as described in methods section. Then the cells were treated with Ad-LacZ (40 MOI), Ad-TRPC1 (40 MOI) or TRPC1 siRNA (20 nmol/L) for 24 h prior to exposure of OGD/R conditions. ROS generation was determined by H₂DCF-DA staining. (O) Quantitative analysis of DCF fluorescence intensity. **P<0.01 vs. control; ##P<0.01 vs. OGD/R; &&P<0.01 vs. OGD/R+TRPC1 siRNA, n=6.

Fig. 4. TRPC1 knockdown enhances NADPH oxidase activity by increasing Nox4 expression. (A) HT22 cells were transfected with siRNA (Si) targeting Nox1, Nox2, Nox3, Nox4 or Nox5, or negative siRNA (Neg) for 24 h. The mRNA expressions of Nox1, Nox2, Nox3, Nox4 and Nox5 were determined by RT-PCR. (B) The cells were co-transfected with TRPC1 siRNA and Nox1, Nox2, Nox3, Nox4 or Nox5 siRNA for 24 h before exposure of OGD/R conditions. The NADPH oxidase activity was measured. ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. OGD/R; && $P < 0.01$ vs. OGD/R+TRPC1 siRNA, $n = 5$. (C) Western blotting analysis of Nox4 expression in brain tissues from wild-type or knockout mice underwent sham or MCAO/reperfusion operation. ** $P < 0.01$ vs. Sham WT; ## $P < 0.01$ vs. I/R WT, $n = 6$. (D and E) HT22 cells were pretreated with TRPC1 siRNA (20 nmol/L) (D) or Ad-TRPC1 (40 MOI) for 24 h prior to exposure of OGD/R conditions. The expression of Nox4 was tested by western blotting. ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. OGD/R, $n = 5$.

transfection with the corresponding siRNA (Fig. 4A). Notably, only Nox4 siRNA inhibited the TRPC1 knockdown-induced increase in NADPH oxidase activity under OGD/R conditions (Fig. 4B). In addition, although the expression of Nox4 in brain tissues was not different between sham knockout mice and sham wild-type mice, the increase in Nox4 expression induced by I/R was more pronounced in knockout mice than in wild-type mice (Fig. 4C). Similarly, in an *in vitro* study, OGD/R markedly increased the expression of Nox4; this increase was potentiated by TRPC1 knockdown and attenuated by TRPC1 overexpression (Fig. 4D and E). These data indicate that Nox4 may be a critical molecular target for TRPC1 in the regulation of ROS generation.

TRPC1 interacts with Nox4 and induces its degradation

To investigate the mechanism by which TRPC1 regulates Nox4 expression, we first tested the effect of TRPC1 inhibition on Nox4 mRNA expression. Although the mRNA expression of Nox4 was increased after 24 h of reperfusion, we did not detect any differences between knockout mice and wild-type mice (Fig. 5A). Consistently, TRPC1 knockdown also had no effect on the OGD/R-induced increase in Nox4 mRNA expression (Fig. 5B), suggesting the occurrence of post-transcriptional regulation. Interestingly, an immunoprecipitation assay clearly revealed that Nox4 was immunoprecipitated with TRPC1 (Fig. 5C), suggesting that

Fig. 5. TRPC1 interacts with Nox4 and facilitates its degradation. (A) RT-PCR analysis of Nox4 mRNA expression in brain tissues from wild-type or knockout mice underwent sham or MCAO/reperfusion operation. (B) TRPC1 siRNA had no effect on the OGD/R-induced increase of Nox4 mRNA expression in HT22 cells. (C) Cell lysates were immunoprecipitated (IP) with TRPC1 antibody and the immunoprecipitated proteins were blotted with Nox4 antibody. (D and E) Western blotting analysis of Nox4 expression. HT22 cells were treated with TRPC1 siRNA (D), Ad-TRPC1 (E) or corresponding negative control for 24 h prior to OGD/R conditions, and then, cycloheximide (CHX) was added at 10 μ g/mL for the indicated times. Densitometric quantification of Nox4 level normalized to GAPDH. ** $P < 0.01$ vs. negative siRNA or Ad-LacZ, $n = 7$.

TRPC1 binds to Nox4. Next, we examined the effect of TRPC1 on Nox4 protein stability by using a protein synthesis inhibitor CHX (10 μ g/mL). The results showed that treatment of HT22 cells with CHX induced a time-dependent decrease in Nox4 protein expression. Compared with the negative siRNA group, TRPC1 knockdown clearly antagonized the degradation of Nox4, increasing the Nox4 protein half-life by more than 9 h (Fig. 5D). In contrast, overexpression of TRPC1 significantly increased the rate of Nox4 degradation (Fig. 5E). Taken together, the results suggest that TRPC1 reduces Nox4 expression by interacting with Nox4 and facilitating its degradation.

TRPC1 prevents OGD/R-induced p47phox and p67phox translocation

NADPH is an enzyme complex composed of membrane subunits (e.g. Nox family and p22phox) and cytosolic subunits (e.g. p47phox and p67phox) [20, 23]. Translocation of p47phox and p67phox from the cytoplasm to the membrane is essential for the activation of NADPH [23]. Western blotting showed that p47phox and p67phox were indeed mainly distributed in the cytoplasm of HT22 cells. OGD/R treatment resulted in a marked translocation of p47phox and p67phox from the cytoplasm to the membrane. This translocation was

Fig. 6. TRPC1 mediates p47phox and p67phox translocation and NADPH oxidase activation. (A–D) The cells were pretreated with TRPC1 siRNA (A and B), Ad-TRPC1 (C and D) or corresponding negative control for 24 h before OGD/R conditions. The content of p47phox and p67phox in membrane (A and C) and cytoplasm (B and D) were detected by western blotting. Na⁺/K⁺ ATPase was used as an internal control of membrane. (E) Representative images of TRPC1, p47phox and p67phox distributions in HT22 cells. (F and G) TRPC1 knockdown (F) or overexpression (G) had no effects on the OGD/R-induced increase of p22phox expression in HT22 cells. (H and I) After treatment as mentioned above, immunoblotting for p47phox or p67phox after immunoprecipitation with Nox4 antibody in HT22 cell membrane extracts. **P<0.01 vs. control; ##P<0.01 vs. OGD/R, n=4–6.

promoted by TRPC1 knockdown but inhibited by TRPC1 overexpression (Fig. 6A–D). These results were further confirmed by immunofluorescent staining using p47phox or p67phox antibody (Fig. 6E). However, the expression of p22phox induced by OGD/R was comparable after inhibition or overexpression of TRPC1 (Fig. 6F and G). Additionally, knockdown of TRPC1 further potentiated the OGD/R-induced interaction between Nox4 and p47phox or p67phox (Fig. 6H). In contrast, upregulation of TRPC1 blocked the increased binding of Nox4 to p47phox or p67phox (Fig. 6I). These results demonstrate that impairment of the translocation of p47phox and p67phox and NADPH oxidase complex formation may underlie the inhibitory effect of TRPC1 on NADPH oxidase activity and ROS generation.

Discussion

This study investigated the effect of TRPC1 on neurological function using *in vivo* and *in vitro* cerebral I/R models. We found that TRPC1 expression and Ca^{2+} entry into neuronal cells were reduced by OGD/R treatment. Knockout of TRPC1 potentiated the I/R-induced Nox4 expression, NADPH oxidase activity, ROS generation, and neurological dysfunction.

Disturbances in Ca^{2+} homeostasis due to TRPC channels have been implicated in many cerebral disorders [9, 10, 14, 15]. In the TRPC family, TRPC1 is highly expressed in the cerebellum, amygdala, substantia nigra, hippocampus, and cerebral cortex [11]. Ca^{2+} entry into brain cells is frequently associated with opening of the TRPC1 channel, which is activated upon store depletion [12, 13]. In the present study, we found that both TRPC1 expression and Ca^{2+} entry were reduced after OGD/R exposure. These results are consistent with those of previous reports that both Ca^{2+} entry and TRPC1 expression are decreased in neuronal cells after neurotoxin stimulation and in patients with Parkinson's disease [8, 14, 24]. Interestingly, the expression of other members of the TRPC family (TRPC2–7) and other important components of SOC channels (e.g. Orai1 and Stim1) were unchanged, indicating that OGD/R specifically inhibits TRPC1 expression. In addition, we further found that upregulation of TRPC1 abolished the decrease in Ca^{2+} entry induced by OGD/R. Although the molecular role of TRPC1 in SOC channels is still controversial and Orai1+Stim1 appear to be sufficient for SOC channels [12, 25], our results suggest that TRPC1 is necessary for the activation of SOC channels in cerebral I/R injury.

Increasing evidence shows that TRPC1 is important for biological and pathological processes in the brain [14, 15, 24, 26, 27]. A recent study reported that TRPC1 ablation dramatically exacerbated memory deficit and neuronal apoptosis induced by amyloid- β [26]. Neuronal loss in brain tissues can be seen in TRPC1-deficient mice [14, 27]. In contrast, TRPC1 overexpression has neuroprotective effects against dopaminergic neurotoxins and cerebral hemorrhage [8, 15]. However, the effects of TRPC1 on cerebral I/R injury are poorly understood. In this study, we found that I/R-induced brain infarction and edema were exacerbated in TRPC1 knockout mice. In addition to the changes in neurological injury, lack of TRPC1 not only further increased the NNS, but also worsened the memory impairment and dyskinesia, as evidenced by their poorer performance of the mice in the Morris water maze test, rotarod test and string test. These findings suggest that TRPC1 may play a neuroprotective role in cerebral I/R injury.

It is worth noting that TRPC1 deficiency is associated with movement deficits under basal conditions due to neuronal cell apoptosis [27], which is consistent with the results in our study. Nevertheless, in I/R mice, knockout of TRPC1 resulted in neurological dysfunction to a significantly larger extent than in sham mice, indicating that other mechanisms may be involved, in addition to the neuronal loss. Although multiple factors contribute to cerebral I/R injury, it is likely that oxidative stress is the major underlying mechanism, resulting from the imbalance between ROS formation and elimination [5, 7]. The elevated level of ROS generation in the brain caused oxidative damage to organelles and was accompanied by increased MDA and reduced GPx and SOD [22]. In this study, along with cerebral I/R injury, ROS generation and MDA levels were significantly increased in the brain, whereas GPx and SOD levels were decreased. These alterations were dramatically potentiated in TRPC1-deficient mice. Furthermore, in an *in vitro* study, the excessive ROS generation induced by OGD/R was inhibited by TRPC1 upregulation. It is generally accepted that the mitochondrial electron transport chain and NADPH oxidase are the two major sources of ROS generation [21, 28]. Here, we found that TRPC1 deficiency had no effect on I/R-induced mROS generation, suggesting that mitochondria-derived ROS generation is not responsible for the increased ROS generation after TRPC1 downregulation. However, the NADPH oxidase inhibitor apocynin abolished the effect of TRPC1 knockdown on ROS generation in HT22 cells. This result was supported by NADPH oxidase activity measurement.

We next explored how TRPC1 regulates NADPH oxidase activity. The Nox family is divided into seven homologs—Nox1, Nox2, Nox3, Nox4, Nox5, Duox1, and Duox2—, all of which play different roles in cellular biology [21, 29]. Nox4 is widely expressed in the central nervous system and has emerged as a potential therapeutic target for cerebral ischemia treatment [16]. Interestingly, we showed that only knockdown of Nox4 blocked the effect of TRPC1 inhibition on NADPH oxidase activity. Moreover, inhibition of TRPC1 enhanced Nox4 expression under I/R conditions, whereas TRPC1 overexpression was associated with reduced Nox expression. This indicates that Nox4 probably specifically accounts for the contribution of TRPC1 to NADPH oxidase activity. Notably, downregulation of TRPC1 had no effect on the increased mRNA expression of Nox4 under I/R conditions. Nox family subunits can be degraded via different protein degradation pathways [29, 30]. This study is, to our knowledge, the first to show that TRPC1 binds to Nox4. Knockdown of TRPC1 prolonged the half-life of Nox4 protein under OGD/R exposure, whereas overexpression of TRPC1 showed the opposite effect. These data suggest that TRPC1 interacts with Nox4 and promotes its protein degradation, leading to decreased Nox4 expression, NADPH oxidase activity, and ROS generation. Nox2-containing NADPH oxidase is a classic multisubunit complex that contains two plasma membrane subunits—Nox2 and p22phox—and three cytosolic subunits—p47phox, p67phox, and Rac1 [21]. In addition to the interaction between Nox2 and p22phox, a recent study showed that Nox4 also interacts with p22phox [23]. On the basis of this finding, we investigated whether TRPC1 affects NADPH complex assembly. The results showed that TRPC1 downregulation promoted p47phox and p67phox translocation from the cytoplasm to the membrane, concomitantly with increased interaction between Nox4 and p47phox or p67phox. However, overexpression of TRPC1 had the opposite effects.

Conclusion

In conclusion, this study demonstrated that TRPC1 deficiency exacerbates cerebral I/R-induced neurological deficits and brain injury by promoting Nox4-containing NADPH-derived ROS generation. Our findings may indicate a protective role of TRPC1 in the pathogenesis of brain ischemic diseases such as stroke.

Disclosure Statement

The authors declare no conflicts of interest.

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