Tanshinone IIA reduces AQP4 expression and astrocyte swelling after oxygen-glucose deprivation and reoxygenation by inhibition of the HMGB1/RAGE/NF-κB/IL-6 pro-inflammatory axis

Zhaohua Tang (✉ drzh01@outlook.com)
The First Affiliated Hospital of Chongqing Medical University

Gang Yang
The First Affiliated Hospital of Chongqing Medical University

Zhengbu Liao
The First Affiliated Hospital of Chongqing Medical University

Feilan Chen
Chongqing Medical University

Song Chen
The First Affiliated Hospital of Chongqing Medical University

Wangwen Tao
The Affiliated Hospital of Northwest University

Gang Huo
The First Affiliated Hospital of Chongqing Medical University

Xiaochuan Sun
The First Affiliated Hospital of Chongqing Medical University

Xiaoshu Wang
The First Affiliated Hospital of Chongqing Medical University

Article

Keywords: tanshinone IIA, astrocyte swelling, aquaporin-4, high mobility group box-1, receptor for advanced-glycation end, interleukin-6

Posted Date: May 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1589622/v1
This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Here, we investigated the role of tanshinone IIA (TSO IIA) in astrocytic swelling caused by ischemia and reperfusion-like injury in an in vitro model and the molecular mechanisms underlying this effect. Primary brain astrocytes were cultured in conditions of glucose and oxygen deprivation and reoxygenation (OGD/R). We investigated the effects of TSO IIA treatment on cell swelling and injury, and the expression levels of AQP4 protein in the plasma membrane. We then studied the involvement of the HMGB1/RAGE/NF-κB/IL-6 pro-inflammatory axis in TSO IIA-mediated protection. Treatment with TSO IIA alleviated OGD/R-induced astrocytic swelling and the over-clustering of AQP4 protein in the plasma membrane. In addition, TSO-IIA significantly reduced over-expression of HMGB1, the high levels of NF-κB protein in the nucleus, and of IL-6 protein in cytoplasm and extracellular media induced by OGD/R. Combination of TSO IIA and recombinant HMGB1 reversed these effects. Inhibition of RAGE, the receptor of HMGB1, induced similar results to those of TSO IIA. In addition, exogenous IL-6 reversed TSO IIA-mediated effect on AQP4 over-clustering and cell swelling. TSO IIA significantly reduced astrocyte swelling after OGD/R injury in vitro, via blocking activation of the HMGB1/RAGE/NF-κB/IL-6 pro-inflammatory axis and thereby decreasing the expression of AQP4 in plasma membrane.

Introduction

Brain edema following stroke, trauma, tumor growth, or infection is closely associated with a variety of severe negative consequences\(^1\)\(^2\). Astrocytes are the main glial cell type in the central nervous system (CNS), and their swelling, due abnormal accumulation of intracellular fluid and caused mainly by pro-inflammatory cytokines, is an important component of cerebral edema\(^3\)\(^4\). Current treatments for brain edema are limited to osmotic therapies and surgical decompression.

Tanshinone IIA (TSO IIA), a herbal medicine with many active ingredients, is widely distributed in Japan and China\(^5\). Because of the extensive preclinical and clinical studies on its anti-inflammatory and anti-atherosclerotic properties, TSO IIA and its derivatives have been widely used as prescription treatments for stroke and angina pectoris\(^6\)\(^7\)\(^8\). Recently, TSO-IIA has been reported to reduce cerebral edema and protect from cerebral ischemia-reperfusion or traumatic injury in rats, through its anti-inflammatory effects\(^10\)\(^11\)\(^12\), but the specific molecular mechanisms underlying these effects remains unclear.

Treatment of astrocyte swelling is important for avoiding the damaging consequences of brain edema\(^13\). Aquaporins (AQPs) are plasma membrane channels that play an integral role in the development of cytotoxic edema for their role in facilitating bidirectional transmembrane water flow\(^14\). AQP4, a major member of the aquaporin family in the central nervous system, is highly expressed in astrocytes\(^15\)\(^16\).

Increasing evidence suggests that neuroinflammation is critical in the pathogenesis of brain edema\(^17\)\(^18\). Inflammation is mainly caused by the secretion of injury-related molecules by local cells (such as microglia and astrocytes) in CNS diseases\(^13\)\(^19\). High-mobility group box protein 1 (HMGB1), a ubiquitous non-histone DNA binding protein, has been recently reported to be an important regulator in
neuroinflammation. During various pathophysiological cellular events, HMGB1 is translocated from the cytoplasm of astrocytes, microglia, and necrotic cells into the extracellular space. HMGB1 activates pattern-recognition receptors, such as receptor for advanced-glycation end products (RAGE), promotes activation of the nuclear factor-kappa B (NF-κB), and triggers inflammatory responses in astrocytes, immune-competent cells, and neurons. Furthermore, HMGB1 and its downstream pro-inflammatory cytokines such as interleukin-6 (IL-6) are associated with the AQP4 expression and brain edema.

To study the mechanisms underlying the effect of TSO and its potential in treating cerebral edema, we investigated the effects of TSO IIA on astrocyte swelling after oxygen-glucose deprivation and reoxygenation (OGD/R) injury in vitro. Further, we examined whether these effects are related to the regulation of AQP4 expression in plasma membrane through the HMGB1/RAGE/NF-κB/IL-6 pro-inflammatory axis.

Results

2.1 Tanshinone IIA reduced astrocytic swelling induced by OGD/R

To determine whether TSO IIA has a protective effect on astrocyte swelling, we measured the change of cell volume 1, 4, 12 and 24 h after OGD/R in different treatment conditions. Following OGD/R treatment, cell volume increased significantly, and reached a peak 4 h into reoxygenation (8.27 ± 0.66 ml/mg in the OGD/R group vs. 5.13 ± 0.55 ml/mg in the control group; P < 0.01, Fig. 1A). Treatment with TSO IIA for 24 h prior to and throughout the OGD/R phase significantly reduced cell volume increase at 4 and 12 h into reoxygenation (both P < 0.05 vs. OGD/R group). As shown in Fig. 1B, TSO IIA was found to be most protective at the concentration of 1 µM/ml (P < 0.01 vs. OGD/R group, Fig. 1B), with no statistical differences between the 1 µM/ml and 10 µM/ml concentrations (P>0.01, Fig. 1B). However, exposure of TSO IIA only in the OGD or reoxygenation phase did not have any effect on cell volume (both P>0.05 vs. OGD/R group, Fig. 1C). Control cultures treated with TSO IIA showed no changes in cell volume (data not shown).

To further confirm the anti-edema protection of TSO IIA, Cell morphological and ultrastructural changes were inspected under a light and electron microscope. As shown in Fig. 1D, TSO IIA treatment significantly alleviated swelling and roundness, led to thickened and shortened processes, edge shrinkage, and significantly increased the refractive index caused by OGD/R. The ultrastructural changes characterized by mitochondrial swelling and increased lysosome number were remarkably reduced by TSO IIA treatment (Fig. 1E). Furthermore, there was a time-dependent increase of LDH activity during the reoxygenation stage, which was also significantly attenuated by TSO IIA (P < 0.01 vs. OGD/R group, Fig. 1F).

2.2 TSO IIA reduced clustering of AQP4 in the plasma membrane after OGD/R
To investigate the effect of TSO IIA on AQP4 clustering in the plasma membrane during astrocytic swelling after OGD/R, we measured the expression of AQP4 protein. Western blot results showed that AQP4 protein expression in the plasma membrane increased by 168.4 ± 33.6% compared with the control group, 4 h into reoxygenation (P < 0.01 vs. control group; Fig. 2A). Treatment with TSO IIA significantly relieved OGD/R-induced AQP4 increase in the plasma membrane (P < 0.01 vs. OGD/R group). AQP4 clustering in plasma membrane was then assessed by immunofluorescence 4 h into reoxygenation. After OGD/R, excessive aggregation of AQP4 protein appeared in the plasma membrane of astrocytes, which was significantly reduced by TSO IIA treatment (Fig. 2B).

2.3 TSO IIA inhibited HMGB1-activated inflammatory axis, AQP4 clustering in the plasma membrane and cell swelling

To determine whether decrease of astrocyte swelling mediated by TSO IIA was due to the regulation of HMGB1-activated inflammatory axis, cells were first treated with or without TSO IIA, and HMGB1 expression in the cytoplasm and surrounding medium was evaluated. Western blot analysis showed that HMGB1 expression in the cytoplasm of astrocytes significantly increased by 138.5 ± 24.6% compared with control cells after OGD/R (P < 0.01 vs. control group, Fig. 3A). Further, ELISA analysis showed that HMGB1 expression in the medium after OGD/R was increased by 375.2 ± 45.5% (P < 0.01 vs. control group, Fig. 3B). TSO IIA treatment blocked the OGD/R-induced increase of HMGB1 protein in the cytoplasm and medium to 144.6 ± 23.7% and 136 ± 49.6% of the control group, respectively (both P < 0.01 vs. OGD/R group).

Cells were then treated with the vehicle, TSO IIA or TSO IIA + rHMGB1, and NF-κB protein in nuclear and IL-6 protein in the cytoplasm and extracellular medium after OGD/R were evaluated. As shown in Fig. 3, the protein levels of NF-κB in the nucleus and IL-6 in the cytoplasm or in extracellular medium were significantly increased by 138.5 ± 24.6%, 77.5 ± 18.6% and 258.9 ± 50.3%, respectively after OGD/R (all P < 0.05 vs. control group, Fig. 3C-E), while TSO treatment inhibited increase in these protein levels (all P < 0.05 vs. OGD/R group). Furthermore, the combined treatment of TSO IIA and rHMGB1 reversed the TSO IIA-mediated changes (all P < 0.05 vs. TSO IIA group). TSO IIA and TSO IIA + rHMGB1 treatment were also found to play similar roles in the regulation of plasma membrane AQP4, cell volume and LDH activity (all P < 0.05 TSO IIA + rHMGB1 group vs. TSO IIA group, Fig. 3F-H).

2.4 Role of RAGE in TSO IIA-mediated reduction of astrocyte swelling

To further clarify the involvement of RAGE, which is one of the HMGB1 receptors in TSO IIA-mediated reduction of astrocyte swelling, cells were either treated with vehicle, TSO IIA, RAGE inhibitor FPS-ZM1, or both FPS-ZM1 and HMGB1. The increase in the levels of NF-κB protein in nucleus and IL-6 protein in the cytoplasm and medium after OGD/R were all inhibited by FPS-ZM1 treatment (all P < 0.05 vs. OGD/R group, Fig. 4A-C). These results were not significantly different from TSO IIA treatment (all P > 0.05). Furthermore, combination of FPS-ZM1 and HMGB1 did not change the above-mentioned effects.
mediated by FPS-ZM1 (all P > 0.05 vs. FPS-ZM1 group) in terms of regulation of AQP4 expression in the plasma membrane, cell volume, and LDH activity (Fig. 4D-F).

2.5 TSO IIA reduced IL-6 mediated AQP4 expression and cell swelling

To evaluate whether IL-6 is a key inflammatory factor in TSO IIA-mediated regulation of AQP4 expression in the plasma membrane and astrocyte swelling, cells were either treated with vehicle, TSO IIA, or TSO IIA and IL-6. Then, the levels of AQP4 protein in the plasma membrane, the cell volume, and LDH activity were examined. Compared with TSO IIA treated cells, cells treated with TSO IIA + IL-6 displayed increased levels of AQP4 protein in the plasma membrane (all P < 0.05, Fig. 5A). Combination of TSO IIA and IL-6 reversed the decreases in cell volume and LDH activity induced by TSO IIA treatment alone (all P < 0.05 vs. TSO IIA group, Fig. 5B and C).

Discussion

Therapeutic options to prevent brain edema in CNS diseases are extremely limited, and neuroprotective therapies are urgently needed. In the current study, we have explore the effects of TSO IIA treatment to show for the first time that: 1) TSO IIA protects against astrocytes swelling after OGD/R injury possibly by suppressing AQP4 expression in the plasma membrane and 2) by blocking the activation of HMGB1/RAGE/NF-κB/IL-6 pro-inflammatory axis.

TSO IIA is a lipophilic diterpene extracted from Salvia miltiorrhiza Bunge\textsuperscript{25}. Due to its anti-inflammatory and anti-atherosclerotic properties, TSO IIA has been shown to prevent or slow progression of a wide spectrum of diseases, including central nervous system, cardiovascular, and cerebrovascular diseases\textsuperscript{7}. Huang et al. found that, using a traumatic brain injury rat model, TSO IIA treatment downregulated the expression levels of inflammatory factors and of AQP4, and decreased brain edema\textsuperscript{11}. In a transient middle cerebral artery occlusion and reperfusion model, also in rats, Song et al. found that TSO IIA was able to significantly reduce neuroinflammation, infarct volume, improve neurological deficits, and reduce brain edema\textsuperscript{10}. However, the specific molecular mechanisms underlying the effect of TSO IIA in alleviating brain edema remain unclear. In the present study, using astrocytes in culture, treatment with TSO IIA significantly inhibited increase in astrocyte volume induced by OGD/R injury. This protective effect in swelling was also consistent with changes in cell morphology, ultrastructure, and cytotoxicity. These results conformed the protective role of TSO IIA in edema in an in vitro model of ischemia and reperfusion-like injury.

In addition, our results showed upregulation of AQP4 in the plasma membrane and its association with astrocytic swelling following OGD/R. Specifically, treatment with TSO IIA resulted in a significant reduction of AQP4 over-clustering in the plasma membrane of astrocytes. AQP4 is the main water channel protein found in astrocytes and has a role in facilitating transmembrane water movement in the CNS\textsuperscript{26}. Furthermore, AQP4 was identified as a major player in astrocyte swelling and brain edema in
various central nervous system diseases\textsuperscript{26}. Under pathological conditions, over-expression of AQP4 in the plasma membrane leads to inappropriate cellular uptake of water in astrocytes, resulting in brain edema\textsuperscript{27}. Prior in vitro and vivo studies reported that blocking increase of membrane AQP4 protein in astrocytes could effectively prevent cell swelling or brain edema\textsuperscript{28}. Thus, our results suggest that TSO IIA-mediated abrogation of AQP4 over-expression in the plasma membrane is important for protecting astrocytes from swelling following OGD/R.

The putative regulatory mechanisms underlying the effect of TSO IIA treatment on AQP4 aggregation in the plasma membrane and astrocyte swelling needs to be further studied. In previous studies, extracellular HMGB1 overexpression activates downstream signaling pathways and enhances the expression of inflammatory cytokines, such as IL-6, TNF-\(\alpha\), and IL-1\(\beta\)\textsuperscript{29,30}. In the brain, HMGB1 and associated downstream inflammatory signaling pathways have been linked to significantly increased brain edema after traumatic brain injury, cerebral ischemia, and hepatic encephalopathy\textsuperscript{31}. Importantly, inhibition of HMGB1 led to decreased inflammation and improvement in brain edema\textsuperscript{24,32,33}. Up-regulation of HMGB1 after OGD/R injury in vitro have been shown to promote NF-\(\kappa\)B activation and IL-6 secretion, and lead to decrease in astrocyte swelling\textsuperscript{34,35}. In agreement with prior studies, we showed that the expression of intracellular and extracellular HMGB1 was significantly raised following OGD/R, and was accompanied by an increase in the nuclear transport of NF-\(\kappa\)B protein and secretion of IL-6. Treatment of cells with TSO IIA abolished the changes induced by OGD/R. Therefore, down-regulation of HMGB1 expression, and consequently of its downstream inflammatory response, play an important role edema. Corroborating this finding, we exposed astrocytes to TSO IIA and exogenous HMGB1. As expected, such co-treatment reversed the effects of TSO IIA on the NF-\(\kappa\)B activation, IL-6 secretion, AQP4 over-aggregating in the plasma membrane, and cell swelling, further suggesting that the target of TSO IIA-mediated neuroprotection is the pro-inflammatory factor HMGB1.

Secreted HMGB1 binds to its receptors RAGE, TLR2, and TLR4 to activate downstream signaling pathways and release inflammatory factors that lead to further damage\textsuperscript{36}. Numerous studies have demonstrated that genetic or pharmacological inhibition of RAGE in animal models attenuate brain edema after injury and inhibit pro-inflammatory response\textsuperscript{37,38}. Thus, we studied whether RAGE was involved in TSO IIA-mediated reduction of astrocyte swelling. Our results are in agreement with this hypothesis and demonstrate a role for RAGE in neuroinflammatory and cell swelling, given our findings that RAGE inhibition alleviates increased nuclear NF-\(\kappa\)B transport, IL-6 release, AQP4 accumulation on the plasma membrane, and astrocyte swelling after OGD/R. In addition, further extra-exposure to exogenous rHMGB1 did not alter these effects. Further, our results shows that the efficacy of TSO IIA is similar to that of as RAGE inhibitors. Taken together, our findings suggest that regulation of HMGB1-mediated pro-inflammation depend of TSO IIA occurs through RAGE.

IL-6 is an important inflammatory cytokine that is mainly synthesized and secreted by astrocytes and microglia\textsuperscript{39,40}. Laird et al. reported that HMGB1 can trigger IL-6 release and that IL-6 enhances AQP4 expression and cellular swelling of brain astrocytes\textsuperscript{24}. Neutralizing IL-6 abrogates the promotion of AQP4
expression in the plasma membrane and the cell swelling induced by HMGB1. Therefore, IL-6 seems to be one of the key inflammatory proteins induced by HMGB1 to promote AQP4 expression and cell edema. In the present study, the increased IL-6 protein expression in cytoplasm and in the extracellular medium after OGD/R was attenuated by treatment with TSO IIA. Co-treatment of astrocytes with TSO IIA and exogenous IL-6 abrogated the benefits of TSO IIA on the over-clustering of AQP4 in the plasma membrane, cell swelling, and LDH release. These results suggested that TSO IIA can alleviate the expression of the pro-inflammatory mediator, HMGB1, and thereby abolish activation of the HMGB1/RAGE/NF-κB/IL-6 inflammatory axis. In turn, this interaction leads to decrease in over-expression of AQP4 in the plasma membrane and astrocyte swelling after OGD/R.

It is worth noting that although this study has several strengths, there are also some inevitable limitations. The brain is a complex system with many different cell types such as neurons, microglia and endothelial cells which interact with each other during the inflammation and edema process. In this study, we only examined the effects of TSO IIA on astrocyte swelling. Further studies should be conducted in co-culture systems in vitro and in vivo to validate our findings. In addition to RAGE, other receptors of HMGB1, such as TLR2 and TLR4, and their downstream signaling factors are involved in the inflammatory response induced by HMGB1, and these factors have not been studied here and should be investigated in future studies.

**Conclusion**

We showed that treatment with TSO IIA can alleviate astrocyte swelling after OGD/R injury in vitro. We suggest that this effect depends on the abrogation of activation of HMGB1/RAGE/NF-κB pathway, down-regulation of expression of the pro-inflammatory factor IL-6, and thus reduction of the excessive accumulation of AQP4 protein in the plasma membrane. TSO IIA therefore has the potential to be used as a new therapeutic option for brain edema after I/R-like injury.

**Materials And Methods**

**3.1 Animals and ethics approval**

Two-day old Sprague-Dawley (SD) rat pups (licensed 21 CAE035; authorization no. 75-776) were supplied by the Laboratory Animal Center of Chongqing Medical University, China. All procedures were approved by the ‘Use Committee’ and ‘Institutional Animal Care’ of Chongqing Medical University, China, and followed the National Institutes of Health (NIH) guidelines (NIH Publication No. 80-23, revised 1996) on animal care and use. All efforts were made to minimize animal suffering, and pain and discomfort were carefully monitored during all the experiments. Animals underwent surgery in random order, and outcome assessments were evaluated by investigators blinded to the experimental groups.

**3.2 Special reagents and antibodies**
Special reagents and antibodies include recombinant HMGB1 (rHMGB1, ProSpec, Rehovot, Israel), IL-6 (PeproTech, NJ, USA), FPS-ZM1 (Sigma-Aldrich, St. Louis, MO, USA), anti-HMGB1 antibody (Abcam, Cambridge, UK), anti-AQP4 antibody (Abcam), anti-NF-κB antibody (Cell Signaling Technology, Boston, MA), anti-IL-6 antibody (Millipore, Billerica, MA, USA), anti-GAPDH antibody (Beyotime, Shanghai, China), anti-Histone H3 antibody (Beyotime), and anti-α-tubulin antibody (Sigma-Aldrich).

3.3 Astrocyte isolation and culture

Preparation of primary rat cortical astrocytes has been described in a previous article. In short, 2-day-old SD rats were deep anesthetized with 5% isoflurane in 70% N₂/30% O₂, and then were decapitated for brains extraction. During the whole operation, the rectal temperature was maintained at 37 °C using a feedback-regulated water heating system. The brains were dissected and treated with 0.25% (v/v) trypsin solution for 10 mins. Cells were cultured in a medium containing DMEM-F12 and 15% (v/v) fetal bovine serum in 95% air and 5% carbon dioxide, and the medium was changed twice a week. After 7-9 days of culture, the solution was agitated for 14 hours to remove oligodendrocytes and microglia. After determination of glial fibrillary acidic protein (GFAP; Santa Cruz, California, USA) using immunofluorescence immunostaining, the culture comprised 95-99% of 15-20 days old primary astrocytes, which were used in subsequent experiments.

3.4 Astrocyte model of OGD/R and treatments

OGD/R injury was used to establish an in vitro model of ischemia reperfusion-like injury. Briefly, primary astrocytes were washed twice, cultured in serum-free DMEM without glucose, at 37 °C in 5% CO₂ and 95% N₂ for 5 hours (oxygen glucose deprivation, OGD). Then, cells were rinsed once and returned to the normal medium of 15% (v/v) fetal bovine serum and DMEM-F-12 in 95% air and 5% CO₂ for 24 hours (reoxygenation, R). Astrocyte cultures were then randomly assigned into seven different treatment groups.

i. TSO IIA group: cells were treated with 1 μM/ml TSO IIA for 24 hours before and throughout the OGD/R process.

ii. TSO IIA + HMGB1 group: cells were co-treated with TSO IIA as described in (i) and 1 ug/mL rHMGB1 during the reoxygenation phase.

iii. FPS-ZM1 group: cells were treated with 0.5 nM FPS-ZM1 for 24 hours before and throughout the OGD/R process.

iv. FPS-ZM1 + HMGB1 group: cells were co-treated with FPS-ZM1 as in (iii) and rHMGB1 as in (ii).

v. TSO IIA +IL-6 group: cells were co-treated with TSO IIA as in (i) and 1 ng/m IL-6 during the reoxygenation stage.

vi. OGD/R group: cells were treated with the corresponding volume of vehicle solution for 24 hours before and throughout the OGD/R process.
vii. Control group: cells were treated with the corresponding volume of vehicle solution without the OGD/R process.

The doses of TSO IIA, HMGB1, FPS-ZM1 and IL-6 were based on those found to be the most effective in preliminary trials (data not shown).

3.5 Cell edema analysis

To estimate cell edema after OGD/R, cell volumes were analyzed at different timepoints (0.5, 2, 8 and 24 hours) during reoxygenation. Briefly, 1 mM 3-O-methylglucose (3-OMG) and 0.5 μCi/ml [3H]-3-OMG (NEN, Boston, MA, USA) were added to the culture for 6 hours. The radioactivity of the culture medium and the cell extract were then determined. Cell volume was expressed in mL/mg protein. A small portion of cell extract was used for protein estimation using the BCA method as previously described.

3.6 Measurement of cell morphology and ultrastructure

The morphology and ultrastructure of edematous astrocytes were observed by phase contrast microscopy and electron microscopy. After reoxygenation for 2 hours, the cell edema was the most obvious and imaging of the cell morphology and ultrastructure was carried out. Samples were prepared for electron microscopy by rinsing cells in phosphate buffered saline (PBS), fixing with 0.1 M cacodylate buffered glutaraldehyde for 1 hour, fixing with osmium tetroxide for 2 hours, dehydrating in graded alcohol, and embedding in Epon. Sections were counterstained and examined using an electron microscope.

3.7 Measurement of lactate dehydrogenase activity

To examine cell damage by OGD/R, the cell culture medium was sampled at 0.5, 2, 8 and 24 hours after reoxygenation. Lactate dehydrogenase (LDH) release rates in the medium were assessed using an LDH assay kit according to the kit manufacturer's instructions (Beyotime).

3.8 Plasma membrane and nucleus isolation

In order to detect the expression of AQP4 protein in the plasma membrane, the plasma membrane was isolated and collected. Briefly, cells were washed with ice cold PBS, harvested and centrifuged. They were then homogenized in Tris-EDTA buffer and centrifuged at 15,000 g for 30 minutes. The particles were resuspended, and centrifuged again at 15,000 g for 30 mins. The remaining particles were then dissolved in 100 mL of RIPA buffer, and proteins were extracted for further analysis. In order to detect the expression of NF-κB protein in the nucleus, nuclear proteins were isolated and collected. Cells were resuspended in nuclear extraction reagent containing phenylmethanesulfonyl fluoride, phosphatase inhibitor, and protease inhibitor, vortexed for 5 seconds and incubated on ice for 30 minutes. The lysate was centrifuged at 12000 g at 4 °C for 10 minutes to obtain nucleoproteins.

3.9 Western blot analysis
Western blot analysis was used to determine the expressions of plasma membrane AQP4 protein, cytoplasmic HMGB1 protein, nuclear NF-κB protein, and cytoplasmic IL-6 at different time points during reoxygenation. Protein concentrations were determined using a BCA Protein Assay Kit (Beyotime). The primary antibodies used were as following: AQP4 (1:250 dilution, Santa Cruz), HMGB1 (1:1000 dilution; abcam), NF-κB (1:1000 dilution; Cell Signaling Technology), IL-6 (1:1000 dilution; Millipore), α-tubulin (1:1000, Beyotime), Histone H3 and GAPDH (1:1000, Beyotime) (25). The ECL system was used for detection, and densitometry was used for scanning membranes for semi-quantitative analysis. The amount of AQP4, HMGB1, NF-κB, and IL-6 protein were quantified after normalization to α-tubulin, Histone H3, and GAPDH protein. The expression levels of proteins in the plasma membrane, cytoplasm and in the nuclear extracts were normalized to levels of of α-tubulin, GAPDH and Histone H3, respectively.

3.10 Fluorescence immunostaining

Immunofluorescence double staining analysis was performed to examine the co-expression of glial fibrillary acidic protein (GFAP) and AQP4 protein in astrocytes after 2 hours of reoxygenation. Briefly, cells were fixed onto a cover glass with cold methanol. Cells were incubated with GFAP (1:500 dilution, Santa Cruz) and AQP4 (1:100 dilution, Santa Cruz) antibodies at 4 ºC overnight. Cells were then incubated with TRITC - or FITC labeled IgG antibody at room temperature for 1 hour. As a negative control, normal rabbit serum instead of primary antibody was used.

3.11 Enzyme-linked immunosorbent assay (ELISA)

The levels of HMGB1 and IL-6 proteins released from astrocytes into the surrounding medium after OGD/R were measured by ELISA according to the manufacturer's instructions (Beyotime). Briefly, culture media were sampled, incubated for 1 h with antibodies targeting HMGB1 or IL-6, followed by incubation with enzymatic working solution for 30 min at 37°C, and incubated with TMB solution for 15 min at 37°C in darkness. Finally, absorbance at 450nm was measured to determine protein levels.

3.12 Statistical Analysis

Measurement data are expressed as mean ± error of mean. One way ANOVA was used to compare the normal distribution data between groups, and Tukey's post hoc test was performed. A P < 0.05 was considered significant. Data analysis was performed using SPSS 17.0.

Declarations

Acknowledgments

This research was supported by the National Natural Science Funds of China (81301630 and 81771355), the Natural Science Foundation of Chongqing·China (cstc2021jcyj-msxmX0262), and the Future Medical Youth Innovation Team Support Plan of Chongqing Medical University, China (W0106). The authors
would like to thank all the reviewers who participated in the review and MJEditor services (www.mjeditor.com) for their linguistic assistance during the preparation of this manuscript.

Authors contributions

ZT, GY and XW conceived and designed the work that led to the submission, and was a major contributor in writing the manuscript. FC contributed to the conception of the study and manuscript review. ZL contributed significantly to data acquisition, data analysis, and manuscript preparation. XS and GH carried out literature search, data analysis, and manuscript editing. WW and SC performed the experiments and contributed to data acquisition. All authors read and approved the final manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep.

Data Availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

References


Figures

Figure 1

**TSO IIA reduced astrocytic swelling and injury after OGD/R.**

(A) Cell volume quantification after OGD/R in untreated cells and cells treated with TSO IIA (1 μmol/ml) (n=5). (B) Dose-dependent effect of TSO IIA on cell volume (n=5). 0.1, 1 or 10 μmol/ml of TSO IIA were added 24 h before and throughout the OGD/R process. (C) Time dependent effect of TSO IIA in astrocytic
swelling. TSO IIA (1 μmol/ml) was added 24 h before and throughout the OGD/R (TSO IIA + OGD/R) process, only during the OGD phase (TSO IIA + OGD), or only during the reoxygenation phase (TSO IIA + R) (n=5). (D) Phase-contrast imaging showing cell morphological changes under the different treatments. Astrocytic swelling is depicted by black arrows (n=5). (E) Stereoscan photographs showing increase in lysosome number (black arrows) and mitochondria swelling (white arrows) (n=5). (F) LDH activity analysis showing the protective effect of TSO IIA on OGD/R injury (n=5). (The data are expressed as mean±SEM, *P<0.05 vs. Control; **P<0.01 vs. Control; #P<0.05 vs. OGD/R; ##P<0.01 vs. OGD/R).

Figure 2

TSO IIA treatment decreased clustering of AQP4 in the plasma membrane after OGD/R.

(A) Western blot analysis showing AQP4 protein in the plasma membrane after OGD/R in untreated cells or cells treated with TSO IIA (n=5). (B) Double-labelling immunofluorescence staining confirmed AQP4 protein clustering in the plasma membrane under different treatment conditions (n=5). (Data are expressed as mean±SEM, **P<0.01 represents vs. Control; ##P<0.01 vs. OGD/R, PM: Plasma membrane).

Figure 3

TSO IIA reduced cell swelling through inhibition of the HMGB1-activated inflammatory axis.

(A and B) HMGB1 expression in the cytoplasm and culture medium after OGD/R, detected by Western Blot and ELISA analysis, respectively, in untreated cells and cells treated with TSO IIA (n=5). (C) NF-κB protein accumulation in the nucleus in astrocytes in response to vehicle, TSO IIA or both TSO IIA and rHMGB1 (n=5). (D and E) IL-6 protein expression in the cytoplasm and medium after OGD/R in cells in response to vehicle, TSO IIA or both TSO IIA and rHMGB1 (all n=5). (F - H) AQP4 clustering in the cytoplasm, cell volume and LDH activity after OGD/R detected from cells in response to vehicle, TSO IIA or both TSO IIA and rHMGB1 (all n=5). (Data are expressed as mean±SEM, **P<0.01 represents vs. Control; ##P<0.01 vs. OGD/R, CM: Plasma membrane).

Figure 4

The effects of TSO IIA and FPS-ZM1 on the HMGB1-induced inflammatory axis, AQP4 clustering in the plasma membrane, and astrocytic swelling.
(F) (A - C) NF-κB protein accumulation in the nucleus and IL-6 protein expression in the cytoplasm and medium as examined by Western blot or ELISA assays after OGD/R in cells exposed to vehicle, TSO IIA, FPS-ZM1, or both FPS-ZM1 and HMGB1. (D - F) AQP4 protein clustering in the plasma membrane, cell volume and LDH activity after OGD/R detected in cells in response to vehicle, TSO IIA, FPS-ZM1, or both FPS-ZM1 and HMGB1. (Data are expressed as mean±SEM, **P<0.01 represents vs. Control; ##P<0.01 vs. OGD/R, CM: Plasma membrane).

**Figure 5**

The role of IL-6 in TSO IIA-mediated inhibition of AQP4 over-clustering in the cytoplasm and astrocyte swelling after OGD/R.

(A) AQP4 expression in the plasma membrane after OGD/R in cells subjected to vehicle, TSO IIA, or both TSO IIA and IL-6. (B and C) Cell volume and LDH activity after OGD/R detected in cells subjected to vehicle, TSO IIA, or both TSO IIA and IL-6. (Data are expressed as mean±SEM, **P<0.01 represents vs. Control; ##P<0.01 vs. OGD/R, PM: Plasma membrane).

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- UncroppedWBimages.pdf