Blocking P2RX7 attenuates ferroptosis in endothelium and reduces HG-induced hemorrhagic transformation after MCAO by inhibiting ERK1/2 and P53 signaling pathways

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Research Article

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Abstract

Background

Hyperglycemia is a risk factor for poor prognosis after acute ischemic stroke and promote the occurrence of hemorrhagic transformation (HT). The activation of P2RX7 play an important role in endotheliocyte damage and BBB disruption. Ferroptosis is a novel pattern of programmed cell death caused by the accumulation of intracellular iron and lipid peroxidation, resulting in ROS production and cell death. This study is to explore the mechanism of P2RX7 could reduce HT pathogenesis after acute ischemic stroke through regulating endotheliocyte ferroptosis.

Methods

Male SD rats were performed to establish middle cerebral artery occlusion (MCAO) model injected with 50% high glucose (HG) and HUVECs were subjected to OGD/R treated with high glucose (30mM) for establishing HT model in vivo and in vitro. P2RX7 inhibitor (BBG) and P2RX7 small interfering RNAs (siRNA) were used to investigate the role of P2RX7 in BBB after MCAO in vivo and OGD/R in vitro, respectively. The neurological deficits, infarct volume, degree of intracranial hemorrhage, integrity of the BBB, immunoblotting and immunofluorescence were evaluated both 24 h after MCAO.

Results

Our study found that the level of P2RX7 was gradually increased after MCAO and/or treated with HG. Our results showed that treatment with HG after MCAO can aggravate neurological deficits, infarct volume, oxidative stress, iron accumulation and integrity of the BBB in HT model, and HG-induced HUVECs damage. The inhibition of P2RX7 reversed the damage effect of HG, significantly downregulated the expression level of P53, HO-1 and p-ERK1/2 and upregulated the level of SLC7A11 and GPX4, which implicated that P2RX7 inhibition attenuated oxidative stress and ferroptosis of endothelium in vivo and in vitro.

Conclusion

Our data provide evidence that the P2RX7 play an important role in HG-associated oxidative stress, endothelial damage and BBB disruption, which regulates HG-induced HT by ERK1/2 and P53 signaling pathways after MCAO.

1. Introduction

Ischemic stroke is a common cerebrovascular disease caused by acute blockage of cerebral vascular circulation, and is the main cause of death in the world. Hemorrhagic transformation (HT) is a common

complication of acute ischemic stroke (AIS), occurring in 10 to 40% of AIS patients. Diabetes is one of the most common clinical risk factors in patients with ischemic stroke, which may promote the pathological process of ischemic stroke and the occurrence of HT[1]. Hence, high glucose (HG) is used to induce the occurrence of HT after cerebral infarction[2]. The mechanisms of HG-induced HT involve oxidative damage and disruption of blood brain barrier (BBB). Although many studies have tried to find ways of reducing HT damage, there are very few effective drugs or therapeutic strategies available to attenuate HG-enhanced HT in animal models. Hence, there is a greatly needed to investigate the mechanisms of HT and potential therapies that can reduce the risk of HT and improve outcomes in patients with AIS.

Ferroptosis is an iron-dependent programmed cell death triggered by lipid peroxides accumulation in the presence of increased production of reactive oxygen species (ROS) and inactivation of glutathione peroxidase 4 (GPX4)[3]. Many regulatory factors, such as SLC7A11, GSH, GPX4, P53, ferroportin and transferrin, are thought to be involved in ferroptosis processes[4]. Some studies have found that ferroptosis is involved in some neurological disease, such as stroke, Alzheimer's disease and Parkinson's disease[5]. Furthermore, ischemic stroke has been revealed to be improved after treatment with the ferroptosis inhibitor[6, 7]. Inhibition of ferroptosis in cerebral ischemia-reperfusion injury to treat stroke is a research hotspot. In addition, high glucose induce cytotoxicity and the accumulation of lipid peroxide, which can be reversed by ferrastatin-1[8]. Some studies showed that ferroptosis plays an important role in the occurrence and development of diabetes complications[9]. Studying the mechanism of ferroptosis has important potential value for AIS patients with diabetes.

The P2X7 receptor is an ATP-gated, non-selective cation channel, belonging to the family of ionotropic P2X receptors[10]. The activation of P2RX7 causes the opening of the channel pore, allowing the passage of small cations (Ca2+, Na + and K+), and activating downstream cascade, such as MAPK signaling pathway[10]. Some evidence showed that the P2X7 receptor plays a important role in central nervous system disorder[10]. Genetic deletion and pharmacological blockade of the P2X7 receptor play neuroprotection in stroke[11–13]. Moreover, inhibition of P2X7 receptor can reduce neuroinflammation, oxidative stress and rescued endothelial dysfunction[14–16]. Therefore, we hypothesized that blockade of the P2X7 receptor protects against HT by limiting ferroptosis-mediated oxidative damage after cerebral ischemia/reperfusion in rat. P2RX7 signaling pathway may be a potential therapeutic strategy to reduce the risk of HT and improve the outcome of patients with AIS.

2. Material And Methods

2.1 Animals models of focal cerebral ischemia

Adult male SD rats weighing 250–280 g were enrolled in this research. The rats were obtained from Animal Center of Wuhan University and housed under standard conditions at 22°C and 50–60% relative humidity, alternating a light/dark cycle of 12:12h. Food and water were supplied ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee of Wuhan University. The investigators were blinded to the treatment of the animals during all surgical procedures.

Focal cerebral ischemia was conducted by intravascular occlusion of the left middle cerebral artery (MCAO) according to previously described protocol[17]. Briefly, SD rats were anesthetized by 4% pentobarbital sodium (50mg/kg) and cut a midline incision in the neck. Then, the left common carotid artery was separated and ligated, the external carotid artery was ligated with 4 – 0 silk thread, and the internal carotid artery was temporarily clipped with an arterial clamp. A silicone-coated nylon monofilament (0.26 mm diameter, Beijing Sunbio Biotech, China) was inserted 18–20 mm into the internal carotid artery, occluding the origin of the MCA. After 3 h, withdrawing the nylon monofilament to restore the blood flow of middle cerebral artery. To established the HT model, 50% glucose was injected intraperitoneally at 6 mL/kg before pulling out the plug to induce acute hyperglycemia in HT group. After the operation, the revived rats were sent back to their cages and given free access to water and food.

2.2 Experimental design and Drugs

All SD rats were randomly divided into four groups: Sham group—rats underwent the MCAO surgical procedure without the filament insertion; MCAO group—rats underwent 3 h of MCAO and received instant reperfusion; HT group—rats underwent 3h of MCAO and received 50% D-glucose (6 ml/kg) intraperitoneally at 30 min before the filament is pulled out; and HT + BBG group—rats underwent MCAO and received 50% D-glucose (6 ml/kg) intraperitoneally, injected intravenous BBG (10 mg/kg, MCExpress, USA) at 60 min before the filament is pulled out.

2.3 Behavioral tests

An investigator blinded to treatment status examined behavioral test at 21 h after reperfusion using neurologic deficit score and corner test. Neurologic deficit score was assessed as described previously[17]. The higher the neurologic deficit score implied more serious injury of neurologic function. For the corner test, SD rats were placed between splints at an Angle of 30 degrees and rats with unilateral brain injury tend to turn to the non-infarcted side. We tested 15 times for each rat and recorded the frequency of right turns.

2.4 Brain water content

Cerebral edema was measured by brain water content with the standard wet–dry method as previously description[18]. Briefly, the brain was separated to the ipsilateral and contralateral hemispheres and respectively weighed by an electronic analytic balance for wet weight. After dry at 100°C for 24 h, the dry weight was weighted. Cerebral water content was calculated as: (wet weight-dry weight)/wet weight×100%.

2.5 TTC staining

Infarct volume was evaluated by 2,3,5-triphe-nyltetrazolium chloride staining (TTC, Sigma, MO) at 21 h after reperfusion. Fresh brain tissue was cut into seven coronal sections (2 mm thick), stained with the 1% TTC solution at 37°C for 30 min, and then fixed in 4% paraformaldehyde for 24 h. TTC-stained sections were photographed, and the infarct volume was analyzed using Image J image-processing software (NIH, Bethesda, MD). The normal tissue was stained red, whereas white or pale represented the

infarct area. The ratio of infarct volume = infarct volume of the ipsilateral hemisphere/total volume of the ipsilateral hemisphere×100%.

2.6 Measurement of MDA and SOD

The level of malondialdehyde (MDA) and superoxide dismutase (SOD) in brain tissue and in HUVECs were measured by MDA assay kit (Jiancheng Bioengineering Institute, China) and SOD assay kit (Jiancheng Bioengineering Institute, China) according to the manufacturer's specifications, respectively.

2.7 Spectrophotometric assay of hemoglobin content

Concentration of hemoglobin in brain tissue was measured with a QuantiChrom Hemoglobin Assay Kit (BioAssay Systems, USA) according to the previous reported[18]. The reslut was showed as milligrams per deciliter.

2.8 Hematoxylin-eosin (HE) staining and Prussian blue dyeing

The left brain tissues of rats were fixed by 4% paraformaldehyde, embedded in paraffin, and then stained by HE (C0105, Beyotime, CA) according to the instructions. Brain sections was stained with Perls' Prussian blue to determine ferric (Fe3+) iron content as described previously[19]. After the sections were sealed, the cerebral sections of each group were observed and photographed under a microscope.

2.9 Cell culture and oxygen-glucose deprivation/reperfusion (OGD/R) model

Human umbilical vein endothelial cells (HUVECs) were cultured in complete medium that contained RPMI1640, 20% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Beyotime Biotechnology, Shanghai, China) at 37°C under 5% CO2. Cells were subcultured every 5–6 days until grown to 80–90% fusion. Culture medium was replaced 24 h after passage and every 2 days thereafter. In order to established OGD/R model in vitro, the normal culture medium of HUVECs was replaced by DMEM medium without glucose, and the cells were placed in an anaerobic chamber at 94% N2/5% CO2/1% O2 and cultured at 37°C for 5h. After oxygen-glucose deprivation injury, cells were cultured in complete RPMI1640 medium or HG media containing 30 mM D-glucose without growth factors for another 19 h.

2.10 siRNA transfection

HUVECs were transfection with lipofectamine 3000 (Invitrogen, USA) using P2RX7 siRNA (5'-AACCAGAAGGGACACACAG-3') or control siRNA (GenePharma, Shanghai, China) as directed.

2.11 Cell viability and dead/live assay

Cell viability was determined by the CCK-8 assay according to the manufacturer's instructions (MCE, USA). HUVECs were seeded on 96-well plates at a density of 5000 cells/well. After treatment of HUVECs, 10 μM of CCK-8 was added to each well and incubated for 1 h. The absorbance at 450 nm was detected

by enzyme labelling apparatus. Cell viability was exhibited as a percentage of the sham group. For dead/live experiment, HUVECs were washed twice with PBS, then cells were incubated with PI (4 μM) and calcein-AM (3 μM) for 30 min at room temperature darkness. Cells were washed again and detected under a fluorescence microscope. PI bound HUVECs produces red fluorescence, indicating dead cells, and calcein-AM bound cells produces green fluorescence for identifying live cells.

2.12 Transendothelial electrical resistance (TEER)

The 24-wells were coated with collagen before implanting. Then, 5*105/ml HUVECs were seeded on corning transwell inserts with a 0.4 μm pore size (Corning 3470) in the luminal side and grown in the complete culture medium as previously described[20]. Total resistance (Ω) of cultured epithelial cells was measured using Millicell-ERS (US) with a STX2 probe daily for 1 week. Group experiment after the resistance is stabilized. All TEER values were normalized to the area of the membrane (0.33 cm^2) and corrected for the resistance without cells.

2.13 Measurement of intracellular iron ion

The level of iron ion in HUVECs were measured using Intracellular Iron Colorimetric Assay Kit (PPLYGEN, China) according to the instructions.

2.14 Immunofluorescence

Rat brains were cut into 25-μm paraffin sections for immunofluorescence. The slices were treated with 0.25% Triton-X 100 for 30 min and sealed with PBS containing 5% bovine serum albumin (BSA) for 1 h. Then, the slices were directly incubated with the primary antibodies at 4°C overnight, such as rabbit anti-P2RX7 (1:200, Proteintech) and mouse anti-Claudin-5 (1:100, Invitrogen). After washing with PBS for 3 times, it was sealed with 5% BSA containing the Alexa 488 or 594-conjugated secondary antibody (1:500, Jackson). The tissue sections were washed twice in PBS and then immersed in 4', 6-diamidino-2-phenylindole (DAPI) with 20min. The sections were detected with a fluorescence microscope (Eclipse 90i; Nikon, Tokyo, Japan).

2.15 Real-time quantitative reverse-transcriptase PCR

Total RNA was extraced with the Trizol reagent (Invitrogen, USA). The concentration was determined by a spectrophotometer. One microgram of total RNA was reversely transcribed with the Hifair® □ 1st Strand cDNA Synthesis SuperMix Kit (Yesen Biotechnology, shanghai, China). The primers s for P2X7R and GAPDH were obtained from (Sangon Biotech, Shanghai, China) as follow: P2RX7, forward primer: AGGTGGCAGTTCAGGGAGGAATC, reverse primer: TGTATTTGGGTTGACAGCGATGGG. GAPDH, forward primer: GGCACAGTCAAGGCTGAGAATG, reverse primer: ATGGTGGTGAAGACGCCAGTA. The amplification was performed in a LightCycler 480 system (Roche, Pleasanton, CA, USA) using the Hieff® qPCR SYBR Green Master Mix (Yesen Biotechnology, shanghai, China). The reaction system with a total volume of 20μl was incubated at 95°C for 5 min, then 40 cycles of 10 s at 95°C and 30s at 60°C. Endogenous

control GAPDH normalized candidate genes mRNA levels in the same sample. The 2 − ΔΔCt method was used to quantify relative gene expression.

2.16 Western blot analysis

Equal amounts of protein were taken for per lane (20 μg) and separated by electrophoresis on 8–12% SDS-PAGE gels. Proteins were transferred to PVDF membranes by electrophoresis and sealed with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1.5 h at room temperature. Thereafter, membranes were incubated with different primary antibodies at 4℃ overnight, including rabbit anti-Occludin-1 (1:1000, Invitrogen), mouse anti-Claudin-5 (1:1000, Invitrogen), and rat anti-ZO-1 (1:500, Santa Cruz Biotechnology), rabbit anti-MMP-9 (1:1000, Abcam), rabbit anti-SLC7A11 (1:1000, Abclone), rabbit anti-HO-1 (1:1000, Abclone), rabbit anti-GPX4 (1:1000, Abcam), rabbit anti-P2X7R (1:1000, Proteintech), rabbit anti-ERK1/2 (1:1000, Cell signaling technology), mouse anti-p-ERK1/2 (1:1000, Cell signaling technology), Rabbit anti-Albumin (1:1000, Proteintech), mouse anti-P53 (1:1000, Proteintech), rabbit anti-Transferrin (1:1000, Proteintech)and rabbit anti-GAPDH (1:3000, Abcam),. After washing with TBST for 3 times, PVDF membranes were immersed with horseradish peroxidase (HRP) - conjugated secondary antibody (1:5000) for 1.5 h at room temperature. Immunolabeling were developed with enhanced ECL kit (Biosharp). Chemiluminescence levels was captured using an imaging system and data were normalized using GAPDH.

2.17 Statistical analysis

The study was conducted by researchers following the principle of randomization and blinding. All results were analyzed by the GraphPad Prism version 5.04 statistical packageas and were showed mean ± standard deviation (SD). Differences of multiple groups were assessed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered statistically significant.

3. Results

3.1 HG increases P2X7R expression in cerebral ischemic/reperfusion area

Immunofluorescence staining was used to detect the protein levels of ZO-1 in cerebral infarct tissue, which suggested BBB damage after MCAO (Fig. 1A-B). We found that the protein level of ZO-1 was reduced in MCAO group compared with in sham group ($P < 0.01$) and further decreased in HT group ($P < 0.01$). To study the changes of P2X7R expression after HT, the brain tissues on the infarcted side were removed for western blot analysis and QPCR analysis at 24h after MCAO in rats. The results indicated that P2X7R expression gradually increase in MCAO group and HT group (Fig. 1A, C and D) compared to Sham group. These results indicated P2RX7 may play an important role in BBB damage and HT pathology.

3.2 Effects of BBG on behavioral tests and cerebral edema after MCAO treated with high glucose

The schematic diagram of the experimental design was exhibited in Fig. 2A. According to the performance tested by neurologic deficit score and corner tests, MCAO group exhibited significant neurologic deficit ($P < 0.01$). Rat treated with hyperglycemia can worsen the abnormal behavior on these tests ($P < 0.05$). Rats conducted with administered BBG and HG performed better than did rats treated with HG alone ($P < 0.01$; Fig. 2B-C). Cerebral edema was detected by dry and wet method, rats treated with HG displayed a trend toward brain edema compared to MCAO-treated rats ($P < 0.01$). BBG treatment decreased cerebral edema in rats treated with HG ($P < 0.05$; Fig. 2D). P2X7 receptor agonist can reduce neurologic deficit and cerebral edema aggravated by HT after MCAO in rats.

3.3 Effect of BBG on infarct volume and hemorrhagic transformation after MCAO treated with hyperglycemia

Cerebral infarction area tested by TTC staining can directly reflect the degree of brain injury. Infarct volume in the HT group was significantly higher than that of the MCAO group ($P < 0.05$). Treatment of BBG reduced the infarct volume compared with that in the HT group ($P < 0.05$, Fig. 3A-B). Moreover, we detected the hemoglobin concentration in ipsilateral hemisphere. The increase in HG-enhanced bleeding was significantly inhibited by BBG administration ($P < 0.01$, Fig. 3C). HE staining also showed the degree of cerebral tissue injury and hemorrhage on the infarct side. In the brain tissue section, tissue edema, degree of blood and nucleus pyknosis increased significantly in the HT group compared to in the MCAO group. BBG can significantly improve the degree of HT and reduce brain tissue damage compared to MCAO group (Fig. 3D).

3.4 Effect of BBG on BBB disruption after MCAO treated with HG

In this experiment, considering the color of the BBG, albumin was used as an indicator of BBB permeability, was mainly concentrated in the regions of the ischemic hemisphere. Immunofluorescent staining and westernblotting showed that albumin extravasation in the HT group was greater than that in the MCAO group ($P < 0.01$, Fig. 4A-C) but was significantly reduced by BBG treatment in rats ($P < 0.01$, Fig. 4A-C). Increased albumin extravasation indicated more severe damage to the BBB. MPO level means the degree of leukocyte infiltration. Our results indicated that the level of MPO was increased in MCAO group than in sham group ($P < 0.01$), and significant higher in HT group than in MCAO group ($P < 0.01$), whereas BBG can reduced the levels of MPO after MCAO treated with HG ($P < 0.01$, Fig. 4D). We also explored the influence of HG on degradation of BBB. Western blotting showed that band density of tight junction protein zonula occludens-1 (ZO-1) and occludin was reduced in the ischemic hemisphere, whereas BBG treatment significantly prevented the HG-enhanced BBB disruption (Fig. 4E-H).

3.5 Effect of BBG on ferroptosis after MCAO treated with HG

Prussian dyeing was used to tested the accumulation of iron in cerebral infraction. The result found that the iron accumulation was significantly higher in HT group than in the MCAO group. rats treated with BBG can decrease the levels of iron compared to BBG group (Fig. 5A). Reactive oxygen species were measured by MDA level and SOD level. The level of MDA was significant higher in HT group than in the MCAO group ($P < 0.01$) and the concentration of SOD of HT group was decreased compared with MCAO group ($P < 0.01$), whereas BBG can reduced the levels of MDA and increased the level of SOD after MCAO treated with HG (Fig. 5B-C). To further explore the relation with ferroptosis, western blot analysis showed that the expression of TFR1, SLC7A11 and GPX4. The expression of SLC7A11 and GPX4 was downregulated, but the expression of TFR1 was increased in HT group compared to MCAO group. BBG treatment can upregulate SLC7A11 and GPX4 and reduce TFR1 expression (Fig. 5D-F), which indicate BBG may regulate ferroptosis in HG-induced HT.

3.6 Effect of BBG on P2RX7-ERK1/2 signaling pathway after MCAO treated with HG

To illustrate the underlying mechanisms of HG-enhanced ferroptosis after cerebral ischemia, we first determined the expression levels of P2RX7, p-ERK1/2 and ERK1/2 in the reperfused brain tissue. The expression of P2RX7 and p-ERK1/2 was increased in HT group compared to MCAO group. BBG treatment can significantly reduce P2RX7 and p-ERK1/2 expression compared to HT group (Fig. 6A-B). P2RX7 and Claudin-5 co-expression detected by immunofluorescence show similar changes (Fig. 6C). Moreover, we also found the expression of MMP-9 apparently up-regulated in MCAO group ($P < 0.01$), and the treatment of hyperglycemia further induced the expression of MMP-9 ($P < 0.01$). BBG can effectively decrease the expression of MMP-9 to improve BBB damage ($P < 0.01$; Fig. 6D-E).

3.7 Effects of P2RX7 inhibition on HUVECs damage after OGD/R with high glucose in vitro

An in vitro BBB model was constructed using HUVECs and OGD model was used to mimic ischemia-reperfusion injury. For further analyzing the degree of HG-induced HUVECs injury after OGD/R, the stability of endothelial cell structure was measured using TEER. We observed the changes of TEER within 7 days after HUVECs inoculation, and found that it reached the maximum value about the 6th day. When the resistance is stable, TEER of each group is detected (Fig. 7A-C). We found that the inhibition of P2RX7 can improve HG-induced HUVECs damage and protect cell integrity (Fig. 7B-C). HG can aggravate HUVECs damage after OGD/R ($P < 0.01$), and inhibition of P2RX7 can improve the viability of HUVECs injury compared to HG + siNC group in vitro ($P < 0.01$; Fig. 7D). Dead/live assay implicated that P2RX7 inhibition can attenuate HG-induced HUVECs damage in vitro (Fig. 7E). To clarified the extent of the damage to the extracellular structure, the protein expression of ZO-1 and occludin was measured by western blot analysis at 24 h after OGD in vitro. The results found that the expression of ZO-1 and occludin was significantly decreased after OGD, and showed a decreasing trend after OGD with high glucose. While the inhibition of P2RX7 can reversed these damage. so, these data indicated that the

inhibition of P2RX7 can improve HUVECs viability, reduce the damage of extracellular structure and protect the integrity of BBB.

3.8 Effects of P2RX7 inhibition on HUVECs ferroptosis after OGD/R with HG in vitro

To clarify the protective mechanism of HUVECs, we further tested that the level of oxidative stress and iron concentration which were related to the ferroptosis. We found that the MDA level of control group was increased compared to sham group ($P < 0.01$), and HG can further aggravate oxidative stress, while the MDA level of HG + si-P2RX7 group was reduced after OGD/R in vitro compared to HG + siNC group ($P < 0.05$; Fig. 8A). We also found that the concentration of iron ions was changed in HUVECs with different treatment. The results indicated that the level of iron ions showed an increased trend in HG group after OGD/R in vitro. The inhibition of P2RX7 can decrease the accumulation of iron ions in HUVECs after OGD/R in vitro ($P < 0.01$; Fig. 8B). In order to identify ferroptosis pathway of HUVECs, we detected ferroptosis-related protein by western blot analysis in HUVECs at 24 h after OGD in vitro. The results showed that the expression level of SLC7A11 and GPX4 was reduced and further decreased after OGD with high glucose. But the expression of P53 was increased after OGD and showed an increasing trend after OGD with high glucose. The inhibition of P2RX7 can significantly increase the expression of SLC7A11 and GPX4 and reduce the level of P53 (Fig. 8C-F). These results implicated that SLC7A11/GPX4 pathway play important role in HUVECs ferroptosis after OGD with high glucose. High expression of P53 may regulate ferroptosis by inhibiting SLC7A11 after OGD with high glucose. P2RX7 may regulate SLC7A11/GPX4 pathway by suppressing P53 in HUVECs after OGD with high glucose.

3.9 Effects of P2RX7 inhibition on ERK1/2-HO-1 signaling pathway in HUVECs after OGD/R treated with high glucose in vitro

To further clarify the potential relationship of P2RX7 and ferroptosis, we also detected the expression of P2RX7, p-ERK1/2, ERK1/2 and HO-1. The data indicated that the expression level of P2RX7 was significantly suppressed by P2RX7 siRNA in OGD/R + HG + si-P2RX7 group compared with in OGD/R + HG group ($P < 0.01$). The level of p-ERK1/2 was up-regulated in control group compared to in sham group, and further increased after OGD/R treated with high glucose. P2RX7 inhibition significantly down-regulated the level of p-ERK1/2. Some studies have found that the activation of p-ERK1/2 can enhance the level of P53[21]. ($P < 0.01$). Our result also implicated that P2RX7 may activate p-ERK1/2 to regulate P53 after OGD/R and HG. In addition, the HO-1 is needed for the import of iron into the cell and is regulated by intracellular iron concentration[22], therefore we analyzed the expression of HO-1. We also found the expression of HO-1 apparently increased in OGD/R group ($P < 0.05$), and the treatment of hyperglycemia further induced the expression of TFR1 ($P < 0.05$). P2RX7 inhibition can significantly reduce the expression of HO-1 after OGD/R and HG treatment ($P < 0.01$, Fig. 9A-E). These results indicated that the high level of HO-1 may be involve in the process of HUVECs ferroptosis.

Discussion

This study investigated the mechanism of P2RX7 in the pathogenesis of HT after MCAO treated with high glucose. Our results indicated that oxidative stress and iron accumulation were significantly increased after MCAO treated with high glucose, which indicated the existence of ferroptosis in endothelium. The inhibition of P2RX7 showed the protective effect on improving neurological deficits, brain edema, infarct volume, BBB disruption and HT in MCAO model treated with rt-PA. Endothelial cell is one of important component of the BBB. Endothelium damage and destruction of extracellular matrix are important causes of BBB breakdown. Moreover, the inhibition of P2RX7 can reverse the reduction of SLC7A11 and GPX4 and reduce endothelium ferroptosis. We also demonstrated a potential mechanism that P2RX7 may regulate ERK1/2-HO-1 and P53 pathway (Fig. 10).

As we all known, the mechanism of HT after ischemic/reperfusion (I/R) was complex, including oxidative stress, inflammation and excitotoxicity. What the brain tissue lack vital nutrients such as oxygen and glucose is very important reason. Multiple mechanisms of cell injury are activated after cerebral I/R[23]. A direct impact of lack of oxygen and glucose delivery is the change of mitochondrial function, which was recognized as the main source of ROS in reperfusion injury[24]. Some evidence suggested that iron is a risk factor in the development of cerebral I/R[25], and the iron content is increased in ischemic brains[26]. Iron-overloaded animals are more susceptible to MCAO, whereas iron chelation or iron deficiency reduces the damage of I/R[27]. Ferroptosis has been suggested to be involve in the process of endothelial cell damage and BBB disruption[27, 28]. Some drugs were showed protective effect by inhibiting ferroptosis via SLC7A11/GPX4 signaling pathway after MCAO[27, 29, 30]. Our result showed the existence of ferroptosis in infract brain tissue after MCAO and in HUVECs after OGD/R, with lipid peroxidation, iron concentration and decreased expression of SLC7A11 and GPX4, which indicated that ferroptosis of endothelium is a potential pathogenic mechanism.

In addition, several studies showed that high glucose levels were related with increased HT in diabetic patients with acute ischemic stroke[31]. Hyperglycemia has been considered an independent predictor of stroke outcome. With the increase of patients with diabetes, it is more and more important to study the effect of hyperglycemia on HT after ischemic stroke. The mechanism of hyperglycemia-induced HT may involve oxidative stress. Accumulation of ROS is also an important hallmark of ferroptosis, which is reduced by the iron-dependent Fenton response and contributes to lipid peroxidation[32]. Some studies indicated that HG can mediate endothelial dysfunction by ferroptosis pathway[33, 34], and some studies showed that antiglycemic therapy can significantly reduce HT[35, 36]. Understanding the molecular mechanism of HG-induced ferroptosis holds promising applications for the protection of BBB in the future. In our study, we found that HG-induced HUVECs damage, and the reduction in TEER value as well as in the expression of tight junction protein, such as ZO-1 and occludin, significantly decrease BBB integrity in vitro, which is associated to lipid peroxidation. MMP-9, as a key protease regulating the BBB, was higher also play an important role in BBB disruption. HG-induced ferroptosis is one of important way of endothelial cell death after MCAO.

Under pathological conditions, the purinergic P2X7R can be activated by increased extracellular ATP concentrations, which can trigger ATP excitotoxic neuronal death caused by calcium (Ca^{2+}) overload and

disturbance of mitochondrial membrane potential[37, 38]. P2X7R-mediated oxidative stress may play a part in the development and progression of neurodegenerative diseases, such as AD and PD[39, 40]. Some studies have found that activation of P2X7R mediates the production of NOX2-dependent ROS by activating extracellular ERK1/2[16]. Some studies implicated that P2RX7 involves damage to vascular endothelium, leading to endothelial dysfunction[41]. Blocking P2RX7 activation can attenuate free radical production[42–44]. P2RX7 activation can regulate the expression of P53[45–47]. Some studies have identified that p53 can promote ferroptosis by suppressing the expression level of SLC7A11/GPX4[48–52], which indicated the relationship of P2RX7 and SLC7A11. SLC7A11 and GPX4 are one of key regulators of ferroptosis. Furthermore, there have found that P2X7R mediated ERK1/2 phosphorylation[53, 54] and ERK1/2 can regulate P53[55, 56]. Some studies indicated that inhibition of ERK1/2 can reduce the apoptosis of hippocampal neuron after SAH[57]. We hypothesized that P2RX7 may act on SLC7A11 by regulating ERK1/2 phosphorylation and activating P53. Furthermore, heme derived from HT after MCAO can further stimulate the expression of HO-1 and TFR1. HO-1 initiate heme catabolism, which releases iron, carbon monoxide, and biliverdin[58]. However, the role of HO-1 in ferroptosis remain controversial. Some studies holded that the upregulation of HO-1 pathway can attenuate ferroptosis[59–61]. Other studies suggested that the overexpression of HO-1 has been identified to significantly promote ferroptosis for the increase in the labile iron pool [62–66]. In the brain, HO-1 and TFR1 are considered to be involved in oxidative stress, iron homeostasis, and cellular adaptive mechanisms[67]. Inhibition of HO-1 can improve cerebral ischemia-reperfusion injury[68]. HO-1 expression level may influence its role in acute injury. Our results showed that ischemia/reperfusion and HG promote ferroptosis in endothelium by excessively inducing HO-1 expression, resulted in the progression of BBB damage in vitro and in vivo, which can be reversed by P2RX7 block. These data elucidated that P2RX7 blocking can regulate SLC7A11/GPX4 by suppressing ERK1/2 and P53 pathway, and plays important role in ferroptosis pathways.

Conclusion

In conclusion, our study showed that the high glucose can promote oxidative stress and iron accumulation, resulting in endothelium ferroptosis and BBB damage, causing HT after MCAO. the P2RX7 play an important role in HG-associated oxidative stress, endothelial damage, BBB disruption, and neurological deficient, inhibition of which reduces HG-induced hemorrhagic transformation after MCAO by inhibiting ERK1/2 and P53. Upregulation of SLC7A11 and GPX4 can reduce the ferroptosis of endothelium. P2RX7 was a potential therapeutic target to reduce the adverse effect of high glucose on BBB through ferroptosis pathway, ultimately reducing neurovascular damage and improving stroke prognosis. These finding have important clinical implications and could be developed to potentially therapeutic strategy for patients with diabetes after AIS.

Declarations

Ethics approval

All animal experiments were approved by the Institutional Animal Care and Use Committee of Wuhan University.

Consent for Publication

Not applicable.

Data and Materials Availability

All data generated or analyzed during this study are included in this article.

Competing interests

The authors declare that there is no conflict of interest for the publication of this article.

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Author's contributions

CL-L and QT designed and complete the study, conducted data analysis, and prepared the manuscript. JF-W, PB-H, YJ-G, CY, GJ-W, SM-H and HW built MCAO models of rats and cultured HUVECs. MC-L reviewed and revised the manuscript.

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References

1. Couret D, Bourane S, Catan A, Nativel B, Planesse C, Dorsemans AC, Ait-Arsa I, Cournot M, Rondeau P, Patche J, Tran-Dinh A, Lambert G, Diotel N, Meilhac O (2018) A hemorrhagic transformation model of mechanical stroke therapy with acute hyperglycemia in mice. J Comp Neurol 526(6):1006–1016. https://doi.org/10.1002/cne.24386
2. Shao A, Gao S, Wu H, Xu W, Pan Y, Fang Y, Wang X, Zhang J (2021) Melatonin Ameliorates Hemorrhagic Transformation via Suppression of ROS-Induced NLRP3 Activation after Cerebral Ischemia in Hyperglycemic Rats. Oxid Med Cell Longev 2021:6659282. https://doi.org/10.1155/2021/6659282

3. Weiland A, Wang Y, Wu W, Lan X, Han X, Li Q, Wang J (2019) Ferroptosis and Its Role in Diverse Brain Diseases. Mol Neurobiol 56(7):4880–4893. https://doi.org/10.1007/s12035-018-1403-3
4. Su L, Jiang X, Yang C, Zhang J, Chen B, Li Y, Yao S, Xie Q, Gomez H, Murugan R, Peng Z (2019) Pannexin 1 mediates ferroptosis that contributes to renal ischemia/reperfusion injury. J Biol Chem 294(50):19395–19404. https://doi.org/10.1074/jbc.RA119.010949
5. Cheng Y, Song Y, Chen H, Li Q, Gao Y, Lu G, Luo C (2021) Ferroptosis Mediated by Lipid Reactive Oxygen Species: A Possible Causal Link of Neuroinflammation to Neurological Disorders. Oxid Med Cell Longev 2021:5005136. https://doi.org/10.1155/2021/5005136
6. Chen B, Chen Z, Liu M, Gao X, Cheng Y, Wei Y, Wu Z, Cui D, Shang H (2019) Inhibition of neuronal ferroptosis in the acute phase of intracerebral hemorrhage shows long-term cerebroprotective effects. Brain Res Bull 153:122–132. https://doi.org/10.1016/j.brainresbull.2019.08.013
7. Feng Y, Madungwe NB, Imam Aliagan AD, Tombo N, Bopassa JC (2019) Liproxstatin-1 protects the mouse myocardium against ischemia/reperfusion injury by decreasing VDAC1 levels and restoring GPX4 levels. Biochem Biophys Res Commun 520(3):606–611. https://doi.org/10.1016/j.bbrc.2019.10.006
8. Ma H, Wang X, Zhang W, Li H, Zhao W, Sun J, Yang M (2020) Melatonin Suppresses Ferroptosis Induced by High Glucose via Activation of the Nrf2/HO-1 Signaling Pathway in Type 2 Diabetic Osteoporosis. Oxid Med Cell Longev 2020:9067610. https://doi.org/10.1155/2020/9067610
9. He J, Li Z, Xia P, Shi A, FuChen X, Zhang J, Yu P (2022) Ferroptosis and ferritinophagy in diabetes complications. Mol Metab 60:101470. https://doi.org/10.1016/j.molmet.2022.101470
10. Sperlágh B, Illes P (2014) P2X7 receptor: an emerging target in central nervous system diseases. Trends Pharmacol Sci 35(10):537–547. https://doi.org/10.1016/j.tips.2014.08.002
11. Zhao H, Zhang X, Dai Z, Feng Y, Li Q, Zhang JH, Liu X, Chen Y, Feng H (2016) P2X7 Receptor Suppression Preserves Blood-Brain Barrier through Inhibiting RhoA Activation after Experimental Intracerebral Hemorrhage in Rats. Sci Rep 6:23286. https://doi.org/10.1038/srep23286
12. Huang C, Chi XS, Li R, Hu X, Xu HX, Li JM, Zhou D (2017) Inhibition of P2X7 Receptor Ameliorates Nuclear Factor-Kappa B Mediated Neuroinflammation Induced by Status Epilepticus in Rat Hippocampus. J Mol Neurosci 63(2):173–184. https://doi.org/10.1007/s12031-017-0968-z
13. Wen Z, Mei B, Li H, Dou Y, Tian X, Shen M, Chen G (2017) P2X7 Participates in Intracerebral Hemorrhage-Induced Secondary Brain Injury in Rats via MAPKs Signaling Pathways. Neurochem Res 42(8):2372–2383. https://doi.org/10.1007/s11064-017-2257-1
14. Furuta T, Mukai A, Ohishi A, Nishida K, Nagasawa K (2017) Oxidative stress-induced increase of intracellular zinc in astrocytes decreases their functional expression of P2X7 receptors and engulfing activity. Metallomics 9(12):1839–1851. https://doi.org/10.1039/c7mt00257b
15. Leng B, Li C, Sun Y, Zhao K, Zhang L, Lu ML, Wang HX (2020) Protective Effect of Astragaloside IV on High Glucose-Induced Endothelial Dysfunction via Inhibition of P2X7R Dependent P38 MAPK Signaling Pathway. Oxid Med Cell Longev 2020:5070415. https://doi.org/10.1155/2020/5070415

16. Deng H, Zhang Y, Li GG, Yu HH, Bai S, Guo GY, Guo WL, Ma Y, Wang JH, Liu N, Pan C, Tang ZP (2021) P2X7 receptor activation aggravates NADPH oxidase 2-induced oxidative stress after intracerebral hemorrhage. Neural Regen Res 16(8):1582–1591. https://doi.org/10.4103/1673-5374.303036
17. Wang W, Li M, Wang Y, Li Q, Deng G, Wan J, Yang Q, Chen Q, Wang J (2016) GSK-3β inhibitor TWS119 attenuates rtPA-induced hemorrhagic transformation and activates the Wnt/β-catenin signaling pathway after acute ischemic stroke in rats. Mol Neurobiol 53(10):7028–7036. https://doi.org/10.1007/s12035-015-9607-2
18. Liu C, Sun S, Xie J, Li H, Li T, Wu Q, Zhang Y, Bai X, Wang J, Wang X, Li Z, Wang W (2022) GLP-1R Agonist Exendin-4 Protects Against Hemorrhagic Transformation Induced by rtPA After Ischemic Stroke via the Wnt/β-Catenin Signaling Pathway. Mol Neurobiol. https://doi.org/10.1007/s12035-022-02811-9
19. Yasmin A, Pitkänen A, Andrade P, Paananen T, Gröhn O, Immonen R (2021) Post-injury ventricular enlargement associates with iron in choroid plexus but not with seizure susceptibility nor lesion atrophy-6-month MRI follow-up after experimental traumatic brain injury. Brain Struct Funct. https://doi.org/10.1007/s00429-021-02395-5
20. Prasad S, Sajja RK, Park JH, Naik P, Kaisar MA, Cucullo L (2015) Impact of cigarette smoke extract and hyperglycemic conditions on blood-brain barrier endothelial cells. Fluids Barriers CNS 12:18. https://doi.org/10.1186/s12987-015-0014-x
21. Malik S, Saha R, Seth P (2014) Involvement of extracellular signal-regulated kinase (ERK1/2)-p53-p21 axis in mediating neural stem/progenitor cell cycle arrest in co-morbid HIV-drug abuse exposure. J Neuroimmune Pharmacol 9(3):340–353. https://doi.org/10.1007/s11481-014-9523-7
22. Park E, Chung SW (2019) ROS-mediated autophagy increases intracellular iron levels and ferroptosis by ferritin and transferrin receptor regulation. Cell Death Dis 10(11):822. https://doi.org/10.1038/s41419-019-2064-5
23. Saralkar P, Arsiwala T, Geldenhuys WJ (2020) Nanoparticle formulation and in vitro efficacy testing of the mitoNEET ligand NL-1 for drug delivery in a brain endothelial model of ischemic reperfusion-injury. Int J Pharm 578:119090. https://doi.org/10.1016/j.ijpharm.2020.119090
24. Tang YC, Tian HX, Yi T, Chen HB (2016) The critical roles of mitophagy in cerebral ischemia. Protein Cell 7(10):699–713. https://doi.org/10.1007/s13238-016-0307-0
25. Ding H, Yan CZ, Shi H, Zhao YS, Chang SY, Yu P, Wu WS, Zhao CY, Chang YZ, Duan XL (2011) Hepcidin is involved in iron regulation in the ischemic brain. PLoS One 6(9):e25324. https://doi.org/10.1371/journal.pone.0025324
26. Tuo QZ, Lei P, Jackman KA, Li XL, Xiong H, Li XL, Liuyang ZY, Roisman L, Zhang ST, Ayton S, Wang Q, Crouch PJ, Ganio K, Wang XC, Pei L, Adlard PA, Lu YM, Cappai R, Wang JZ, Liu R, Bush AI (2017) Tau-mediated iron export prevents ferroptotic damage after ischemic stroke. Mol Psychiatry 22(11):1520–1530. https://doi.org/10.1038/mp.2017.171
27. Chen W, Jiang L, Hu Y, Tang N, Liang N, Li XF, Chen YW, Qin H, Wu L (2021) Ferritin reduction is essential for cerebral ischemia-induced hippocampal neuronal death through p53/SLC7A11-

mediated ferroptosis. Brain Res 1752:147216. https://doi.org/10.1016/j.brainres.2020.147216

28. Abdul Y, Li W, Ward R, Abdelsaid M, Hafez S, Dong G, Jamil S, Wolf V, Johnson MH, Fagan SC, Ergul A (2021) Deferoxamine Treatment Prevents Post-Stroke Vasoregression and Neurovascular Unit Remodeling Leading to Improved Functional Outcomes in Type 2 Male Diabetic Rats: Role of Endothelial Ferroptosis. Transl Stroke Res 12(4):615–630. https://doi.org/10.1007/s12975-020-00844-7
29. Fu C, Wu Y, Liu S, Luo C, Lu Y, Liu M, Wang L, Zhang Y, Liu X (2022) Rehmannioside A improves cognitive impairment and alleviates ferroptosis via activating PI3K/AKT/Nrf2 and SLC7A11/GPX4 signaling pathway after ischemia. J Ethnopharmacol 289:115021. https://doi.org/10.1016/j.jep.2022.115021
30. Zhai QY, Ren YQ, Ni QS, Song ZH, Ge KL, Guo YL (2022) Transplantation of Human Umbilical Cord Mesenchymal Stem Cells-Derived Neural Stem Cells Pretreated with Neuregulin1β Ameliorate Cerebral Ischemic Reperfusion Injury in Rats. Biomolecules 12(3). https://doi.org/10.3390/biom12030428
31. Kunte H, Busch MA, Trostdorf K, Vollnberg B, Harms L, Mehta RI, Castellani RJ, Mandava P, Kent TA, Simard JM (2012) Hemorrhagic transformation of ischemic stroke in diabetics on sulfonylureas. Ann Neurol 72(5):799–806. https://doi.org/10.1002/ana.23680
32. Kuang F, Liu J, Tang D, Kang R (2020) Oxidative Damage and Antioxidant Defense in Ferroptosis. Front Cell Dev Biol 8:586578. https://doi.org/10.3389/fcell.2020.586578
33. Luo EF, Li HX, Qin YH, Qiao Y, Yan GL, Yao YY, Li LQ, Hou JT, Tang CC, Wang D (2021) Role of ferroptosis in the process of diabetes-induced endothelial dysfunction. World J Diabetes 12(2):124–137. https://doi.org/10.4239/wjd.v12.i2.124
34. Zhang J, Qiu Q, Wang H, Chen C, Luo D (2021) TRIM46 contributes to high glucose-induced ferroptosis and cell growth inhibition in human retinal capillary endothelial cells by facilitating GPX4 ubiquitination. Exp Cell Res 407(2):112800. https://doi.org/10.1016/j.yexcr.2021.112800
35. Chen F, Wang W, Ding H, Yang Q, Dong Q, Cui M (2016) The glucagon-like peptide-1 receptor agonist exendin-4 ameliorates warfarin-associated hemorrhagic transformation after cerebral ischemia. J Neuroinflammation 13(1):204. https://doi.org/10.1186/s12974-016-0661-0
36. Kuroki T, Tanaka R, Shimada Y, Yamashiro K, Ueno Y, Shimura H, Urabe T, Hattori N (2016) Exendin-4 Inhibits Matrix Metalloproteinase-9 Activation and Reduces Infarct Growth After Focal Cerebral Ischemia in Hyperglycemic Mice. Stroke 47(5):1328–1335. https://doi.org/10.1161/STROKEAHA.116.012934
37. Arbeloa J, Pérez-Samartín A, Gottlieb M, Matute C (2012) P2X7 receptor blockade prevents ATP excitotoxicity in neurons and reduces brain damage after ischemia. Neurobiol Dis 45(3):954–961. https://doi.org/10.1016/j.nbd.2011.12.014
38. Seeland S, Kettiger H, Murphy M, Treiber A, Giller J, Kiss A, Sube R, Krähenbühl S, Hafner M, Huwyler J (2015) ATP-induced cellular stress and mitochondrial toxicity in cells expressing purinergic P2X7 receptor. Pharmacol Res Perspect 3(2):e00123. https://doi.org/10.1002/prp2.123

39. Ferrazoli EG, de Souza HD, Nascimento IC, Oliveira-Giacomelli Á, Schwindt TT, Britto LR, Ulrich H (2017) Brilliant Blue G, But Not Fenofibrate, Treatment Reverts Hemiparkinsonian Behavior and Restores Dopamine Levels in an Animal Model of Parkinson's Disease. Cell Transplant 26(4):669–677. https://doi.org/10.3727/096368917X695227
40. Cieślak M, Wojtczak A (2018) Role of purinergic receptors in the Alzheimer's disease. Purinergic Signal 14(4):331–344. https://doi.org/10.1007/s11302-018-9629-0
41. Wu XM, Zhang N, Li JS, Yang ZH, Huang XL, Yang XF (2022) Purinergic receptors mediate endothelial dysfunction and participate in atherosclerosis. Purinergic Signal. https://doi.org/10.1007/s11302-021-09839-x
42. Sathanoori R, Swärd K, Olde B, Erlinge D (2015) The ATP Receptors P2X7 and P2X4 Modulate High Glucose and Palmitate-Induced Inflammatory Responses in Endothelial Cells. PLoS One 10(5):e0125111. https://doi.org/10.1371/journal.pone.0125111
43. Zhang QL, Wang W, Alatantuya, Dongmei, Lu ZJ, Li LL, Zhang TZ (2018) Down-regulated miR-187 promotes oxidative stress-induced retinal cell apoptosis through P2X7 receptor. Int J Biol Macromol 120(Pt A):801–810. https://doi.org/10.1016/j.ijbiomac.2018.08.166
44. da Silva CS, Calió ML, Mosini AC, Pires JM, Rêgo D, Mello LE, Leslie A (2019) LPS-Induced Systemic Neonatal Inflammation: Blockage of P2X7R by BBG Decreases Mortality on Rat Pups and Oxidative Stress in Hippocampus of Adult Rats. Front Behav Neurosci 13:240. https://doi.org/10.3389/fnbeh.2019.00240
45. Schulze-Lohoff E, Hugo C, Rost S, Arnold S, Gruber A, Brüne B, Sterzel RB (1998) Extracellular ATP causes apoptosis and necrosis of cultured mesangial cells via P2Z/P2X7 receptors. Am J Physiol 275(6):F962-971. https://doi.org/10.1152/ajprenal.1998.275.6.F962
46. Mello Pde A, Filippi-Chiela EC, Nascimento J, Beckenkamp A, Santana DB, Kipper F, Casali EA, Nejar Bruno A, Paccez JD, Zerbini LF, Wink MR, Lenz G, Buffon A (2014) Adenosine uptake is the major effector of extracellular ATP toxicity in human cervical cancer cells. Mol Biol Cell 25(19):2905–2918. https://doi.org/10.1091/mbc.E14-01-0042
47. Zhang Y, Yuan F, Cao X, Zhai Z, GangHuang, Du X, Wang Y, Zhang J, Huang Y, Zhao J, Hou W (2014) P2X7 receptor blockade protects against cisplatin-induced nephrotoxicity in mice by decreasing the activities of inflammasome components, oxidative stress and caspase-3. Toxicol Appl Pharmacol 281(1):1–10. https://doi.org/10.1016/j.taap.2014.09.016
48. Jiang L, Kon N, Li T, Wang SJ, Su T, Hibshoosh H, Baer R, Gu W (2015) Ferroptosis as a p53-mediated activity during tumour suppression. Nature 520(7545):57–62. https://doi.org/10.1038/nature14344
49. Kang R, Kroemer G, Tang D (2019) The tumor suppressor protein p53 and the ferroptosis network. Free Radic Biol Med 133:162–168. https://doi.org/10.1016/j.freeradbiomed.2018.05.074
50. Hong T, Lei G, Chen X, Li H, Zhang X, Wu N, Zhao Y, Zhang Y, Wang J (2021) PARP inhibition promotes ferroptosis via repressing SLC7A11 and synergizes with ferroptosis inducers in BRCA-proficient ovarian cancer. Redox Biol 42:101928. https://doi.org/10.1016/j.redox.2021.101928

51. Kuang H, Wang T, Liu L, Tang C, Li T, Liu M, Wang T, Zhong W, Wang Y (2021) Treatment of early brain injury after subarachnoid hemorrhage in the rat model by inhibiting p53-induced ferroptosis. Neurosci Lett 762:136134. https://doi.org/10.1016/j.neulet.2021.136134
52. Zhu K, Zhu X, Sun S, Yang W, Liu S, Tang Z, Zhang R, Li J, Shen T, Hei M (2021) Inhibition of TLR4 prevents hippocampal hypoxic-ischemic injury by regulating ferroptosis in neonatal rats. Exp Neurol 345:113828. https://doi.org/10.1016/j.expneurol.2021.113828
53. Gendron FP, Neary JT, Theiss PM, Sun GY, Gonzalez FA, Weisman GA (2003) Mechanisms of P2X7 receptor-mediated ERK1/2 phosphorylation in human astrocytoma cells. Am J Physiol Cell Physiol 284(2):C571-581. https://doi.org/10.1152/ajpcell.00286.2002
54. Sun X, Zhou R, Lei Y, Hu J, Li X (2020) The ligand-gated ion channel P2X7 receptor mediates NLRP3/caspase-1-mediated pyroptosis in cerebral cortical neurons of juvenile rats with sepsis. Brain Res 1748:147109. https://doi.org/10.1016/j.brainres.2020.147109
55. Lin HY, Tang HY, Davis FB, Davis PJ (2011) Resveratrol and apoptosis. Ann N Y Acad Sci 1215:79–88. https://doi.org/10.1111/j.1749-6632.2010.05846.x
56. Lee HJ, Oh SY, Jo I (2021) Zearalenone Induces Endothelial Cell Apoptosis through Activation of a Cytosolic Ca(2+)/ERK1/2/p53/Caspase 3 Signaling Pathway. Toxins (Basel) 13(3). https://doi.org/10.3390/toxins13030187
57. Feng D, Wang B, Ma Y, Shi W, Tao K, Zeng W, Cai Q, Zhang Z, Qin H (2016) The Ras/Raf/Erk Pathway Mediates the Subarachnoid Hemorrhage-Induced Apoptosis of Hippocampal Neurons Through Phosphorylation of p53. Mol Neurobiol 53(8):5737–5748. https://doi.org/10.1007/s12035-015-9490-x
58. Li C, Lönn ME, Xu X, Maghzal GJ, Frazer DM, Thomas SR, Halliwell B, Richardson DR, Anderson GJ, Stocker R (2012) Sustained expression of heme oxygenase-1 alters iron homeostasis in nonerythroid cells. Free Radic Biol Med 53(2):366–374. https://doi.org/10.1016/j.freeradbiomed.2012.03.007
59. Li J, Lu K, Sun F, Tan S, Zhang X, Sheng W, Hao W, Liu M, Lv W, Han W (2021) Panaxydol attenuates ferroptosis against LPS-induced acute lung injury in mice by Keap1-Nrf2/HO-1 pathway. J Transl Med 19(1):96. https://doi.org/10.1186/s12967-021-02745-1
60. Li S, Zhou C, Zhu Y, Chao Z, Sheng Z, Zhang Y, Zhao Y (2021) Ferrostatin-1 alleviates angiotensin II (Ang II)- induced inflammation and ferroptosis in astrocytes. Int Immunopharmacol 90:107179. https://doi.org/10.1016/j.intimp.2020.107179
61. Lv Z, Wang F, Zhang X, Zhang X, Zhang J, Liu R (2021) Etomidate Attenuates the Ferroptosis in Myocardial Ischemia/Reperfusion Rat Model via Nrf2/HO-1 Pathway. Shock 56(3):440–449. https://doi.org/10.1097/SHK.0000000000001751
62. Chang LC, Chiang SK, Chen SE, Yu YL, Chou RH, Chang WC (2018) Heme oxygenase-1 mediates BAY 11-7085 induced ferroptosis. Cancer Lett 416:124–137. https://doi.org/10.1016/j.canlet.2017.12.025
63. Fernández-Mendívil C, Luengo E, Trigo-Alonso P, García-Magro N, Negredo P, López MG (2021) Protective role of microglial HO-1 blockade in aging: Implication of iron metabolism. Redox Biol

38:101789. https://doi.org/10.1016/j.redox.2020.101789

64. Tang Z, Ju Y, Dai X, Ni N, Liu Y, Zhang D, Gao H, Sun H, Zhang J, Gu P (2021) HO-1-mediated ferroptosis as a target for protection against retinal pigment epithelium degeneration. Redox Biol 43:101971. https://doi.org/10.1016/j.redox.2021.101971
65. Wu A, Feng B, Yu J, Yan L, Che L, Zhuo Y, Luo Y, Yu B, Wu, Chen D (2021) Fibroblast growth factor 21 attenuates iron overload-induced liver injury and fibrosis by inhibiting ferroptosis. Redox Biol 46:102131. https://doi.org/10.1016/j.redox.2021.102131
66. Feng YD, Ye W, Tian W, Meng JR, Zhang M, Sun Y, Zhang HN, Wang SJ, Wu KH, Liu CX, Liu SY, Cao W, Li XQ (2022) Old targets, new strategy: Apigenin-7-O-β-d-(-6"-p-coumaroyl)-glucopyranoside prevents endothelial ferroptosis and alleviates intestinal ischemia-reperfusion injury through HO-1 and MAO-B inhibition. Free Radic Biol Med. https://doi.org/10.1016/j.freeradbiomed.2022.03.033
67. Ozen M, Kitase Y, Vasan V, Burkhardt C, Ramachandra S, Robinson S, Jantzie LL (2021) Chorioamnionitis Precipitates Perinatal Alterations of Heme-Oxygenase-1 (HO-1) Homeostasis in the Developing Rat Brain. Int J Mol Sci 22(11). https://doi.org/10.3390/ijms22115773
68. Zhao J, Zhao X, Tian J, Xue R, Luo B, Lv J, Gao J, Wang M (2020) Theanine attenuates hippocampus damage of rat cerebral ischemia-reperfusion injury by inhibiting HO-1 expression and activating ERK1/2 pathway. Life Sci 241:117160. https://doi.org/10.1016/j.lfs.2019.117160

Figures

Figure 1

HG increases expression of P2RX7 and disruption of ZO-1 in ischemic brain tissue.

(A-B) Protein expression levels of ZO-1 at 24h after MCAO (n = 4 rats/group). (A, C-D) Protein and mRNA expression levels of P2X7 receptor at 24h after MCAO (n = 4 rats/group). * mean $P < 0.05$, ** mean $P < 0.01$, *** mean $P < 0.001$.

Figure 2

P2X7 receptor agonist reduces neurologic deficit and encephaledema after MCAO.

(A) Schematic diagram of the experimental design was exhibited in this study. (B-C) Bar graphs showed rat performance change by neurologic deficit scores and corner test scores at 24 h after MCAO in rats (n = 15 rats/group). (D) Bar graphs show cerebral edema on the infarct side at 24 h after MCAO (n = 5 rats/group). * mean $P < 0.05$, ** mean $P < 0.01$, *** mean $P < 0.001$.

Figure 3

P2X7 receptor agonist reduces HG-induced infarct volume and hemorrhagic transformation after MCAO in rats.

(A-B) Bar graphs displyed infarct volumes at 24 h after MCAO (n = 5 rats/group). (C) Hemorrhage volume as evaluated by hemoglobin spectrophotometric assay (n = 5 rats/group). (D) Histopathological changes detected by H&E staining in infarcted cerebral medulla (original magnification ×400) (n=4 rats/group). * mean $P < 0.05$, ** mean $P < 0.01$, *** mean $P < 0.001$.

Figure 4

P2RX7 receptor agonist reduces HG-induced BBB damage after cerebral ischemic in rat.

(A) The level of albumin detected by immunofluorescent staining in infarcted cerebral medulla at 24h after MCAO (original magnification ×400). (B-C) Western blotting images and quantitative data of albumin extravasation at 24 h after MCAO (n=4 rats/group). (D) MPO level of infarcted cerebral tissue in each group at 24 h after MCAO (n=4 rats/group). (E-H) Western blotting images and quantitative data of expression level of ZO-1 and occludin after MCAO in rats (n=4 rats/group). * mean $P < 0.05$, ** mean $P < 0.01$, *** mean $P < 0.001$.

Figure 5

P2RX7 receptor agonist reduces HG-induced ferroptosis after cerebral ischemic in rat.

(A) Iron accumulation confirmed by Perl's Prussian blue in infarcted cerebral medulla (original magnification ×400). (B) MDA level of infarcted cerebral tissue in each group at 24 h after MCAO (n=4 rats/group). (C) SOD level of infarcted cerebral tissue in each group at 24 h after MCAO (n=4 rats/group). (D-F) Western blotting images and quantitative data of relative expression level of ferroptosis-related protein at 24 h after MCAO (n=4 rats/group). * mean $P < 0.05$, ** mean $P < 0.01$, *** mean $P < 0.001$.

Figure 6

P2RX7-ERK1/2 signaling pathway and MMP-9 play important role in HG-induced hemorrhagic transformation after cerebral ischemic in rat.

(A-B) Immunoblot images and data of expression level of P2RX7, p-ERK1/2 and ERK1/2 at 24 h after MCAO (n=4 rats/group). (C) Representative images of P2RX7 and Claudin-5 co-expression in infarcted cerebral medulla (original magnification ×400). (D-E) Immunoblot images and quantitative data of the protein level of the MMP-9 at 24 h after MCAO (n=4 rats/group). * mean $P < 0.05$, ** mean $P < 0.01$, *** mean $P < 0.001$.

Figure 7

The inhibition of P2RX7 reduce the damage of HUVECs and extracellular structure after OGD/R with high glucose in vitro.

(A) Diagram of the blood-brain barrier in vitro. (B) The TEER value of HUVECs within 7 days after inoculation (n=6). (C) The TEER value of HUVECs at 24 h after OGD in vitro (n=6/group). (D) The viability of HUVECs treated by different way was accessed by CCK-8 kit at 24 h after OGD in vitro (n=5/group). (E) The viability of HUVECs tested by dead (red)/live (green) assay kit at 24 h after OGD in vitro; (F-G) Immunoblot images and quantitative data of the expression of the ZO-1 and occludin at 24 h after OGD (n=4/group). * mean $P < 0.05$, ** mean $P < 0.01$, *** mean $P < 0.001$.

Figure 8

The inhibition of P2RX7 reduce HUVECs ferroptosis after OGD/R with high glucose in vitro.

(A) The oxidative stress levels of HUVECs tested by MDA assay kit at 24 h after OGD in vitro (n=4 /group). (B) The iron ions levels of HUVECs tested by intracellular iron colorimetric assay kit at 24 h after OGD in vitro (n=4 /group). (C-F) Immunoblot images and quantitative data of the expression of the P53, SLC7A11 and GPX4 in HUVECs at 24 h after OGD (n=4/group). * mean $P < 0.05$, ** mean $P < 0.01$, *** mean $P < 0.001$.

Figure 9

The inhibition of P2RX7 regulate HUVECs ferroptosis by ERK1/2-HO-1 pathway after OGD/R with HG in vitro.

(A-E) Immunoblot images and quantitative data of the expression of P2RX7, p-ERK1/2, ERK1/2 and HO-1 in HUVECs at 24 h after OGD (n=4/group). * mean $P < 0.05$, ** mean $P < 0.01$, *** mean $P < 0.001$.

Figure 10

The mechanism of endotheliocyte damage and HG-induced hemorrhagic transformation after ischemic stroke

HG: high glucose; ROS: reactive oxygen species; P2X7R: purinergic receptor P2X7; SLC7A11: solute carrier family 7 member 11; GSH: glutathione; GPX4: glutathione peroxidase-4; ERK1/2: extracellular regulated protein kinases; HO-1: heme oxygenase-1; P53: tumor protein 53; TFR1: transferrin receptor 1; MMP-9: matrix metalloproteinase-9.