

Inhibition of GPR4 attenuates SH-SY5Y cell injury in cerebral ischemia/reperfusion *via* anti-apoptotic pathways

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Cerebral ischemia/reperfusion injury (CIRI) can lead to increased vascular endothelial permeability and blood-brain barrier damage in patients with stroke. G protein-coupled receptor 4 (GPR4) is a functional pH sensor that plays a key role in renal ischemia-reperfusion-induced apoptosis. However, whether GPR4 has a role in cerebral ischemia remains to be further studied. Our study found that after oxygen-glucose deprivation/reoxygenation (OGD/R) treatment, the levels of GPR4 and CHOP in SH-SY5Y cells were significantly increased, which was accompanied by a decrease in cell viability, and an increase in LDH release and apoptosis. After knockdown of GPR4 using shRNA, CHOP levels in SH-SY5Y cells were also decreased, which unexpectedly increased cell activity and decreased LDH release and apoptosis rate. Interestingly, CHOP overexpression reversed the effect of GPR4 knockdown, suggesting that OGD/R-induced CIRI may involve endoplasmic reticulum stress-related apoptosis. In conclusion, our study provided a basis for further research on the mechanism of CIRI.

Keywords: G protein-coupled receptor 4; Cerebral ischemia/reperfusion injury; oxygen-glucose deprivation/reoxygenation; Apoptosis; SH-SY5Y cell line injury

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Abbreviations: CIRI, Cerebral ischemia/reperfusion injury; CST, Cell Signaling Technology; CHOP, C/EBP-homologous protein; ECs, endothelial cells; ER, Endoplasmic reticulum; ERS, Endoplasmic reticulum stress; FBS, fetal bovine serum; GPR4, G protein-coupled receptor 4; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, Lactate dehydrogenase; OGD/R, oxygen-glucose deprivation/ reoxygenation; shNC, shRNA negative control;

INTRODUCTION

Cerebral ischemia/reperfusion injury (CIRI) is a condition in which blood perfusion recovers after short-term ischemia and hypoxia, which can aggravate brain tissue damage (Zhao *et al.*, 2018). Oxidative stress, apoptosis and activated inflammation are a series of common reactions after cerebral ischemia-reperfusion injury (Hu *et al.*, 2017; Wu *et al.*, 2018). Therefore, inhibition of oxidative stress, apoptosis and activated inflammation is of great significance for the treatment and prognosis of cerebral ischemia-reperfusion injury (Pfeilschifter *et al.*, 2011). However, the mechanism of CIRI causing this series of reactions is complicated and still needs further exploration.

Nuclear transcription factor C/EBP-homologous protein (CHOP) exists widely in mammalian cells, and proteins regulated by CHOP are related to a variety of cellular activities, such as proliferation (Zhou *et al.*, 2015), differentiation (Shang *et al.*, 2017) and apoptosis (Hu *et al.*, 2018), and it is

also a specific transcription factor for endoplasmic reticulum stress. When endoplasmic reticulum stress occurs, apoptosis can be induced by increased expression of CHOP. A study demonstrated that in cortical neuronal injury induced by OGD/R, the expression of CHOP is up-regulated, indicating that the overexpression of CHOP is related to CIRI (Zhang *et al.*, 2019).

G protein-coupled receptor 4 (GPR4) is a functional pH sensor, which is mainly expressed in endothelial cells (ECs) (Sun *et al.*, 2010). Studies showed that GPR4 activation caused by acidosis is a mediating factor of endoplasmic reticulum stress (Dong *et al.*, 2017). The activation of GPR4 caused by acidosis also increases the expression of some stress response genes, such as CHOP (Dong *et al.*, 2013). Notably, GPR4 plays a key role in HR/acidification-induced CHOP expression and apoptosis induction (Dong *et al.*, 2014). Recent studies showed that GPR4 has a certain effect on tissue ischemia-reperfusion. The expression of GPR4 increases during renal ischemia-reperfusion. GPR4 gene knockdown can improve renal ischemia-reperfusion injury and decrease cell apoptosis by inhibiting the expression of CHOP (Dong *et al.*, 2017). However, whether GPR4 has a role in cerebral ischemia remains to be further studied.

In the current study, the role of GPR4 in OGD/R-treated SH-SY5Y cells and its potential molecular mechanisms were investigated *in vitro*, and our study provided a basis for further exploration of the mechanism of CIRI.

MATERIAL AND METHODS

Cell culture and oxygen-glucose deprivation/reoxygenation (OGD/R) treatment

Human-derived neuroblastoma SH-SY5Y cells were purchased from ATCC and cultured in DMEM (Gibco; Thermo Fisher Scientific, Massachusetts, USA) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere. SH-SY5Y cells were incubated in DMEM medium without glucose and serum for 2, 4, 6 and 12 h at 37°C under hypoxic conditions (1% O₂, 95% N₂ and 5% CO₂), followed by rapid reoxygenation (95% air and 5% CO₂), and then cultured in the conventional medium for 24 h to establish I/R injury cell model.

Western blotting

The levels of proteins of interest were detected by Western blotting. Proteins (20 µg) resolved in 10% SDS-PAGE gels were transferred onto PVDF membranes. Thereafter, the membranes were probed with primary an-

tibodies: anti-GPR4 (ab97271, 1:1000, Abcam, Cambridge, UK), anti-CHOP (#5554, 1:1000, Cell Signaling Technology (CST), Danvers, MA, USA), anti-GAPDH (ab9485, 1:2500, Abcam, Cambridge, UK), anti-cleaved caspase 3 (ab32042, 1:500, Abcam, Cambridge, UK) and anti-cleaved PARP (#5625, 1:1000, CST, Danvers, MA, USA) overnight at 4°C. After that, the membranes probed with primary antibodies were incubated with secondary antibody (Cell Signaling Technology, Massachusetts, USA) for 1 h at room temperature. Bands were visualized with ECL chemiluminescent detection kit (Solarbio, Beijing, China) and data were analyzed using an ImageQuant 350 analyzer (GE Healthcare, Pennsylvania, USA).

GPR4 knockdown

GPR4 was knocked down using shRNA. In brief, *shgpr4#1*, *shgpr4#2* and *shRNA negative control (shNC)* were cloned into pRNA-H1.1, an shRNA expression vector, to construct recombinant plasmids (GenScript, Piscataway, NJ, USA). After that, SH-SY5Y cells were transfected with the recombinant plasmids (2 µg) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for GPR4 silencing (*shGPR4#1* or *shGPR4#2*). 24 h after transfection, the cells were cultured and screened with 400 µg/ml G418. Transfection efficiency was determined by qPCR. shRNAs for GPR4 silencing were designed as two pairs of annealed small interfering RNA (siRNA) fragments. The sequences were as follows:

- shRNA#1 (i) 5'-AAGGCTAATGCTAGCGTCTT-GCCTGTCTC-3' (sense), and 5'-AAGACT-GCTAGCTGAACGTACCCTGTCTC-3' (antisense); (ii) 5'-AACCCTAGTCGATCTGACTGACCTGTCTC-3' (sense) and 5'-AACTTGCTGATGCTC-GACCGTCCTGTCTC-3' (antisense);
- shRNA#2 (i) 5'-AAGGTTGCTGATCTACGTAG-GCCTGTCTC-3' (sense), and 5'-AAGTTCGAT-GCTAGTACTGGCCTGTCTC-3' (antisense); (ii) 5'-AACAAGCTGACTAGCTGGTCACCTGTCTC-3' (sense) and 5'-AACCCGTAGTCGTCGTAGCTTC-CTGTCTC-3' (antisense).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cell viability was measured using MTT assay. SH-SY5Y cells were inoculated into 24-well plates (1×10^5 cells/well) and after 24 h subjected to OGD/R for 2, 4, 6 and 12 h. Then, MTT reagent (50 µg/ml) was added for another 6 h of incubation. Subsequently, the cells were treated with DMSO for 5 min, and then the absorbance at 570 nm was measured using a microplate reader.

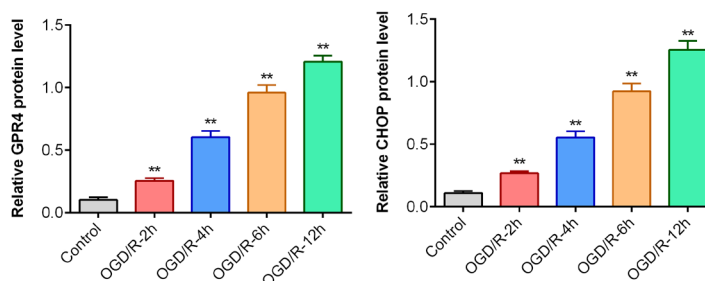
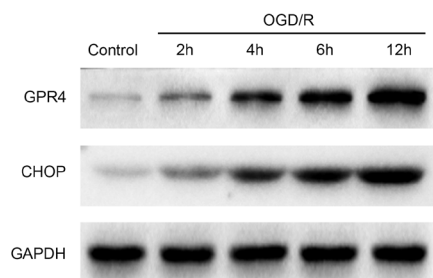


Figure 1. SH-SY5Y cells injury induced by OGD/R is related to the high expression of GPR4 and CHOP.

SH-SY5Y cells were cultured in DMEM medium without glucose and serum for 2, 4, 6 and 12 h at 37°C under hypoxic conditions (1% O₂, 95% N₂ and 5% CO₂), followed by rapid reoxygenation (95% air and 5% CO₂). The protein levels of GPR4 and CHOP were measured by Western blotting. ***p*<0.01 vs control group.

Lactate dehydrogenase (LDH) measurement

Cytotoxicity was evaluated by the determination of LDH activity. LDH release was measured using cytotoxicity detection kit (Beyotime, Shanghai, China) according to the manufacturer's instructions by measuring the absorbance at 490 nm on a microplate reader (Perkin-Elmer, California, USA).

Flow cytometry analysis

Apoptosis was assessed using flow cytometry. SH-SY5Y cells were digested with trypsin and stained with Alexa Fluor® 488 dye-labeled anti-BrdU antibody according to the manufacturer's operating procedures (APO-BrdU™ TUNEL assay kit; Invitrogen; Thermo Fisher Scientific, Inc.). After that, apoptosis determination was carried out using Cytomics FC 500 flow cytometer (Beckman Coulter, California, USA).

CHOP overexpression

Adenovirus vectors (OE-CHOP) for overexpression of CHOP and negative control (OE-NC) were obtained from GenePharma (Shanghai, China). SH-SY5Y cells were cultured with OE-CHOP/OE-NC adenovirus (MOI=100) at 37°C for 6 h. Then, the medium was removed and the culture was continued in a fresh medium at 37°C for 24 h. Cells with stable and high expression of CHOP were banked.

Statistical analysis

SPSS 19.0 software (International Business Machines Corporation, Armonk, New York, USA) was applied for statistical analysis. Experimental data are presented as the mean ± 182S.D. The data between two groups were compared using an unpaired Student's *t*-test, while the data from multiple groups were compared using a One-Way ANOVA followed by Dunnett's posthoc test. *p*<0.05 indicated a statistically significant difference.

RESULTS

Nerve injury induced by OGD/R is related to the high expression of GPR4 and CHOP

Firstly, we investigated the expression of GPR4 and CHOP in SH-SY5Y cells after different treatment time. As shown in Fig. 1, compared to the control group, the protein levels of GPR4 and CHOP in the cells after exposure to OGD/R increased rapidly and in a time-de-

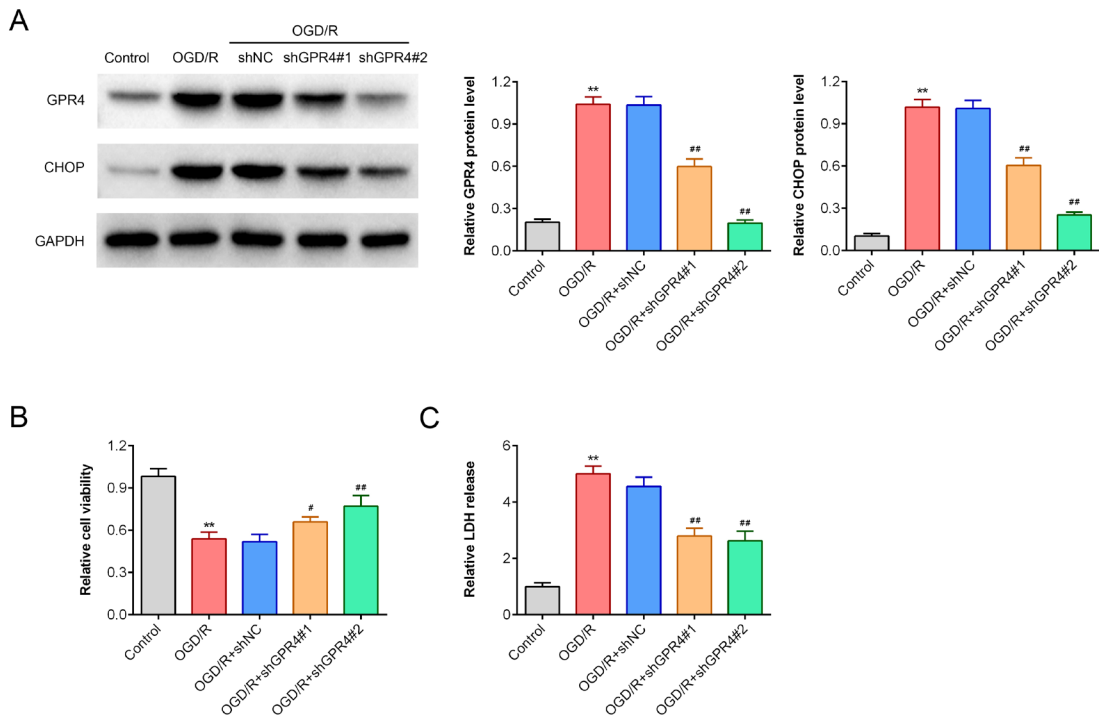


Figure 2. Knockdown of GPR4 reduces SH-SY5Y cell damage induced by OGD/R. SH-SY5Y cells exposed to OGD/R were transfected with shRNA negative control (shNC) or shGPR4#1 or shGPR4#2 for 24 h. (A) The protein levels of GPR4 and CHOP were measured by Western blotting 24 h after transfection. ** $p < 0.01$ vs control group; # $p < 0.01$ vs OGD/R group. (B) Cell viability was measured by MTT assay 24 h after transfection. ** $p < 0.01$ vs control group; # $p < 0.05$, ## $p < 0.01$ vs OGD/R group. (C) LDH release was measured 24 h after transfection. ** $p < 0.01$ vs control group; ## $p < 0.01$ vs OGD/R group.

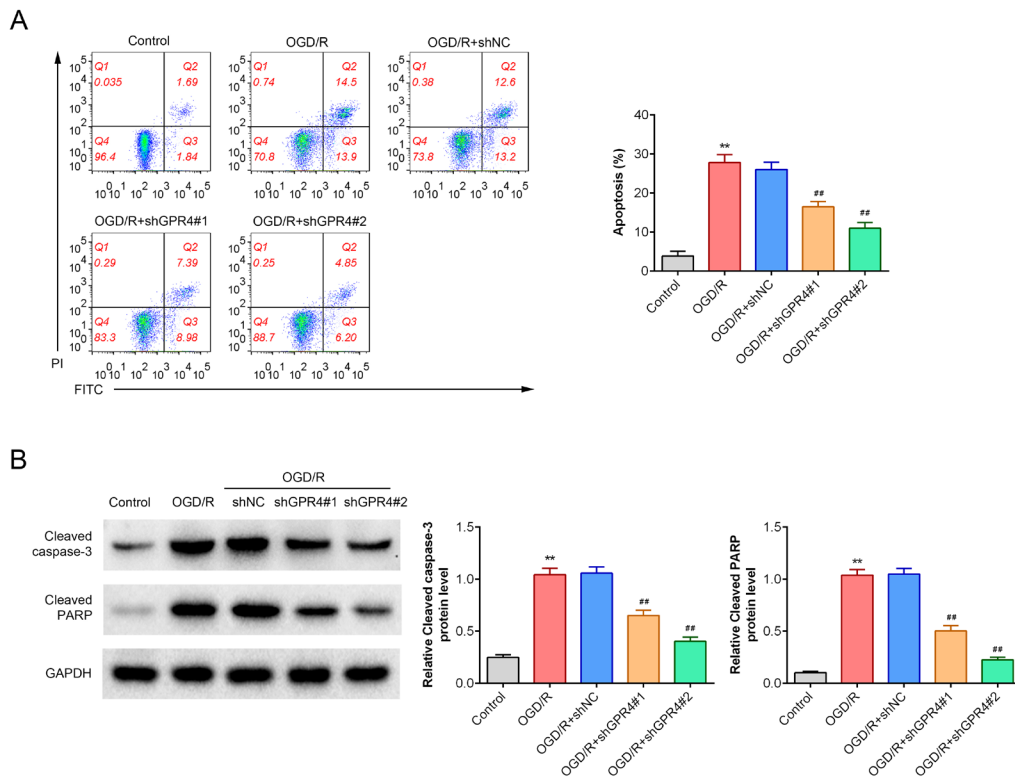


Figure 3. Knockdown of GPR4 reduces SH-SY5Y cells apoptosis induced by OGD/R. SH-SY5Y cells exposed to OGD/R were transfected with shRNA negative control (shNC) or shGPR4#1 or shGPR4#2. (A) Apoptosis was measured by flow cytometry 24 h after transfection. ** $p < 0.01$ vs control group; ## $p < 0.01$ vs OGD/R group. (B) The protein levels of cleaved caspase 3 and cleaved PARP were measured by Western blotting 24 h after transfection. ** $p < 0.01$ vs control group; ## $p < 0.01$ vs OGD/R group.

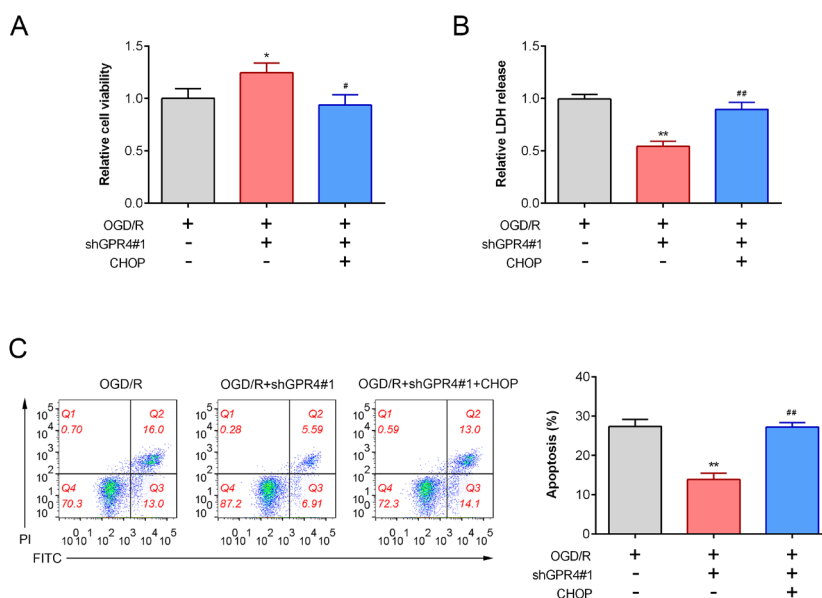


Figure 4. GPR4 promotes OGD/R-induced SH-SY5Y cells damage via CHOP.

SH-SY5Y cells exposed to OGD/R were transfected with shGPR4#1 or infected with Ad-CHOP. (A) Cell viability was measured using MTT assay 24 h after transfection. * $p < 0.05$ vs OGD/R group; * $p < 0.05$ vs OGD/R + shGPR4#1 group. (B) LDH release was measured 24 h after transfection. ** $p < 0.01$ vs OGD/R group; ** $p < 0.01$ vs OGD/R + shGPR4#1 group. C. Apoptosis was measured using flow cytometry 24 h after transfection. ** $p < 0.01$ vs OGD/R group; ** $p < 0.01$ vs OGD/R + shGPR4#1 group.

pendent manner, indicating that the nerve injury induced by OGD/R is related to the high expression of GPR4 and CHOP.

Knockdown of GPR4 reduces nerve cell damage induced by OGD/R

Surprisingly, we found that knockdown of GPR4 by two different shRNAs (shGPR4#1 and shGPR4#2) significantly reduced the levels of GPR4 and CHOP in SH-SY5Y cells exposed to OGD/R, and shGPR4#2 performed better than shGPR4#1 (Fig. 2A). In addition, we further investigated cell viability and LDH release. As shown in Fig. 2B, SH-SY5Y cells exposed to OGD/R showed a significant decrease in cell viability, which was significantly increased after knockdown of GPR4 with shRNA (shGPR4#1 and shGPR4#2), and the performance of shGPR4#2 was superior to shGPR4#1. Conversely, LDH release in SH-SY5Y cells exposed to OGD/R increased sharply compared to the control group, while LDH release was significantly reduced by knockdown of GPR4 with shRNA (shGPR4#1 and shGPR4#2), and the effect of shGPR4#2 was comparable to that of shGPR4#1. Taken together, these results indicated that knockdown of GPR4 reduces nerve cell damage induced by OGD/R.

Knockdown of GPR4 reduces SH-SY5Y cells apoptosis induced by OGD/R

In addition to exploring the effect of GPR4 on cell viability and LDH release, we also studied the effect of GPR4 on cell apoptosis. As shown in Fig. 3A, compared to the control group, the apoptosis rate of SH-SY5Y cells exposed to OGD/R was significantly increased. However, after using different shRNAs to knock down GPR4 (shGPR4#1 and shGPR4#2), the apoptosis rate was significantly reduced. Further analysis showed that OGD/R treatment promoted the expression of cleaved caspase 3 and cleaved PARP in SH-SY5Y cells while knocking down GPR4 prevented this process (Fig. 3B).

In summary, our results showed that knockdown of GPR4 reduces SH-SY5Y cells apoptosis induced by OGD/R.

GPR4 promotes OGD/R-induced SH-SY5Y cell damage via CHOP

To clarify the role of CHOP we overexpressed CHOP and investigated the effects on cell viability, LDH release and apoptosis. As shown in Fig. 4A, compared to the OGD/R group, after GPR4 was knocked down by shGPR4#1, the viability of SH-SY5Y cells was significantly increased. It is worth noting that CHOP overexpression reversed the increase of cell viability caused by GPR4 knockdown (Fig. 4B and C). Moreover, CHOP overexpression also reversed the inhibitory effect of GPR4 knockdown on LDH release and apoptosis (Fig. 4B and C). In summary, these results indicate that GPR4 promotes OGD/R-induced SH-SY5Y cells damage via CHOP.

DISCUSSION

Cerebral ischemia can cause sudden limitation of limb movement, language and sensory disorders, tissue damage and organ dysfunction, and even infarction of key brain regions (Zhang *et al.*, 2019). In the process of rapid recovery of blood flow to the ischemic tissue, reperfusion injury occurs and causes secondary neuron injury (Ferlito *et al.*, 2014; Caltagirone *et al.*, 2016). The pathological process of CIRI involves many mechanisms. There is evidence that apoptosis plays an important role in the development of CIRI (Xiao *et al.*, 2017; Zhao *et al.*, 2018). In this study, the OGD/R model of SH-SY5Y cells was established by subjecting SH-SY5Y cells to OGD/R. Our results showed that CIRI induced by OGD/R caused decreased cell activity, increased LDH release, and increased apoptosis, which might be related to the overexpression of GPR4. Further experiments suggested that OGD/R-induced CIRI may involve endo-

plasmic reticulum stress-related apoptosis (Yuqing Yang, 2020).

Endoplasmic reticulum (ER) is widely found in eukaryotic cells and is involved in lipid metabolism, steroid metabolism synthesis and calcium storage (Fabre *et al.*, 2019). Endoplasmic reticulum stress (ERS) is a pathological state that causes the disorder of ER physiological function. CHOP gene plays an important role in the apoptosis induced by ERS (Lei *et al.*, 2017). Under physiological conditions, CHOP expression is low in cells. During ERS response, the activation of IRE-1, PERK, and ATF6 induce the expression of CHOP, thus inducing apoptosis (Isodono *et al.*, 2010). Studies showed that apoptosis induced by ERS is involved in CIRI (Liu *et al.*, 2018). Pan *et al.*, found that OGD/R induced ERS and further promoted apoptosis in cerebral ischemia-reperfusion by increasing the expression of GRP78 and CHOP in SH-SY5Y cells (Pan *et al.*, 2020). Similarly, in this study, we found that after ischemia, the expression of CHOP in cells increased significantly, which further reduced cell activity and increased LDH release and apoptosis. Cell viability was increased after lowering CHOP levels, while LDH release and apoptosis were significantly reduced, which was reversed by the forced overexpression of CHOP. Therefore, our results suggest that CHOP is involved in OGD/R-induced CIRI, and its mechanism of action may be related to ERS-induced apoptosis.

Acidic pH can stimulate “proton-activated” G protein-coupled receptors (GPCRs, including GPR4, GPR68, GPR65, etc.) to transmit the relevant information (Yang *et al.*, 2007; Sun *et al.*, 2010). GPR4 was found to play a key role in HR/acidic-induced CHOP expression and apoptosis induction and is involved in endothelial cells adhesion and vascular endothelial cells inflammation (Chen *et al.*, 2011). It was recently found that GPR4 expression is also increased in renal ischemia-reperfusion, and knockdown of GPR4 can improve renal ischemia-reperfusion injury and inhibit apoptosis by inhibiting CHOP expression (Dong *et al.*, 2017). Surprisingly, our study found that GPR4 also had a certain effect on CIRI. We found a high, time-dependent GPR4 expression in cells subjected to OGD/R, which reduced cell activity, increased LDH release, and increased apoptosis. After GPR4 depletion using shRNA, cell viability was increased, while LDH release and apoptosis were decreased. It is worth noting that the high expression of GPR4 was also accompanied by the high expression of CHOP, suggesting that the abnormal expression of GPR4 may be related to the apoptosis caused by ERS. More importantly, forced CHOP overexpression reversed the effects of GPR4 knockdown on cell viability, LDH release, and apoptosis. Taken together, our results suggest that CIRI is associated with high GPR4 expression and ERS-related apoptosis.

In conclusion, we investigated the role of GPR4 in cerebral ischemia-reperfusion and its underlying molecular mechanisms *in vitro*. It was found that ischemia-reperfusion decreased cell activity, increased LDH release and apoptosis, which may be related to the overexpression of GPR4 and CHOP. Collectively, our study provides a basis for further exploration of the mechanism of CIRI.

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Competing interests

The authors state that there are no conflicts of interest to disclose.

Ethics approval

Not applicable.

Statement of Informed Consent

Not applicable.

Availability of data and materials

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

Authors' contributions

Chunli Xing designed the study, supervised the data collection, Guizhen Yan analyzed the data, interpreted the data, Qishuai Liu prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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