



Didymin Alleviates Cerebral Ischemia-Reperfusion Injury by Activating the PPAR Signaling Pathway

Qiang Li, Hongting Zhang, and Xiumei Liu

ICU Department, Jiyang People's Hospital of Jinan, Jinan, China.

Purpose: Cerebral ischemia-reperfusion (IR) injury is a severe secondary injury induced by reperfusion after stroke. Didymin has been reported to have a protective effect on intracerebral hemorrhage. However, the underlying mechanism of didymin on regulating cerebral IR injury remains largely unknown.

Materials and Methods: A rat cerebral IR model and oxygen-glucose deprivation/reperfusion (OGD/R) model in PC12 cells were established. Hematoxylin and eosin (H&E) was used to detect the pathological changes in brain tissues, and TUNEL staining was performed to detect apoptosis of brain tissues. MTT and flow cytometry were used to measure the viability and apoptosis of PC12 cells. QRT-PCR and western blot were used to detect inflammation cytokines in PC12 cells. Western blot was used to measure the expression of PPAR- γ , RXRA, Bax, c-caspase-3, and Bcl-2.

Results: Didymin pretreatment decreased apoptotic rates, reduced levels of Bax and c-caspase-3, and increased Bcl-2 level in vivo and in vitro. Additionally, didymin pretreatment increased viability and decreased the inflammation levels [interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α , and monocyte chemoattractant protein (MCP)-1] of OGD/R treated PC12 cells. Moreover, didymin activated the peroxisome proliferator-activated receptors (PPAR) signaling pathway and increased the expression of PPAR- γ and RXRA in OGD/R treated PC12 cells. Inhibition of PPAR- γ eliminated the protective effect of didymin on OGD/R treated cells.

Conclusion: Didymin protected neuron cells against IR injury in vitro and in vivo by activation of the PPAR pathway. Didymin may be a candidate drug for IR treatment.

Key Words: Ischemia-reperfusion injury, didymin, PPAR, inflammation

INTRODUCTION

Stroke is a form of brain injury caused by the rupture of cerebral vessels or the interruption of blood supply to the brain, causing death and disability.^{1,2} Ischemic stroke is the main type of stroke.² Reperfusion is the best method to restore blood supply to cerebral ischemia and protect the brain from ischemic injury.³ However, reperfusion may cause more severe secondary injury to brain tissue, known as ischemia-reperfusion (IR)

injury.^{4,5} Therefore, research on drugs with a therapeutic effect on cerebral IR injury is essential to reduce brain injury and improve brain function after cerebral IR injury.

Inflammation after stroke plays a key role in IR injury.^{6,7} Ischemia followed by reperfusion enhanced the levels of inflammatory cytokines and increased the activation of inflammatory cells and infiltration in the brain tissues.⁸ Kuai, et al.⁹ found that the inflammation levels were dramatically increased in cerebral IR rats. In clinical trials, subcutaneous injection of interleukin (IL)-1 antagonists significantly reduced the plasma levels of inflammatory cytokines in patients with ischemic stroke.¹⁰ Treatment with anti-inflammatory cytokines IL-22 and IL-35 attenuated neuronal apoptosis after cerebral IR injury.^{11,12} Therefore, changes of the inflammation levels in cerebral IR injury reflect the reduction or aggravation of brain injury.

Didymin, a flavonoid, is derived from citrus fruits, such as oranges, bergamot, and lemons.¹³ Its chemical structure is shown in Fig. 1. Early studies found that didymin inhibits the development of non-small-cell lung cancer, breast cancer, and neuro-

Received: March 21, 2022 **Revised:** June 10, 2022

Accepted: August 1, 2022 **Published online:** September 5, 2022

Corresponding author: Qiang Li, MD, ICU Department, Jiyang People's Hospital of Jinan, No.17, Xinyuan Street, Jiyang District, Jinan 251400, China.
E-mail: qll33215@163.com

•The authors have no potential conflicts of interest to disclose.

© Copyright: Yonsei University College of Medicine 2022

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<https://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

blastoma.^{14,15} Shukla, et al.¹⁶ found that didymin prevented the release of inflammatory cytokines and chemokines in the high glucose-induced human umbilical vein endothelial cells. The findings of Gu, et al.¹⁷ showed that didymin suppressed pyroptosis and inflammation of microglia in experimental intracerebral hemorrhage. However, the effect of didymin in cerebral IR injury is still unclear.

Based on these previous studies, we speculated that didymin might play a protective role in the cerebral IR injury. Our study investigated the effect of didymin on apoptosis and inflammation in cerebral IR injury. We discovered that didymin is dramatically inhibited cell apoptosis and suppresses inflammation *in vivo* and *in vitro*, and that the neuroprotective mechanisms may be related to the activation of the peroxisome proliferator-activated receptors (PPAR) signal pathway. This research provides a potential effective drug for therapeutic intervention of cerebral IR injury.

MATERIALS AND METHODS

Bioinformatics analysis

The pathway enrichment of didymin was analyzed by the SymMap database (<http://www.symmap.org>).¹⁸ Briefly, we opened the URL of SymMap: <http://www.symmap.org>, selected “search,” clicked “Ingredient,” input “didymin” in the search box and clicked “research” to perform the search. The search results showed that the Ingredient ID of didymin was “SMIT07548.” After clicking to enter the search result, we pulled down the webpage to “Related components for SMIT07548” and clicked “Target.” Then, the analysis results appeared.

Ethics approval and consent to participate

The experimental protocol of our study was performed in accordance with the Guide for the Care and Use of Laboratory Animals and approved by Jiyang People’s Hospital of Jinan.

Animals and groups

A total of 20 male SD rats (9 weeks old and weighing 300–320 g, Jinan Pengyue Experimental Animal Breeding Co., LTD, Shandong, China) had free access to food and water at 21°C. The rats were divided into four groups: Sham (n=5), middle cerebral artery occlusion (MCAO) (n=5), MCAO+didymin 0.5 mg/kg (n=5), and MCAO+didymin 1.0 mg/kg (n=5). The MCAO+

didymin groups received intraperitoneal injections of didymin (MedChemExpress, Shanghai, China) at the dose of 0.5 mg/kg/d or 1.0 mg/kg/d for 7 days before the MCAO surgery. The chemical structure of didymin is shown in Fig. 1.

MCAO model

The MCAO model was established as previously described.¹⁹ In brief, rats were anesthetized with pentobarbital sodium (60 mg/kg) by intraperitoneal injection, and the heart rate was monitored. After being fixed with adhesive tape, an incision was made on the necks of rats to expose and separate the left common carotid artery (CCA), external CA, and internal CA. Then, the left CCA was ligated with monofilament nylon suture with spherical end (diameter 0.32 mm). After 2 h of occlusion, the nylon suture was removed, and reperfusion was administered for 24 h. The rats in Sham group underwent similar surgery without ligation of the left CCA, and the incision was directly sutured.

Neurological score

After of 24 h of MCAO, the neurological deficit score was assessed by two investigators blinded to the rat groupings, as previously described.²⁰ Briefly, neurological deficits were assessed using a five-point neurological deficit score as follows: 0, no neurologic deficit; 1, failure to fully extend the contralateral forepaw; 2, failure to extend the contralateral forepaw; 3, circling to the contralateral side; and 4, falling to the contralateral side.

Pathological histology of brain tissues

After 24 h of reperfusion, rats were sacrificed with pentobarbital sodium (100 mg/kg). The brains were removed and treated with 4% paraformaldehyde for 24 h, and then embedded in the paraffin. Subsequently, brain tissues were sliced into 5- μ m-thick sections and stained with hematoxylin and eosin (H&E, Beyotime, Shanghai, China). The pathological changes in brain tissues were analyzed under a light microscope (Nikon, Tokyo, Japan).

TUNEL staining

Paraffin sections of brain tissues were dewaxed and dehydrated with gradient ethanol and washed with PBS (Thermo Fisher Scientific, Shanghai, China) for 5 min \times 3 times. Then, the sections were incubated with proteinase K at 37°C for 30 min, followed by washing with PBS for 5 min \times 3 times. Subsequently, the TUNEL reagent (Beyotime) was added and incubated for 30 min at 37°C. After washing with PBS, the sections were developed with peroxidase solution and DAB, and then counterstained by hematoxylin. The TUNEL-positive cells were observed under a fluorescence microscope.

Brain water content of brain tissues

After the rats were sacrificed, the brains were collected and weighted as the wet weight. Subsequently, brains were dried at

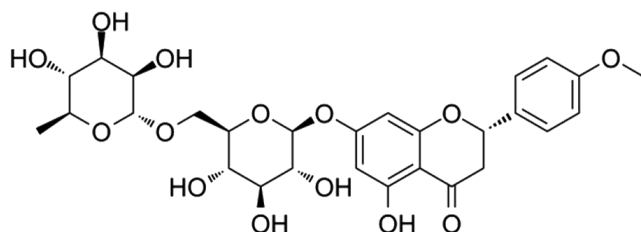


Fig. 1. Chemical structure of didymin.

110°C for 24 h. Then, the dried brains were weighed as the dry weight. The brain water content was calculated as follows:

$$\text{Brain water content (\%)} = \frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100$$

OGD/R model and drug treatment

PC12 cells were cultured in DMEM containing 10% FBS at 37°C. For similar cerebral IR injury in vitro, oxygen-glucose deprivation/reperfusion (OGD/R) model was established as previously described.²¹ In brief, PC12 cells were cultured in glucose-free and serum-free DMEM (Thermo Fisher Scientific), and incubated in a hypoxic environment with 94% N₂, 1% O₂, and 5% CO₂ at 37°C for 6 h. Then, the medium was replaced with complete DMEM (Thermo Fisher Scientific), and the cells were cultured in a normal environment with 95% air and 5% CO₂ at 37°C for 24 h. PC12 cells were divided into the control group (untreated), OGD/R group (OGD/R treatment), and OGD/R+didymin group (PC12 cells were pretreated with 10–40 μM didymin 24 h before OGD/R treatment).

MTT assay

PC12 cells were cultured into a 24-well plate at the density of 5×10⁴ cells per well. After the cells were treated with didymin or OGD/R, MTT (Beyotime) was added into the well and cultured at 37°C for 4 h. Subsequently, the medium was removed from the well and replaced by DMSO to dissolve the formazan crystals. The absorbance was measured on a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm. The cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{absorbance of treated group}}{\text{absorbance of control group}} \times 100$$

Flow cytometry assay

PC12 cells were centrifuged at 1000 g for 5 min to discard the supernatant. Then, the cells were collected to resuspend with PBS for preparing the cell suspension. The resuspended cells (5×10⁴ cells) were centrifuged at 1000 g for 5 min to discard the supernatant, and 195 μL of Annexin V-FITC binding solution (Beyotime) was added to resuspend the cells. Subsequently, PC12 cells were incubated with 5 μL Annexin-FITC and 10 μL propidium iodide staining solution at room temperature (20–25°C) for 15 min in the dark. The apoptotic cells (early apoptotic cells and late apoptotic cells) were detected by flow cytometry (BD Biosciences, San Jose, CA, USA). The results were analyzed by using the FlowJo software version 10 (TreeStar Inc., Ashland, OR, USA).

Western blot assay

Total proteins were extracted from PC12 cells and brain tissues by the RIPA lysis buffer (Beyotime). The protein concentration was measured by the BCA kit (Beyotime). Then, the protein samples (20 μg) were separated by the 10% SDS-PAGE, and then transferred onto the PVDF membranes (EMD Millipore,

Billerica, MA, USA). Subsequently, the membranes were incubated with primary antibodies [Bax, ab32503; c-caspase-3, ab32042; Bcl-2, ab32124; PPAR-γ, ab272718; RXRA, ab125001; IL-1β, ab254360; IL-6, ab259341; tumor necrosis factor (TNF)-α, ab205587; monocyte chemotactic protein (MCP)-1, ab7202; β-actin, ab8226, Abcam, Cambridge, UK] at 4°C overnight, followed by incubation with secondary antibody (ab6721, Abcam) for 1 h. The bands were visualized with an ECL kit (Beyotime) and quantified with the ImageJ software (National Institute of Health).

QRT-PCR assay

A total RNA of PC12 cells were isolated by the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesized by using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific), and qRT-PCR was performed using the SYBR Premix ExTaq (Takara, Tokyo, Japan). The reaction conditions were as follows: 95°C for 3 min, followed by 40 cycles at 95°C for 3 s and 55°C for 20 s. The expression levels of mRNAs were normalized to GAPDH, and then calculated using the 2^{-ΔΔCt} method. The primer sequences used for PCR are as follows: IL-1β, F: 5'-AGGAGAGACAAGCAACGACA-3'; R: 5'-TTGTTTGGGATCCACACTCTCC-3'. IL-6, F: 5'-AGAGACTTCCAGCCAGTTGC-3'; R: 5'-TGCCATTGCACAACCTTTTC-3'. TNF-α, F: 5'-ATGGGCTCCCTCTCATCAGT-3'; R: 5'-GCTTGGTGGTTTGCTACGAC-3'. MCP-1, F: 5'-TGTCTCAGCCAGATGCAGTT-3'; R: 5'-CAGCCGACTCATTGGGATCA-3'. GAPDH, F: 5'-CCGCATCTTCTTGTGCAGTG-3'; R: 5'-CGATACGGCCAAATCCGTTC-3'.

Statistical analysis

GraphPad V7.0 (GraphPad Software Inc., San Diego, CA, USA) was used to conduct the statistical analyses. One-way analysis of variance was applied to evaluate the differences among multiple groups, followed by Tukey's multiple comparisons test. *P*<0.05 was considered to indicate statistically significant difference. All data are displayed as the mean±SD from three experiments.

RESULTS

Didymin pretreatment protects neurological injury and reduces apoptosis in rats

To explore the effect of didymin on rats with cerebral IR injury, H&E staining was used to observe the brain pathological changes of MCAO rats. As shown in Fig. 2A, the hippocampus showed normal structures in Sham group. Obviously, severe brain injury occurred in MCAO group with destroyed cell membranes, swollen cell morphology, cell necrosis, and neuron atrophy. Didymin pretreatment markedly decreased the injury induced by MCAO modeling. The brain water content was markedly decreased by didymin pretreatment compared with

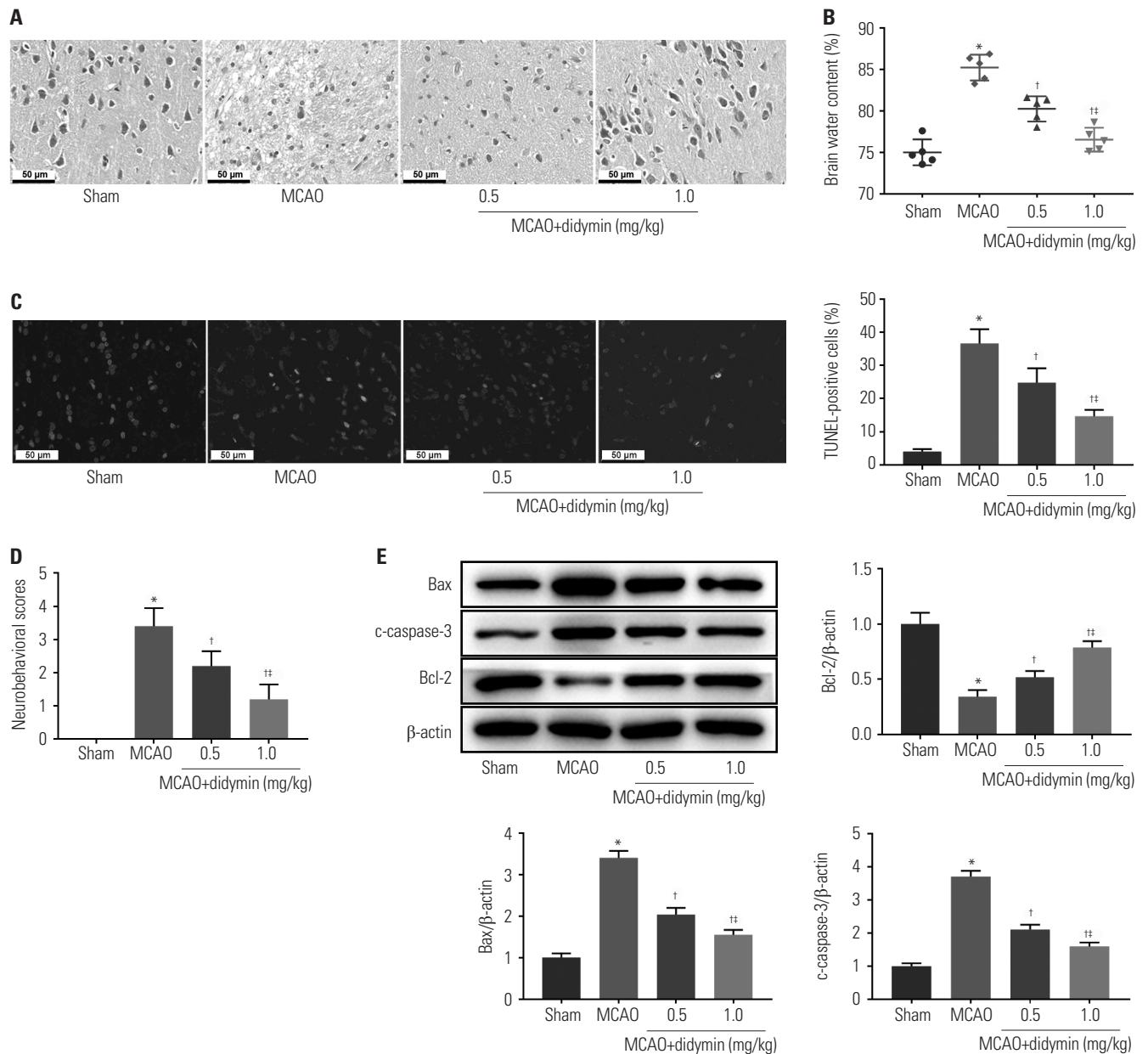


Fig. 2. Didymin pretreatment protects neurological injury and reduces apoptosis in rats. (A) H&E staining was performed to observe the pathological changes of MCAO rats ($n=5$ /group). (B) The brain water content was detected ($n=5$ /group). (C) TUNEL staining was performed to detect the apoptosis of brain tissues ($n=5$ /group). (D) Measurements of neurological scores in mice ($n=5$ /group). (E) Western blot was performed to detect the protein expression of Bax, c-caspase-3, and Bcl-2 ($n=5$ /group). * $p<0.05$ vs. Sham group; † $p<0.05$ vs. MCAO group; ‡ $p<0.05$ vs. MCAO+0.5 mg/kg didymin group. MCAO, middle cerebral artery occlusion.

MCAO group (Fig. 2B). The TUNEL staining showed that MCAO modeling markedly increased the apoptosis of brain tissues. Didymin pretreatment obviously decreased the percentages of TUNEL-positive cells compared with MCAO group (Fig. 2C). Besides, we observed that neurological scores notably decreased by didymin pretreatment compared to MCAO group (Fig. 2D). Furthermore, we detected the expression levels of apoptosis-related proteins. The results showed that didymin pretreatment significantly reduced expression levels of Bax and c-caspase-3 and enhanced Bcl-2 expression levels compared to MCAO group (Fig. 2E).

Didymin decreases OGD/R-induced neuronal apoptosis

We detected the effect of didymin alone on the cell viability of PC12 cells. The data showed that didymin at the dose of 0–40 μ M had no significant effect on the cell viability of PC12 cells (Fig. 3A). As shown in Fig. 3B, OGD/R treatment markedly decreased cell viability. The cell viability in didymin pretreated OGD/R cells was significantly higher than those of untreated OGD/R cells (Fig. 3B). We then detected the effect of didymin pretreatment on apoptosis of OGD/R-treated PC12 cells. We found that OGD/R treatment significantly promoted apopto-

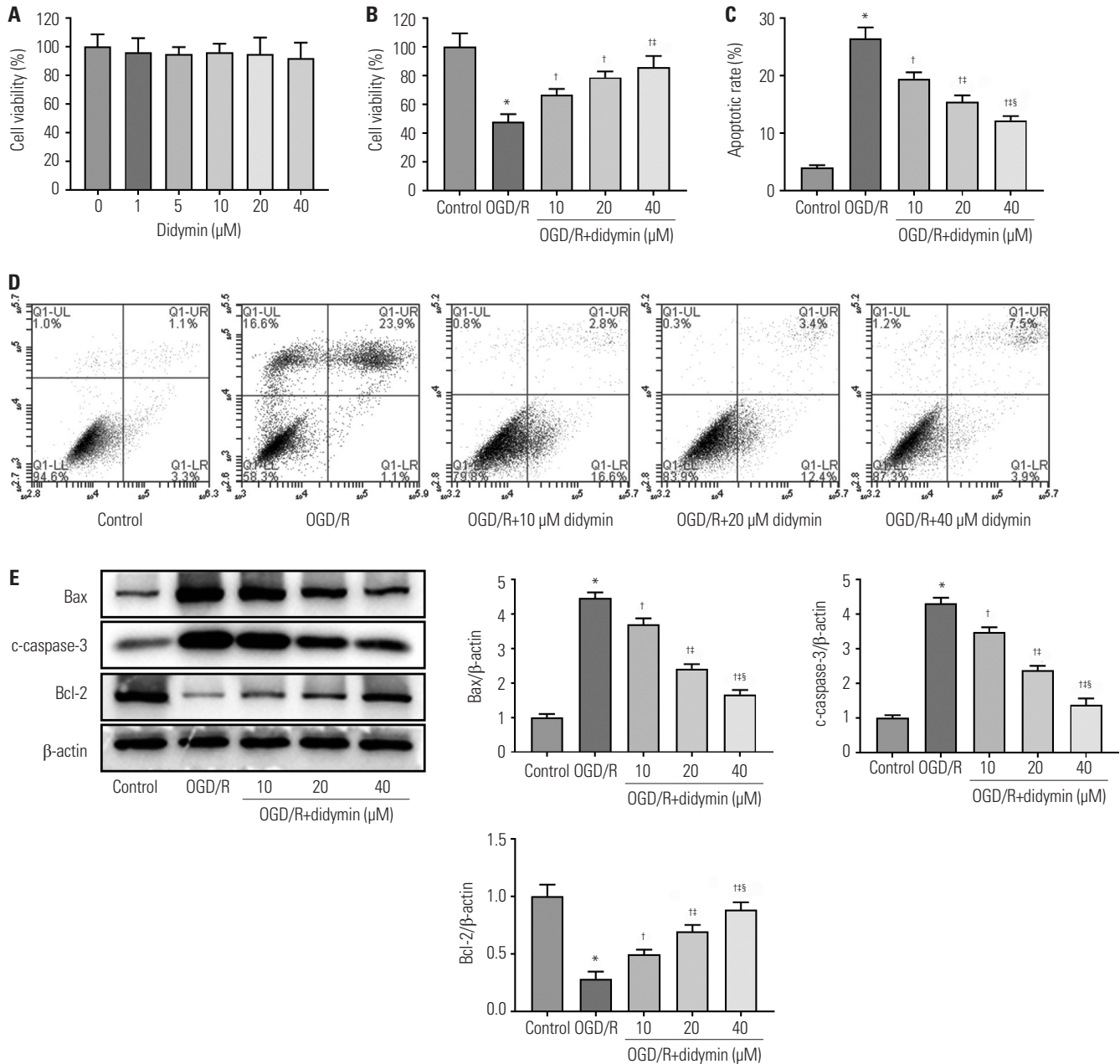


Fig. 3. Didymin protects cell injury against OGD/R-induced neuronal injury. (A) MTT assay was performed to detect the cell viability of PC12 cells. (B) MTT assay was performed to detect the cell viability of OGD/R treated PC12 cells. (C and D) Flow cytometry was performed to detect the apoptosis of OGD/R treated PC12 cells. (E) Western blot was performed to detect the protein expression of Bax, c-caspase-3, and Bcl-2 in OGD/R treated PC12 cells. * $p < 0.05$ vs. control group; † $p < 0.05$ vs. OGD/R group; ‡ $p < 0.05$ vs. OGD/R+10 μM didymin group; § $p < 0.05$ vs. OGD/R+20 μM didymin group. OGD/R, oxygen-glucose deprivation/reperfusion.

sis of PC12 cells compared to the control group. Didymin pretreatment markedly reduced the apoptotic rates of OGD/R treated cells (Fig. 3C and D). The western blot showed that didymin pretreatment significantly decreased the expression levels of Bax and c-caspase-3, while increasing Bcl-2 expression level in OGD/R cells (Fig. 3E).

Didymin pretreatment reduces OGD/R-induced inflammation levels

Inflammation response played an important role in cerebral

IR injury. We detected the effect of didymin on inflammation levels by performing qRT-PCR and western blot. The results showed that OGD/R treatment significantly enhanced the levels of IL-1β, IL-6, TNF-α, and MCP-1 compared to the control group (Fig. 4). However, didymin pretreatment significantly reduced the levels of IL-1β, IL-6, TNF-α, and MCP-1 induced by OGD/R treatment (Fig. 4).

Didymin activates the PPAR signaling pathway

To further investigate the mechanism of didymin on cerebral

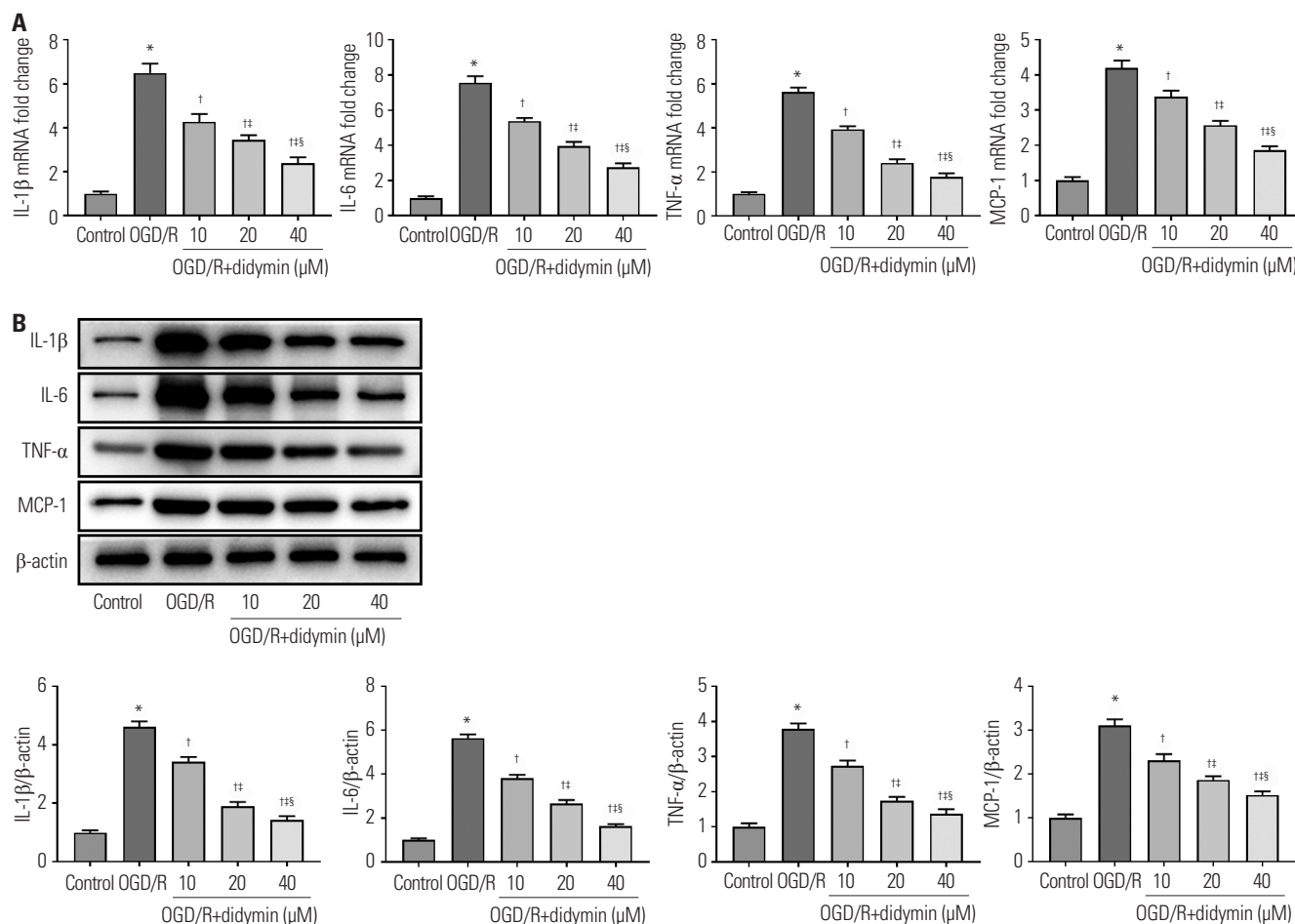


Fig. 4. Didymin pretreatment reduces OGD/R-induced inflammation levels. (A) QRT-PCR was performed to detect the mRNA expression levels of IL-1 β , IL-6, TNF- α , and MCP-1 in OGD/R treated PC12 cells. (B) Western blot was performed to detect the protein expression levels of IL-1 β , IL-6, TNF- α , and MCP-1 in OGD/R treated PC12 cells. * p <0.05 vs. control group; † p <0.05 vs. OGD/R group; ‡ p <0.05 vs. OGD/R+10 μ M didymin group; ‡‡ p <0.05 vs. OGD/R+20 μ M didymin group. IL, interleukin; OGD/R, oxygen-glucose deprivation/reperfusion; TNF, tumor necrosis factor; MCP, monocyte chemotactic protein.

IR injury, we analyzed the signaling pathways regulated by didymin in cerebral IR injury. The SymMap analysis showed that didymin activated the PPAR signaling pathway (Fig. 5A). PPAR- γ has been reported to play a protective role in cerebral IR injury.²² Therefore, we then detected the expression of PPAR- γ and RXRA after didymin pretreatment in PC12 cells. The data showed that didymin obviously increased the levels of PPAR- γ and RXRA (Fig. 5B). We also found that OGD/R treatment markedly decreased the levels of PPAR- γ and RXRA compared to the control group. However, the levels of PPAR- γ and RXRA were significantly higher in OGD/R+didymin groups than in OGD/Rgroup (Fig. 5C).

Inhibition of PPAR signaling pathway eliminates the effects of didymin pretreatment on OGD/R treated PC12 cells

To determine whether didymin protects IR injury by activating the PPAR pathway, GW9662 (5 μ M, ShanghaiyuanyeBio-Technology Co.,Ltd, Shanghai, China), a PPAR- γ antagonist, was used to treat the PC12 cells. The results of western blot

showed that didymin significantly increased the protein expression of PPAR- γ and RXRA. Compared with OGD/R+didymin group, GW9662 markedly reduced the levels of PPAR- γ and RXRA in OGD/R+didymin+GW9662 group (Fig. 6A). Then, the MTT and flow cytometry analysis showed that GW9662 significantly inhibited cell viability and promoted cell apoptosis compared to OGD/R+didymin group (Fig. 6B-D). Besides, didymin significantly decreased the protein expression of Bax and c-caspase-3, while increasing Bcl-2 protein expression. Compared with OGD/R+didymin group, GW9662 significantly increased the protein expression of Bax and c-caspase-3, while decreasing Bcl-2 protein expression (Fig. 6E).

DISCUSSION

In the present study, we identified the effect of didymin pretreatment on apoptosis and neuroinflammation on cerebral IR injury in vivo and in vitro. Our results indicated that didymin alleviated cerebral injury and decreased inflammation levels

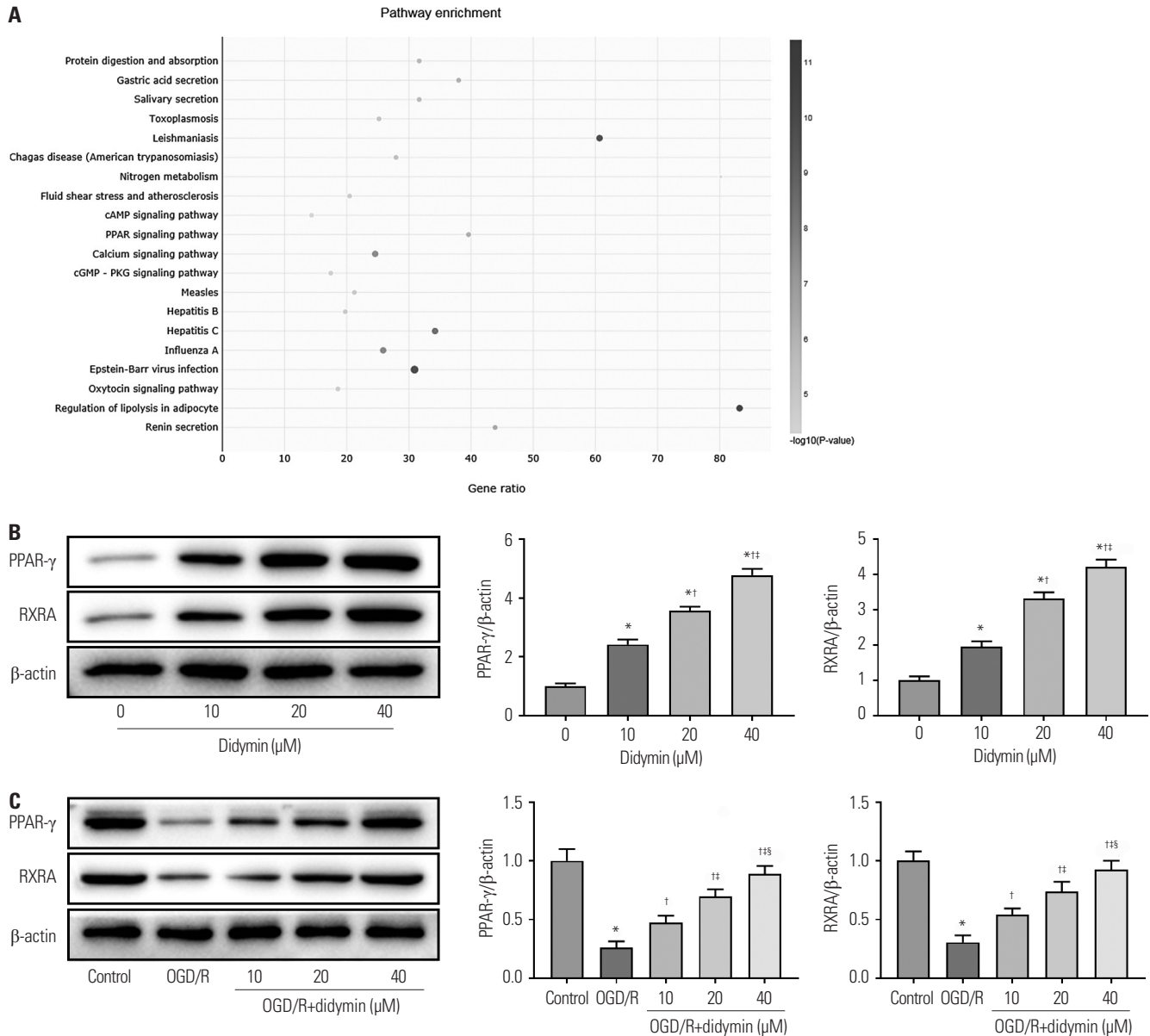


Fig. 5. Didymin activates the PPAR signaling pathway. (A) SymMap analysis showed that didymin activated the PPAR signaling pathway. (B) Western blot was performed to detect the protein expression of PPAR-γ and RXRA in PC12 cells. **p*<0.05 vs. 0 μM group; †*p*<0.05 vs. 10 μM group; ‡*p*<0.05 vs. 20 μM group. (C) Western blot was performed to detect the protein expression of PPAR-γ and RXRA in OGD/R treated PC12 cells. **p*<0.05 vs. control group; †*p*<0.05 vs. OGD/R group; ‡*p*<0.05 vs. OGD/R+10 μM didymin group; §*p*<0.05 vs. OGD/R+20 μM didymin group. PPAR, peroxisome proliferator-activated receptors; OGD/R, oxygen-glucose deprivation/reperfusion.

induced by MCAO or OGD/R treatment. Our study demonstrated that didymin exerted its neuroprotective effect by activating the PPAR signaling pathway.

Inflammation response is an important factor in cerebral IR injury.^{23,24} Cerebral IR injury triggers the infiltration of inflammatory cells, causing a strong inflammatory response that leads to neuronal apoptosis and death.^{25,26} ILs, a family of multipotent cytokines, have been demonstrated to regulate cerebral IR injury.^{4,27} In MCAO rats, IR injury was accompanied by a dramatic increase in IL-1β and IL-6 levels.²⁸ TNF-α is an inflammatory cytokine that can participate in systemic inflammation and plays an important role in the inflammatory response to IR in-

jury.²⁹ The TNF-α level was significantly increased in IR-damaged rat brain tissues and cells.^{30,31} Additionally, MCP-1 was also an important inflammatory cytokine in cerebral IR injury.^{32,33} In our study, the levels of IL-1β, IL-6, TNF-α, and MCP-1 were markedly increased after OGD/R treatment. Significantly, didymin pretreatment reduced the inflammation levels in OGD/R treated cells. In line with our data, Feng, et al.³⁴ found that didymin markedly decreased the levels of IL-1β, IL-6, and TNF-α of mice with non-alcoholic fatty liver disease.

The Bcl-2 family consists of many important apoptosis regulators of programmed cell death, including both anti-apoptotic molecules (such as Bcl-2) and pro-apoptotic molecules (such

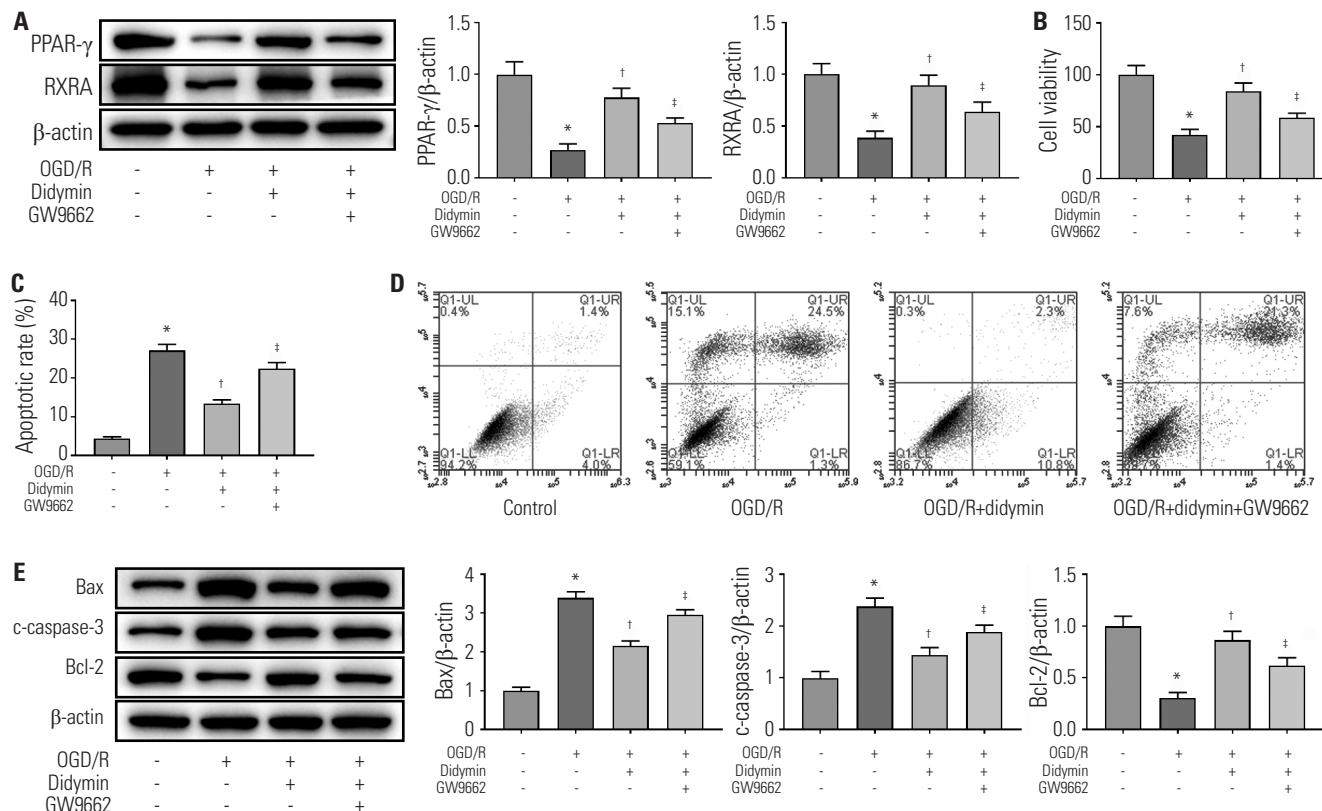


Fig. 6. Inhibition of PPAR signaling pathway eliminates the effects of didymium pretreatment on OGD/R treated PC12 cells. (A) Western blot was performed to detect the protein expression of PPAR- γ and RXRA in PC12 cells. (B) MTT assay was performed to detect the cell viability of PC12 cells. (C and D) Flow cytometry analysis was performed to detect the apoptosis of PC12 cells. (E) Western blot was performed to detect the protein expression of Bax, c-caspase-3, and Bcl-2 in PC12 cells. * $p < 0.05$ vs. control group; † $p < 0.05$ vs. OGD/R group; ‡ $p < 0.05$ vs. OGD/R+didymium group. PPAR, peroxisome proliferator-activated receptors; OGD/R, oxygen-glucose deprivation/reperfusion.

as Bax).³⁵ Caspases are a family of intracellular cysteine proteases that play important roles in the process of apoptosis.^{36,37} Caspase-3 is one of the important members and plays an important role in cerebral IR injury.^{38,39} In this study, we found that didymium pretreatment significantly decreased the expression of pro-apoptotic proteins Bax and c-caspase-3, and increased the expression of anti-apoptotic protein Bcl-2. The apoptotic cells were markedly decreased after didymium pretreatment. Didymium played a protective role on cerebral IR injury, which may be achieved by inhibiting the expression of Bax, caspase-3 and increasing Bcl-2.

PPARs are a family of ligand-regulated nuclear receptors, and PPAR- γ is a member of the PPAR family.⁴⁰ Studies have found that PPAR- γ has a protective effect in ischemic injury.^{41,42} Diminished expression of PPAR- γ exacerbated injury in liver IR mice.⁴³ In myocardial IR injury, PPAR- γ can alleviate cardiomyocyte apoptosis by inhibiting JNK activity.⁴² Moreover, studies have reported that PPAR- γ has a complex neuroprotective mechanism, including inhibiting inflammation, decreasing apoptosis, and reducing oxidative stress level.^{44,45} We found that didymium pretreatment activated the PPAR- γ signaling pathway. The levels of PPAR- γ and RXRA were significantly increased by didymium. Moreover, didymium pretreatment enhanced the levels of PPAR- γ and RXRA in PC12 cells decreased by OGD/R treatment. Then,

the PPAR- γ antagonist GW9662 was used to further confirm the role of PPAR- γ in cerebral IR injury. We found that the inhibition of PPAR- γ eliminated the protective effect of didymium on OGD/R treated cells. Therefore, didymium may alleviate cerebral IR injury by activating the PPAR- γ signaling pathway.

In conclusion, the present study demonstrated the protective role of didymium on cerebral IR injury. Our findings also suggested that didymium pretreatment decreased apoptosis, reduced inflammation levels, and alleviated IR injury by activating the PPAR- γ signal pathway.

AVAILABILITY OF DATA AND MATERIAL

All of the data used to support the findings of this study may be released upon application to the correspondence author.

AUTHOR CONTRIBUTIONS

Conceptualization: Qiang Li. **Data curation:** Hongting Zhang. **Formal analysis:** Xiumei Liu. **Funding acquisition:** Xiumei Liu. **Investigation:** Qiang Li. **Methodology:** Qiang Li. **Project administration:** Xiumei Liu. **Resources:** Hongting Zhang. **Software:** Hongting Zhang. **Supervision:** Qiang Li. **Validation:** Hongting Zhang. **Visualization:** Xiumei Liu. **Writing—original draft:** Hongting Zhang. **Writing—review & editing:** Qiang Li. **Approval of final manuscript:** all authors.

ORCID iDs

Qiang Li <https://orcid.org/0000-0002-0423-2410>
 Hongting Zhang <https://orcid.org/0000-0002-5423-9055>
 Xiumei Liu <https://orcid.org/0000-0003-3081-8252>

REFERENCES

- Sacco RL. Stroke disparities: from observations to actions: inaugural Edward J. Kenton Lecture 2020. *Stroke* 2020;51:3392-405.
- Campbell BCV, De Silva DA, Macleod MR, Coutts SB, Schwamm LH, Davis SM, et al. Ischaemic stroke. *Nat Rev Dis Primers* 2019; 5:70.
- Li L, Li Y, Miao C, Liu Y, Liu R. Coriolus versicolor polysaccharides (CVP) regulates neuronal apoptosis in cerebral ischemia-reperfusion injury via the p38MAPK signaling pathway. *Ann Transl Med* 2020;8:1168.
- Shi Y, Yi Z, Zhao P, Xu Y, Pan P. MicroRNA-532-5p protects against cerebral ischemia-reperfusion injury by directly targeting CXCL1. *Aging (Albany NY)* 2021;13:11528-41.
- Jia L, Chen Y, Tian YH, Zhang G. MAPK pathway mediates the anti-oxidative effect of chicoric acid against cerebral ischemia-reperfusion injury in vivo. *Exp Ther Med* 2018;15:1640-6.
- Chamorro Á, Dirnagl U, Urra X, Planas AM. Neuroprotection in acute stroke: targeting excitotoxicity, oxidative and nitrosative stress, and inflammation. *Lancet Neurol* 2016;15:869-81.
- Yu G, Sun W, Wang W, Le C, Liang D, Shuai L. Overexpression of microRNA-202-3p in bone marrow mesenchymal stem cells improves cerebral ischemia-reperfusion injury by promoting angiogenesis and inhibiting inflammation. *Aging (Albany NY)* 2021;13: 11877-88.
- Elango C, Devaraj SN. Immunomodulatory effect of Hawthorn extract in an experimental stroke model. *J Neuroinflammation* 2010;7:97.
- Kuai F, Zhou L, Zhou J, Sun X, Dong W. Long non-coding RNA THRIL inhibits miRNA-24-3p to upregulate neuropilin-1 to aggravate cerebral ischemia-reperfusion injury through regulating the nuclear factor κ B p65 signaling. *Aging (Albany NY)* 2021;13:9071-84.
- Smith CJ, Hulme S, Vail A, Heal C, Parry-Jones AR, Scarth S, et al. SCIL-STROKE (subcutaneous interleukin-1 receptor antagonist in ischemic stroke) a randomized controlled phase 2 trial. *Stroke* 2018;49:1210-6.
- Xu C, Zhu H, Shen R, Feng Q, Zhou H, Zhao Z. IL-35 is a protective immunomodulator in brain ischemic injury in mice. *Neurochem Res* 2018;43:1454-63.
- Dong Y, Hu C, Huang C, Gao J, Niu W, Wang D, et al. Interleukin-22 plays a protective role by regulating the JAK2-STAT3 pathway to improve inflammation, oxidative stress, and neuronal apoptosis following cerebral ischemia-reperfusion injury. *Mediators Inflamm* 2021;2021:6621296.
- Mouly P, Gaydou EM, Auffray A. Simultaneous separation of flavanone glycosides and polymethoxylated flavones in citrus juices using liquid chromatography. *J Chromatogr A* 1998;800:171-9.
- Hung JY, Hsu YL, Ko YC, Tsai YM, Yang CJ, Huang MS, et al. Didymin, a dietary flavonoid glycoside from citrus fruits, induces Fas-mediated apoptotic pathway in human non-small-cell lung cancer cells in vitro and in vivo. *Lung Cancer* 2010;68:366-74.
- Singhal J, Nagaprasanthan LD, Vatsyayan R, Ashutosh, Awasthi S, Singhal SS. Didymin induces apoptosis by inhibiting N-Myc and upregulating RKIP in neuroblastoma. *Cancer Prev Res (Phila)* 2012; 5:473-83.
- Shukla K, Sonowal H, Saxena A, Ramana KV. Didymin prevents hyperglycemia-induced human umbilical endothelial cells dysfunction and death. *Biochem Pharmacol* 2018;152:1-10.
- Gu L, Sun M, Li R, Zhang X, Tao Y, Yuan Y, et al. Didymin suppresses microglia pyroptosis and neuroinflammation through the Asc/Caspase-1/GSDMD pathway following experimental intracerebral hemorrhage. *Front Immunol* 2022;13:810582.
- Wu Y, Zhang F, Yang K, Fang S, Bu D, Li H, et al. SymMap: an integrative database of traditional Chinese medicine enhanced by symptom mapping. *Nucleic Acids Res* 2019;47:D1110-7.
- Fu D, Liu H, Zhu J, Xu H, Yao J. [D-Ala², D-Leu⁵] enkephalin inhibits TLR4/NF- κ B signaling pathway and protects rat brains against focal ischemia-reperfusion injury. *Mediators Inflamm* 2021;2021: 6661620.
- Sun L, Ji D, Zhi F, Fang Y, Zhu Z, Ni T, et al. MiR-494-3p upregulation exacerbates cerebral ischemia injury by targeting Bhlhe40. *Yonsei Med J* 2022;63:389-98.
- Zhan J, Li X, Luo D, Yan W, Hou Y, Hou Y, et al. Polydatin attenuates OGD/R-induced neuronal injury and spinal cord ischemia/reperfusion injury by protecting mitochondrial function via Nrf2/ARE signaling pathway. *Oxid Med Cell Longev* 2021;2021: 6687212.
- Wu XJ, Sun XH, Wang SW, Chen JL, Bi YH, Jiang DX. Mifepristone alleviates cerebral ischemia-reperfusion injury in rats by stimulating PPAR γ . *Eur Rev Med Pharmacol Sci* 2018;22:5688-96.
- Cruz SA, Hari A, Qin Z, Couture P, Huang H, Lagace DC, et al. Loss of IRF2BP2 in microglia increases inflammation and functional deficits after focal ischemic brain injury. *Front Cell Neurosci* 2017;11:201.
- McCarter KD, Li C, Jiang Z, Lu W, Smith HA, Xu G, et al. Effect of low-dose alcohol consumption on inflammation following transient focal cerebral ischemia in rats. *Sci Rep* 2017;7:12547.
- Wang J, Mao J, Wang R, Li S, Wu B, Yuan Y. Kaempferol protects against cerebral ischemia reperfusion injury through intervening oxidative and inflammatory stress induced apoptosis. *Front Pharmacol* 2020;11:424.
- Wang G, Han B, Shen L, Wu S, Yang L, Liao J, et al. Silencing of circular RNA HIPK2 in neural stem cells enhances functional recovery following ischaemic stroke. *EBioMedicine* 2020;52:102660.
- Zhang B, Zhang HX, Shi ST, Bai YL, Zhe X, Zhang SJ, et al. Interleukin-11 treatment protected against cerebral ischemia/reperfusion injury. *Biomed Pharmacother* 2019;115:108816.
- Guo H, Zhu L, Tang P, Chen D, Li Y, Li J, et al. Carthamin yellow improves cerebral ischemia-reperfusion injury by attenuating inflammation and ferroptosis in rats. *Int J Mol Med* 2021;47:52.
- Eder P, Linke K, Witowski J. Update on the mechanisms of action of anti-TNF- α antibodies and their clinical implications in inflammatory bowel disease. *Pol Arch Med Wewn* 2016;126:772-80.
- Zhao M, Hou S, Feng L, Shen P, Nan D, Zhang Y, et al. Vinpocetine protects against cerebral ischemia-reperfusion injury by targeting astrocytic connexin43 via the PI3K/AKT signaling pathway. *Front Neurosci* 2020;14:223.
- Zheng Y, Wang L, Chen M, Liu L, Pei A, Zhang R, et al. Inhibition of T cell immunoglobulin and mucin-1 (TIM-1) protects against cerebral ischemia-reperfusion injury. *Cell Commun Signal* 2019;17: 103.
- Yin P, Wei Y, Wang X, Zhu M, Feng J. Roles of specialized pro-resolving lipid mediators in cerebral ischemia reperfusion injury. *Front Neurol* 2018;9:617.
- Zhang Y, Cao RY, Jia X, Li Q, Qiao L, Yan G, et al. Treadmill exercise promotes neuroprotection against cerebral ischemia-reperfusion injury via downregulation of pro-inflammatory mediators. *Neuropsychiatr Dis Treat* 2016;12:3161-73.

34. Feng Z, Pang L, Chen S, Pang X, Huang Y, Qiao Q, et al. Didymin ameliorates dexamethasone-induced non-alcoholic fatty liver disease by inhibiting TLR4/NF- κ B and PI3K/Akt pathways in C57BL/6J mice. *Int Immunopharmacol* 2020;88:107003.
35. Levine B, Sinha S, Kroemer G. Bcl-2 family members: dual regulators of apoptosis and autophagy. *Autophagy* 2008;4:600-6.
36. Kim R, Emi M, Tanabe K. Caspase-dependent and -independent cell death pathways after DNA damage (review). *Oncol Rep* 2005;14:595-9.
37. Denault JB, Salvesen GS. Caspases: keys in the ignition of cell death. *Chem Rev* 2002;102:4489-500.
38. Cai Y, Yang E, Yao X, Zhang X, Wang Q, Wang Y, et al. FUNDC1-dependent mitophagy induced by tPA protects neurons against cerebral ischemia-reperfusion injury. *Redox Biol* 2021;38:101792.
39. Du SJ, Zhang Y, Zhao YM, Dong YJ, Tang JL, Zhou XH, et al. Astragaloside IV attenuates cerebral ischemia-reperfusion injury in rats through the inhibition of calcium-sensing receptor-mediated apoptosis. *Int J Mol Med* 2021;47:302-14.
40. Wójtowicz S, Strosznajder AK, Jeżyna M, Strosznajder JB. The novel role of PPAR alpha in the brain: promising target in therapy of Alzheimer's disease and other neurodegenerative disorders. *Neurochem Res* 2020;45:972-88.
41. Akahori T, Sho M, Hamada K, Suzaki Y, Kuzumoto Y, Nomi T, et al. Importance of peroxisome proliferator-activated receptor-gamma in hepatic ischemia/reperfusion injury in mice. *J Hepatol* 2007;47:784-92.
42. Zhong CB, Chen X, Zhou XY, Wang XB. The role of peroxisome proliferator-activated receptor γ in mediating cardioprotection against ischemia/reperfusion injury. *J Cardiovasc Pharmacol Ther* 2017;23:46-56.
43. Kuboki S, Shin T, Huber N, Eismann T, Galloway E, Schuster R, et al. Peroxisome proliferator-activated receptor- γ protects against hepatic ischemia/reperfusion injury in mice. *Hepatology* 2008;47:215-24.
44. Ding Y, Kang J, Liu S, Xu Y, Shao B. The protective effects of peroxisome proliferator-activated receptor gamma in cerebral ischemia-reperfusion injury. *Front Neurol* 2020;11:588516.
45. Wu JS, Cheung WM, Tsai YS, Chen YT, Fong WH, Tsai HD, et al. Ligand-activated peroxisome proliferator-activated receptor-gamma protects against ischemic cerebral infarction and neuronal apoptosis by 14-3-3 epsilon upregulation. *Circulation* 2009;119:1124-34.