

FABP4 alleviates endoplasmic reticulum stress-mediated ischemia-reperfusion injury in PC12 cells via regulation of PPAR γ

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Abstract. Ischemic stroke is a life-threatening complication with a high rate of morbidity. Circulating fatty acid binding protein 4 (FABP4) has been reported to be associated with the outcome of acute ischemic stroke. The present study aimed to illustrate the function of FABP4 in ischemic stroke. PC12 cells exposed to oxygen glucose deprivation/reoxygenation (OGD/R) were used to mimic ischemia-reperfusion (I/R) injury in ischemic stroke. Cell viability was estimated using a Cell Counting Kit-8 assay. The expression of FABP4 in PC12 cells under OGD/R was detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). PC12 cells were transfected with FABP4 small interfering RNA (siRNA), inflammatory cytokines and reactive oxygen species (ROS) were determined via RT-qPCR and ROS assay kit. Western blotting was performed to detect endoplasmic reticulum stress (ERS)-related proteins and peroxisome proliferator-activated receptor γ (PPAR γ). Flow cytometry was used to evaluate the cell apoptotic rate. The expression of FABP4 increased gradually with the prolongation of reoxygenation within 8 h. FABP4-knockdown inhibited the transcription of inflammatory cytokines, the production of ROS and decreased cell apoptosis. Furthermore, decreased ERS-related proteins and increased PPAR γ were estimated in PC12 cells transfected with FABP4 siRNA. PPAR γ inhibitor GW9662 weakened the anti-apoptotic effect of FABP4-knockdown. Taken together, these results indicated that FABP4-knockdown suppressed cell apoptosis via relieving ERS; this effect was reversed by treatment of GW9662.

Introduction

As the leading cause of mortality and disability worldwide, stroke refers to cerebral vascular injury, local or whole brain tissue damage caused by various reasons, resulting in clinical symptoms for >24 h or sudden death (1,2). Stroke is commonly classified into two categories, ischemic stroke (cerebral infarction) and hemorrhagic stroke (parenchymal hemorrhage, intraventricular hemorrhage and subarachnoid hemorrhage), of which ischemic stroke accounts for ~80% (3,4). To date, few drugs have been approved for the treatment of ischemic stroke (5), and there is still considerable capacity and demand for the development of effective drugs to cure ischemic stroke. An improved understanding of the pathophysiology of ischemic stroke could provide basis for clinical research.

Cerebral ischemia-reperfusion (I/R) injury is generally considered as a complex pathophysiological process of ischemic stroke (6). Cerebral ischemia initiates a series of cascades, including inflammation, followed by the production of reactive oxygen species (ROS) and apoptosis, which aggravate the damage of brain cells, destroy the extracellular matrix and blood-brain barrier (7).

FABPs are lipid-binding chaperones that are expressed in various tissue types. FABPs may regulate the transport of fat, promote the formation of blood vessels and cell differentiation, and participate in the occurrence of various metabolic diseases (8-10). FABP4 participates in ERS and inflammation, contributing toward the release of inflammatory cytokines (11,12). FABP4 protects the kidney from I/R-induced acute kidney injury (13). A recent study has demonstrated that FABP4 may be a novel therapeutic target for hepatic I/R injury (14). In addition, high levels of FABP4 in plasma were associated with poor prognosis in patients with acute ischemic stroke (15). However, the role of FABP4 in cerebral I/R injury has rarely been investigated. Therefore, the aim of the present study was to investigate the function of FABP4 in the pathogenesis of ischemic stroke. The PC12 cell line is a differentiated cell line of rat adrenal medulla pheochromocytoma. It is widely used in neurophysiological and neuropharmacological research because it possesses the general characteristics of neuroendocrine cells and can be passaged. In the present study, PC12 cells were selected to establish an OGD/R model *in vitro*.

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Materials and methods

Cell culture. PC12 cell line (Type Culture Collection of the Chinese Academy of Sciences) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS) at 37°C in an atmosphere of 5% CO₂. Cells were transfected with 10 μ M FABP4 small interfering RNA (siRNA; Santa Cruz Biotechnology, Inc.) or negative control siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. A total of 6 h post-transfection, fresh DMEM, supplemented with 10% fetal bovine serum, was replaced, followed by incubation for 48 h. GW9662 (MedChemExpress Co., Ltd.), a selective PPAR γ antagonist was used to treat cells 2 h prior to OGD/R. ERS agonist tunicamycin (MedChemExpress Co., Ltd.) was obtained to incubate cells 2 h prior to OGD/R to induce ERS.

OGD/R model. PC12 cells that have been exposed to OGD/R have been widely used to simulate cerebral I/R injury *in vitro* (16,17). Cells were maintained in a glucose-deprivation buffer (Gibco; Thermo Fisher Scientific, Inc.) and incubated in an anaerobic chamber in an atmosphere of 95% N₂/5% CO₂ for 2, 4, 6, 8 or 10 h, followed by fresh DMEM (containing 10% fetal bovine serum) under normoxic conditions with 95% air/5% CO₂ for 24 h.

CCK-8 assay. PC12 cells were resuspended in culture medium and then planted onto a 96-well plate (5x10³ cells per well). Following reoxygenation for 2, 4, 6, 8 or 12 h, 10 μ l CCK-8 reagent (MedChemExpress Co., Ltd.) was added to each well and incubated for 2 h. Subsequently, the absorbance value of each well was recorded using a microplate reader (BioTek Instruments, Inc.) at 450 nm wavelength.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from PC12 cells. The PrimeScript RT reagent kit (Takara Biotechnology, Inc.) was used for reverse transcription of RNA (42°C for 30 min; 99°C for 5 min). Amplification reaction was performed using SYBR-Green PCR kit (Qiagen, Inc.) and subjected to Applied Biosystems 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences were listed as follows: FABP4 sense, 5'-GCCAGGAATTTGACGAAGTCAC-3' and antisense, 5'-TTCTGCACATGTACCAGGACAC-3'; IL-6 sense, 5'-ATTGTATGAACAGCGATGATGCAC-3' and antisense, 5'-CCAGGTAGAAACGGAACTCCAGA-3'; IL-1 β sense, 5'-CCCTGAAGTCAACTGTGAAATAGC A-3' and antisense, 5'-CCCAAGTCAAGGGCTTGGAA-3'; TNF- α sense, 5'-TCAGCCTCTTCTCATTCCTGC-3' and antisense, 5'-TTGGTGGTTTGCTACGACGTG-3'; β -actin sense, 5'-GGAGATTAGTCCCTGGCTCCTA-3' and antisense, 5'-GACTCATCGTACTCCTGCTTGCTG-3'. β -actin served as an endogenous control. PCR reaction conditions were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 20 sec and 65°C for 40 sec. Expression levels of target genes were analyzed using the 2^{- $\Delta\Delta$ C_q} method (18).

Cell apoptosis assay. A total of 1x10⁶/ml PC12 cells were collected and apoptosis was detected by double staining with fluorescein isothiocyanate (FITC)-Annexin V and propidium iodide (PI) for 10 min in the dark at room temperature. Subsequently, cells were subjected to a FACSCalibur flow cytometer (BD Biosciences) to detect the apoptotic rate. Data were analyzed using flow cytometry software (iSort™ Automated Cell Sorter; version A.0; Thermo Fisher Scientific, Inc.).

Western blotting. Total protein was extracted from PC12 cells using RIPA lysis buffer (Beyotime Institute of Biotechnology). Following centrifugation for 10 min at 4°C (12,000 x g), cell supernatant was collected and the protein concentration was estimated using a BCA assay kit (Beyotime Institute of Biotechnology). A total of 20 μ g protein per lane was separated by 10% SDS-PAGE gel and transferred onto PVDF membranes. Membranes were blocked with 5% skimmed milk at room temperature for 2 h followed by incubation with primary antibodies at 4°C overnight. FABP4 (1:5,000; cat. no. ab92501), cleaved-caspase-3 (1:500; cat. no. ab49822) and caspase-3 (1:2,000; cat. no. ab184787) antibodies were obtained from Abcam, GRP78 (1:1,000; cat. no. sc-13539) and CHOP (1:1,000; cat. no. sc-7351) antibodies were purchased from Santa Cruz Biotechnology, Inc. β -actin (1:5,000; A1978) antibody was obtained from Sigma-Aldrich (Merck KGaA). Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; cat. no. ab6721; Abcam) and goat anti-mouse IgG secondary antibodies (1:5,000; cat. no. ab6789; Abcam) were used for detection (room temperature; 2 h). The protein bands were visualized with an Enhanced Chemiluminescence Detection kit (Thermo Fisher Scientific, Inc.). Protein expression levels were semi-quantified using Image-Pro Plus software version 6.0 (Roper Technologies, Inc.).

Determination of ROS. The production of ROS was estimated using a ROS assay kit (Beyotime Institute of Biotechnology). PC12 cells (5x10⁶ cells/ml) were collected and suspended in the diluted DCFH-DA (probe) at a final concentration of 10 μ M. Next, cells were incubated at 37°C for 20 min and mixed every 5 min to enable the probe to be in full contact with the cells. The cells were washed three times with DMEM, and ROS was determined at wavelengths of 488 and 525 nm using a fluorescence microplate reader (Omega Bio-Tek, Inc.).

The measurement of SOD activity. SOD activity was determined using an SOD assay kit (Beyotime Institute of Biotechnology). In brief, SOD sample preparation solution was used to lyse PC12 cells, and the solution was centrifuged for 5 min at 4°C (12,000 x g) to collect cell supernatant. Corresponding solution was added for 30 min at 37°C and the absorbance value was recorded using a microplate reader (Omega Bio-Tek, Inc.) at 450 nm wavelength.

Statistical analysis. All the experiments were performed in triplicate and results are expressed as the mean \pm standard deviation of data from triplicate experiments. A t-test or one-way analysis of variance followed by Tukey's test was used for comparisons between two groups or multiple groups, respectively. P<0.05 was considered to indicate a statistically significant difference.

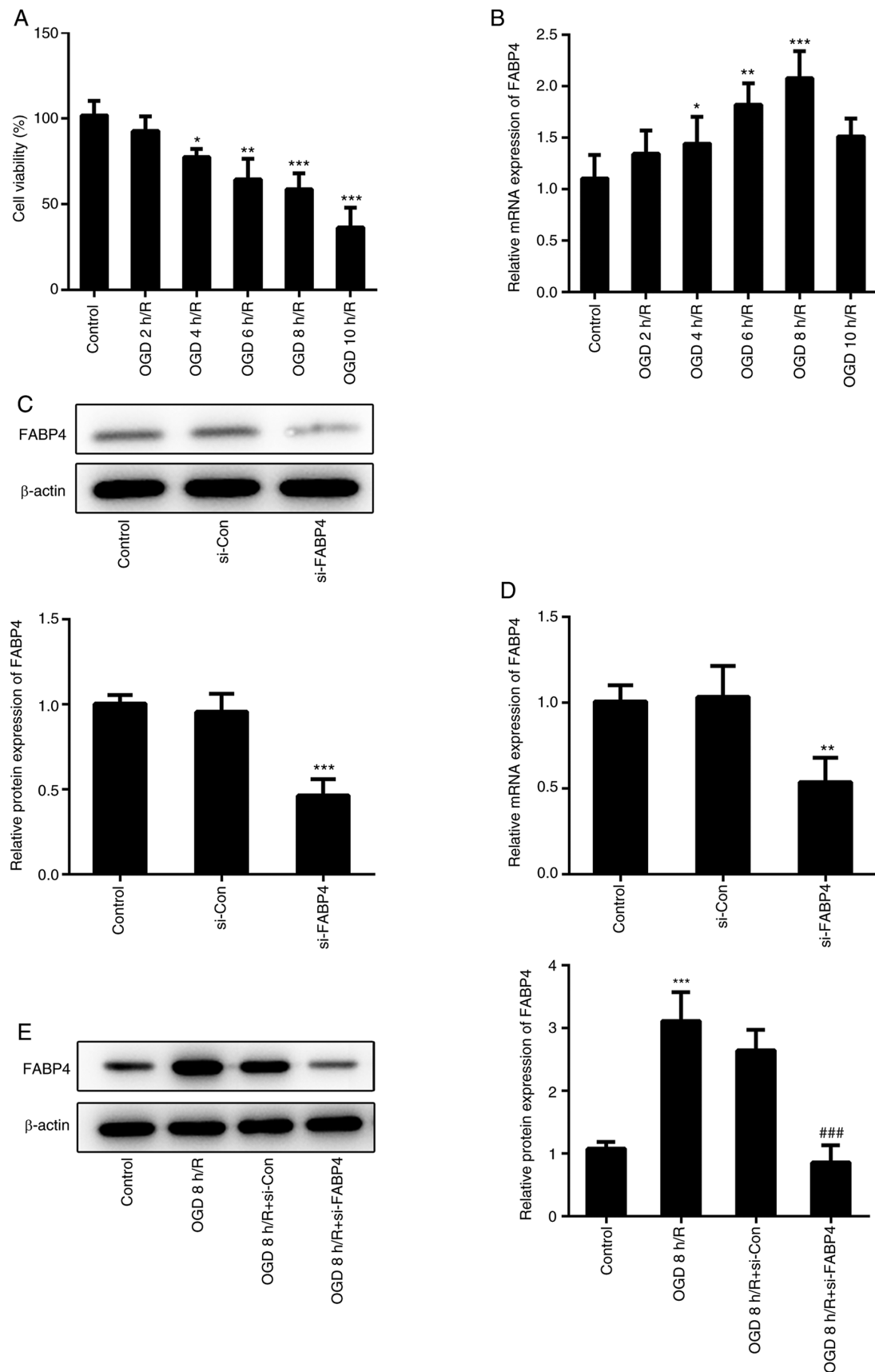


Figure 1. Downregulation of FABP4 in PC12 cells under OGD/R conditions. (A) PC12 cells were maintained under OGD for 2, 4, 6, 8 or 10 h followed by reoxygenation for 24 h. Cell viability was estimated using a Cell Counting Kit-8 assay. (B) mRNA expression of FABP4 in PC12 cells exposed to OGD/R were determined by RT-qPCR. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control. The (C) protein and (D) mRNA expression of FABP4 in PC12 cells were determined by RT-qPCR and western blotting following transfection with FABP4 siRNA or control siRNA. ** $P < 0.01$, *** $P < 0.001$ vs. si-Con. (E) The protein expression of FABP4 in PC12 cells exposed to OGD 8 h/R (OGD for 8 h and reoxygenation for 24 h) was determined by western blotting. *** $P < 0.001$ vs. control; ### $P < 0.001$ vs. OGD 8 h/R+si-Con. FABP4, fatty acid binding protein 4; siFABP4, FABP4 small interfering RNA; siCon, control small interfering RNA; OGD/R, oxygen glucose deprivation/reoxygenation; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

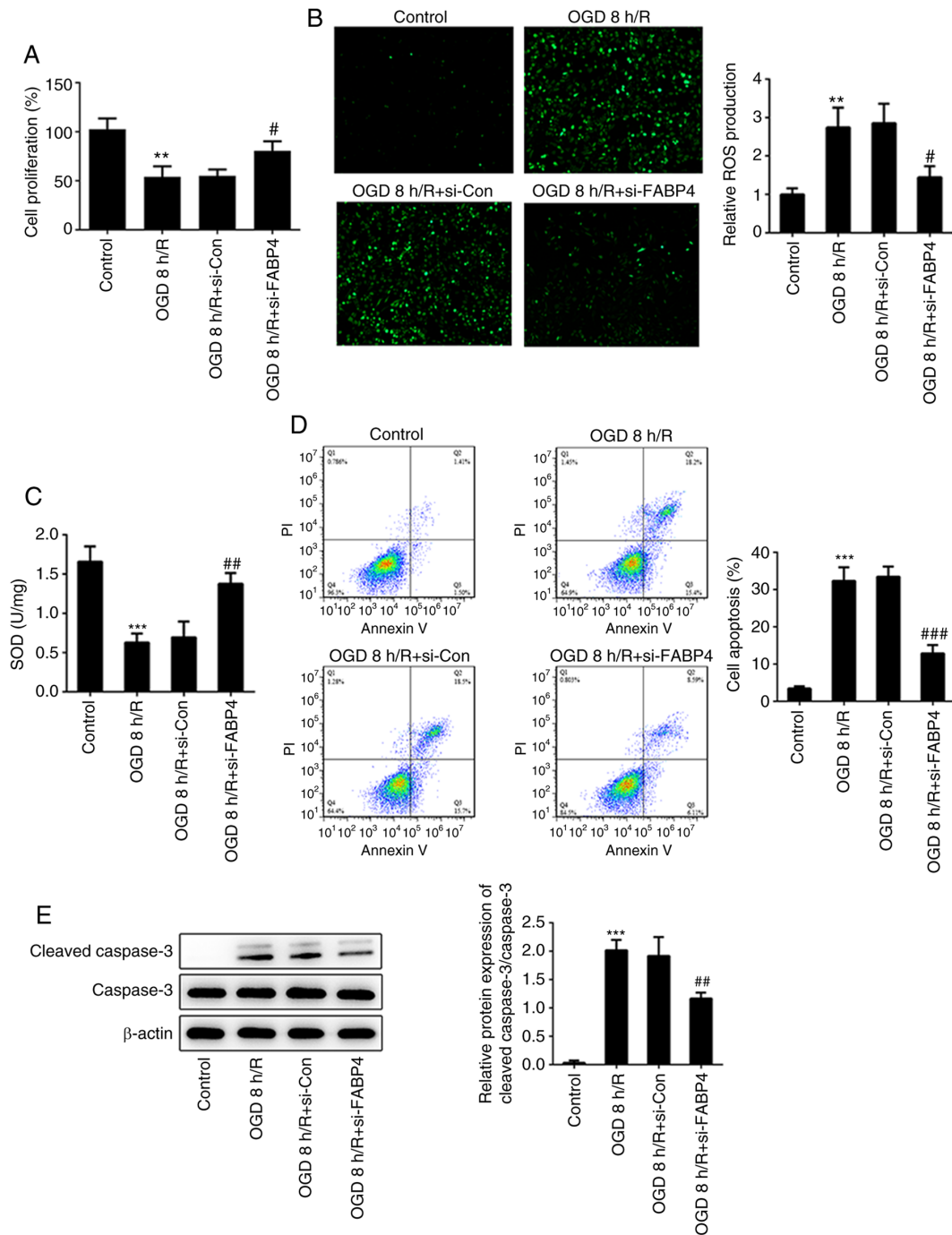


Figure 2. FABP4-knockdown inhibits oxidative stress and apoptosis. (A) Cell proliferation was measured by Cell Counting Kit-8 assay. (B) ROS production and (C) SOD activity were assessed using a ROS assay kit (magnification, x100) or SOD assay kit, respectively. (D) Cell apoptosis was detected by flow cytometry. (E) Cleaved caspase-3, caspase-3 and β -actin were estimated by western blotting. ** $P < 0.01$, *** $P < 0.001$ vs. control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. OGD 8 h/R+si-Con. FABP4, fatty acid binding protein 4; ROS, reactive oxygen species; SOD, superoxide dismutase; siCon, control small interfering RNA; OGD/R, oxygen glucose deprivation/reoxygenation.

Results

Downregulation of FABP4 in PC12 cells under OGD/R. PC12 cells subjected to 2, 4, 6, 8 or 10 h OGD, respectively, followed by 24 h reoxygenation. As shown in Fig. 1A, prolonged duration of OGD led to a decrease in cell activity. The expression of FABP4 in PC12 cells exposed to OGD/R was gradually increased, which matched the decrease in cell viability. However, FABP4 was downregulated when cells were exposed to OGD for 10 h, compared with OGD

for 8 h, which may involve several underlying mechanisms (Fig. 1B). Therefore, OGD for 8 h and reoxygenation for 24 h served as an invariant OGD/R condition for subsequent experiments. PC12 cells under normal conditions were transfected with FABP4 siRNA, and the transcription and translation of FABP4 was significantly decreased (Fig. 1C and D). Additionally, FABP4 siRNA decreased the protein expression of FABP4 under OGD/R (Fig. 1E). Furthermore, FABP4 was downregulated in PC12 cells exposed to OGD/R.

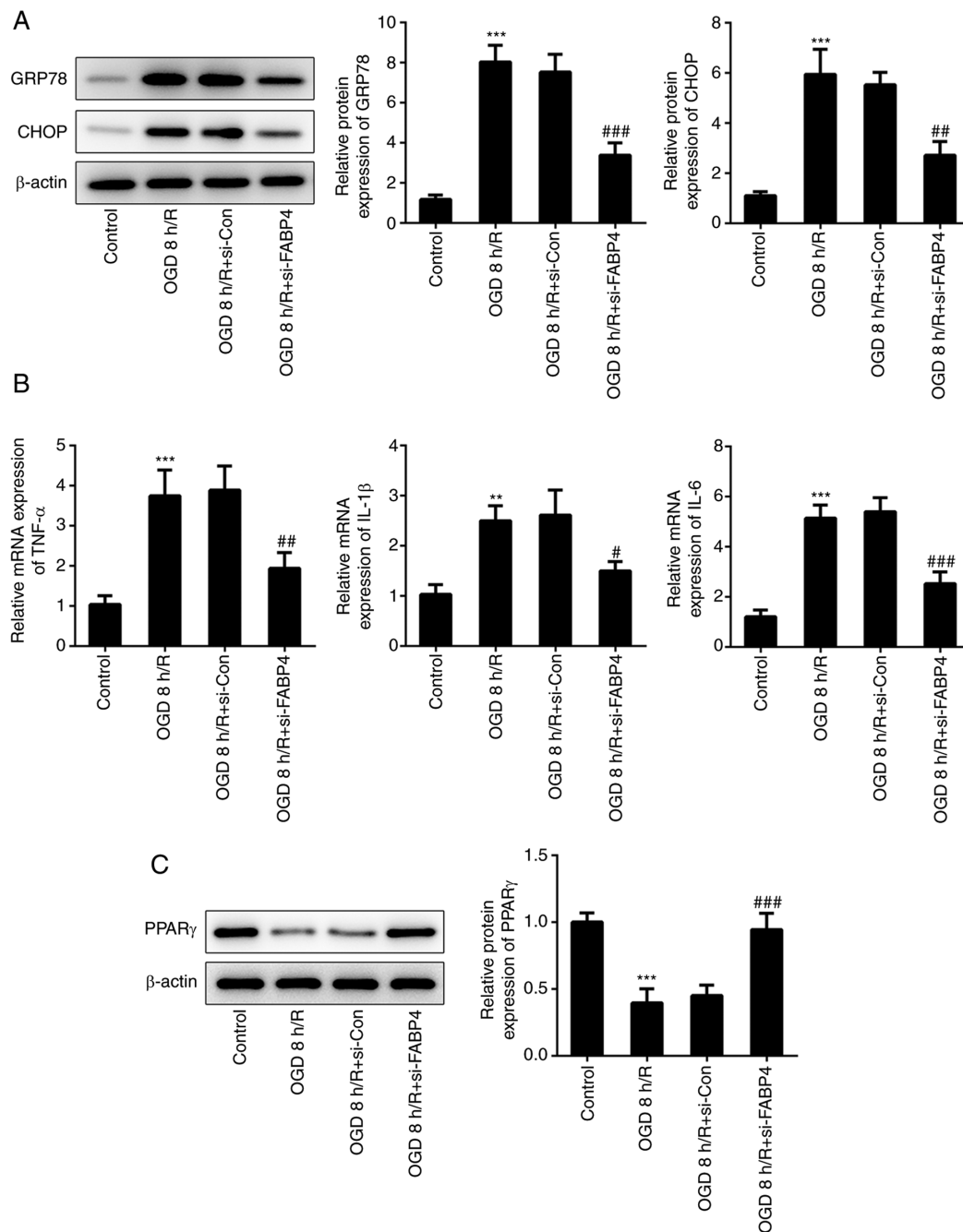


Figure 3. Interference of FABP4 suppresses inflammation and ERS and activates PPAR γ . (A) GRP78, CHOP and β -actin expression was estimated by western blotting. (B) mRNA expression of TNF- α , IL-1 β and IL-6 were determined by reverse transcription-quantitative polymerase chain reaction. (C) Protein expression of PPAR γ was detected by western blotting. ** $P < 0.01$, *** $P < 0.001$ vs. control; # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ vs. OGD 8 h/R+si-Con. FABP4, fatty acid binding protein 4; ERS, endoplasmic reticulum stress; PPAR γ , peroxisome proliferator-activated receptor γ ; GRP78, glucose regulating protein 78; CHOP, C/EBP homologous protein; TNF- α , tumor necrosis factor α ; IL, interleukin; OGD/R, oxygen glucose deprivation/reoxygenation.

FABP4 knockdown inhibits oxidative stress and apoptosis. Oxidative stress is the main event in the pathogenesis of I/R injury (19). The present study demonstrated that OGD/R inhibited cell proliferation and induced a potent increment of ROS production. FABP4 siRNA caused a significant increase in cell proliferation and decrease in the ROS level, compared with control siRNA under OGD/R (Fig. 2A and B). On the other hand, the decrease in SOD activity under OGD/R, compared with the control group, demonstrated that oxidative stress occurred under OGD/R conditions (Fig. 2C). Apoptotic rates of PC12 cells under different conditions were determined

by flow cytometry. OGD/R induced marked cell apoptosis, while FABP4 siRNA potentially decreased the apoptotic rate (Fig. 2D). Furthermore, increased cleaved caspase-3 under an OGD/R and FABP4 knockdown-mediated decrease in cleaved caspase-3 under OGD/R further suggested an anti-apoptotic effect of FABP4-knockdown (Fig. 2E). Therefore, FABP4 siRNA protected PC12 cells against OGD/R-induced oxidative stress and apoptosis.

Interference of FABP4 suppresses inflammation, ERS and activated PPAR γ . A recent study illustrated that FABP4

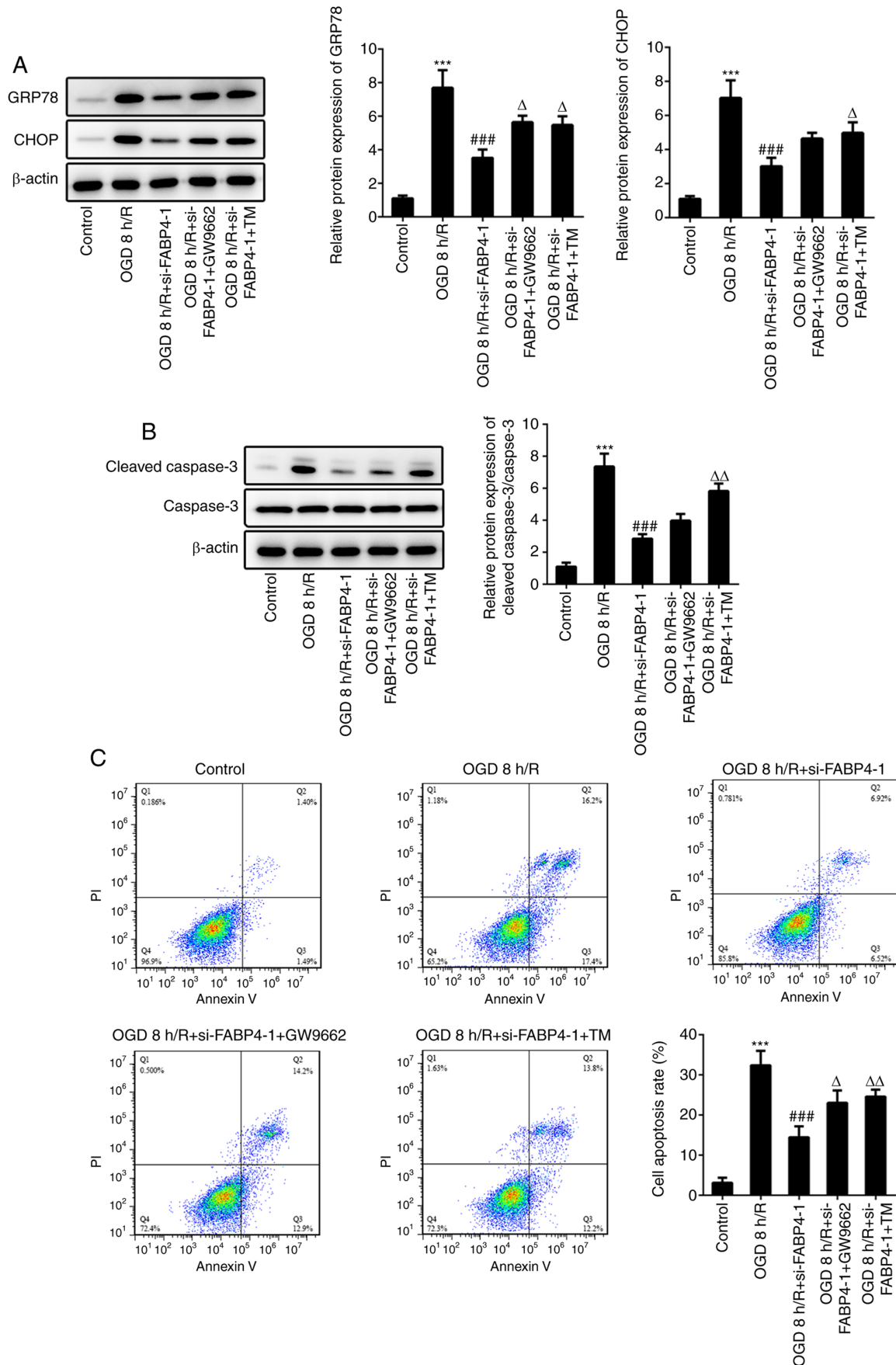


Figure 4. FABP4 modulates ERS and apoptosis via regulation of PPAR γ . (A) PC12 cells exposed to OGD 8 h/R. Cells were pretreated with 1 μ M GW9662 or 1 μ g/ml TM for 2 h before OGD 8 h/R. GRP78, CHOP and β -actin expression was estimated by western blotting. (B) Cleaved caspase-3, caspase-3 and β -actin expression was estimated by western blotting. (C) Cell apoptosis was detected by flow cytometry. *** P <0.001 vs. control; ### P <0.001 vs. OGD 8 h/R; Δ P <0.05, $\Delta\Delta$ P <0.01 vs. OGD 8 h/R+si-FABP4-1. FABP4, fatty acid binding protein 4; ERS, endoplasmic reticulum stress; PPAR γ , peroxisome proliferator-activated receptor γ ; TM, tunicamycin; OGD/R, oxygen glucose deprivation/reoxygenation; GRP78, glucose regulating protein 78; CHOP, C/EBP homologous protein.

regulated the apoptosis of tubular epithelial cells via inactivating ERS (20). ERS is generally considered to contribute toward neuronal apoptosis following cerebral IR (21). ERS-related proteins, GRP78 and CHOP, were enhanced under OGD/R (Fig. 3A), accompanied by upregulation of inflammatory factors TNF- α , IL-1 β and IL-6 (Fig. 3B). Notably, FABP4 siRNA alleviated OGD/R-induced ERS and inflammation. Activation of PPAR γ may protect the brain from cerebral I/R injury via inhibiting ERS (22). As shown in Fig. 3C, the protein expression of PPAR γ was markedly decreased under OGD/R; however, it was reinforced in PC12 cells transfected with FABP4 siRNA. Taken together, these finding suggested that FABP4 siRNA repressed inflammation, ERS and activated PPAR γ under OGD/R.

FABP4 modulates ERS and apoptosis via regulation of PPAR γ . PPAR γ antagonist GW9662 was employed in subsequent experiments to further investigate the role of PPAR γ in FABP4-mediated ERS and apoptosis. The levels of GRP78 and CHOP were decreased following transfection with FABP4 siRNA under OGD/R. GW9662 reversed the function of FABP4 siRNA, showing a similar result to the ERS agonist, tunicamycin (Fig. 4A). Cleaved caspase-3 was decreased in PC12 cells transfected with FABP4 siRNA, compared with that of the OGD/R group, and cleaved caspase-3 in cells treated with GW9662 or tunicamycin on the basis of FABP4 siRNA transfection was increased, compared with FABP4 siRNA transfection only (Fig. 4B). Furthermore, apoptotic rates of PC12 cells indicated that the anti-apoptotic effects of FABP4 siRNA were abolished by GW9662 or tunicamycin (Fig. 4C). These results implied that PPAR γ involved FABP4-regulated ERS and apoptosis.

Discussion

Cerebral I/R injury is a core pathophysiological process of ischemic stroke (6). FABP4 has been investigated in I/R injury of the kidney, liver and myocardium (13,14,23). The roles of FABP4 in cerebral I/R injury are largely unknown. In the present study, cerebral I/R injury was simulated *in vitro* to investigate the effects of FABP4 in PC12 cells under OGD/R conditions.

During 8 h of OGD, cell viability was gradually down-regulated and FABP4 was gradually increased, suggesting that FABP4 had a harmful effect on PC12 cells under OGD/R conditions, which was consistent with the results reported in myocardial IR injury (23). Oxidative stress and inflammation serve as the main pathogenesis of I/R injury, and effective prevention of oxidative stress and inflammation is conducive to ameliorate I/R injury (24). In the present study, FABP4-silencing decreased the expression of inflammatory factors, IL-1 β , IL-6 and TNF- α , and the decrease in ROS and increase in SOD demonstrated that FABP4 siRNA relieved oxidative stress.

Bosquet *et al* (25) reported that exogenous FABP4 promoted ERS in HepG2 cells. In addition, another study reported by Bosquet *et al* (26) described that the FABP4 inhibitor prevented lipid-induced ERS-associated inflammation in skeletal muscle. The present study demonstrated that FABP4-silencing ameliorated ERS and ERS-associated

apoptosis. ERS contributes toward the development of cerebral I/R injury (25).

PPAR γ accelerated the elimination of additional superoxide by promoting the transcription of SOD. Inhibition of PPAR γ promoted neuron apoptosis in ischemia by upregulating ROS scavenging genes (26). FABP4 siRNA repressed inflammation and enhanced PPAR γ signaling in proximal tubular epithelial cells (27). In PC12 cells, FABP4 siRNA contributed toward the increase in PPAR γ expression. The PPAR γ antagonist, GW9662, counteracted the effects of FABP4 siRNA on ERS and apoptosis.

In summary, the present study demonstrated that FABP4 was overexpressed in PC12 cells under OGD/R, and FABP4-silencing inhibited ERS-associated inflammation, oxidative stress and apoptosis via upregulating PPAR γ signaling.

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Availability of data and materials

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

Authors' contributions

XLY performed the experiments and drafted the manuscript, JHM analyzed and interpreted the data and QD designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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