

Downregulation of TNFAIP1 alleviates OGD/R-induced neuronal damage by suppressing Nrf2/GPX4-mediated ferroptosis

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Abstract. TNF α -induced protein 1 (TNFAIP1) serve a role in neurovascular disease. However, the potential role and molecular mechanism of TNFAIP1 in cerebral ischemia-reperfusion (I/R) remains elusive. In the present study, reverse transcription-quantitative PCR and western blotting were used to assess TNFAIP1 mRNA and protein expression levels in PC12 cells. Furthermore, using Cell Counting Kit-8, flow cytometry and western blotting, cell viability and apoptosis were evaluated. Oxidative stress was evaluated using DCFH-DA staining and ELISA was used for assessment of inflammatory factors. Expression of components in the nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathway and ferroptosis were assessed using western blotting analysis and an iron assay kit. TNFAIP1 expression was significantly upregulated in oxygen glucose deprivation and reperfusion (OGD/R)-injured PC12 cells. However, knocking down TNFAIP1 expression restored PC12 cell viability and decreased apoptosis following OGD/R-challenge. Furthermore, TNFAIP1 silencing significantly suppressed OGD/R-induced oxidative stress and inflammatory damage in PC12 cells. TNFAIP1 knockdown inhibited ferroptosis via activation of the Nrf2 signaling pathway in OGD/R-injured PC12 cells. Erastin treatment reversed the beneficial effects of TNFAIP1 knockdown on PC12 cell viability, apoptosis alleviation, oxidative stress and inflammation following OGD/R treatment. These results suggested that TNFAIP1 knockdown could alleviate OGD/R-induced neuronal cell damage by suppressing Nrf2-mediated ferroptosis, which might lay

the foundation for the investigation of targeted-therapy for cerebral I/R injury in clinic.

Introduction

Stroke was reported to cause 3.94 million (95% uncertainty interval 3.43-4.58) new cases in China in 2019 (1). It has been previously reported that ischemic stroke constitutes >80% of all cases of stroke (2). This disease has become a key threat to public health with high rates of disability and mortality (3). At present, the primary therapeutic method for ischemic stroke is to recanalize occluded arteries through thrombolysis or thrombectomy (4). However, it has been reported that the restoration and reperfusion of blood flow into previously blood-deficient area has the potential to aggravate brain tissue injury and is frequently accompanied by cerebral ischemia-reperfusion (I/R) injury (CIRI) (5). CIRI is a dynamic and complex pathophysiological process, which is caused by a range of cellular and external physiological factors (6). Intracellular Ca²⁺ overload, overproduction of free radicals, excitatory amino acids, inflammatory cascade activation, acidosis, increased mitochondrial permeability and cell apoptosis are reported to be the primary factors of CIRI (7). Although thrombolysis and embolectomy restore blood flow to the infarcted brain tissue, such therapy also results in I/R (8,9). Therefore, it is important to elucidate the molecular mechanisms underlying CIR to develop novel strategies with significant efficacy for treatment of CIR.

TNF α -induced protein 1 (TNFAIP1), which is also called B12 or BACURD2, has numerous biological functions (10). It has been reported that TNFAIP1 is a key mediator of inflammation by activating NF- κ B activity (11). Furthermore, TNFAIP1 has been reported to stimulate DNA polymerase δ activity and interact with proliferating cell nuclear antigen, which suggests that TNFAIP1 regulates the inflammatory response, cell proliferation and cell cycle progression (12). TNFAIP1 may also promote neurotoxicity (13). Gladwyn-Ng *et al* (14) previously reported that TNFAIP1 hinders neuronal migratory capabilities in the embryonic cortex and changes the morphology of the immature neuron (14). Another recent study reported that TNFAIP1 expression is upregulated following myocardial I/R and that TNFAIP1 knockdown ameliorates myocardial

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I/R injury (MIRI) via the Akt/GSK-3 β /nuclear factor erythroid 2-related factor 2 (Nrf2) pathway (15). However, the role of TNFAIP1 in CIR and its underlying mechanism remains poorly understood. Therefore, the present study evaluated the effects of TNFAIP1 on CIR and investigated how TNFAIP1 may regulate the pathophysiological process of CIRI.

Materials and methods

Cell culture and treatment. The rat adrenal gland cancer PC12 cell line was purchased from BioVector NTCC. DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; Cytiva) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) were used to cultivate the cells at 37°C with 5% CO₂ for 2 h. To establish the oxygen glucose deprivation and reperfusion (OGD/R) model *in vitro*, PC12 cells were cultured in glucose-free DMEM (Procell Life Science & Technology Co., Ltd.) in an oxygen-free incubator supplied with 5% CO₂ and 95% N₂ at 37°C for 2 h. Following hypoxia treatment, the media was replaced with normoxic glucose-containing medium and cells were transferred to an incubator supplied with 95% air and 5% CO₂ at 37°C for 24 h. Subsequently, cells were treated with 5 μ M ML385 at room temperature for 6 h or 0.75 μ M Erastin at room temperature for 24 h.

Cell transfection. For knockdown of TNFAIP1 expression, short hairpin RNAs (shRNAs) targeting TNFAIP1 with a pRNAU6.1 vector backbone (sh-TNFAIP1#1, 5'-GGAAGT GCTGACCGACAAA-3'; sh-TNFAIP1#2, 5'-GATTGCAGA TAGCTAGCTA-3') and appropriate scrambled sequence negative control (sh-NC, 5'-GGTACGCAATAGGAGTGT GTG-3') were synthesized by Shanghai GenePharma Co., Ltd. The transfection of 100 nM of recombinants into PC12 cells was performed at 37°C for 48 h using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.). The transfection of cells with sh-TNFAIP1 was performed 24 h prior to OGD/R treatment. After 48 h, the cells were collected for subsequent experiments.

Cell Counting Kit-8 (CCK-8) assay. Following OGD/R treatment, cells were seeded into 96-well plates at a density of 5x10³ cells and cultivated in DMEM with 10% FBS (HyClone; Cytiva) at room temperature for 24 h. Each well was treated with 10 μ l CCK-8 solution (Sangon Biotech Co., Ltd.) added to each well for further incubation at 37°C with 5% CO₂ for 4 h. A microplate reader was used for the assessment of the optical density at 450 nm.

Flow cytometry. A total of 200 μ l PC12 cells were rinsed with 1 ml pre-cold PBS twice and centrifuged. Cells were then resuspended into 100 μ l binding buffer. Following incubation with 5 μ l Annexin V-FITC on ice for 15 min, cells were stained with 10 μ l propidium iodide (10 mg/ml) at 4°C for 30 min in the dark. A flow cytometer (BD Biosciences) was used for detection. Flowjo vX.0.7 software (FlowJo LLC) was used to assess the rates of apoptosis.

Reactive oxygen species (ROS) detection. ROS Assay kit containing DCFH-DA (MilliporeSigma) was used to evaluate

ROS generation. PC12 cells were probed using DCFH-DA (10 μ M) at 37°C in the dark for 30 min. Subsequently, PBS-rinsed cells were imaged using an Axio Observer D1 fluorescence microscope (Carl Zeiss AG; magnification, x200).

Assessment of oxidative stress markers. PC12 cells were inoculated into six-well plates at a density of 4x10⁵/well. Following aforementioned treatment, levels of intracellular glutathione (GSH) and malondialdehyde (MDA) were assessed using GSH Assay kit (cat. no. S0073; Beyotime Institute of Biotechnology) and MDA Assay Kit (cat. no. S0131S; Beyotime Institute of Biotechnology) according to the manufacturer's protocol. A microplate reader was used for colorimetric analysis.

ELISA. Following aforementioned treatment, the protein expression levels of inflammatory factors TNF- α , IL-1 β and IL-6 in cell supernatant were assessed using corresponding ELISA kits (cat. nos. EK0526, EK0393 and EK0412, respectively; all Wuhan Boster Biological Technology, Ltd.). A microplate reader was used to assess the absorbance at 450 nm.

Assessment of Fe²⁺ levels. Following 48-h transfection, exposure of cells to OGD/R treatment with or without 5 μ M ML385 was performed. Iron Assay kit (cat. no. ab83366; Abcam) was used to assess levels of Fe²⁺ in PC12 cells according to the manufacturer's protocol. A microplate reader was used to assess the absorbance at 593 nm of each well.

Reverse transcription-quantitative PCR (RT-qPCR). RNA was isolated from cells using RNAiso Plus (Takara Bio, Inc.) and quantified using QuickDrop (Molecular Devices LLC) at 260 and 280 nm. cDNA was produced using PrimeScript™ RT Master Mix (Takara Bio, Inc.) before qPCR using SYBR® Premix Ex Taq™ II kit (Takara Bio, Inc.) using an ABI 7500 (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The following thermocycling conditions were used for qPCR: 95°C for 10 min; followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec. The primer sequences used for PCR were as follows: TNFAIP1 forward (F), 5'-ATCATCATC TTGCCTGGCCC-3' and reverse (R), 5'-GAACAAAGCTGT TCCCGTGC-3' and GAPDH F, 5'-GTCGTGGAGTCTACT GCGTCTTCA-3' and R, 5'-TCGTGGTTCACACCCATC ACAACA-3'. GAPDH was used as an internal reference and the 2^{- $\Delta\Delta$ C_q} method (16) was used for the calculation of relative mRNA expression levels.

Western blotting. The proteins were quantified using a BCA protein assay kit (Thermo Fisher Scientific, Inc.) after the extraction of total proteins from the indicated PC12 cells using RIPA lysis buffer reagent (Beijing Solarbio Science & Technology Co., Ltd.). Following the separation of protein (30 μ g/lane) using 10% SDS-PAGE, the proteins were transferred onto PVDF membranes. Membranes were blocked using 5% non-fat milk at room temperature for 2 h. Overnight incubation of membranes was performed at 4°C with primary antibodies, supplied by Abcam, as follows: TNFAIP1 (1:1,000; cat. no. ab86934), Bcl-2 (1:1,000; cat. no. ab196495), Bax (1:1,000; cat. no. ab32503), cleaved caspase 3 (1:5,000; cat. no. ab214430), Nrf2 (1:1,000; cat. no. ab92946), Lamin B1 (1:1,000; cat. no. ab16048), heme oxygenase-1 (HO-1;

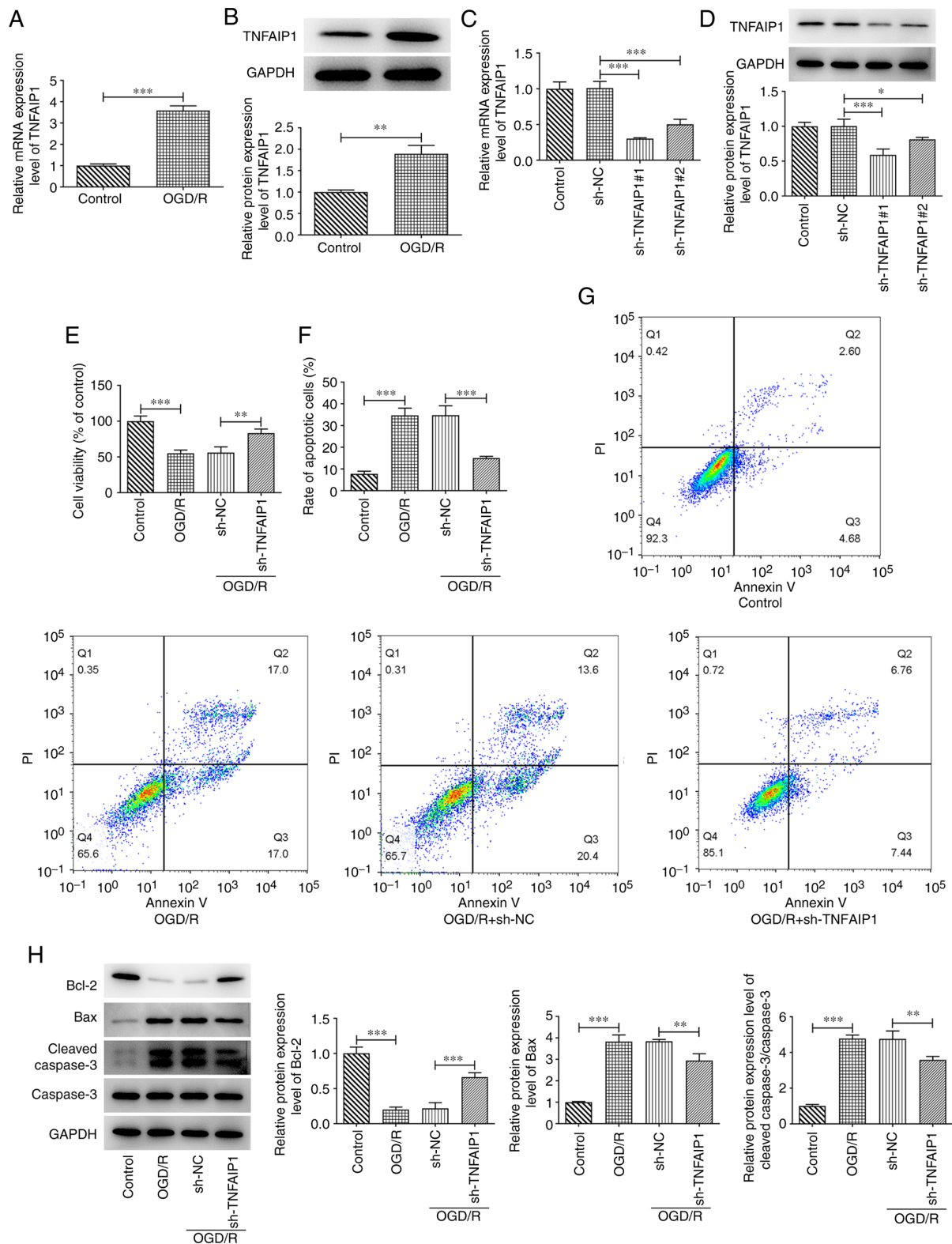


Figure 1. TNFAIP1 silencing alleviates OGD/R-induced PC12 cell injury. (A) mRNA and (B) protein expression levels of TNFAIP1 in OGD/R-induced PC12 cells were assessed using RT-qPCR and western blotting. (C) mRNA and (D) protein expression levels of TNFAIP1 following transfection with sh-TNFAIP1#1 and 2 were assessed using RT-qPCR and western blotting. (E) Cell proliferation was assessed using Cell Counting Kit-8 assay. (F) Cell apoptosis was assessed using (G) flow cytometry. (H) Western blotting was used to assess expression levels of apoptosis-associated protein. Data are presented as the mean \pm SD. * P <0.05, ** P <0.01 and *** P <0.001. TNFAIP1, TNF α -induced protein 1; OGD/R, oxygen glucose deprivation and reperfusion; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin RNA; NC, negative control; PI, propidium iodide.

1:1,000; cat. no. ab68477), NADPH quinone dehydrogenase 1 (NQO-1; 1:1,000; cat. no. ab80588), GSH peroxidase 4 (GPX4; 1:1,000; cat. no. ab125066), ferritin heavy chain (1:1,000; cat.

no. ab183781), ferroportin (FPN; 1:1,000; cat. no. ab239511), transferrin receptor 1 (TFR1; 1:1,000; cat. no. ab84036) and GAPDH (1:1,000; cat. no. ab8245). The membranes were

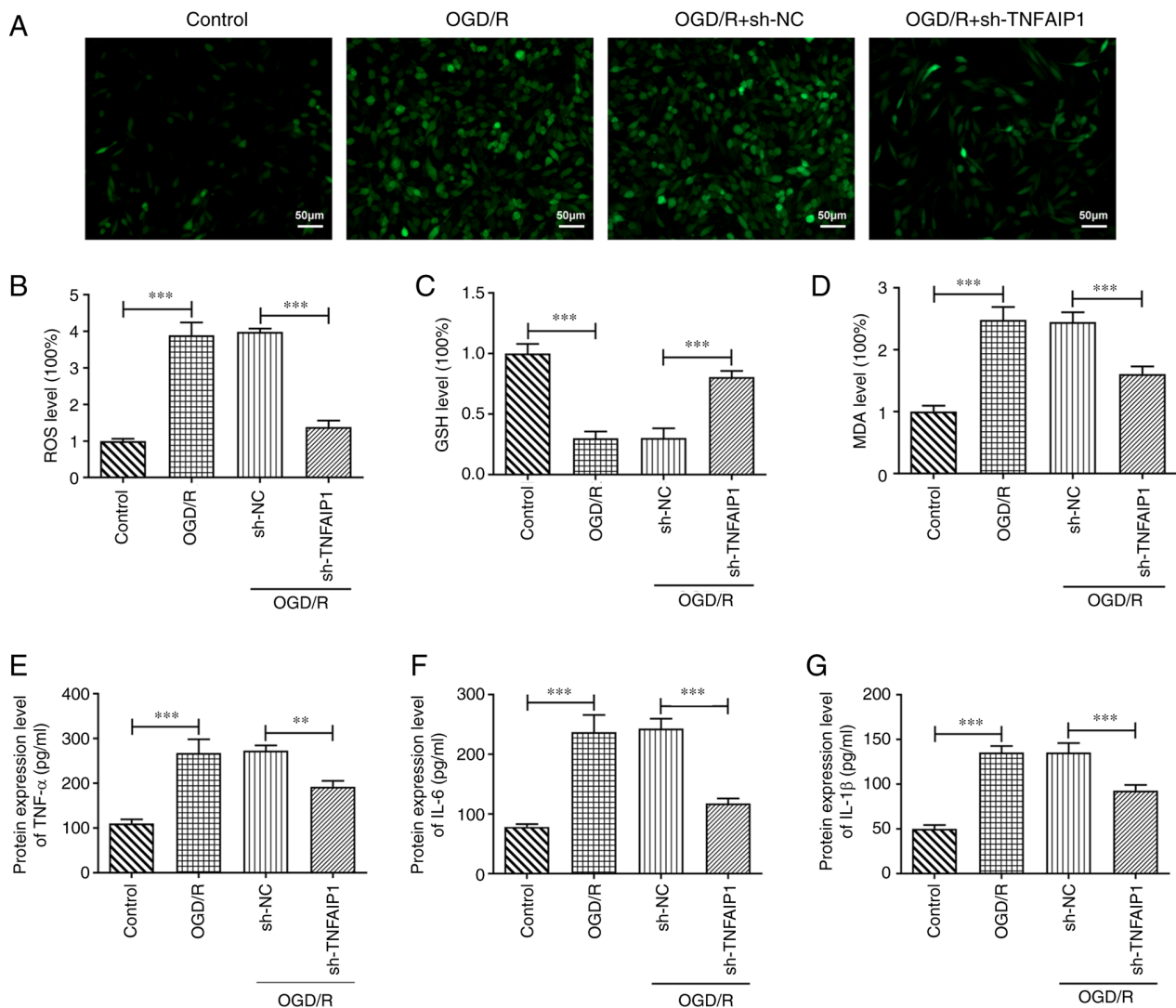


Figure 2. TNFAIP1 silencing decreases oxidative stress and the inflammatory response in OGD/R-induced PC12 cells. (A) DCFH-DA staining was used to assess (B) ROS levels. Scale bar, 50 μ m. Levels of (C) GSH and (D) MDA were evaluated using commercially available kits. The protein expression levels of (E) TNF- α , (F) IL-6 and (G) IL-1 β were assessed using ELISA. Data are presented as the mean \pm SD. ** $P < 0.01$ and *** $P < 0.001$. TNFAIP1, TNF α -induced protein 1; OGD/R, oxygen glucose deprivation and reperfusion; ROS, reactive oxygen species; GSH, glutathione; MDA, malondialdehyde; sh, short hairpin RNA; NC, negative control.

washed with PBS for three times and then incubated with the HRP-labeled rabbit anti-mouse secondary antibodies (1:2,000; cat. no. ab6728; Abcam) at room temperature for 2 h. The antibody-labeled proteins were visualized using the ECL Detection Reagent (Shanghai Yeasen Biotechnology Co., Ltd.) and analyzed using ImageJ (version 1.49; National Institutes of Health).

Statistical analysis. All the experiments should be repeated at least three times. Data are presented as the mean \pm standard deviation and were analyzed using SPSS 22.0 software (IBM Corp.). Unpaired Student's *t* test or one-way ANOVA followed by Bonferroni's post hoc test were used for comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Downregulation of TNFAIP1 decreases OGD/R-induced PC12 cell injury. To evaluate the role of TNFAIP1 in

OGD/R-insulted PC12 cells, TNFAIP1 mRNA and protein expression levels were assessed in PC12 cells. The mRNA and protein expression levels of TNFAIP1 were significantly increased in OGD/R-treated PC12 cells compared with those in the control group (Fig. 1A and B). TNFAIP1 expression was knocked down in PC12 cells by transfection with sh-TNFAIP1#1 and #2. RT-qPCR and western blotting were performed to assess transfection efficiency. It was revealed that the mRNA and protein expressions of TNFAIP1 were significantly reduced compared with those in sh-NC after transfection with sh-TNFAIP1 (Fig. 1C and D). sh-TNFAIP1#1 exhibited superior transfection efficiency; therefore, sh-TNFAIP1#1 was used for subsequent experiments and was referred to as sh-TNFAIP1 thereafter. OGD/R treatment significantly suppressed PC12 cell viability compared with the control, which was significantly reversed by TNFAIP1 silencing compared with sh-NC group (Fig. 1E). Furthermore, the proportion of apoptotic cells was significantly elevated after OGD/R

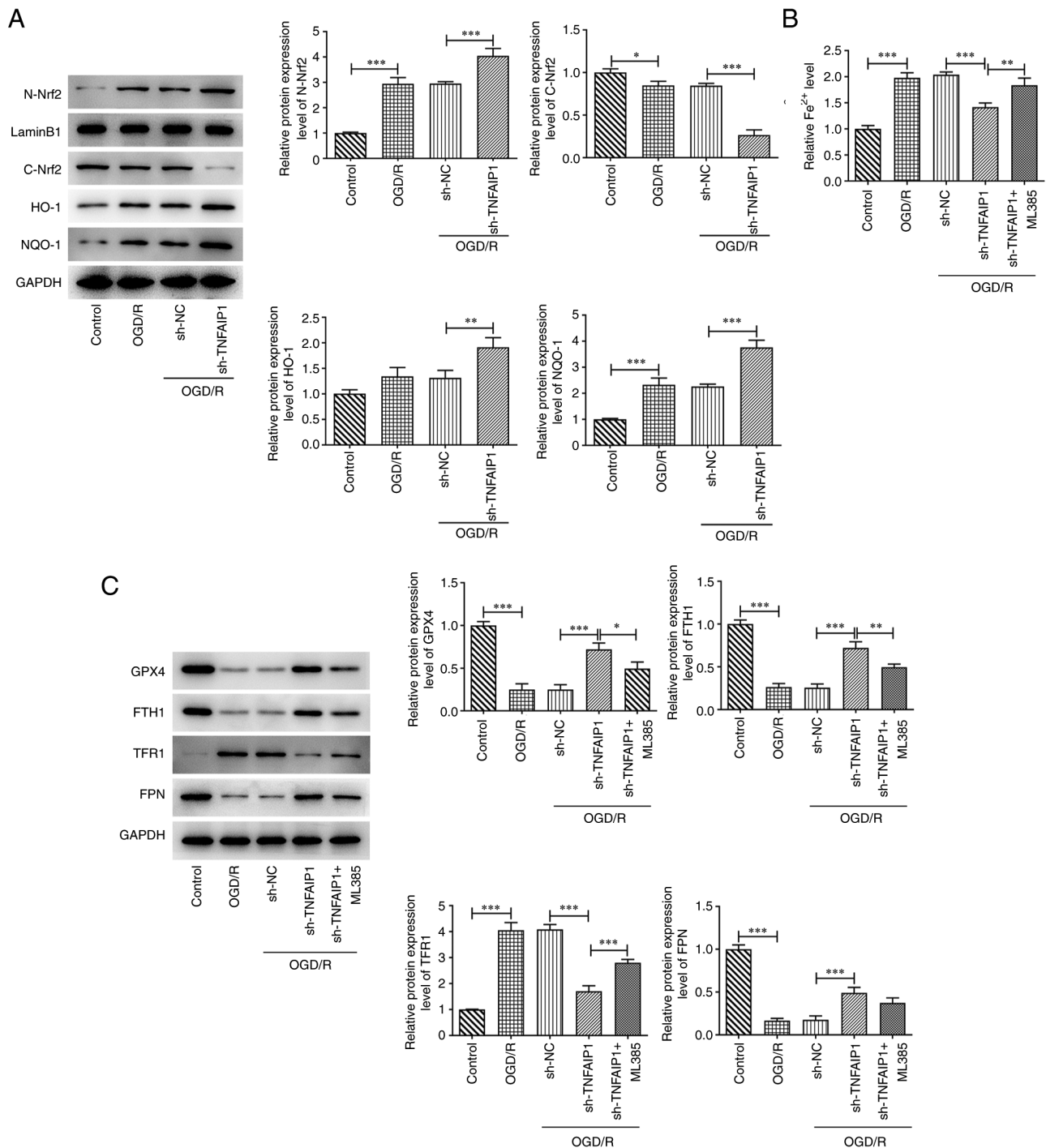


Figure 3. Knockdown of TNFAIP1 inhibits ferroptosis via activation of the Nrf2 signaling pathway in OGD/R-induced PC12 cells. (A) Western blotting was performed to assess protein expression levels of C-Nrf2, N-Nrf2, HO-1 and NQO-1. (B) Iron Assay kit was used to assess Fe^{2+} levels. (C) Western blotting was performed to assess protein expression levels of GPX4, FTH1, FPN and TFR1. Data are presented as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. TNFAIP1, TNF α -induced protein 1; OGD/R, oxygen glucose deprivation and reperfusion; Nrf2, nuclear factor erythroid 2-related factor 2; C, cytoplasmic; N, nuclear; HO-1, heme oxygenase 1; NQO-1, NADPH quinone dehydrogenase 1; GPX4, glutathione peroxidase 4; FTH1, ferritin heavy chain; FPN, ferroportin; TFR1, transferrin receptor 1; sh, short hairpin RNA; NC, negative control.

treatment compared with the control and significantly reversed by TNFAIP1 knockdown compared with the sh-NC group (Fig. 1F and G). Consistently, OGD/R significantly decreased Bcl-2 protein expression levels while significantly increasing those of Bax and cleaved caspase 3 compared with the control. However, TNFAIP1 knockdown significantly reversed the effects of OGD/R on expression levels of these proteins in PC12 cells (Fig. 1H).

TNFAIP1 silencing alleviates oxidative stress and the inflammatory response in OGD/R-induced PC12 cells. To assess the impact of TNFAIP1 knockdown in PC12 cells following OGD/R, the levels of oxidative stress and inflammation were evaluated. OGD/R significantly elevated levels of ROS compared with the control whereas TNFAIP1 silencing significantly decreased this enhancement compared with the sh-NC group (Fig. 2A and B). OGD/R treatment significantly

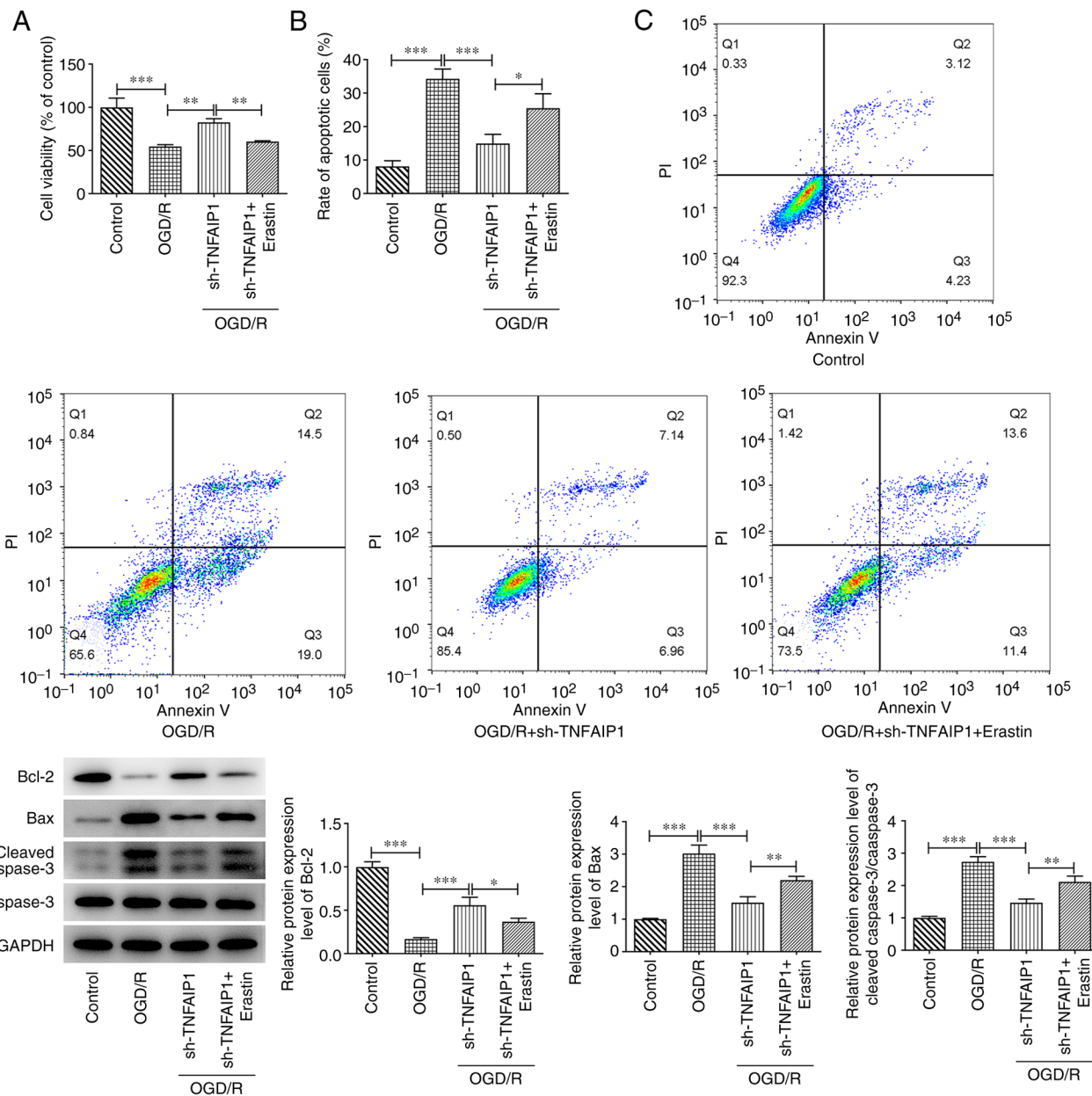


Figure 4. Erastin reverses the effect of TNFAIP1 silencing on OGD/R-induced PC12 cell injury. (A) Cell viability was assessed using Cell Counting Kit-8 assay. (B) Cell apoptosis was assessed using (C) flow cytometry. (D) Western blotting was performed to assess expression levels of apoptosis-associated protein. Data are presented as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. TNFAIP1, TNF α -induced protein 1; OGD/R, oxygen glucose deprivation and reperfusion; sh, short hairpin RNA; NC, negative control; PI, propidium iodide.

decreased GSH but significantly increased MDA protein expression levels in PC12 cells compared with the control; however, knocking down TNFAIP1 expression significantly reversed OGD/R-induced oxidative stress compared with the sh-NC group (Fig. 2C and D). Furthermore, protein expression levels of TNF- α , IL-6 and IL-1 β were significantly elevated following OGD/R compared with the control, which was significantly reversed after TNFAIP1 knockdown compared with the sh-NC group (Fig. 2E-G).

Knocking down TNFAIP1 expression suppresses ferroptosis via activation of the Nrf2 signaling pathway in OGD/R-injured PC12 cells. The potential mechanism by which TNFAIP1 regulated OGD/R-induced PC12 cells was assessed. Results obtained from western blotting demonstrated that OGD/R

treatment significantly decreased cytoplasmic (C)-Nrf2 protein expression levels but significantly elevated those of nuclear (N)-Nrf2 and NQO-1 and markedly elevated those of HO-1 compared with the control. TNFAIP1 silencing significantly decreased C-Nrf2 protein expression levels further whereas it significantly increased those of N-Nrf2, HO-1 and NQO-1 compared with sh-NC group (Fig. 3A). To assess the association between the Nrf2 signaling pathway and ferroptosis downstream of TNFAIP1 in PC12 cells induced with OGD/R, the Nrf2 inhibitor ML385 was used to treat cells. OGD/R resulted in significantly elevated Fe²⁺ levels compared with the control and transfection with sh-TNFAIP1 significantly reversed the enhancement in Fe²⁺ levels that resulted from OGD/R compared with the sh-NC group. However, ML385 treatment significantly reversed the

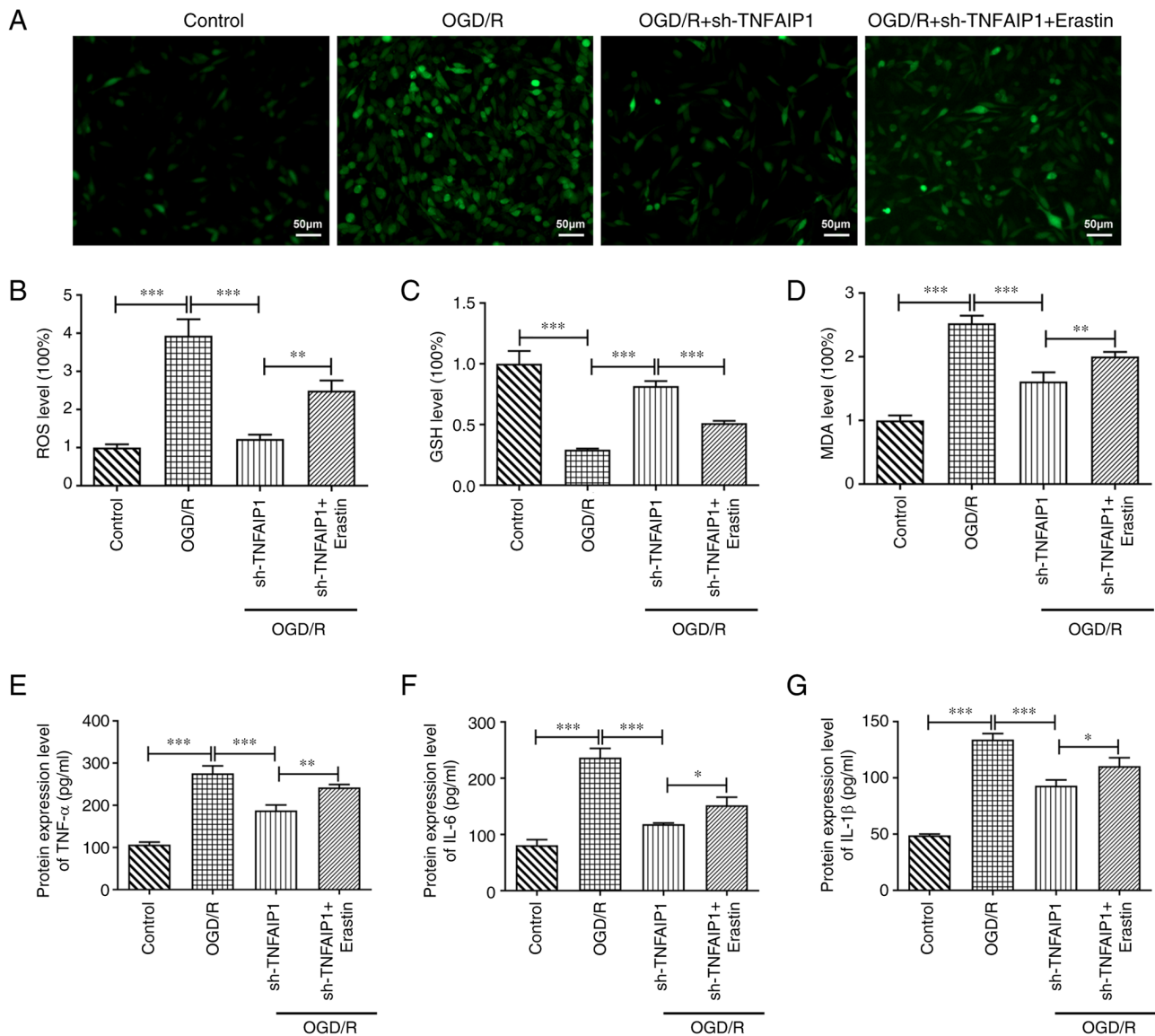


Figure 5. Erastin reverses the effects of TNFAIP1 silencing on oxidative stress and inflammation in PC12 cells following OGD/R injury. (A) DCFH-DA staining was performed to assess (B) ROS levels. Scale bar, 50 μ m. The levels of (C) GSH and (D) MDA were assessed using commercially available kits. The protein expression levels of (E) TNF- α , (F) IL-6 and (G) IL-1 β were assessed using ELISA. Data are presented as the mean \pm SD. * P <0.05, ** P <0.01 and *** P <0.001. TNFAIP1, TNF α -induced protein 1; OGD/R, oxygen glucose deprivation and reperfusion; sh, short hairpin RNA; NC, negative control; ROS, reactive oxygen species.

inhibitory effects of sh-TNFAIP1 on levels of Fe²⁺ in PC12 cells compared with the sh-TNFAIP1 group. It was also demonstrated that protein expression levels of GPX4, FTH1 and FPN were significantly decreased whilst those of TFR1 were significantly increased by OGD/R compared with the control and this was significantly reversed by TNFAIP1 silencing compared with the sh-NC group. The effects of TNFAIP1 knockdown on protein expression levels of GPX4, FTH1 and TFR1 were significantly reversed and the effect on the protein expression levels of FPN was markedly reversed by ML385 treatment compared with sh-TNFAIP1 group (Fig. 3C).

Erastin reverses the impact of TNFAIP1 silencing on OGD/R-induced PC12 cell injury. To assess the role of ferroptosis in TNFAIP-mediated OGD/R injury, erastin was added to the PC12 cells as a ferroptosis promoter. Treatment with

erastin significantly decreased viability of OGD/R-induced PC12 cells with TNFAIP1 expression knocked down compared with sh-TNFAIP1 group (Fig. 4A). Erastin significantly increased cell apoptosis after treatment with OGD/R and transfection with sh-TNFAIP1 compared with sh-TNFAIP1 group (Fig. 4B and C). Significantly decreased Bcl-2 protein expression levels and significantly increased protein expression levels of Bax and cleaved caspase 3 were observed in OGD/R-stimulated PC12 cells with TNFAIP1 knockdown after treatment with erastin compared with the sh-TNFAIP1 group (Fig. 4D).

Erastin counteracts the impact of TNFAIP1 knockdown on oxidative stress and inflammation in PC12 cells following OGD/R injury. Erastin treatment significantly increased the production of ROS in PC12 cells transfected with sh-TNFAIP1 following OGD/R injury compared with sh-TNFAIP1 group

(Fig. 5A and B). Furthermore, co-treatment with erastin and sh-TNFAIP1 significantly decreased protein expression levels of GSH and significantly increased MDA protein expression levels compared with those in the sh-TNFAIP1 group (Fig. 5C and D). Moreover, ELISA demonstrated that erastin significantly increased the protein expression levels of TNF- α , IL-6 and IL-1 β in OGD/R-stimulated PC12 cells transfected with sh-TNFAIP1 compared with sh-TNFAIP1 group (Fig. 5E-G).

Discussion

Evidence has demonstrated that the production of certain cytokines, inflammatory cell infiltration and ROS production occur in ischemic injury, which can exacerbate the damage due to cerebral ischemia (17,18). When reperfusion occurs, recovery of blood supply may result in ROS production and inflammation, inducing CIRI (19). Therefore, novel effective therapeutic targets are required for treatment for ischemic stroke. In the present study, TNFAIP1 knockdown was demonstrated to increase cell viability and decrease oxidative stress and inflammation, in addition to suppressing ferroptosis in PC12 cells insulted with OGD/R, via the Nrf2 signaling pathway. This indicated the potential to use TNFAIP1 for the attenuation of CIRI.

OGD/R has been frequently used in research for induction of CIRI and has been reported to stimulate inflammation, apoptosis, autophagy and endoplasmic reticulum stress in cultured cortical neurons (20,21). In the present study, the OGD/R model was established to simulate CIRI *in vitro*. OGD/R stimulation was demonstrated to significantly suppress cell viability, aggravate apoptosis and oxidative stress and induce inflammatory damage in PC12 cells. TNFAIP1 is an evolutionarily conserved single-copy gene in humans, mice, rats and nematode worm that was first reported in umbilical vein endothelial cells (22). TNFAIP1 has been reported to serve a key role in neurodevelopment and a number of neurological diseases (23). For example, TNFAIP1 is overexpressed in the neurons of the cortex and hippocampus in the brains of APP/PS1 mice and the upregulation of TNFAIP1 can induce apoptosis in mice with Alzheimer's disease (24). A previous study reported that knocking down TNFAIP1 expression inhibits formaldehyde-induced neurotoxicity by suppression of cell apoptosis whilst increasing cell viability and neurite outgrowth by the inhibition of the AKT/cAMP response element binding protein (CREB) signaling pathway (25). Qiu *et al* (26) reported that TNFAIP1 knockdown elevates cell viability and suppresses apoptosis to prevent di(2-ethylhexyl) phthalate-induced neurotoxicity by triggering the CREB signaling pathway. In the present study, TNFAIP1 mRNA and protein expression levels were significantly upregulated in PC12 cells following OGD/R. TNFAIP1 silencing significantly reversed the OGD/R-induced decrease in cell viability while reducing cell apoptosis. Furthermore, oxidative stress and inflammatory response caused by OGD/R induction were attenuated by TNFAIP1 knockdown, which supported the protective effects of TNFAIP1 knockdown in PC12 cells following OGD/R induction. TNFAIP1 has been reported as being induced by TNF- α (27); however, in the present study it was demonstrated that the inhibition of TNFAIP1

significantly reduced TNF- α protein expression levels. This may be linked to previous reports that OGD/R can cause TNFAIP1 to regulate TNF- α in turn (15,28). Furthermore, Yi *et al* (25) reported that clearance of ROS suppresses formaldehyde-mediated upregulation of TNFAIP1 expression; however in the present study, it was demonstrated that TNFAIP1-silencing inhibited production of ROS in OGD/R-treated PC12 cells.

It has been previously reported that the Nrf2 signaling pathway is associated with I/R process in numerous types of tissue (29,30). Zhao *et al* (31) reported that sulforaphane attenuates liver injury from intestinal I/R via the Nrf2/antioxidant response element pathway. Wei *et al* (32) reported that Nrf2 protects against neuronal and capillary degeneration following retinal I/R injury. Furthermore, another study reported that TNFAIP1 expression is increased following I/R injury and TNFAIP1 knockdown alleviates MIRI via the AKT/GSK3 β /Nrf2 signaling pathway (15). Therefore, it was hypothesized that TNFAIP1 is involved in neuron injury from CIRI. In the present study, C-Nrf2 protein expression levels were significantly decreased and N-Nrf2 protein expression levels were significantly enhanced following OGD/R treatment, whereas transfection with sh-TNFAIP1 significantly decreased C-Nrf2 protein expression levels and significantly increased protein expression levels of N-Nrf2 further. It can be hypothesized that excessive oxidative stress due to OGD/R promoted separation of Kelch-like ECH-associated protein 1 from Nrf-2 to activate Nrf2 and that silencing of TNFAIP1 expression may prolong activation of the Nrf2 signaling pathway following OGD/R. The Nrf2 signaling pathway is a key pathway that leads to ferroptosis (33). Yuan *et al* (34) reported that kaempferol inhibits OGD/R-induced ferroptosis in neurons via the AKT/Nrf2/GPX4 signaling pathway. The present study demonstrated that OGD/R significantly enhanced levels of Fe²⁺ and TFR1 protein expression levels but significantly diminished protein expression levels of GPX4, FTH1 and FPN. TNFAIP1 silencing significantly reversed this trend and ML385 treatment prevented this reversal. Erastin was added to the PC12 cells to promote ferroptosis, which demonstrated that erastin reversed the beneficial effects of TNFAIP1 on viability, oxidative stress and inflammatory damage in OGD/R-induced PC12 cells. These results suggested that downregulation of TNFAIP1 expression may alleviate OGD/R-induced neuronal cell damage by inhibition of ferroptosis via regulation of the Nrf2 signaling pathway.

In summary, the present study demonstrated the potential protective role of TNFAIP1 silencing in OGD/R-injured neurocytes and the key role of Nrf2-mediated ferroptosis in the viability, oxidative stress, apoptosis and inflammatory damage of PC12 cells following OGD/R stimulation, which suggested that TNFAIP1 may be a promising therapeutic target for CIRI. However, there are also some limitations of the present study. First, the function of TNFAIP1 in CIRI in a clinical setting was not explored. Second, an *in vivo* CIRI model was not established to investigate the role of TNFAIP1 in CIRI.

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Availability of data and materials

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

Authors' contributions

LX and KL designed the present study and drafted and revised the manuscript. LX, JZ, HS, GZ and XJ performed the experiments. ML and PZ reviewed the literature and analyzed the data. LX and KL confirmed the authenticity of all the raw data. All authors have 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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