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Glutaredoxin 1 protects neurons from oxygen-glucose deprivation/reoxygenation (OGD/R)-induced apoptosis and oxidative stress via the modulation of GSK-3β/Nrf2 signaling

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Abstract

Increasing evidence has indicated that glutaredoxin 1 (GRX1) is a potent antioxidant protein that promotes cell survival under conditions of oxidative stress. Oxidative stress-induced neuronal injury contributes to cerebral ischemia/reperfusion injury. However, the role of GRX1-mediated antioxidant defense against neuronal damage during cerebral ischemia/reperfusion injury has not been thoroughly investigated. Thus, the objective of this study was to evaluate whether GRX1 protects neurons against oxygen-glucose deprivation/reoxygenation (OGD/R)-evoked oxidative stress injury in an *in vitro* model of cerebral ischemia/reperfusion injury. Our data revealed that GRX1 was induced by OGD/R treatment in neurons. Functional assays indicated that loss of GRX1 exacerbated OGD/R-induced apoptosis and the generation of reactive oxygen species (ROS), while GRX1 up-regulation protected against OGD/R-evoked neuronal injury. Further investigation revealed that GRX1 promoted the nuclear expression of nuclear factor erythroid 2-related factor 2 (Nrf2) and enhanced transcription of the Nrf2/antioxidant response element (ARE) in GOD/R-exposed neurons. Furthermore, GRX1 promoted the activation of Nrf2/ARE associated with the modulation of glycogen synthase kinase-3β (GSK-3β). GSK-3β inhibition blocked GRX1 knockdown-mediated suppression of Nrf2 activation. Notably, the suppression of Nrf2 partially reversed GRX1-mediated anti-oxidative stress injury in OGD/R-exposed neurons. In summary, these findings indicate that GRX1 protects neurons against OGD/R-induced oxidative stress injury by enhancing Nrf2 activation via the modulation of GSK-3β. Our study suggests that GRX1 is a potential neuroprotective protein that protects against cerebral ischemia/reperfusion injury.

Keywords GRX1 · GSK-3β · Oxygen-glucose deprivation/reoxygenation · Oxidative stress · Nrf2.

Introduction

Cerebral ischemia/reperfusion injury is a life-threatening pathological process that has been the leading cause of disability and mortality for the last few years (Benjamin et al. 2018). Insufficient blood supply caused by blood vessel occlusion in the brain leads to irreversible ischemic damage that can be further exacerbated by blood reperfusion. The process is called cerebral ischemia/reperfusion injury (Lo et al. 2003). The pathological effects of cerebral ischemia/reperfusion injury are accompanied by the excessive production of reactive oxygen species (ROS), which causes oxidative stress in neurons and contributes to brain damage (Saito et al. 2005). Thus, enhancing our understanding of the mechanisms involved in neuronal death and oxidative stress could provide novel therapeutic strategies for the management of cerebral ischemia/reperfusion injury.

Glutaredoxin 1 (GRX1), a member of the sulfhydryl disulfide oxidoreductases, catalyzes the removal of glutathione from cysteine residues, which helps to maintain steady-state protein functioning (Gravina and Mieyal 1993; Allen and Mieyal 2012). GRX1 modulates various cell functions, including cell survival, apoptosis, differentiation and transcription (Sun et al. 2017; Yang et al. 2018; Madusanka et al. 2020). The dysregulation of GRX1 contributes to the pathogenesis of numerous disorders, including cancer, osteoarthritis

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and cardiovascular disease (Qi et al. 2016; Chen et al. 2017; Sun et al. 2017). Notably, GRX1 plays a vital role in maintaining the cellular redox state (Zhu et al. 2017). The overexpression of GRX1 prevented hydrogen peroxide-induced apoptosis and oxidative stress in retinal pigment epithelial cells (Liu et al. 2015). The up-regulation of GRX1 inhibited oxidative stress and apoptosis in osteoarthritis chondrocytes (Sun et al. 2017). A deficiency of GRX1 enhanced the excessive accumulation of intracellular oxidants, including ROS in HeLaS3 cells with exposure to γ-ray irradiation, heat shock and hydrogen peroxide (Zhao and Zhang-Akiyama 2020). Therefore, GRX1 represents an attractive target for the modulation of cellular protection in response to oxidative stress.

Cellular redox homeostasis is principally maintained by nuclear factor erythroid 2-related factor (Nrf2) (Itoh et al. 1997). Nrf2 is a Cap 'n' Collar basic leucine zipper transcription factor that is activated in response to oxidative stress (Kensler et al. 2007). Generally, Nrf2 translocates from the cytoplasm to the nucleus, where it interacts with anti-oxidant response element (ARE) within the promoter region of target genes. This up-regulates the transcription of a group of cytoprotective genes (Ma and He 2012). Nrf2/ARE activation is controlled by multiple regulators at different levels (Silva-Islas and Maldonado 2018). Glycogen synthase kinase-3β (GSK-3β) modulates Nrf2/ARE activation (Rada et al. 2012; Cuadrado 2015). The activation of GSK-3β results in Nrf2 degradation, and blocks the activation of Nrf2/ARE (Rada et al. 2012; Cuadrado 2015). Importantly, the GSK-3β/Nrf2 axis regulates oxidative stress during cerebral ischemia/reperfusion injury (Cai et al. 2017; Rana and Singh 2018). Moreover, multiple studies have reported that the GSK-3β/Nrf2 axis is regulated by various factors and mechanisms during cerebral ischemia/reperfusion injury (Liu et al. 2018; Park et al. 2018; Bao and Gao 2020). Thus, elucidating the molecular mechanism by which the GSK-3β/Nrf2 axis is activated has the potential to facilitate the development of promising approaches for preventing the oxidative stress-induced cerebral ischemia/reperfusion injury.

GRX1 is capable of protecting against oxidative stress and enhancing cell survival under various conditions of stress (Li et al. 2014; Liu et al. 2015, 2016). Emerging evidence revealed that GRX1 plays a crucial role in maintaining neuronal survival under different destructive stresses (Johnson et al. 2015). Interestingly, a recent study reported that GRX2, another member of GRX protein family, is also capable of preventing OGD/R-induced neuronal injury (Wen et al. 2020), indicating that the GRX protein family may have critical roles in cerebral ischemia/reperfusion injury. Moreover, GRX1 plays a key role in mediating myocardial ischemia/reperfusion injury (Burns et al. 2020). To date, whether GRX1 participates in the modulation of neuronal survival and oxidative stress during cerebral ischemia/reperfusion injury remains unknown. Thus, the objective of this study was to evaluate whether GRX1 protects neurons against oxygen-glucose deprivation/reoxygenation (OGD/R)-evoked injury in an *in vitro* model of cerebral ischemia/reperfusion injury. We demonstrated that GRX1 was induced by OGD/R treatment in neurons, and that a loss of GRX1 exacerbated OGD/R-induced apoptosis and ROS generation. Notably, the up-regulation of GRX1 protected against OGD/R-evoked neuronal injury. Further investigation revealed that GRX1 enhanced the nuclear expression of Nrf2 and enhanced the transcription of Nrf2/ARE in GOD/R-exposed neurons. Moreover, GRX1 promoted Nrf2/ARE activation via the modulation of GSK-3β phosphorylation. The suppression of Nrf2 partially reversed GRX1-mediated protective effects in OGD/R-exposed neurons. In summary, these findings indicate that GRX1 protected neurons from OGD/R-induced injury through the modulation of GSK-3β/Nrf2 signaling.

Materials and methods

Neuron culture

HT22 hippocampal neurons were provided by the BeNa Culture Collection (Kunshan, Jiangsu Province, China) and cultured using recommended methods. Briefly, HT22 neurons were plated on Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10 % fetal bovine serum and cultivated in 95 % air/5 % CO_2 at 37 °C. Cells at the second passage were utilized for the experiments.

Induction of OGD/R injury in HT22 neurons

HT22 neurons were washed with phosphate-buffered saline (PBS), plated in glucose-free medium and cultured under a hypoxic conditions (5 % CO_2/3 % O_2/92 % N_2 at 37 °C) for 8 h, which stimulated ischemic-like conditions *in vitro*. Then, media was replaced with fresh media supplemented with 4.5 g/l glucose, and neurons were cultured for 24 h under normoxic conditions (95 % air/5 % CO_2).

Real-time quantitative RCR (RT-qPCR)

HT22 neurons were collected and homogenized in TRIzol Reagent (Thermo Fisher Scientific) for total RNA extraction. The purified RNA was converted to cDNA using the EasyScript First-Strand cDNA Synthesis SuperMix (TransGen, Beijing, China). RT-qPCR was performed to quantify transcript levels using PerfectStartT Green qPCR SuperMix (TransGen). The recommended thermal cycle program was used, as follows: 94°C for 30 s and 45 cycles of 94°C for 5 s and 60°C for 30 s. RT-qPCR data were assessed using the $2^{-\Delta\Delta Ct}$ method, and gene expression was

determined using β-actin as a reference gene. The primer sequences were as follows: GRX1 sense: 5'-GCTCAGGA GTTTGTGAACTGC-3' and antisense: 5'-AGAAGACC TTGTTTGAAAGGCA-3'; β-actin sense: 5'-GGCTGTAT TCCCCTCCATCG-3' and anti-sense: 5'-CCAGTTGG TAACAATGCCATGT-3'.

Western blot

HT22 neurons were collected and homogenized in Western Lysis Buffer (Beyotime, Shanghai, China) containing a proteinase inhibitor cocktail and phenylmethanesulfonyl fluoride for protein extraction. After determination of protein concentration by the bicinchoninic acid (BCA) method, equivalent amounts of proteins were resolved via sodium dodecyl sulfate polyacrylamide gel electrophoresis. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes using an Electrophoretic Blotting System (Bio-Rad Laboratories, Shanghai, China). PVDF membranes were blocked with 5 % nonfat milk, and incubated with appropriate antibodies including rabbit polyclonal anti-GRX1 (1:500), rabbit polyclonal anti-β-actin (1:1000), rabbit polyclonal anti-Nrf2 (1:500), rabbit polyclonal anti-Histone H3 (1:1000), mouse monoclonal anti-phospho-GSK-3β (1:1000) (Sanying Biotech, Wuhan, China). Immunoreactive proteins were developed by ECL Western Blotting Substrate (Thermo Fisher Scientific) after incubation with goat anti-rabbit or goat anti-mouse secondary antibodies (Sanying Biotech). Protein expression was determined by measuring band intensities using Image-Pro Plus 6.0.

Cell transfection

The siRNA sequences targeting GRX1 were designed and synthesized by GenePharma (Shanghai, China). GRX1 coding sequences were subcloned into a pcDNA3.1 vector to construct a GRX1 expression vector. Lipofectamine 3000 Reagent (Thermo Fisher Scientific) was used to transfect siRNAs or vectors into HT22 neurons in accordance with the manufacturer's recommendations. In brief, cells were grown to 70 % confluence at the time of transfection. The vectors or siRNAs were mixed with Lipofectamine 3000 Reagent and then added to cells. Transfected cells were incubated for 48 h at 37 °C before subsequent detections.

Cell viability assay

Cell counting kit-8 (CCK-8) which can be converted into orange formazan by intracellular dehydrogenase was utilized to measure the viability of cells. HT22 neurons were seeded into a ninety-six-well plate (1×10^4) and cultivated overnight prior to transfection. After transfection and OGD/R exposure, 10 μl per well CCK-8 reagent was added to cells. After an incubation period of 2 h, cell viability was determined by measuring the absorbance of each solution at 450 nm with a Microplate Reader (Bio-Tek Instruments, Winooski, VA, USA).

Cell apoptosis assay

Apoptosis in HT22 neurons was assessed by flow cytometry using an Annexin V-FITC/PI Apoptosis Detection Kit (Beyotime, Shanghai, China) in accordance with the manufacturer's protocols. In brief, cells were digested by trypsin to obtain single cell suspension. A total of 5×10^4 cells were collected and suspended into 195 μl of Annexin V-FITC binding buffer. Then, 5 μl of Annexin V-FITC and 10 μl of PI reagents were added to the cells. Cells were incubated for 10 min in the dark. Afterwards, cells were detected by flow cytometry.

ROS assay

Intracellular ROS levels were quantitatively determined using a ROS-sensitive DCFH-DA probe that was capable of being converted to fluorescent DCF when oxidized. DCFH-DA (Beyotime) was diluted in serum-free medium to produce a final concentration of 10 μmol/l. HT22 neurons were harvested after they were treated appropriately, and were resuspended using DCFH-DA-containing media. Cells were cultivated for 20 min in an incubator at 37 °C. Thereafter, cells were washed with serum-free medium to completely remove DCFH-DA that had not entered cells. Fluorescence was quantitatively determined using flow cytometry.

Nrf2 transcriptional activity assay

Nrf2 transcriptional activity was evaluated using an Nrf2/ARE luciferase reporter vector. The Nrf2/ARE luciferase reporter vector (Beyotime, Shanghai, China), *Ranilla* luciferase reporter vector, and GRX1 siRNA or the GRX1 expression vector were co-transfected into HT22 neurons and cultivated for 48 h. After OGD/R exposure, cells were collected and analyzed using a Dual Luciferase Reporter Gene Assay Kit (Beyotime) to measure luciferase activities within cells.

Statistical analysis

Each individual experiment was repeated at least three times. The means and standard deviations of the data were obtained by using GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA). All of the data were expressed as means ± standard deviations. Differences were determined using the Student's *t* test or one-way analysis of variance (ANOVA) followed by Bonferroni's *post-hoc* test. Values of $p < 0.05$ were considered significant.

Results

GRX1 was an OGD/R-responsive factor in neurons

To evaluate whether GRX1 modulates OGD/R-induced neuronal injury, we assessed changes in the expression of GRX1 in response to OGD/R exposure in HT22 neurons. RT-qPCR findings indicated that OGD/R exposure induced GRX1 mRNA expression in HT22 neurons (Fig. 1a). Moreover, protein expression of GRX1 was also induced by OGD/R exposure (Fig. 1b and c). These data imply that GRX1 acts as an OGD/R-responsive factor in neurons.

Loss of GRX1 increased the sensitivity of neurons to OGD/R-induced injury

To elucidate the potential function of GRX1 in the regulation of OGD/R-induced injury, loss-of-function experiments involving GRX1 silencing were carried out in HT22 neurons using GRX1-specific siRNA expression. The transfection of GRX1 siRNA markedly depleted GRX1 expression in HT22 neurons in an OGD/R-exposure-independent manner (Fig. 2a–c). HT22 neuron viability, which was significantly impaired by OGD/R exposure, was further decreased by GRX1 knockdown (Fig. 2d). The OGD/R-induced apoptosis of neurons was markedly exacerbated by GRX1 knockdown (Fig. 2e and f). In addition, the loss of GRX1 also enhanced OGD/R-mediated ROS generation (Fig. 2g and h). Therefore, these data reveal that the loss of GRX1 enhances the sensitivity of neurons to OGD/R-induced injury.

GRX1 overexpression protects against OGD/R-induced neuronal injury

To verify whether GRX1 has neuroprotective effects on OGD/R-exposed neurons, gain-of-function experiments involving GRX1 overexpression in HT22 neurons were performed. Transfection HT22 neurons with the GRX1 expression vector markedly up-regulated the expression of GRX1 in an OGD/R exposure-independent manner (Fig. 3a and b). Interestingly, the up-regulation of GRX1 restored the viability of OGD/R-exposed neurons (Fig. 3c). Notably, GRX1 up-regulation markedly alleviated OGD/R-induced apoptosis (Fig. 3d and e) and ROS generation (Fig. 3f and g) in HT22 neurons. Collectively, these results reveal that GRX1 has neuroprotective effects on OGD/R-exposed neurons.

GRX1 overexpression enhanced the activation of Nrf2 antioxidant signaling

To reveal the molecular mechanism responsible for GRX1-mediated neuroprotection, we explored the effect of GRX1 expression on Nrf2 antioxidant signaling. We found that GRX1 knockdown markedly decreased OGD/R-mediated nuclear translocation of Nrf2 in HT22 neurons (Fig. 4a and b). Moreover, GRX1 knockdown down-regulated the transcriptional activity of Nrf2/ARE (Fig. 4c). In contrast, GRX1 overexpression markedly up-regulated the OGD/R-induced nuclear translocation of Nrf2 (Fig. 4d and e) and increased transcriptional activity of Nrf2/ARE (Fig. 4f). These results indicate that GRX1 modulates Nrf2 antioxidant signaling in OGD/R exposed neurons.

GRX1 regulates Nrf2 signaling associated with modulation of GSK-3β phosphorylation

To further explore the molecular basis of GRX1-mediated modulation of Nrf2 signaling, we assessed the effect of activating GSK-3β, a vital regulator of Nrf2. The results showed that GRX1 knockdown markedly decreased GSK-3β phosphorylation, which indicated that GRX1 knockdown enhanced GSK-3β activation (Fig. 5a and b). To confirm whether GSK-3β contributes to the regulation of GRX1 knockdown-induced suppression of Nrf2 signaling, we detected the effect of GSK-3β inhibition on GRX1 knockdown-mediated effects. We utilized SB216763, a chemical inhibitor of GSK-3β (Coghlan et al. 2000), to decrease the activity of GSK-3β. Notably, the inhibition of GSK-3β by SB216763 markedly abolished GRX1 knockdown-induced suppressive effects on Nrf2/ARE activation (Fig. 5c). Furthermore, GSK-3β inhibition reversed the GRX1 knockdown-mediated exacerbation of OGD/R-induced neuronal apoptosis (Fig. 5d and

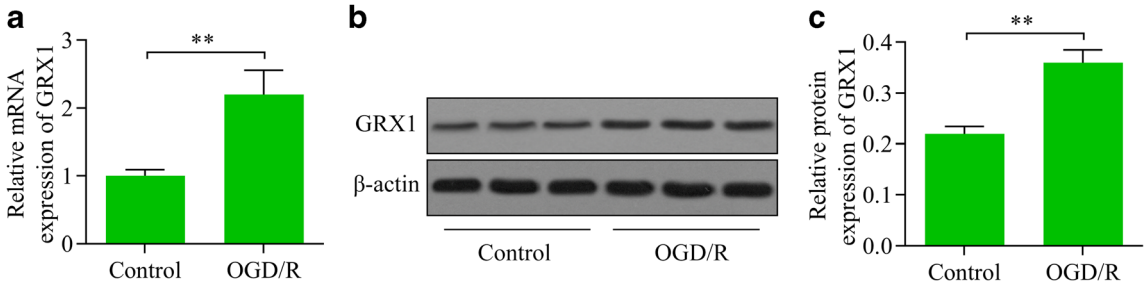

Fig. 1 Effect of OGD/R exposure on GRX1 expression. **a** Relative mRNA expression of GRX1 was examined using RT-qPCR (n = 3). **b** Protein expression of GRX1 was determined via Western blot, and **c** the quantification data (n = 3). Data were expressed as means ± standard deviation. Differences were assessed by the Student's t test. **p < 0.01

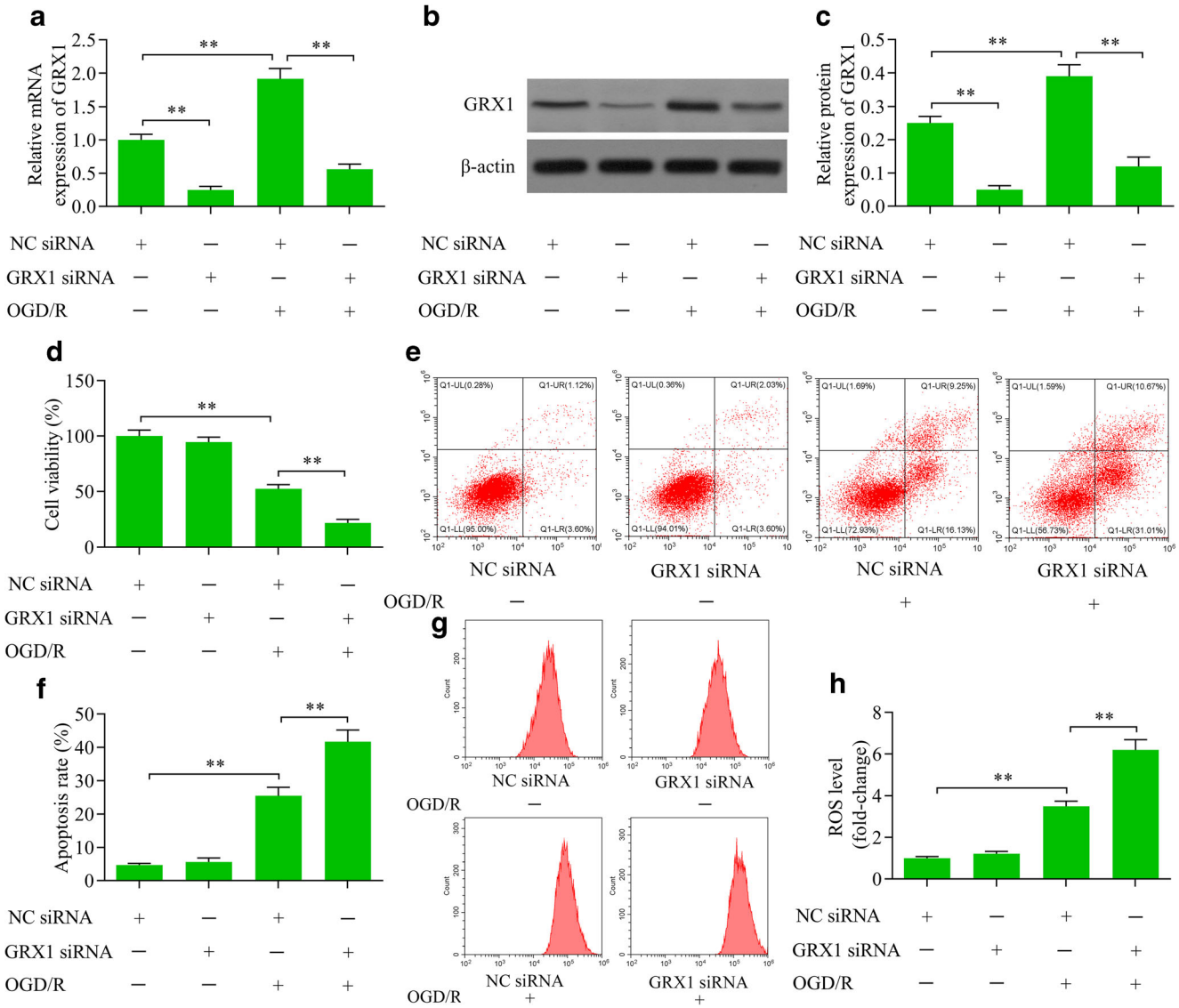

Fig. 2 GRX1 knockdown exacerbated OGD/R-induced neuronal injury. HT22 neurons were transfected with either negative control (NC) siRNA or GRX1 siRNA for 48 h and subjected to OGD/R exposure. **a** Relative mRNA expression of GRX1 was examined via RT-qPCR (n = 3). **b** Protein expression of GRX1 was determined via Western blot, and **c** the quantification data (n = 3). (D) Cell viability was assessed using a CCK-8 assay (n = 5). **e**, **f** Apoptosis was evaluated using an Annexin V-FITC/PI apoptosis assay (n = 3). Data in the Annexin V-FITC+/PI- and Annexin V-FITC+/PI + were quantified. **g**, **h** ROS generation was monitored using a ROS detection assay (n = 3). Data were expressed as means ± standard deviation. Differences were assessed by ANOVA followed by Bonferroni's post-hoc test. **p < 0.01

e) and ROS generation (Fig. 5f and g). Taken together, these data suggest that GRX1 regulates Nrf2 activation via the modulation of GSK-3β.

Suppression of Nrf2 reversed GRX1-mediated neuroprotective effect

To verify whether Nrf2 modulates GRX1-mediated neuroprotective effects, we investigated whether Nrf2 inhibition altered GRX1 overexpression-mediated effects on OGD/R-exposed neurons. We utilized ML385, a chemical inhibitor of Nrf2 (Singh et al. 2016), to suppress the activity of Nrf2. The inhibition Nrf2 by ML385 markedly decreased Nrf2/ARE-mediated transcriptional activity and blocked GRX1 overexpression-mediated Nrf2 activation (Fig. 6a). Notably, the suppressive effect of GRX1 overexpression on OGD/R-induced neuronal apoptosis and ROS generation was markedly reversed by Nrf2 inhibition (Fig. 6b and c). Collectively, these results confirm that GRX1 exerts neuroprotective effects against OGD/R-induced injury by enhancing Nrf2 activation.

Discussion

In the present research, we report that GRX1 acts as a pro-survival protein in neurons. Our data showed that GRX1

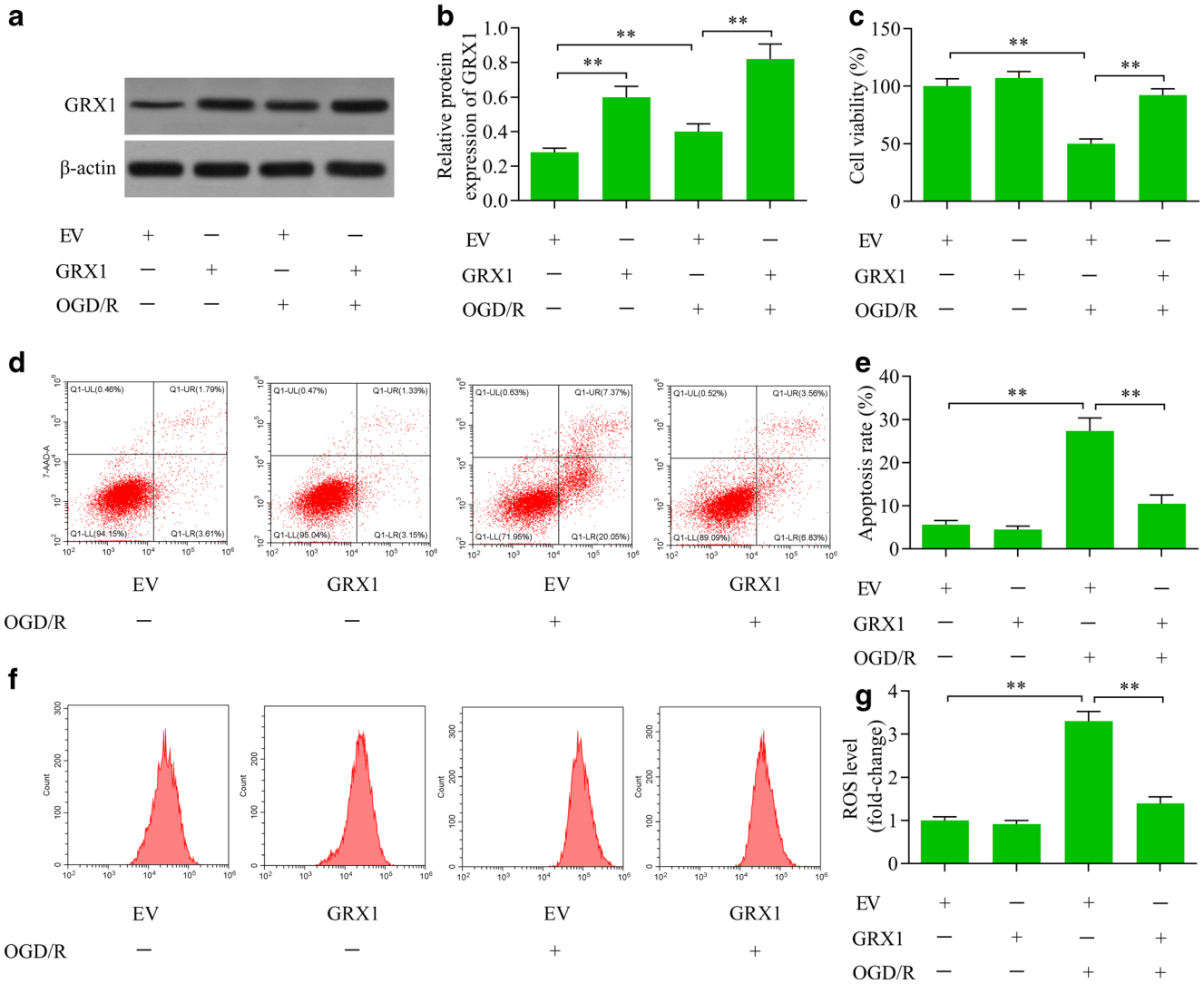

Fig. 3 GRX1 overexpression ameliorated OGD/R-induced neuronal injury. HT22 neurons were transfected with either an empty vector (EV) or GRX1 expression vector for 48 h and subjected to OGD/R exposure. **a** Protein expression of GRX1 was determined via Western blot, **b** the quantification data (n = 3). **c** Cell viability was determined using a CCK-8 assay (n = 5). **d**, **e** Apoptosis was assessed using an Annexin V-FITC/PI apoptosis assay (n = 3). Data in the Annexin V-FITC$^+$/PI$^-$ and Annexin V-FITC$^+$/PI$^+$ were quantified. **f**, **g** ROS levels were measured using a ROS detection assay (n = 3). Data were expressed as means ± standard deviation. Differences were assessed by ANOVA followed by Bonferroni's post-hoc test. **p < 0.01

facilitated neuronal survival under conditions of OGD/R exposure *in vitro*. The protective effect of GRX1 was associated with the activation of Nrf2 antioxidant signaling. We demonstrated that GRX1 inactivated GSK-3β by increasing GSK-3β phosphorylation, which enhanced the nuclear translocation of Nrf2 and Nrf2/ARE activation. These processes exert anti-apoptotic and anti-oxidant effects on OGD/R-exposed neurons (Fig. 6d). Collectively, these findings indicate that GRX1/GSK-3β/Nrf2 signaling plays a key role in regulating oxidative stress in neurons, which may modulate cerebral ischemia/reperfusion injury *in vivo*.

GRX1 has previously been reported to have a key role in the regulation of cell survival. The up-regulation of GRX1 protected cardiomyocytes from nitric oxide-induced apoptosis (Inadomi et al. 2012). Moreover, GRX1 protects cardiomyocytes and coronary artery endothelial cells against high glucose level-induced damage (Li et al. 2014; Qi et al. 2016). Notably, GRX1 participates in the modulation of ischemia/reperfusion injury (Godoy et al. 2011). The up-regulation of GRX1 ameliorates adverse ventricular remodeling induced by myocardial ischemia/reperfusion injury (Bubb et al. 2017). Furthermore, GRX1 overexpression attenuates OGD-induced injury in kidney epithelial cells *in vitro*, indicating the protective role of GRX1 in kidney ischemia (Yin et al. 2019). Interestingly, in this study, we determined that the loss of GRX1 enhanced OGD/R-induced apoptosis in neurons, while GRX1 overexpression protected neurons from OGD/R-induced apoptosis. In accordance with previous findings, our study confirms a cytoprotective role for GRX1, and suggests that GRX1 may participate in the regulation of

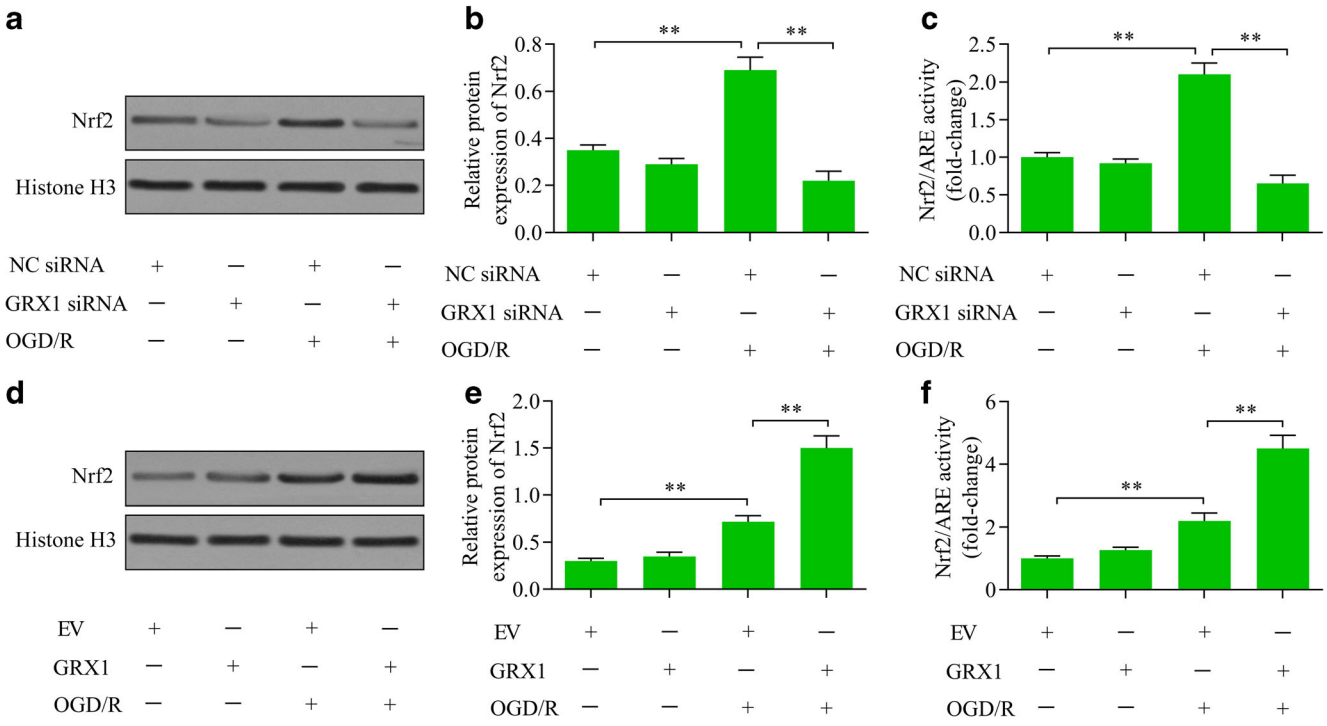

Fig. 4 GRX1 is involved in the modulation of Nrf2 antioxidant signaling. **a** The effect of GRX1 knockdown on Nrf2 nuclear expression was assessed via Western blot, and **(b)** the quantification data (n = 3). **c** The effect of GRX1 knockdown on Nrf2/ARE transcriptional activity was assessed using a luciferase reporter assay (n = 5). **d** The effect of GRX1 overexpression on Nrf2 nuclear expression was examined via Western blot, and (**e**) the quantification data (n = 3). **f** The effect of GRX1 overexpression on Nrf2/ARE transcriptional activity was assessed using a luciferase reporter assay (n = 5). Data were expressed as means ± standard deviation. Differences were assessed by ANOVA followed by Bonferroni's post-hoc test. **p < 0.01

cerebral ischemia/reperfusion injury. Interestingly, a recent study reported that GRX2, another member of the GRX protein family, is also capable of preventing OGD/R-induced apoptosis in neurons (Wen et al. 2020), indicating that the GRX protein family may have critical roles in cerebral ischemia/reperfusion injury. GRX1 and GRX2 have a significant Cys-X-X-Cys active site motif and these two cysteine residues act as redox sensors and allow for a monothiol mechanism using the N-terminal cysteine to reduce the reduced sulfhydryl groups of the cysteines of proteins (Holmgren 1979; Lundberg et al. 2001). However, these two protein have different cellular localizations (Holmgren 1979; Lundberg et al. 2001). These two proteins have shown similar effects on OGD/R injury of neurons. However, whether these proteins functions interactively in mediating OGD/R injury is unknown. Therefore, further studies should be performed to determine their specific different roles in mediating OGD/R injury of neurons.

Emerging evidence has revealed that GRX1 has a notable neuroprotective function (Johnson et al. 2015). It has been reported that GRX1 mRNA and protein expression are both up-regulated in mouse brain cells in response to chemical insult (Kenchappa and Ravindranath 2003). The treatment of neurons with 6-hydroxydopamine significantly enhanced GRX1 expression, and GRX1 knockdown exacerbated 6-hydroxydopamine-induced cytotoxic effects (Arodin et al. 2014). The up-regulation of GRX1 protected neurons from MPTP- or copper-induced apoptosis (Kenchappa et al. 2004; Cater et al. 2014). These findings indicate that GRX1 is induced in response to stress in neurons. Consistent with these findings, our results demonstrated that GRX1 was induced by OGD/R exposure in HT22 neurons and GRX1 overexpression protected against OGD/R-induced neuronal injury. Therefore, our study confirms that GRX1 is essential for neuronal survival and may participate in the modulation of ischemia/reperfusion-induced neuronal injury.

GRX1 confers resistance to oxidative stress. The up-regulation of GRX1 attenuates oxidative stress injury induced by hydrogen peroxide in retinal pigment epithelial cells (Liu et al. 2015, 2016). The steady laminar flow-induction of GRX1 protected endothelial cells from oxidative stress-induced apoptosis (Li et al. 2017). Moreover, GRX1 overexpression alleviated oxidative stress and apoptosis in osteoarthritic chondrocytes (Sun et al. 2017). Therefore, GRX1 functions as a potent antioxidant protein. Considering that OGD/R-evoked oxidative stress contributes to neuronal injury, we evaluated whether GRX1 was involved in regulating OGD/R-mediated oxidative stress in neurons. Herein, we found that the loss of GRX1 enhanced OGD/R-induced ROS production, while GRX1 overexpression suppressed ROS production in

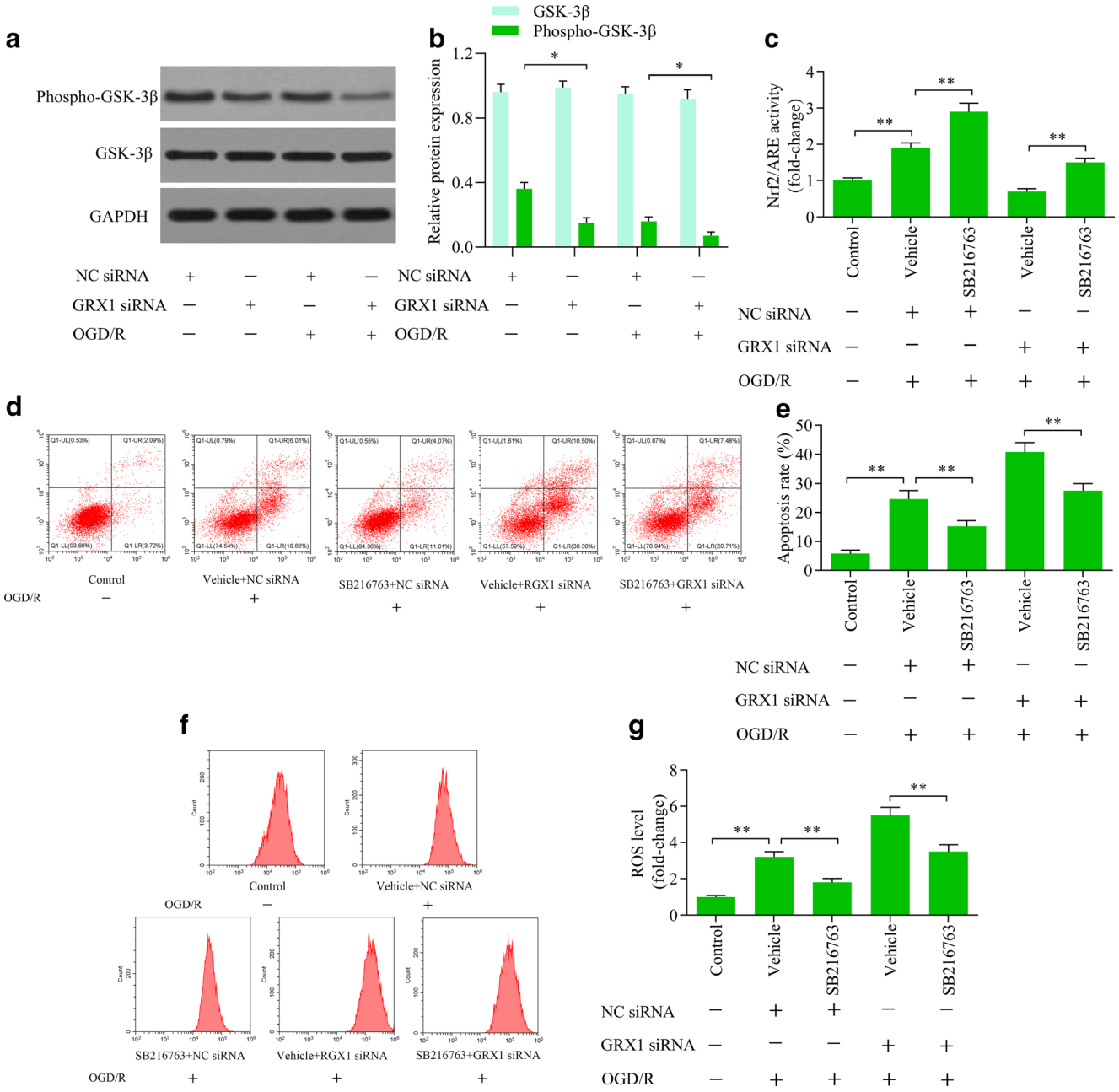

Fig. 5 GSK-3β inhibition reversed GRX1 knockdown-mediated modulation of Nrf2 signaling. **a** The effect of GRX1 knockdown on GSK-3β phosphorylation was determined via Western blot, and (**b**) the quantification data (n = 3). HT22 neurons were transfected with GRX1 siRNA and incubated for 48 h with or without SB216763 (3 μM), a GSK-3β inhibitor, prior to OGD/R exposure (n = 3). **c** The effect of GSK-3β inhibition on Nrf2/ARE transcriptional activity was monitored using a luciferase reporter assay (n = 5). **d**, **e** The effect of GSK-3β inhibition on neuronal apoptosis was assessed using an Annexin V-FITC/PI apoptosis assay (n = 3). Data in the Annexin V-FITC$^+$/PI$^-$ and Annexin V-FITC$^+$/PI$^+$ were quantified. **f**, **g** The effect of GSK-3β inhibition on intracellular ROS generation was evaluated using a ROS detection assay (n = 3). Data were expressed as means ± standard deviation. Differences were assessed by ANOVA followed by Bonferroni's post-hoc test. **p < 0.01

OGD/R-exposed neurons. Our findings suggest that GRX1 also participates in the regulation of OGD/R-associated oxidative stress in neurons and may serve as a promising target for neuroprotective therapy.

It has been reported that GRX1 regulates cell survival by affecting multiple signaling pathways (Li et al. 2017; Sun et al. 2017). It has been reported that GRX1 prevents the myocardial ischemia/reperfusion-induced reduction of cardioprotective proteins including Akt and HO-1(Lekli et al. 2010), which are key mediators in Nrf2 signaling. In this study, we identified GRX1 as a novel regulator of Nrf2 antioxidant signaling. Our data showed that GRX1 promoted Nrf2 activation through the inactivation of GSK-3β. The phosphorylation of GSK-3β by Akt inactivates GSK-3β

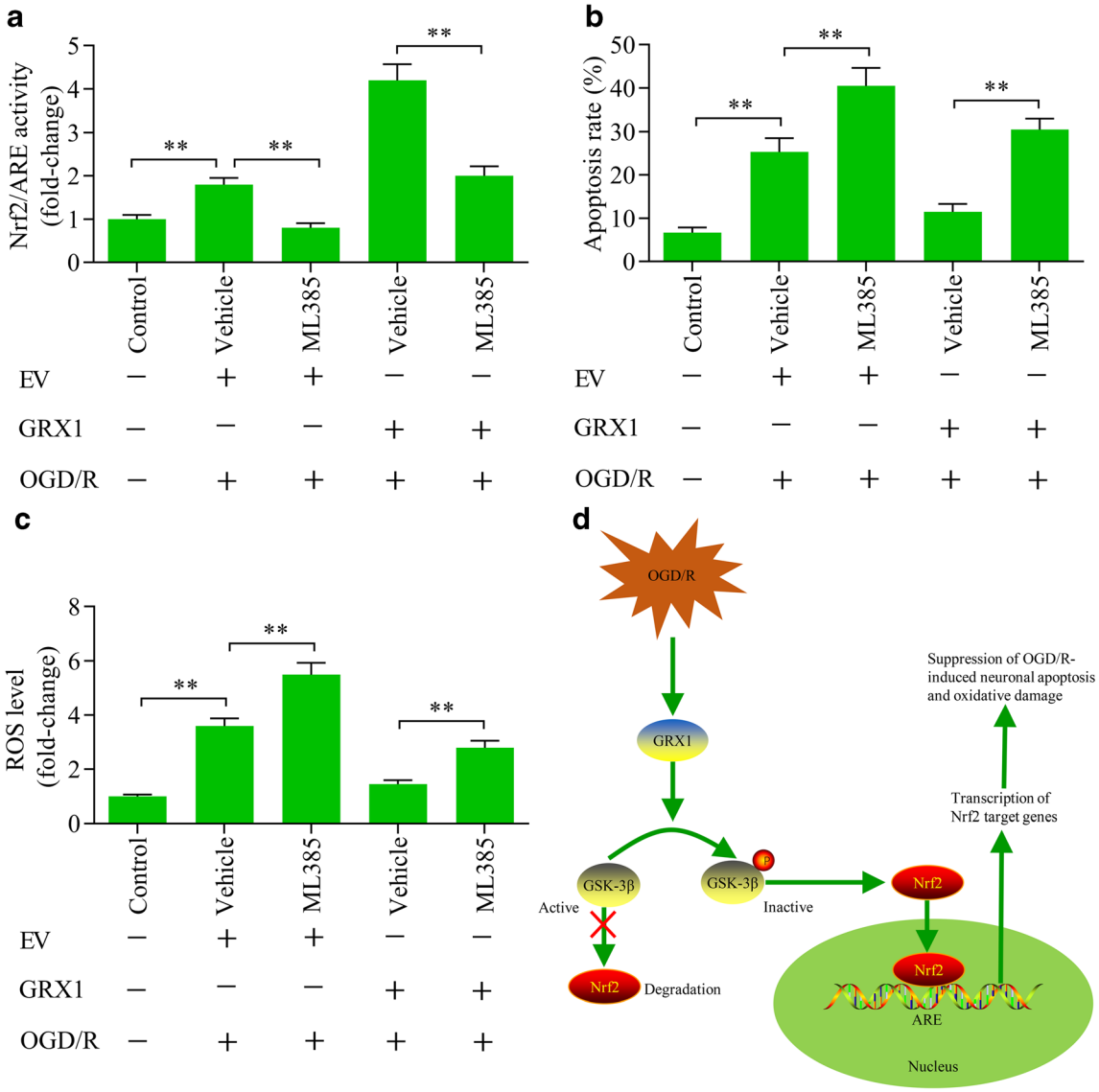

Fig. 6 GRX1 protects against OGD/R-induced neuronal injury via Nrf2. HT22 neurons were transfected with GRX1 expression vectors and incubated for 48 h either with or without ML385 (5 μM), an Nrf2 inhibitor, before OGD/R exposure. **a** Nrf2/ARE transcriptional activity was monitored using a luciferase reporter assay (n = 5). **b** Neuronal apoptosis was assessed using an Annexin V-FITC/PI apoptosis assay (n = 3). Data in the Annexin V-FITC$^+$/PI$^-$ and Annexin V-FITC$^+$/PI$^+$ were quantified. **c** Intracellular ROS levels was monitored using ROS detection assay (n = 3). Data were expressed as means ± standard deviation. Differences were assessed by ANOVA followed by Bonferroni's post-hoc test. **p < 0.01. **d** A graphical model of GRX1-mediated regulation of Nrf2 activation and its role in the regulation of OGD/R-induced neuronal injury

(Golpich et al. 2015; Lee et al. 2020). Interestingly, GRX1 has been reported to be a critical regulator of Akt. GRX1 can prevent the oxidative modification of Akt, which leads to increased Akt phosphorylation (Ahmad et al. 2014; Liu et al. 2015). However, whether GRX1 regulates GSK-3β/Nrf2 axis via Akt requires further investigation. Nevertheless, our work showed that GRX1 may contribute to Nrf2 activation through the inactivation of GSK-3β.

Certain limitations of this work should be noted. The effects of GRX1 were mainly investigated *in vitro* using a cellular model. However, the precise effects of GRX1 *in vivo* are unknown. Therefore, further investigation using animal models of cerebral ischemia/reperfusion injury is required. It would be better to see the *in vivo* effects of GRX1 overexpression by virus vector mediated gene transfer on cerebral ischemia/reperfusion-induced infarct size, neurological dysfunction, neuronal apoptosis and oxidative stress in rodent models. Only through these studies can the neuroprotective effect of GRX1 in cerebral ischemia/reperfusion injury be further confirmed.

In conclusion, our data demonstrate that GRX1 is induced by OGD/R exposure in neurons and that the up-regulation of GRX1 alleviates OGD/R-induced neuronal apoptosis and oxidative stress by enhancing the activation of Nrf2/ARE.

Moreover, we revealed that GRX1 contributes to Nrf2/ARE activation via the inactivation of GSK-3β. Thus, our study suggests that GRX1 is a novel regulator of the GSK-3β/Nrf2 axis and suggests that the GRX1/GSK-3β/Nrf2 axis may represent a novel mechanism modulating the oxidative stress response induced by cerebral ischemia/reperfusion injury.

Author contributions Zhengguo Qiu designed the work, performed the experiment, and drafted the article. Xu Li performed the experiment. Chongzhen Duan collected the data. Rui Li collected the data. Lifeng Ha designed the work and reviewed the article. The authors read and approved the final manuscript.

Data availability The data and material used to support the findings of this study are available from the corresponding author upon request.

Code availability Not applicable.

Declarations

Conflicts of interest The authors declare no conflict of interest.

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication All authors have approved for the publication of this manuscript.

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