Life Sciences 223 (2019) 88–94

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

The protective effect of glutaredoxin 1/DJ-1/HSP70 signaling in renal tubular epithelial cells injury induced by ischemia

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ARTICLE INFO

Keywords:
GRX1
DJ-1
HSP70
OGD
HK-2

ABSTRACT

Aims: Gluaredoxin1 (GRX1) is an important protein of the cellular antioxidant defense system, but its role in renal epithelial cell injury caused by ischemia remains unclear. In this study, we aimed to gain insight into the role of GRX1 in HK-2 cells with oxygen glucose deprivation (OGD) injury, which served as an in vitro cell model of renal epithelial cell ischemic injury. We investigated the underlying regulation of GRX1, DJ-1, and HSP70 as well as the role of the GRX1/DJ-1/HSP70 signaling pathway in this model.

Materials and methods: The protein and mRNA expressions were measured by Western blot and qRT-PCR assays, respectively. GRX1 was overexpressed by transfection of pcDNA.3.1-GRX1 and DJ-1 was inhibited by transfection with DJ-1 siRNA. Cell apoptosis, caspase-3 activity, lactate dehydrogenase (LDH) leakage, or superoxide dismutase (SOD) content was tested by the related detection kit. Reactive oxygen species (ROS) level was detected via carboxy-H_2DCF-DA.

Key findings: We found that GRX1 was distinctly down-regulated in HK-2 cells after incubation under the OGD condition. GRX1 overexpression markedly constrained cell apoptosis, caspase-3 activity, LDH leakage, and the ROS level, while SOD content was elevated. GRX1 up-regulation increased DJ-1 and HSP70 protein expression, while DJ-1 inhibition significantly offset the effect of GRX1 overexpression on HSP70, indicating that GRX1 could regulate HSP70 via control of DJ-1. Moreover, we observed that HSP70 inhibition removed the constraints imposed by GRX1 overexpression on ROS level, LDH leakage, and caspase-3 activity.

Significance: Overall, this study showed that GRX1 minimizes cell injury and apoptosis in HK-2 cells under OGD conditions via regulation of DJ-1 and HSP70 expression.

1. Introduction

The kidney is prone to ischemic injury, which can be caused by numerous factors, including severe trauma, extensive burns, major surgery, massive blood loss, obstetric hemorrhage, severe infection, sepsis, and dehydration [11,28,35]. The renal tubular epithelial cells on the outer medulla are sensitive to ischemia, which can cause renal tubular injury [32]. Ischemia always leads to oxidative stress, which is a toxic process caused by the accumulation of reactive oxygen species (ROS) [38]. Superoxide dismutase (SOD) rapidly removes oxygen free radicals and prevents their accumulation under physiological conditions [27]. We investigated the underlying molecular mechanisms of oxygen glucose deprivation (OGD) in human proximal tubular cells from the HK-2 cell line to develop a theoretical basis for the prevention and treatment of renal ischemia.

Gluaredoxin1 (GRX1), a common thiol/disulfide oxidoreductase in many organisms, is an important protein of the cellular antioxidant defense system [10]. It catalyzes redox reactions between glutathione and protein disulfide bonds and participates in various cell functions [36], including antioxidation, anti-apoptosis, protein folding, cell differentiation, and regulation of the activity of transcription factors [24,34]. GRX1 has been reported to prevent cell apoptosis and injury caused by oxidative stress in retinal pigment epithelial cells, chondrocytes, and coronary arteries endothelial cells [19,22,34], and it may play a key role in ROS scavenging in oral carcinogenesis [6]. Moreover, Grx1 has been demonstrated to ameliorate cell apoptosis in cardiomyocytes, embryonic fibroblasts, and articular chondrocytes [14,30]. Currently, the role of GRX1 in renal tubular epithelial cell injury caused by OGD remains unclear.

DJ-1, a member of the peptidase protein C56 family encoded by the

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https://doi.org/10.1016/j.lfs.2019.03.015
Received 14 February 2019; Accepted 7 March 2019
Available online 08 March 2019
0024-3205/

PARK7 gene, has an effective antioxidative stress function and could be regulated by GRX1 in Parkinson's disease [16]. DJ-1 has a variety of biological functions, such as inhibition of cellular oxidative stress and regulation of mitochondrial function, autophagy, and apoptosis [8,26]. DJ-1 has been shown to reduce cell death induced by oxidative stress in neurons, retinal pigment epithelium, and HepG2 cells [13,15,33]. In addition, DJ-1 inhibition has been reported to elevate ROS production in mouse renal proximal tubule cells [7]. Therefore, we hypothesized that GRX1 could regulate the expression of DJ-1 in HK-2 and control OGD-induced cell apoptosis.

DJ-1 has also been found to positively regulate HSP70 expression in neurons [4]. HSP70 is a molecular chaperone, and it has been shown to increase the synthesis and release of endogenous antioxidants [25]. HSP70 also has an anti-apoptotic function in various cells, such as cardiomyocytes, airway epithelial cells, and neurons [1,12,31], and it was reported to alleviate renal ischemia/reperfusion injury [29].

We hypothesized that GRX1 could control renal injury induced by OGD by regulating DJ-1 and HSP70. In this study, we focused on identifying the role and molecular mechanisms of GX1 in HK-2 cells with OGD injury. We investigated GRX1 expression in these cells as well as the effect of GRX1 overexpression on ROS level, SOD, lactate dehydrogenase (LDH) leakage, and cell apoptosis. We found that GRX1 controlled the effects of OGD injury through regulation of DJ-1 and HSP70 in HK-2 cells.

2. Materials and methods

2.1. Cell culture

The human kidney epithelial cell line HK-2 (ATCC, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 culture medium (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco) at 37 °C in an atmosphere of 95% air and 5% CO_2.

2.2. ODG injury

The normal or transfected HK-2 cells with OGD injury were incubated in Earle's balanced salt solution under the condition of 95% N_2 and 5% CO_2. Cells without OGD were cultured with Earle's balanced salt solution supplemented with glucose in a non-anoxic environment of 95% air and 5% CO_2 at 37 °C for 9 h. Thereafter, all cells were incubated in serum-free DMEM/F12 medium with 95% air and 5% CO_2 at 37 °C for 15 h.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total mRNA in cells was extracted with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and then reverse-transcribed using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA). qRT-PCR was performed in triplicate utilizing the CFX Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and Absolute Blue QPCR SYBR Green Mix (Applied Biosystems, Foster City, CA, USA). The procedure was 94 °C for 30 s; 40 cycles of 93 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. The housekeeping gene *GAPDH* was used as an internal reference. The primer pairs for *GRX1* were 5′-TTT TCA TCA AGC CCA CC-3′ (sense) and 5′-CCA CCT ATA CAC TCT TTA CCG-3′ (anti-sense), and for *GAPDH*, 5′-CGT CTT CAC CAC CAT GGA GA-3′ (sense) and 5′-CGG CCA TCA CGC CAC AGT TT-3′ (anti-sense). The relative levels of gene expression were measured using the $2^{-\Delta\Delta Ct}$ method.

2.4. Western blot analysis

Proteins were extracted from cells, followed by measurement of protein concentration for each sample with an assay dye reagent (Bio-Rad, Hercules, CA, USA). A total of 20 μg of protein from each sample was loaded and separated by SDS-PAGE electrophoresis, and the separated proteins were then transferred onto a nitrocellulose membrane (Amersham, Little Chalfont, UK). After incubation with 2% non-fat dry milk in Tris-buffered saline (TBS) at room temperature for 1 h, the membrane was incubated with primary antibodies, anti-GRX1 (1:600, Abcam Inc., Cambridge, MA, USA), GAPDH (1:900, Abcam Inc), DJ-1 (1:500, Abcam Inc), and HSP70 (1:600, Abcam Inc), overnight at 4 °C. Subsequently, the membrane was incubated in horseradish peroxidase-conjugated secondary antibodies (CST, Danvers, MA, USA) (1:1000) at room temperature for 1 h. Finally, the blot was quantified using a Bio-Rad ChemiDoc apparatus. GAPDH was the reference protein.

2.5. Recombinant plasmid construction and cell transfection

The full-length cDNA of human *GRX1* (NCBI accession no. W79548) was synthesized by reverse transcription PCR and digested with *Eco*RI and *Bam*HI enzymes (New England Biolabs, Beverly, MA). Next, the *GRX1* fragment was ligated into pcDNA.3.1 vector (Novagen, Madison, WI, USA) after the digestion of pcDNA.3.1 with *Eco*RI and *Bam*HI enzymes. The recombinant plasmids were transformed into DH5α competent cells (Takara Biotechnology, Dalian, China) and amplified overnight at 37 °C. The recombinant vectors were extracted from DH5α by using TaKaRa MiniBEST Plasmid Purification Kit Ver.4.0 (Takara Biotechnology) and sequenced. The correct plasmids were named pcDNA.3.1-GRX1.

Cells (3×10^4 cells/well) were plated in a 24-well culture plate in an incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C with 5% CO_2. The transfection was conducted when the cells reached 70% confluence according to the specification. Briefly, pcDNA.3.1-GRX1 (1 μg), pcDNA.3.1 (1 μg), DJ-1 siRNA (5′-AGG CGC GGC TGC AGT CTT TAA-3′, 1 μg), HSP70 siRNA (5′-CGACGGAGACAAGCCC AAG-3′, 1 μg), or nonspecific siRNA (1 μg) was diluted in 100 μL of FBS-free DMEM/F12 medium with 3 μL of TurboFect (Thermo Fisher Scientific) per well followed by mixing. The mixture was then added to the wells and incubated for 24 h. The transfection efficiencies were measured by qRT-PCR and Western blot.

2.6. Annexin V-FITC/PI apoptosis assay and caspase-3 activity test

Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) apoptosis detection kit (Beckman Coulter, Brea, CA, USA) was used to test cell apoptosis. The procedure was done in accordance with the manufacturer's instructions. Cells were precooled in cold phosphate-buffered saline and then resuspended in binding buffer. Annexin V stock solution (10 μL) was added, and the mixture was incubated at 4 °C for 30 min. Subsequently, PI (10 μL) was added, followed by incubation for 8 min. Finally, cells were characterized with a FACS analyzer (BD Biosciences, San Jose, CA, USA).

Caspase-3 activity detection was conducted according to the specifications of the Caspase-3 activity assay kit (Beyotime, Nantong, China). Cells were homogenized in 100 mL of reaction buffer (1% NP-40, 20 mM Tris-HCl [pH 7.5], 137 mM Nad, and 10% glycerol) containing 10 mL of caspase-3 substrate and incubated at 37 °C for 2 h. Results were measured by an ELISA reader at 405 nm (Thermo Fisher Scientific).

2.7. LDH leakage detection

Membrane leakage of LDH in culture medium supernatant was determined using an LDH cytotoxicity detection kit (Beyotime). Briefly, the transfected cells were incubated with chrysophanol for 24 h, and the LDH in the culture medium was detected according to standard instructions.

2.8. ROS and SOD detection

The ROS level was tested following the incubation of cells with carboxy-H_2DCF-DA (Sigma, St. Louis, MO, USA) for 30 min at 37 °C in the dark. Fluorescence-positive cells were detected by a Multi-Detection microplate reader (Biotek, Winooski, VT, USA) at the excitation and emission filters of 488 and 530 nm. An SOD assay kit (Beyotime) was used to determine SOD release in the culture medium based on the supplier's instructions. The absorbance was analyzed by an ELISA reader (Molecular Devices, Sunnyvale, CA, USA) at 500 nm.

2.9. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Statistical analyses were processed with SPSS version 22.0 software (SPSS Inc., Chicago, IL, USA) with one-way analysis of variance followed by LSD and Bonferroni test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. GRX1 is down-regulated in HK-2 cells with OGD injury

We assessed the expression of GRX1 mRNA and protein in HK-2 cells with OGD injury and found that the mRNA (Fig. 1A) and protein (Fig. 1B) were both significantly reduced.

3.2. GRX1 overexpression inhibits oxidative stress and LDH leakage in HK-2 cells under OGD conditions

To explore the role of GRX1 in HK-2 cells with OGD injury, we first overexpressed GRX1 by transfecting pcDNA.3.1-GRX1 into normal HK-2 cells. The data demonstrate that both GRX1 mRNA (Fig. 2A) and protein (Fig. 2B) levels were increased 2-fold after pcDNA.3.1-GRX1 transfection, indicating that it was successful. Thereafter, we cultured the transfected cells under OGD conditions and tested the ROS level, SOD content, and LDH leakage. Results showed that the ROS level (Fig. 2C) and LDH leakage (Fig. 2D) were obviously reduced when GRX1 was up-regulated, while the SOD content (Fig. 2E) increased.

3.3. GRX1 overexpression suppresses OGD-induced apoptosis

In order to understand the function of GRX1 overexpression in HK-2 cells with OGD-induced apoptosis, we measured cell apoptosis with an Annexin V-FITC/PI apoptosis assay and a caspase-3 activity test in transfected HK-2 cells with OGD injury. In the Annexin V-FITC/PI and caspase-3 activity assays, cell apoptosis (Fig. 3A) and caspase-3 activity (Fig. 3B) were both markedly decreased after GRX1 overexpression.

3.4. GRX1 regulates HSP70 via controlling DJ-1 expression

To gain insight into the molecular mechanism of GRX1 in HK-2 cells under OGD conditions, we measured the expression of DJ-1 and HSP70 proteins in HK-2 cells with GRX1 overexpression. Expression (Fig. 4A) of DJ-1 and HSP70 proteins was doubled and tripled, respectively. In addition, we performed a loss-of-function experiment for DJ-1 in HK-2 cells with GRX1 overexpression and found that expression of HSP70 and DJ-1 proteins was markedly inhibited (Fig. 4B). Thus, the decrease of DJ-1 appeared to significantly offset the promotion of HSP70 protein expression associated with GRX1 overexpression in HK-2 cells. We then evaluated the ROS level (Fig. 4C), caspase-3 activity (Fig. 4D), and LDH leakage (Fig. 4E) after DJ-1 inhibition in HK-2 cells with GRX1 overexpression under OGD conditions, and we found that they were all clearly elevated.

3.5. GRX1 protects HK-2 cells from OGD injury via regulation of HSP70

We found that GRX1 affects HSP70 expression via control of DJ-1, and we hypothesized that GRX1 protects HK-2 cells from OGD injury via regulation of HSP70. Thus, we suppressed HSP70 by transfecting HSP70 siRNA into HK-2 cells with GRX1 overexpression. Protein expression (Fig. 5A) of HSP70 was obviously reduced following the HSP70 siRNA transfection, indicating that it was successful. In addition, the ROS level (Fig. 5B), caspase-3 activity (Fig. 5C), and LDH leakage (Fig. 5D) were increased after HSP70 suppression in HK-2 cells with GRX1 up-regulation and OGD injury.

4. Discussion

Renal ischemia is a complication of renal transplant, cardiac surgery, acute kidney injury, partial nephrectomy, and other types of trauma [2]. Studies have confirmed that renal tubular epithelial cells tend to have structural and functional damage as a result of ischemia. In our study, we established a cell ischemia model by culturing HK-2 cells under OGD conditions and then explored the role of GRX1/DJ-1/HSP70 signaling in this model. The GRX system helps protect cells against oxidative stress injury. Masaldan et al. found that increased GRX1 protects senescent cells against the effects of oxidative stress [23].

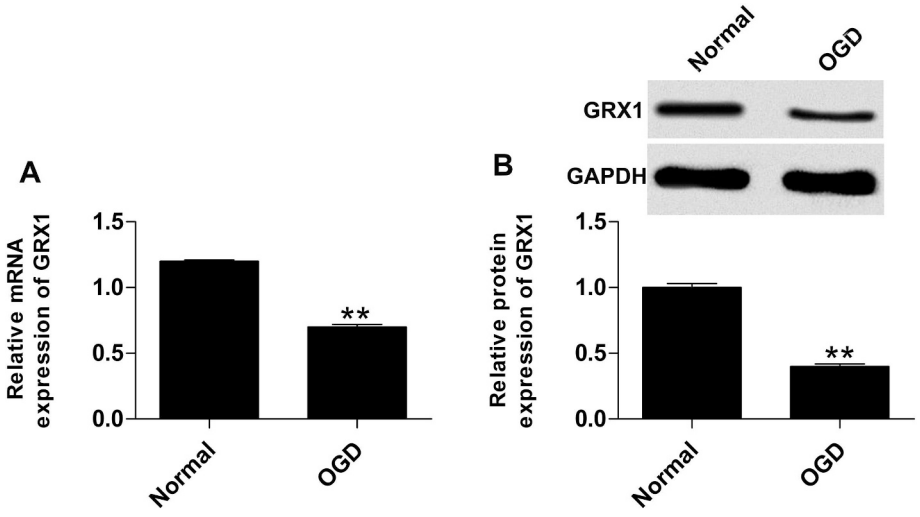

Fig. 1. Effect of OGD injury on GRX1 expression in HK-2 cells. The relative GRX1 mRNA (A) and protein levels (B) were detected by qRT-PCR and Western blot analysis, respectively. Normal, HK-2 cells without transfection or OGD injury; OGD, HK-2 cells with OGD injury. GAPDH served as the control. N = 3, **P < 0.01 for the OGD group vs. the Normal group.

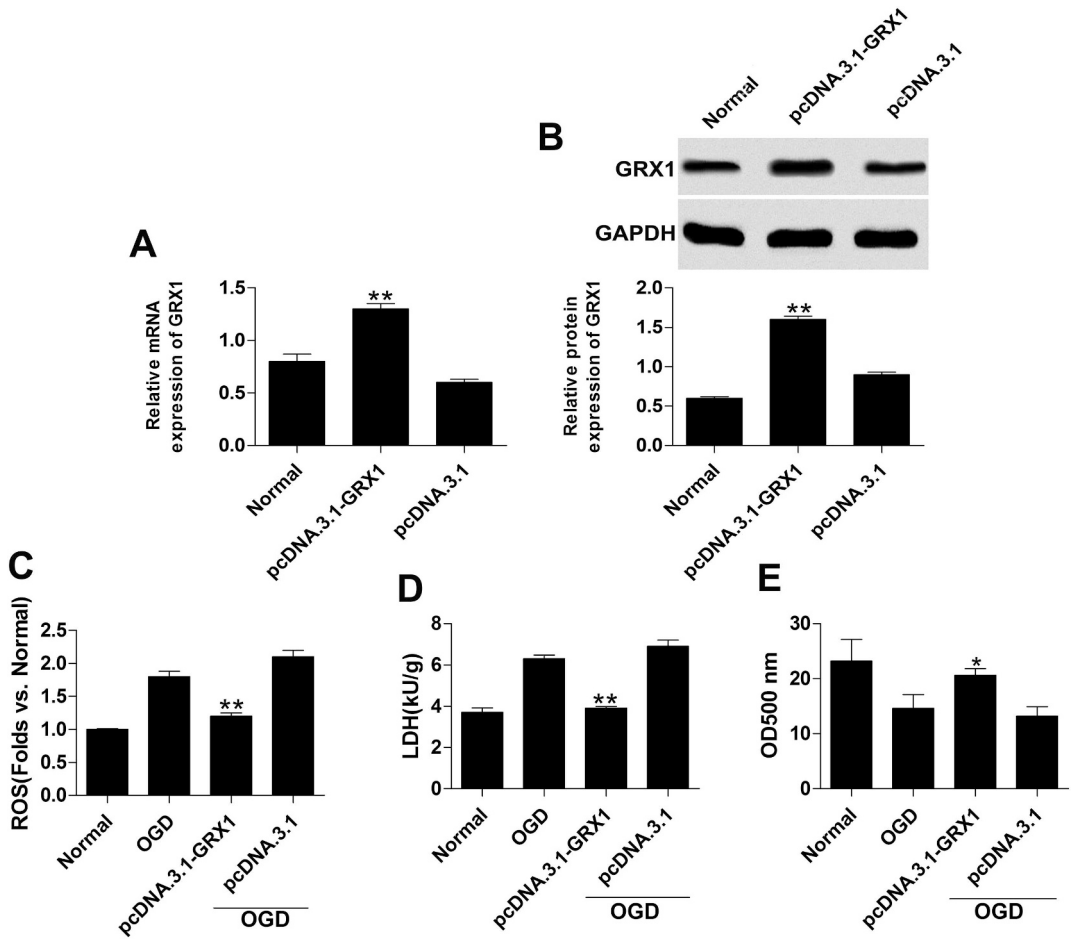

Fig. 2. Effects of GRX1 overexpression on ROS level, LDH leakage, and SOD content. The relative GRX1 mRNA (A) and protein levels (B) were detected by qRT-PCR and Western blot analysis in HK-2 cells without OGD injury, respectively. GAPDH was used as the control. pcDNA.3.1-GRX1, HK-2 cells transfected with pcDNA.3.1-GRX1 without OGD injury; pcDNA.3.1, HK-2 cells transfected with pcDNA.3.1 without OGD injury. Subsequently, the HK-2 cells with GRX1 overexpression were cultured under OGD conditions. (C) ROS level was measured by carboxy-H_2DCF-DA in HK-2 cells under OGD conditions. (D) LDH leakage was tested by LDH cytotoxicity detection kit in HK-2 cells with GRX1 overexpression under OGD conditions. (E) SOD content was tested by the SOD assay kit in HK-2 cells with GRX1 overexpression under OGD conditions. pcDNA.3.1-GRX1, HK-2 cells transfected with pcDNA.3.1-GRX1 with OGD injury; pcDNA.3.1, HK-2 cells transfected with pcDNA.3.1 with OGD injury. N = 3, *P < 0.05, **P < 0.01 for the pcDNA.3.1-GRX1 group vs. the pcDNA.3.1 group.

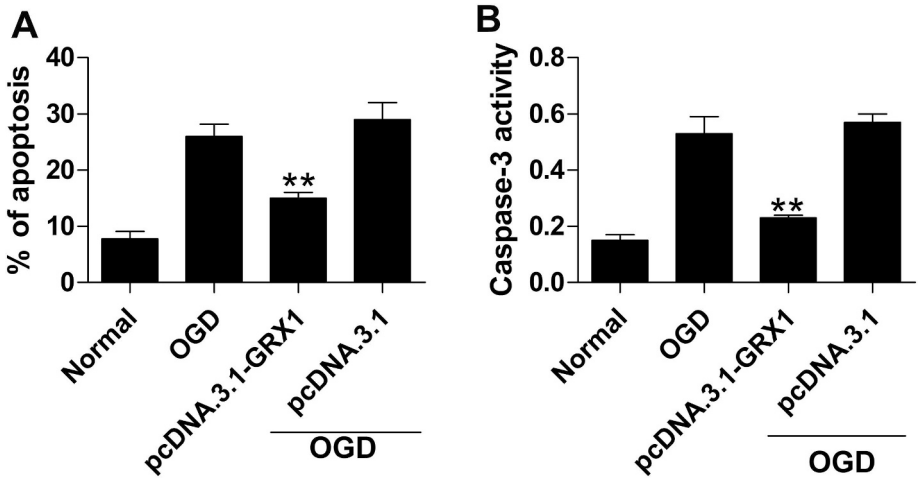

Fig. 3. Effects of GRX1 overexpression on cell apoptosis and caspase-3 activity. Cell apoptosis (A) and caspase-3 activity were tested with an Annexin V-FITC/PI apoptosis detection kit and a Caspase-3 activity assay kit in HK-2 cells with GRX1 overexpression under OGD conditions, respectively. N = 3, **P < 0.01 for the pcDNA.3.1-GRX1 group vs. the pcDNA.3.1 group.

GRX1 has also been demonstrated to regulate the protective function of steady laminar flow on endothelial cells against cell injury induced by oxidative stress [20]. Liu et al. reported that GRX1 was increased by H_2O_2 and protected retinal pigment epithelial cells from oxidative stress injury [21]. Interestingly, our data indicated that GRX1 was decreased by OGD in HK-2 cells. Moreover, GRX1 overexpression significantly constrained the ROS level, caspase-3 activity, LDH leakage, and cell apoptosis, while increasing SOD content in HK-2 cells under OGD conditions. Our study is the first to report the role of GRX1 in renal epithelial cells with OGD injury.

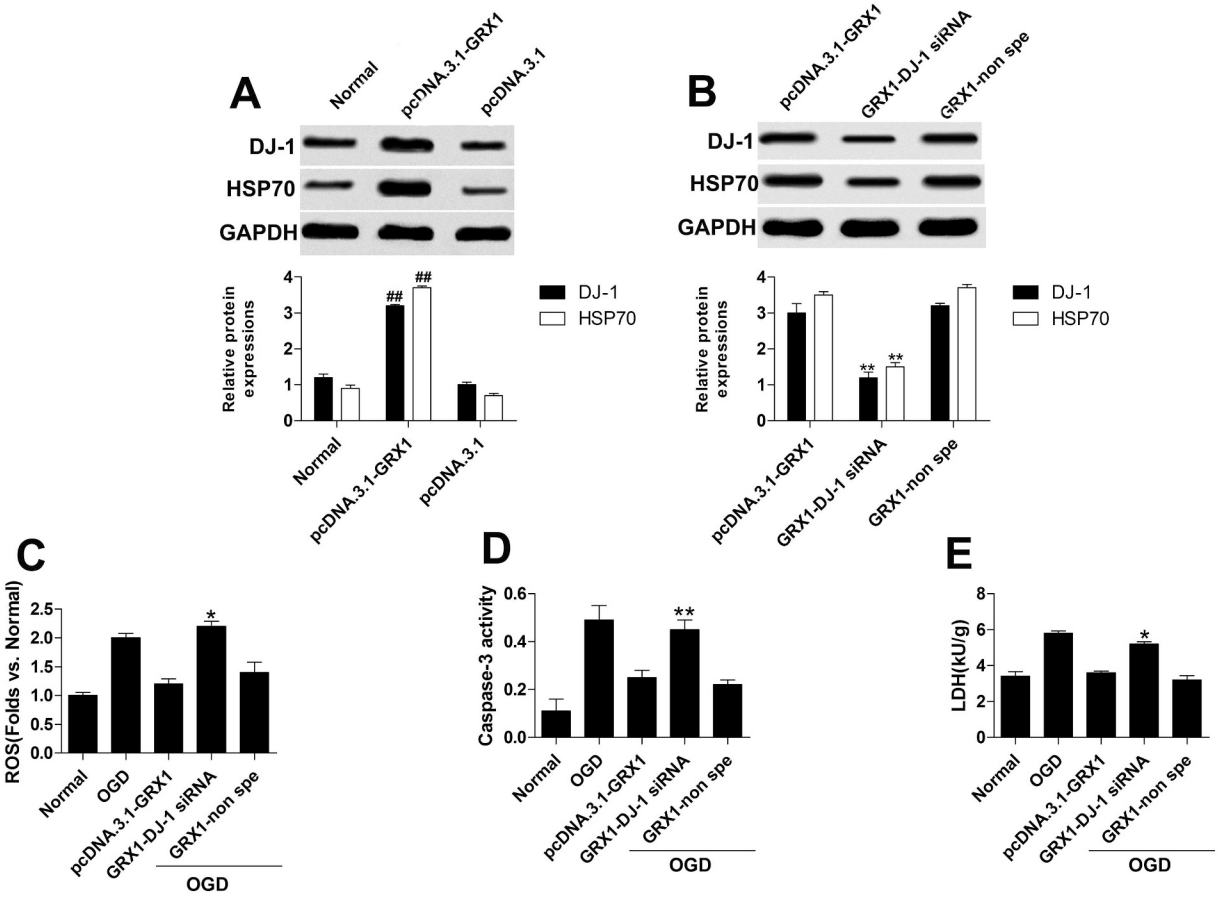

Fig. 4. GRX1 regulates HSP70 through control of DJ-1. (A) Levels of DJ-1 and HSP70 proteins were detected by Western blot analysis in HK-2 cells with GRX1 overexpression without OGD injury. (B) Levels of DJ-1 and HSP70 proteins were detected by Western blot analysis in HK-2 cells with GRX1 overexpression and DJ-1 inhibition without OGD injury. (C), (D), and (E) ROS level, caspase-3, and LDH leakage were tested with carboxy-H2DCF-DA, an LDH cytotoxicity detection kit, and an SOD assay kit in HK-2 cells with GRX1 overexpression and DJ-1 inhibition with OGD injury, respectively. GRX1-DJ-1 siRNA, HK-2 cells with GRX1 overexpression transfected with DJ-1 siRNA and incubated under OGD conditions; GRX1-non spe, HK-2 cells with GRX1 overexpression transfected with nonspecific siRNA and incubated under OGD conditions. N = 3, $^{\#\#}P < 0.01$ for the pcDNA.3.1-GRX1 group vs. the pcDNA.3.1 group. $^{*}P < 0.05$, $^{**}P < 0.01$ for the GRX1-DJ-1 siRNA group vs. the GRX1-non spe siRNA group.

Previously, GRX1 was shown to regulate DJ-1 in the brains of patients with Parkinson's disease [16]. Kolisek et al. reported that DJ-1 exerts a protective role against mitochondrial oxidative stress [17], and Bonilha et al. showed that inhibition of DJ-1 led to oxidative stress and subsequent age-related retinal abnormalities [5]. DJ-1 up-regulation has been shown to constrain apoptosis of human primary alveolar type II cells [3]. Eltoweissy et al. demonstrated that the antioxidative function of DJ-1 is important in renal cells [9], but the relation between GRX1 and DJ-1 in HK-2 cells with OGD injury was unclear. We hypothesized that GRX1 could regulate DJ-1 expression to protect HK-2 cells from oxidative stress induced by OGD. In our study, we found that GRX1 overexpression markedly increased expression of DJ-1 protein, while inhibition of the protein by DJ-1 siRNA significantly reversed the effect of GRX1 overexpression on caspase-3 activity, LDH leakage, and the ROS level in HK-2 cells under OGD conditions. Thus, GRX1 could potentially regulate DJ-1, decreasing OGD injury in HK-2 cells. HSP70, which is associated with oxidative stress and cell apoptosis, was reported to be modulated by DJ-1 in neurons [4]. Yurinskaya et al. found that HSP70 protected human neuroblastoma cells against apoptosis and oxidative stress caused by amyloid peptide isoAsp7-Aβ [37], and Lazarev et al. demonstrated that HSP70 could rescue glioblastoma cells from oxidative stress [18]. HSP70 overexpression has also been shown to depress renal ischemia/reperfusion injury [29].

In our study, we also hypothesized that DJ-1 could regulate HSP70 expression in HK-2 cells with OGD injury. The results demonstrated that increased GRX1 expression was associated with increased expression of HSP70. In addition, inhibition of DJ-1 obviously blocked this effect, indicating that GRX1 could regulate HSP70 via control of DJ-1. Further, HSP70 suppression promoted caspase-3 activity, LDH leakage, and ROS level in HK-2 cells with OGD injury. This investigation is the first to show that GRX1 can protect renal cell from OGD injury via modulating DJ-1/HSP70.

Overall, our data indicated that GRX1 is decreased in HK-2 cells after OGD injury. GRX1 overexpression effectively attenuated cell injury and apoptosis in such cells. Moreover, the protection of HK-2 cells by GRX1 under OGD conditions could be realized by modulating DJ-1/HSP70. Therefore, the results of this study reveal the potential role of GRX1/DJ-1/HSP70 in HK-2 cells with OGD injury and provide novel insight into the treatment of renal ischemia.

5. Conclusion

GRX1 may be an important regulator of the protection on HK-2 against OGD injury. Our data indicated that GRX1 overexpression attenuated cell injury and apoptosis by regulating DJ-1/HSP70. We define a novel molecular mechanism underlying renal ischemia.

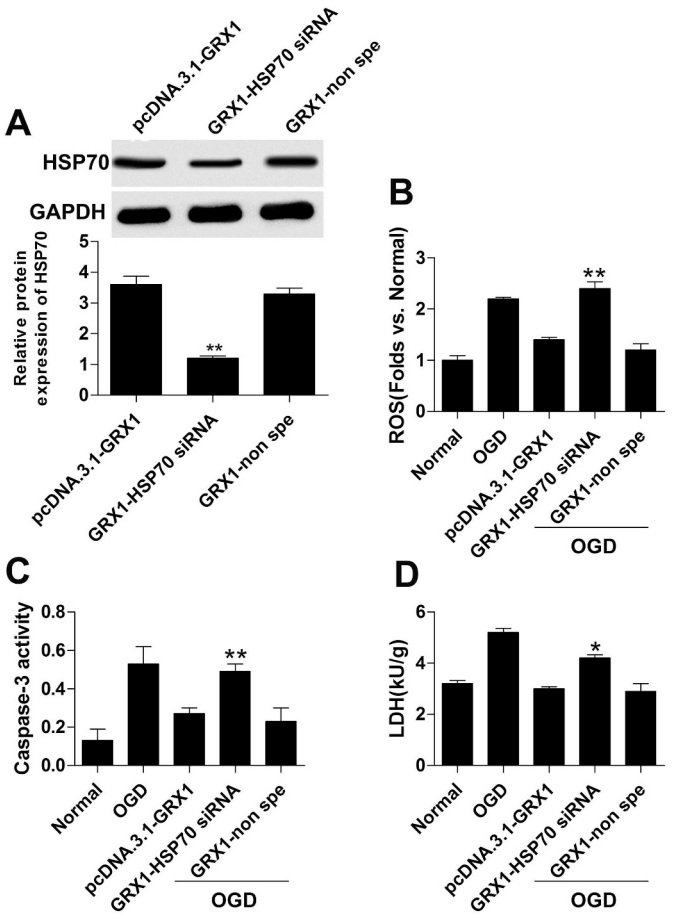

Fig. 5. The effect of HSP70 inhibition on ROS level, Caspase-3 activity and LDH leakage. (A) Protein levels of HSP70 were detected by Western blot analysis in HK-2 after GRX1 overexpression and HSP70 inhibition without OGD injury. (B), (C) and (D) ROS level, Caspase-3 and LDH leakage was tested by carboxy-H2DCF-DA, LDH cytotoxicity detection kit and SOD assay kit in HK-2 after GRX1 overexpression and HHSP70 inhibition with OGD injury, respectively. GRX1-DJ-1 siRNA, HK-2 with GRX1 overexpression transfected with HSP70 siRNA and incubated under OGD condition. *P < 0.05, **P < 0.01 for the GRX1-HSP70 siRNA group vs. the GRX1-non spe siRNA group.

Abbreviations

GRX1	Gluaredoxin1
OGD	oxygen glucose deprivation
LDH	lactate dehydrogenase
ROS	reactive oxygen species
FBS	fetal bovine serum
qRT-PCR	Quantitative real-time polymerase chain reaction
Annexin V-FITC/PI	Annexin V-fluorescein isothiocyanate/propidium iodide

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Acknowledgements

None.

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