Downregulation of FOXO4 promotes neuronal survival by mediating oxidative-stress–induced apoptosis after cerebral ischemia/reperfusion injury

Chunying Deng1,2, Peilan Zhang1*, Yun Zhang1,3

1Department of Neurology, Tianjin Huanhu Hospital, Tianjin 300350; 2Department of Neurology, North China University of Science and Technology Affiliated Hospital; 3Department of Geriatrics, Tangshan Gongren Hospital, Tangshan City, Hebei Province 063000, China

*For correspondence: Email: zhangpeilan666@163.com; Tel: +86-022-59065241

Sent for review: 13 October 2020 Revised accepted: 28 December 2020

Abstract

Purpose: To investigate the effect of FOXO4 on cerebral ischemia/reperfusion (CIR) injury and the underlying mechanism.

Methods: An in vitro ischemia/reperfusion (IR) model was achieved using oxygen-glucose deprivation/reoxygenation (OGD/R). Expression of RNA and protein was determined using quantitative real time polymerase chain reaction (qRT-PCR) and western blotting, respectively. Cell viability and apoptosis were determined using MTT assay and flow cytometry, respectively. Commercial kits were used to measure lactate dehydrogenase (LDH), reactive oxygen species (ROS), chloramphenicol acetyltransferase (CAT), malondialdehyde (MDA), and superoxide dismutase (SOD).

Results: Following OGD/R, FOXO4 mRNA and protein expressions were upregulated in SH-SY5H human neuroblastoma cells. OGD/R reduced cell proliferation and increased the proportion of apoptotic cells, and these effects were inhibited by knockdown of FOXO4 (p < 0.05). Levels of cleaved caspase 3 and cleaved poly(ADP-ribose) polymerases (PARPs) were increased after OGD/R and these increases were inhibited by FOXO4 knockdown. ROS content and levels of LDH and MDA were increased after OGD/R and decreased by knockdown of FOXO4 (p < 0.05). Levels of CAT and SOD were reduced after OGD/R, and this reduction was reversed by knockdown of FOXO4 (p < 0.05).

Conclusion: The results demonstrate that knockdown of FOXO4 promotes cell proliferation and inhibits cellular apoptosis via reduction of oxidative stress after CIR injury, indicating a new therapeutic target for the treatment of CIR injury.

Keywords: FOXO4, Neuronal survival, Oxidative stress, Cerebral ischemia/reperfusion injury

INTRODUCTION

Cerebral ischemia/reperfusion (CIR) injury occurs when blood supply to the brain is suspended and subsequently restored [1]. Cerebral IR injury induces brain dysfunction, which contributes to high mortality and disability [2,3]. According to the World Health Organization (WHO), 15 million strokes occur worldwide each year, with more than 6 million deaths and
another 5 million disabled [4]. The mechanism of CIR injury is complicated and unclear. Thus, the pathological process of CIR injury should be investigated to understand and treat CIR injury in the future.

Many biological processes are involved in CIR injury. Oxidative stress has been reported to occur in almost all cases of CIR injury, resulting in excessive production of reactive oxygen species (ROS) [5,6]. Excessive ROS may cause cell death and tissue necrosis by inducing DNA damage, lipid peroxidation, cytoskeletal structural injury, and chemotaxis [5]. Poly(ADP-ribose) polymerases (PARPs) are a class of zinc-finger DNA-binding proteins that are essential for cellular response to DNA damage [7]. Among the PARPs, PARP-1 has been reported to regulate inflammation in central nervous system disorders, including CIR injury [8]. Overactivation of PARP-1 has been detected in the brain after brain ischemia and cardiac arrest in response to oxidative DNA damage [9]. Ischemic injury was prevented in a PARP knockout mouse model compared with wild-type mice, indicating that inhibition of PARP is a potential new therapeutic strategy for CIR injury [10].

FOXO4 is a member of the Forkhead (Fox) transcription factor O family, which regulates multiple pathophysiological processes, including cellular apoptosis, oxidative stress, and cell cycle arrest [11]. In a rat model of myocardial IR injury, upregulation of FOXO4 promoted myocardial apoptosis through increasing ROS generation [12]. During renal IR injury, production of hydrogen peroxide was induced through overexpression of FOXO4, resulting in renal cell apoptosis in mice [13]. However, there are no data on the role of FOXO4 in CIR injury. Thus, the purpose of this study was to investigate the effects of FOXO4 on CIR injury and provide a new treatment target for CIR injury.

EXPERIMENTAL

Cell culture

SH-SY5H human neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum (FBS, Gibco, Grand Island, NY, USA), Glutamax (Gibco), 100 units/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), and 1× MEM non-essential amino acids (Gibco) in a humidified atmosphere of 5 % CO₂ at 37 °C.

The in vitro IR model was achieved by oxygen-glucose deprivation/reoxygenation (OGD/R) [14]. Briefly, the SH-SY5H cells were seeded in 12-well plates at a density of 1 × 10^5 cells/well in glucose-free DMEM with 1 % O₂, 94 % N₂, and 5 % CO₂ (hypoxic conditions) and incubated at 37 °C for 3 h. The medium was changed to standard DMEM with 95 % O₂ and 5 % CO₂ (normoxic conditions) and cells were incubated for up to 48 h. SH-SY5H cells cultured in standard medium under normoxic conditions were used as the control group.

Small hairpin RNA (shRNA) targeting FOXO4 (shFOXO4) and negative control shRNA (shNC, Ibsbio, China) were mixed with Lipofectamine 2000 (Invitrogen) according to manufacturer's protocols. The SH-SY5H cells were cultured in 6-well plates at a density of 1 × 10^5 cells/well in serum-free medium. The transfection mixture was added to the cells and incubated for 6 – 8 h. The medium was then changed to standard culture medium. SH-SY5H cells were cultured for 24 h at 37 °C and collected for further analysis.

MTT assay

The SH-SY5H cells were cultured in 96-well plates. After transfection, MTT (5 mg/ml in PBS; Abcam, Cambridge, UK) was added and incubated for 4 h according to the manufacturer's instructions. The formazan was dissolved using dimethyl sulfoxide. The absorbance value at 490 nm was measured using a spectrophotometer (BioTek, Winooski, VT, USA).

Extraction of RNA and quantitative (real-time) PCR (qPCR)

Total RNA extraction was performed using TRIzol reagent (Thermo Fisher Scientific Waltham, MA, USA). Reverse transcription was performed with 1 µg RNA using the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany). RNA expression level was measured using the QuantiTect SYBR Green RT-PCR Kit (QIAGEN) with the StepOnePlus system (Applied Biosystems, Foster City, CA, USA). Relative RNA expression was quantified using the 2^(-ΔΔCt) method [15]. Sequences of primers (Sigma-Aldrich, St. Louis, MO, USA) used in this study are shown in Table 1.

Western blotting

Cell lysates were prepared by extraction of cells in RIPA cell lysis buffer (Beyotime, Shanghai, China). Proteins were separated through a 7.5 % SDS-PAGE gel. The separated proteins were then transferred to PVDF membranes, followed
by blocking of membranes with 5% fat-free milk. The blocked membranes were probed with the appropriate primary antibody overnight at 4°C and then incubated with secondary antibodies for 2 h at room temperature. Protein bands were detected using ECL Detection reagents (Sigma-Aldrich). The primary antibodies used in this study (all from Cell Signaling Technology, Danvers, MA, USA) were anti-FOXO4 (#9472, 1:1000 dilution), anti-cleaved caspase 3 (#9664, 1:1000 dilution), anti-cleaved PARP (#5625, 1:1500 dilution), anti-β-actin (#4970, 1:5000 dilution), and anti-GAPDH (#5174, 1:5000 dilution).

Table 1: Sequences of primers used in this study

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXO4</td>
<td>Forward: 5'-GGCTGCCGCGATCATAGAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGCTGGTTAGCGATCTCTGG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-TGTGGGCATCAATGGATTTGG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-ACACCATGTATTCCGGGTCAAT-3'</td>
</tr>
</tbody>
</table>

**Flow cytometry**

After transfection with shRNA for 24 h, SH-SY5H cells were collected and resuspended at a concentration of 1 × 10⁷ cells/ml. The cell suspension was stained with Annexin-V–FITC and propidium iodide (Sigma-Aldrich) for 15 minutes. Apoptosis was detected using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA). Cells (2 ×10⁴) were collected, recorded, and the data were presented as two-parameter dot-plots.

**Measurement of biochemical parameters**

*Lactate dehydrogenase* (LDH), reactive oxygen species (ROS), catalase (CAT), *malondialdehyde* (MDA), and *superoxide dismutase* (SOD) were measured using the LDH assay kit (Abcam), ROS detection assay kit (Abcam), CAT assay kit (GenScript, Piscataway, NJ, USA), MDA assay kit (Abcam), and SOD activity assay kit (Abcam) according to the manufacturers’ instruction.

**Statistical analysis**

GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA) was used to carry out all data analysis. Student’s *t*-test was used to compare differences between two groups. One-way ANOVA was used to compare multiple groups. All data are expressed as mean ± standard deviation (SD). *P* < 0.05 was considered statistically significant.

**RESULTS**

**FOXO4 expression was upregulated by OGD/R in SH-SY5H cells**

After OGD/R, expression of FOXO4 mRNA was significantly upregulated in SH-SY5H cells compared with the control group (Figure 1 A). FOXO4 protein was also upregulated after OGD/R (Figure 1 B).

**Knockdown of FOXO4 facilitated OGD/R-induced survival of SH-SY5H cells**

Expression of FOXO4 mRNA was decreased in SH-SY5H cells transfected with shFOXO4, compared with cells transfected with shNC in both control and OGD/R groups (Figure 2 A). Protein expression of FOXO4 was significantly downregulated in SH-SY5H cells transfected with shFOXO4, compared with cells transfected with shNC in both control and OGD/R groups (Figure 2 B). Cell viability was increased in SH-SY5H cells transfected with shFOXO4, compared with cells transfected with shNC in the OGD/R group (Figure 2 C). In cells cultured in standard medium under normoxic conditions, there was no difference in viability between cells transfected with shFOXO4 and shNC (Figure 2 C). In cells cultured in standard medium under normoxic conditions, there was no significant change after transfection with shFOXO4 in cells cultured in standard medium under normoxic conditions (Figure 2 D). In the OGD/R group, LDH activity was decreased in cells transfected with shFOXO4, compared with its negative control group (Figure 2 D).

**Knockdown of FOXO4 prevented OGD/R-induced apoptosis in SH-SY5H cells**

The results of flow cytometric analysis demonstrated that OGD/R promoted apoptosis,
compared with the control group, whereas downregulation of FOXO4 reduced the proportion of apoptotic cells induced by OGD/R (Figure 3 A). Levels of cleaved caspase 3 and cleaved PARP were increased after OGD/R, whereas knockdown of FOXO4 reduced the increased levels of cleaved caspase 3 and cleaved PARP induced by OGD/R (Figure 3 B).

Knockdown of FOXO4 reduced OGD/R-induced oxidative stress in SH-SY5H cells

After OGD/R, ROS content was significantly increased, whereas the increase in ROS content was inhibited by knockdown of FOXO4 (Figure 4 A). CAT activity was reduced after OGD/R and this reduction was suppressed by knockdown of FOXO4 (Figure 4 B). After OGD/R, MDA was significantly increased, whereas this increase was inhibited by knockdown of FOXO4 (Figure 4 C). SOD activity was reduced after OGD/R and this reduction was reversed by shFOXO4-induced downregulation of FOXO4 (Figure 4 D).

DISCUSSION

OGD is a common in vitro IR model that is usually used to investigate ischemia-induced cellular dysfunction [16]. Sustained OGD (> 1 h) followed by re-oxygenation (OGD/R) contributes to mitochondrial dysfunction, ROS production, and cellular necrosis (but not apoptosis) [16]. In this study, expression of FOXO4 mRNA and protein was upregulated in the OGD/R neuroblastoma cells. Further exploration of the mechanism underlying this effect demonstrated that knockdown of FOXO4 inhibits OGD/R-induced cell proliferation and ROS production and promotes apoptosis, indicating that inhibition
of FOXO4 is a potential new therapeutic target for CIR injury.

As mentioned above, FOXO4 regulated ROS generation after myocardial IR injury and renal IR injury [12,13]. In this study, ROS content was increased after OGD/R, and downregulation of FOXO4 reduced OGD/R-induced ROS production, indicating that FOXO4 controlled ROS generation in CIR injury, which is consistent with observations in myocardial IR injury and renal IR injury. SODs are a class of enzymes catalyzing the conversion of superoxide into oxygen and hydrogen peroxide and poison of ROS [17]. MDA has been widely used as a biomarker of oxidative stress [18].

In this study, SOD activity was reduced, and MDA was increased after OGD/R in neuroblastoma cells; inhibition of FOXO4 expression prevented this phenomenon, suggesting that upregulation of FOXO4 promoted an increase in oxidative stress during CIR injury. In addition, it is well known that oxidative stress is associated with cellular apoptosis [19]. Overproduction of ROS induced neuronal cell damage through an apoptotic or a necrotic pathway, promoting cell death [20]. The results of this study demonstrated that cell viability was reduced after OGD/R and negatively correlated with FOXO4 expression, indicating that overexpression of FOXO4 accelerated apoptosis after CIR injury. Taken together, the data show that in this in vitro CIR model, upregulation of FOXO4 contributed to oxidative stress-induced apoptosis in neuroblastoma cells. Inhibition of FOXO4 demonstrated the protective effects on neuroblastoma cells, implying a new therapeutic strategy for CIR injury.

Caspase 3 is a crucial enzyme in the process of programmed cell death (PCD), such as in apoptosis and pyrosis [21]. Caspase 3 is activated through cleavage by upstream caspases and translocated from the cytoplasm into the nucleus in cells undergoing PCD [21]. In this study, cleaved caspase 3 increased after OGD/R, and knockdown of FOXO4 reduced the level of increased cleaved caspase 3, indicating that FOXO4 mediated PCD in CIR injury. PARP-1 is a major substrate of caspase 3 [21]. Caspase 3 cleaves PARP-1 at position 214/215 to generate the 85-kDa fragment in hepatocytes undergoing apoptosis [22]. In this study, cleaved PARP levels were increased by OGD/R and decreased by FOXO4 knockdown. This change in the level of cleaved PARP coincided with the change in cleaved caspase 3, further suggesting that inhibition of FOXO4 reduced apoptosis in CIR injury.

CONCLUSION

The findings of this study demonstrate that FOXO4 is upregulated after OGD/R. This upregulation of FOXO4 promotes apoptosis and inhibits cell proliferation, whereas FOXO4 knockdown inhibits apoptosis induced by oxidative stress after CIR injury, providing a new therapeutic target for CIR injury.

DECLARATIONS

Acknowledgement

This work was supported by Key R & D Self-Financing Projects in Hebei Province (Grant no. 172777151).

Competing interests

There is no conflict of interest to disclose.

Authors’ contributions

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Chunying Deng designed the study, supervised the data collection, and analyzed the data. Peilan Zhang interpreted the data and prepared the manuscript for publication. Yun Zhang supervised the data collection, analyzed the data, and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

Open Access

This is an Open Access article that uses a funding model which does not charge readers or their institutions for access and distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0) and the Budapest Open Access Initiative (http://www.budapestopenaccessinitiative.org/), which permit unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited.

REFERENCES

2. Yang J, Chen M, Cao RY, Li Q, Zhu F. The Role of Circular RNAs in Cerebral Ischemic Diseases: Ischemic


7. Pascal JM. The comings and goings of PARP-1 in response to DNA damage. DNA Repair 2018; 71: 177-182.


