Original Article
Effects of GCN2/eIF2α on myocardial ischemia/hypoxia reperfusion and myocardial cells injury

Yan Pu¹, Dong Wu², Xiaoe Lu³, Linjun Yang⁴

¹Department of Emergency Centre, Hanzhong Central Hospital, Hanzhong 723000, Shaanxi, China; Departments of ²Physical Emergency, ³Critical Medicine, Shaanxi Provincial People’s Hospital, Xi’an 710068, Shaanxi, China; ⁴Department of Emergency, Hanzhong People’s Hospital, Hanzhong 723000, Shaanxi, China

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Abstract: Myocardial ischemia/hypoxia-reperfusion injury is a common and severe cardiovascular disorder. General control non-derepressible 2 (GCN2) plays an important role in the role of cardiomyocyte glucose metabolism. Therefore, our study focused on the expression of GCN2/eIF2α in myocardial ischemia/hypoxia-reperfusion injury and its mechanism of myocardial cell injury. In the volunteers and patients with myocardial ischemia and hypoxia-reperfusion, the expression of GCN2 and eIF2α on serum were detected by RT-qPCR. The GCN2, eIF2α interference and GCN2 overexpression plasmids were constructed and transfected into cells. Then, the level of TNF-α, IL-1β, IL-6, IFN-γ were detected by ELISA and the level of ROS, MDA, LDH and SOD were measured by the corresponding kits, respectively. Besides, the expression of GCN2/eIF2α signaling pathway (p-eIF2α, ATF4, CHOP, UCP2 and eIF2α) and apoptosis-related proteins (Bax, Bcl-2 and cleaved caspase3) was detected by western blot. Flow cytometry was performed to value cell apoptosis. The expression of GCN2 is increased in oxygen-glucose deprivation/reoxygenation (OGD/R) model cells and GCN2 interference reduces the inflammation and oxidative stress in H9C2 cells after OGD/R. GCN2 interference reduced the level of apoptosis in OGD/R model cells and inhibited the expression of GCN2/eIF2α signaling pathway. We found that eIF2α interference could offset the effects of GCN2 overexpression on oxidative stress and apoptosis in H9C2 cells, and verified that GCN2 is produced by eIF2α phosphorylation. Together, GCN2/eIF2α signaling pathway plays an important role in myocardial ischemia/hypoxia-reperfusion injury, which could provide a new idea for the treatment of myocardial infarction on clinical.

Keywords: Myocardial ischemia/hypoxia reperfusion injury, GCN2, eIF2α

Introduction

Myocardial ischemia/hypoxia-reperfusion refers to the interruption of myocardial blood supply and oxygen supply in a short time, and restore blood supply and oxygen supply within a certain period time, so that the original ischemic myocardium is more severe than before blood supply and oxygen supply recover [1]. Myocardial ischemia/hypoxia-reperfusion injury can induce myocardial cell apoptosis and increase myocardial infarct size, thus explaining the cause of acute myocardial infarction patients with high mortality after myocardial reperfusion is still more than 10% [2]. Myocardial ischemia/hypoxia-reperfusion injury leads to the cell death process involving many pathological mechanisms, which are related to changes in the expression levels of some proteins or kinases. These proteins or kinases play a key role in the pathological process of myocardial ischemia/hypoxia reperfusion injury [3]. When a specific agonist or inhibitor is used to increase or decrease its activity or to alter the expression of these proteins or kinases by transgenic methods, it may have an important or even decisive influence on the survival of cells after myocardial ischemia/hypoxia reperfusion.

Therefore, a better understanding of the cellular and molecular mechanisms of myocardial ischemia/hypoxia reperfusion injury and its complex signaling pathways facilitate clinically better treatment of such diseases.

General control non-derepressible 2 (GCN2) is a kinase that senses the absence of one or more amino acids by virtue of direct binding to uncharged cognate tRNAs [4]. Studies have
shown that GCN2 in the liver is activated in fatty liver and that GCN2 deficiency can protect the obese diet-induced liver degeneration and insulin resistance. GCN2 has complex dependence in lipid metabolism regulation. Other studies have shown that GCN2 deficiency can attenuate cross-sectional aortic systolic (TAC) or doxorubicin-induced myocardial impairment by reducing cardiomyocyte apoptosis and oxidative stress [5]. Moreover, GCN2 plays an important role in cardiomyocyte glucose metabolism and lipid metabolism. GCN2 knockout mice can alleviate diabetes-induced cardiomyocyte injury [6]. Other studies have shown that GCN2 deficiency can attenuate cross-sectional aortic systolic (TAC) or doxorubicin-induced myocardial impairment by reducing cardiomyocyte apoptosis and oxidative stress [5]. Moreover, GCN2 plays an important role in cardiomyocyte glucose metabolism and lipid metabolism. GCN2 knockout mice can alleviate diabetes-induced cardiomyocyte injury [6]. Recent studies demonstrate that GCN2 kinase plays an essential role in reducing inflammation [7]. In addition to regulating amino acid starvation response, GCN2 is also involved in memory formation [8], immune response [9] and muscle atrophy [10].

So far, plenty of work has been done on myocardial ischemia/hypoxia-reperfusion. However, to date, no research has investigated the role of GCN2 in H9C2 OGD/R model cells, and the mechanisms of myocardial ischemia/hypoxia-reperfusion development have not been fully elucidated. In this study, we hypothesized that expression of GCN2/eIF2α in myocardial ischemia/hypoxia-reperfusion is increased and the GCN2/eIF2α signaling pathway plays an important role in myocardial ischemia/hypoxia-reperfusion injury.

Materials and methods

Patients and specimen

The blood samples of 30 patients with myocardial ischemia/hypoxia-reperfusion were collected from Hanzhong Central Hospital. The mean age of patients was 40 years old (ranging from 21 to 68). Another 30 blood samples were collected from volunteers (mean age of volunteers was 43 years old, ranging from 30 to 64). These samples were obtained for RT-qPCR to detect the expression of GCN2 and eIF2α. All patients received no chemotherapy or radiotherapy before surgery. All patients signed written informed consent, and ethical consent was granted from the Committee for Ethical Review of Research Involving Human Subjects of the Hanzhong Central Hospital.

Cell culture and OGD/R model

H9C2 cells were obtained from Cell Bank of the Chinese Academy of Sciences, and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% inactivated fetal bovine serum, 2 mL-glutamine, 100 U/ML penicillin and streptomycin at 37°C with 5% CO₂. After 2 h oxygen-glucose deprivation (OGD) treatment according to the experimental needs, the cells were collected by reoxygenation for 12 h to construct the OGD/R model cell.

Transfection

Construction of GCN2 interference plasmid, eIF2α-1 interference plasmid and GCN2 overexpression plasmid, the cells were plated in six-well plates and transfection was conducted at 70-80% confluence, respectively. Transfection used Opti-MEM, serum-free RMPI DMEM and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s instruction. The cells were cultured in six-well plates under normal conditions for 1-3 days at 37°C after transfection.

Reverse transcription-quantitative PCR (RT-qPCR)

The total RNA was extracted from blood and H9C2 cells using TRIzol reagent (TaKara, Dalian, China) and the cDNA was synthesized with RNA Transcription Kit (TaKara, Dalian, China). Then, PCR was performed using GeneAmp PCR System 9700 (Applied Biosystems; Thermo Fisher Scientific, Inc.). Conditions for RT-qPCR was set as the following: 95°C for 10 min, 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s and a final extension of 10 min at 72°C. The mRNA expression of GCN2 and eIF2α were analyzed using the 2-ΔΔCq method. The primers used were as follows: GCN2 forward, 5’-TGCCAACTTACATCAGAAAAGC-3’, and reverse 5’-TTTGAGGTATATTTGCTTTGG-3’; eIF2α forward, 5’-GCAGGTAGTTTGTACCATTTA-3’, and reverse 5’-GCCAGAGAATAGATCAGTATT-3’; GAPDH forward, 5’-CCAGGGGTGCCTTCTCTT-3’, and reverse 5’-CCGTGGGTAGAGTCATACTGG.

Western-blot

The expression of GCN2/eIF2 signaling pathway (p-eIF2α, ATF4, CHOP, UCP2 and eIF2α) and apoptosis-related proteins (Bax, Bcl-2 and cleaved caspase3) was detected by Western-blot. Total protein from the cells was extracted using RIPA lysis buffer and protein concentrations were determined using a BCA protein kit (Bio-rad Laboratories, Inc.). Then separated by SDS-PAGE, and transferred to PVDF membr-
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rates was assessed by flow cytometric analysis.

Statistical analysis

The data are expressed as the mean ± SD and were analyzed with SPSS v11.5. Statistical differences between the groups were assessed by one-way ANOVA statistical analysis followed by a Tukey’s test if necessary. The statistical analyses were performed using two-tailed Student’s-test (GraphPad Prism 5). All experiment was repeat at least three times. Differences with P<0.05 were considered significant.

Results

GCN2 and eIF2α expression is increased in the blood of patients with myocardial ischemia and hypoxia reperfusion (acute myocardial infarction)

Fresh blood samples were obtained to determine the expression of GCN2 and eIF2α in the blood of patients with myocardial ischemia and hypoxia-reperfusion. The RT-qPCR results showed that the expression of GCN2 and eIF2α mRNA in the blood of patients with myocardial ischemia and hypoxia-reperfusion was much higher than that in the blood of volunteers (Figure 1).

Reduction of inflammatory and oxidative stress in OGD/R cells after GCN2 interference

We investigated the effect of GCN2 in H9C2 cell by western blot and RT-qPCR. The results of western blot showed that the expression of SH-RNA-GCN2-1 and SH-RNA-GCN2-2 group was much lower than the control group and SH-RNA-NC group (Figure 3B). The RT-qPCR assay showed similar results to those of western blot (Figure 3A). The results indicate that the interference plasmid successfully interfered with GCN2.

anes. The Membranes were blocked with 5% nonfat milk and 0.1% Tween-20 for 1 h at room temperature. Samples were then incubated with the primary antibodies overnight at 4°C. After three washes with TBST buffer, the membranes were incubated with secondary antibodies mouse anti-rabbit (1:10000, Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-mouse (1:10000 Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C for 3 h and visualized by enhanced chemiluminescence (ECL) reaction reagents. The primary antibodies used were as followed: anti-p-eIF2α (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-ATF4 (1:1000; Abcam, Cambridge, MA), anti-CHOP (1:1000; Abcam, Cambridge, MA), anti-UCP2 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-eIF2α (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bax (1:1000; Abcam, Cambridge, MA), anti-Bcl-2 (1:1000; Abcam, Cambridge, MA) and anti-cleaved caspase3 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA).

Detection of ELISA, reactive oxygen species assay (ROS), and malondialdehyde (MDA), lactate dehydrogenase (LDH)

The expression of TNF-α, IL-1β, IL-6 and IFN-γ was detected by ELISA kits (Andihuatai Technology Co. Ltd., Beijing, China) by a microplate reader (MULTISKAN MK3, Thermo, San Jose, CA, USA) according to the manufacturer’s instructions. LDH, ROS, SOD and MDA were measured using commercial kits, respectively (Invitrogen, Carlsbad, CA).

Flow cytometry apoptosis detection assay

Cells were seeded in 6 cm culture dish for about 24 h, then washed in 4°C PBS and resuspended in 500 μl of hypotonic fluorochrome solution. Place the centrifuge tube in a 1000 rpm centrifuge, centrifuge for 5 minutes, discard the supernatant, add 195 μl of Annexin V-FITC binding solution, add 5 μl of Annexin V-FITC, mix gently, incubate at room temperature for 10 minutes in the dark, 1000 rpm. The supernatant was discarded by centrifugation for 5 minutes, and 195 μl of Annexin V-FITC binding solution was added thereto, and the cells were gently suspended. Add 10 μl of propidium iodide staining solution, mix gently, place in the ice bath and avoid the light. Apoptosis
Moreover, Transfect SH-RNA-GCN2-1 into OGD/R model cells, the results of ELISA showed that SH-RNA-GCN2-1 significantly downregulated the expression of TNF-α, IL-6, IL-1β and IFN-γ (Figure 3C-F). These data indicated that knockdown of GCN2 inhibited the inflammatory cytokine release both in OGD/R model cell.

Oxidative stress plays a significant role in cardiac injury. Hence, we investigated the effect of GCN2 interference on ROS, LDH and MDA generation. Infection with SH-RNA-GCN2-1 decreased ROS and MDA generations was compared with control group (Figure 3G, 3H). These implied that interfered of GCN2 ameliorated OGD/R induced cardiomyocyte injury. LDH release is a crucial indicator of cellular injury. As shown in Figure 3I, OGD/R treatment increased the LDH release, which was significantly decreased by knockdown of GCN2. Also, SH-RNA-GCN2-1 group significantly increased SOD generation compared to the control group (Figure 3J). The results indicated that the reduction of inflammatory and oxidative stress in OGD/R cells after GCN2 interference.

Decreased apoptosis level of OGD/R model cells after GCN2 interference

Flow cytometry to detect apoptosis rate: the apoptosis rate of SH-RNA-GCN2-1 group was significantly lower than that of Control group,
Figure 3. Reduction of inflammatory and oxidative stress in OGD/R cells after GCN2 interference. (A) The expression of GCN2 mRNA was examined by RT-qPCR in H9C2 cell after transfect SH-RNA-GCN2. (B) Western blot to show the protein levels of GCN2 in OGD/R model cells after transfect SH-RNA-GCN2. Quantification as-
there was a significant difference (P<0.05) (Figure 4A). Western-blotting was used to detect the expression levels of Bcl-2, cleaved caspase3 and Bax apoptotic genes, as shown in Figure 4B. The expression of Bcl-2 protein in SH-RNA-GCN2-1 group was significantly higher than that in control group (P<0.05). The expression of Bax protein in SH-RNA-GCN2-1 group protein was significantly lower than that of SH-RNA-NC group (P<0.01). The experimental results confirmed that the activity of the Cleaved caspase 3 protein in the SH-RNA-GCN2-1 group was lower than that in the control group (P<0.05), and the SH-RNA-NC group. There was a statistically significant difference between the above groups (Figure 4B). These results indicate that interfered GCN2 can decrease the apoptosis level of OGD/R model cells.

**GCN2 interference inhibits the expression of GCN2/eIF2α signaling pathway**

The interference plasmid was transfected into OGD/R model cells, and the expression of GCN2/eIF2 signaling pathway protein (p-eIF2α, ATF4, CHOP, UCP2 and eIF2α) was detected by western blot. The results show that the expression of p-eIF2α, ATF4, CHOP in SH-RNA-GCN2-1 group is lower than the control group and SH-RNA-NC group, the expression of eIF2α is not changed, and the expression of UCP2 in SH-RNA-GCN2-1 group is higher than control group and SH-RNA-NC group (Figure 5).

**The role of GCN2 is through phosphorylation of eIF2α**

Transfection of eIF2α-1 interference plasmid SH-RNA-eIF2α-1 and GCN2 overexpression plasmid PcDNA-GCN2 into H9C2 cells, respectively. As expected, the expression of eIF2α-1 was decreased in stable SH-RNA-eIF2α-1 transfected H9C2 cells (Figure 6A, 6B). Meanwhile, GCN2 overexpression markedly stimulated the expression of GCN2 compared to the control of H9C2 cells (Figure 6C, 6D). The eIF2α-1 interference plasmid SH-RNA-eIF2α-1 and GCN2 overexpression plasmid PcDNA-GCN2 were transfected into OGD/R model cells. We investigated the effect of eIF2α-1 interference plasmid SH-RNA-eIF2α-1 and GCN2 overexpression plasmid PcDNA-GCN2 on ROS, MDA and LDH generation. As shown in Figure 6E, Interference with eIF2α-1 and overexpress GCN2 can significantly reduce ROS, MDA and LDH release. Meanwhile, the relative expression of SOD was significantly increased in SH-RNA-eIF2α-1 and SH-RNA-eIF2α-1+PcDNA-GCN2 (Figure 6E). Then, flow cytometry revealed a lower apoptosis in SH-RNA-eIF2α-1, decrease of eIF2α-1 significantly suppressed the apoptosis rate. Also, transfection of eIF2α-1 interference plasmid and GCN2 overexpression plasmid into OGD/R cells significantly inhibited apoptosis rate (Figure 7A). We also detected the expression of p-eIF2α, ATF4, CHOP, UCP2, eIF2α in OGD/R model cells. The result of western blot showed that p-eIF2α, ATF4 and CHOP protein decreased after transfected SH-RNA-eIF2α-1 and SH-RNA-eIF2α-1+PcDNA-GCN2. And the expression of UCP2 is significantly increased, but there is no change in the expression of eIF2α-1 (Figure 7B). These data indicated that the role of GCN2 is produced by phosphorylation of eIF2α.

**Discussion**

Myocardial ischemia-reperfusion injury is the main pathological change after myocardial infarction and cardiopulmonary bypass. Its pathogenesis reflects the convergence of multiple pathways, including reactive oxygen species, ion channels, inflammation and endothelial dysfunction. The model of myocardial ischemia reperfusion injury is of great significance for studying the physiological processes of the disease at the cellular level.

Increasing evidence has indicated that general control nonderepressible 2 (GCN2) controls inflammation by inhibiting inflammatory activation. Under conditions of amino acid deprivation, GCN2 maintains amino acid homeostasis by phosphorylating eukaryotic initiation factor 2α at Ser51 (eIF2αSer51) and selectively stimulating the expression of amino acid biosynthetic genes [11, 12]. GCN2 was found to promote sodium salicylate or histone deacetylase inhibitor-induced apoptosis [13, 14], and it has...
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been suggested that GCN2 expression levels determine the sensitivity of cancer cells to Na+, K+-ATPase ligand-induced apoptosis [15].

The role of tumor necrosis factor TNF-α is mainly to regulate immune cells, which are mainly produced by activated macrophages. Under normal physiological conditions, the expression level of TNF-α in the myocardium is low, and myocardial ischemia 2 and reperfusion injury occurs in cardiomyocytes. At the time, the damaged cells release a large amount of the pro-inflammatory factor TNF-α, which induces the synthesis of other inflammatory factors such as IL-1β. The expression can affect the synthesis of proteins in cardiomyocytes. As one of the interleukin-1 families, it is an important inflammatory response. The effect of GCN2 on the inflammatory response of myocardial ischemia-reperfusion injury in OGD/R model cells was detected by enzyme-linked immunosorbent assay (ELISA). It was found that GCN2 significantly reduced the expression of pro-inflammatory factors TNF-α and IL-1β and induced cardiomyocytes to produce sputum.

Here, we focused on its biological role in myocardial IR injury and it was dramatically increased in myocardial IR injury. The knockdown of GCN2 inhibited cell apoptosis in vivo. In addition, LDH release, MDA contents, and ERS-regulated cardiomyocyte apoptosis were also significantly repressed by the downregulation of GCN2. The production of ROS can trigger leukocyte chemotaxis and inflammation, leading to severe cardiac damage [16], and is one of the important factors in studying the pathogenesis of myocardial IR injury [17]. We found that knockdown of GCN2 inhibited ROS production and release of inflammatory cytokines. It has been reported that various cytokines can be released during inflammation, which is one of the most important aspects of myocardial IR injury [18].

Apoptosis, also known as programmed cell death (PCD), is a genetically controlled process of active cell death that plays an important role in regulating organogenesis and maintaining homeostasis in the body, but excessive apoptosis can cause damage to the body. Activation of
intracellular caspase (caspase) is a key step in initiating apoptosis. Misao [19] found that Bcl-2 was significantly increased in cardiomyocytes of acute myocardial infarction, and Bax decreased correspondingly, indicating that Bcl-2 protein plays an important role in the rescue of infarcted myocardium. Nakamura et al [20] found that ischemia-reperfusion injury can reduce Bcl-2 protein and increase Bax in myocardial tissue. These findings demonstrate that
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Figure 7. Decreased apoptosis level of OGD/R model cells after eIF2α interference and GCN2 overexpressed. A. Flow cytometry to detect apoptosis rate. B. Examined expression of p-eIF2α, eIF2α, CHOP, ATF4 and UCP2 protein by western blot. *P<0.05, **P<0.01, ***P<0.001 vs. Control group.
Bcl-2 and Bax can play a role in myocardial ischemia-reperfusion by regulating apoptosis. The Bcl-2 protein family plays an essential role in cell proliferation, differentiation, and apoptosis. Bax acts as an important regulatory protein in the apoptotic mitochondrial pathway, releasing cytochrome c to the cytoplasm by disrupting the integrity of the mitochondrial membrane. In the cytoplasm, cytochrome activates caspase-9 by interacting with apoptotic protease-activating factor 1 (apaf-1), and caspase-9 induces apoptosis by activating its downstream caspase-3. Caspase-3 is thought to act as a key protease in the caspase family. When caspase-3 is activated, it can cause DNA damage repair enzyme degradation, and activate endonuclease, leading to apoptosis. In our study, the apoptosis-related proteins were detected by western blot and apoptosis was detected by flow cytometry. The results showed that GCN2 interference reduced the apoptosis level of OGD/R cells.

Research has shown that GCN2 promoted eIF-2α phosphorylation response that includes protein synthesis attenuation and an increase in the expression of ATF4 [12]. Based on our results that the GCN2 interference plasmid was transfected into OGD/R model cells, and the expression of p-eIF2α, ATF4, CHOP, UCP2 and eIF2α protein was detected by western blot. The results showed that after eIF2α or ATF4 was inhibited, GCN2 levels increased significantly. We observed that the role of GCN2 is produced by phosphorylation of eIF2α and inhibition of GCN2 repressed myocardial IR injury via inactivating GCN2/eIF2α signaling pathway.

Of course, in this experimental study, there are certain limitations and shortcomings. This study is based on in vitro cell levels. The ischemia-reperfusion study has certain differences in the preparation of animal models of ischemia-reperfusion. The specific differences need to be further research.

Conclusion

To conclude, it is a first report of expression of GCN2/eIF2α in myocardial ischemia-reperfusion reperfusion and its mechanism of myocardial cell injury, which is expected to be an attractive target for the development of new drugs for the treatment of OGD/R.

Acknowledgements

All patients signed written informed consent, and ethical consent was granted from the Committee for Ethical Review of Research Involving Human Subjects of the Hanzhong Central Hospital. Protective effect of esmolol on myocardial mitochondrial dysfunction in septic rats (2018SF-023).

Disclosure of conflict of interest

None.

Address correspondence to: Linjun Yang, Department of Emergency, Hanzhong People’s Hospital, Hanzhong 723000, Shaanxi, China. Tel: 86-0916-2681190; E-mail: bmlinjunyang@163.com

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