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Glaucocalyxin A Protects H9c2 Cells Against Hypoxia/Reoxygenation-Induced Injury Through the Activation of Akt/Nrf2/HO-1 Pathway

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Abstract

Myocardial infarction (MI) is one of the most serious cardiovascular diseases associated with myocardial ischemia/reperfusion (I/R) injury. Glaucocalyxin A (GLA) is a biologically active ent-kauranoid diterpenoid that has been found to ameliorate myocardial I/R injury in mice. However, the mechanism has not been fully investigated. In the present study, we aimed to investigate the effect of GLA on rat cardiomyocytes H9c2 cells exposed to hypoxia/reoxygenation (H/R). The results showed that GLA treatment improved cell viability of H/R-stimulated H9c2 cells. Administration with GLA suppressed the H/R-stimulated reactive oxygen species (ROS) production in H9c2 cells. GLA also elevated the activities of antioxidant enzymes, including superoxide dismutase and glutathione peroxidase in H/R-stimulated H9c2 cells. Moreover, GLA prevented H/R-stimulated cell apoptosis in H9c2 cells, as evidenced by increased bcl-2 expression, decreased bax expression, as well as reduced caspase-3 activity. Furthermore, GLA enhanced the activation of protein kinase B (Akt)/nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway in H9c2 cells exposed to H/R. Additionally, treatment with LY294002 reserved the protective effects of GLA on H/R-stimulated oxidative injury in H9c2 cells. In conclusion, these findings suggested that GLA protected H9c2 cells from H/R-stimulated oxidative damage, which was mediated by the Akt/Nrf2/HO-1 signaling pathway. Thus, GLA might be a promising therapeutic agent for the prevention and treatment of myocardial I/R.

Keywords

myocardial infarction (MI), glaucocalyxin A (GLA), myocardial ischemia/reperfusion (I/R) injury, oxidative stress, Akt/Nrf2/HO-1 signaling pathway

Introduction

Myocardial infarction (MI) is one of the most serious cardiovascular diseases which is resulted from the formation of plaques in the interior walls of arteries[1,2]. During the MI process, reduced blood flow to the heart causes a lack of oxygen supply and heart attack. MI is a leading cause of death among all cardiovascular diseases[3,4]. A comprehensive understanding of the molecular mechanisms may be helpful for the development of an effective intervention strategy for MI.

Acute myocardial ischemia/reperfusion (I/R) injury is a potent event in the development of MI[5]. A variety of pathological processes and mediators are involved in the I/R-related cell injury, including the generation of reactive oxygen species (ROS), changes in the pH, acute inflammatory response, and intracellular calcium overload[6]. Among these, overproduction of ROS is proposed to be the major intracellular event that occurred during I/R injury[7]. ROS are the most likely contributors to reperfusion-induced oxidative stress, which may contribute to myocardial injury and cardiomyocyte death

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through a variety of mechanisms, such as inducing the activation of proapoptotic pathways[8,9]. Therefore, attenuating I/R-mediated oxidative stress and apoptosis may be beneficial for preventing MI.

Glaucocalyxin A (GLA) is a biologically active ent-kauranoid diterpenoid that has been widely studied for some important biological activities, including antitumor, antibacterial, antioxidative, anti-inflammatory, anticoagulative, antithrombotic, and immune regulatory activities[10–13]. In addition, GLA might be an attractive candidate for improving the prognosis of acute MI by controlling cardiac fibrosis[14]. Additionally, GLA was reported to ameliorate myocardial I/R injury in mice through the attenuation of microvascular thrombosis[15]. However, the effects of GLA on I/R-mediated oxidative stress and apoptosis in cardiomyocytes remain unclear. The aim of this study was to investigate the role of GLA in H9c2 cells exposed to hypoxia/reoxygenation (H/R) and explore the underlying mechanism.

Materials and Methods

Cell Culture

The rat cardiomyocyte-derived H9c2 cell line (Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultivated in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% (v/v) penicillin/streptomycin (Gibco) at 37 °C in a 5% carbon dioxide (CO_2) atmosphere.

Establishment of H/R Model

To simulate myocardial I/R injury *in vitro*, H9c2 cells were cultured in a hypoxic environment with 1% oxygen (O_2), 94% nitrogen (N_2), and 5% CO_2 in modular gas chambers for 24 h, followed by reoxygenation for 2 h in a 21% O_2, 5% CO_2, and 74% N_2 incubator at 37 °C. Cells were pretreated with or without GLA for 2 h before H/R stimulation.

Cell Cytotoxicity Assay

After incubation with a series concentration of GLA (0, 5, 10, 20, 40 μM) for 24 h, the culture supernatants were collected for the determination of lactate dehydrogenase (LDH) leakage using a commercial LDH detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's directions.

Cell Viability Assay

Cell viability was evaluated using the cell counting kit-8 (CCK-8) solution assay (Dojindo, Kumamoto, Japan). Briefly, H9c2 cells were seeded at a density of 1×10^4 cells per well in 96-well plates. After various treatments, 10 μl of CCK-8 solution was added to the cells and incubated for 2 h following the manufacturer's specifications. The cell viability was assessed by measuring the optical density at 450 nm using a microplate reader (BioTek, Winooski, VT, USA).

Measurement of Cellular ROS Production

The production of intracellular ROS was tested by flow cytometry using 2′-7′dichlorofluorescin diacetate (DCFH-DA) as the fluorescence probe. In short, H9c2 cells with different treatments were washed with phosphate-buffered saline and then incubated with 10 μM DCFH-DA (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 30 min in the dark. H9c2 cells were then analyzed by the flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) using a 488 nm excitation filter and a 525 nm emission filter.

ELISA

H9c2 cells were lysed in lysis buffer (Invitrogen, Carlsbad, CA, USA) for 30 min at 4 °C, and then the cell lysates were collected for the determination of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities with relevant detection kits (Nanjing Jiancheng Bioengineering Institute) per the manufacturer's instructions.

Western Blot Analysis

Total and nuclear proteins of H9c2 cells were, respectively, extracted by protein extraction kits (Beyotime Institute of Biotechnology, Shanghai, China) following the manufacturer's protocol. After the determination of the protein concentration using a bicinchoninic acid kit (Beyotime), protein fractions were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then blotted onto polyvinylidene difluoride membranes (pore size: 0.45 μm; Millipore Billerica, MA, USA). The membranes were blocked with 5% (w/v) skim milk dissolved in Tris-buffered saline with tween 20 (TBST) buffer for 2 h at room temperature. Subsequently, membranes were incubated overnight at 4 °C with primary antibodies against nuclear factor erythroid 2-related factor 2 (Nrf2), lamin B1 (Abcam, Cambridge, MA, USA); β-actin, heme oxygenase 1 (HO-1), bax, bcl-2 (Invitrogen), protein kinase B (Akt) or p-Akt (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:1000 dilution in TBST. Then, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) for 1 h at room temperature. The signals were visualized by Image Lab software (Bio-Rad Laboratories, Hercules, CA, USA) after exposing the membranes to enhanced chemiluminescence solution (Millipore).

Caspase-3 Activity

The caspase-3 activity in H9c2 cells was analyzed using a caspase-3 activity assay kit (Beyotime Biotechnology, China) based on the catalytic activity of caspase-3 on Ac-DEVD-*p*NA. The formation of *p*NA was detected using

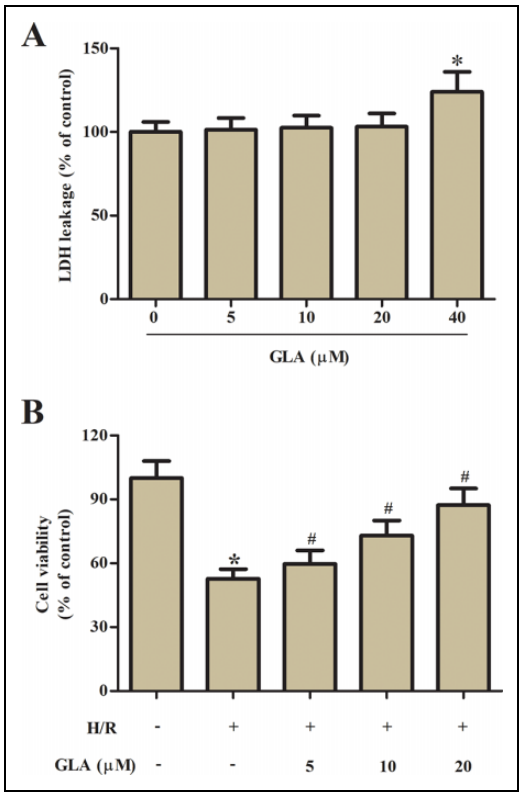

Fig. 1. Effect of GLA on cell viability of H9c2 cells. (A) LDH leakage assay was performed to assess the cytotoxic effect of GLA on H9c2 cells after incubation with a series concentration of GLA (0, 5, 10, 20, and 40 μM) for 24 h. (B) MTT assay was performed to assess the protective effect of GLA on H/R-stimulated H9c2 cells. H9c2 cells were preincubated with GLA (5, 10, and 20 μM) for 2 h, and then subjected to H/R stimulation. $^{*}P < 0.05$ indicates the significant difference compared with control H9c2 cells. $^{\#}P < 0.05$ indicates the significant difference compared with H/R-stimulated H9c2 cells. GLA: glaucocalyxin A; H/R: hypoxia/reoxygenation; LDH: lactate dehydrogenase; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

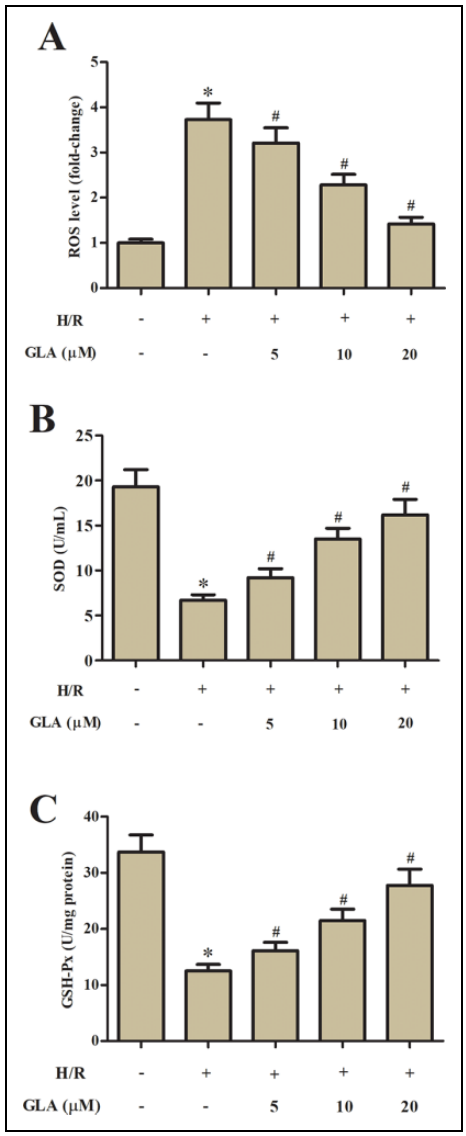

Fig. 2. Effect of GLA on oxidative stress in H9c2 cells. H9c2 cells were preincubated with GLA (5, 10, and 20 μM) for 2 h, and then subjected to H/R stimulation. (A) Flow cytometry was performed to evaluate the ROS production in H9c2 cells using the fluorescence probe DCFH-DA. (B, C) ELISA was carried out to assess the SOD and GSH-Px activities in H9c2 cells. $^{*}P < 0.05$ indicates the significant difference compared with control H9c2 cells. $^{\#}P < 0.05$ indicates the significant difference compared with H/R-stimulated H9c2 cells. DCFH-DA: 2′-7′dichlorofluorescin diacetate; GLA: glaucocalyxin A; GSH-Px: glutathione peroxidase; H/R: hypoxia/reoxygenation; ROS: reactive oxygen species; SOD: superoxide dismutase.

a microplate reader (BioTek) at 405 nm to reflect the caspase-3 activity.

Statistical Analysis

Results from three independent experiments were analyzed by SPSS software 19.0 (SPSS Inc, Chicago, IL, USA) and presented as means ± SD. Comparisons among groups were determined by one-way analysis of variance followed by the least significant difference test. Differences were proposed to be statistically significant when $P < 0.05$.

Results

GLA Improves the Cell Viability in H/R-Stimulated H9c2 Cells

To assess the cytotoxic effect of GLA on H9c2 cells, the cells were incubated with a series concentration of GLA (0, 5, 10, 20, 40 μM) for 24 h. LDH leakage assay showed that GLA at the concentration of 40 μM caused a significant increase in LDH leakage (Fig. 1A). Therefore, the concentrations of 5, 10, and 20 μM were selected in the following experiments. Subsequently, we examined the effect of GLA on cell viability of H/R-stimulated H9c2 cells. Results from 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay demonstrated that preincubation with

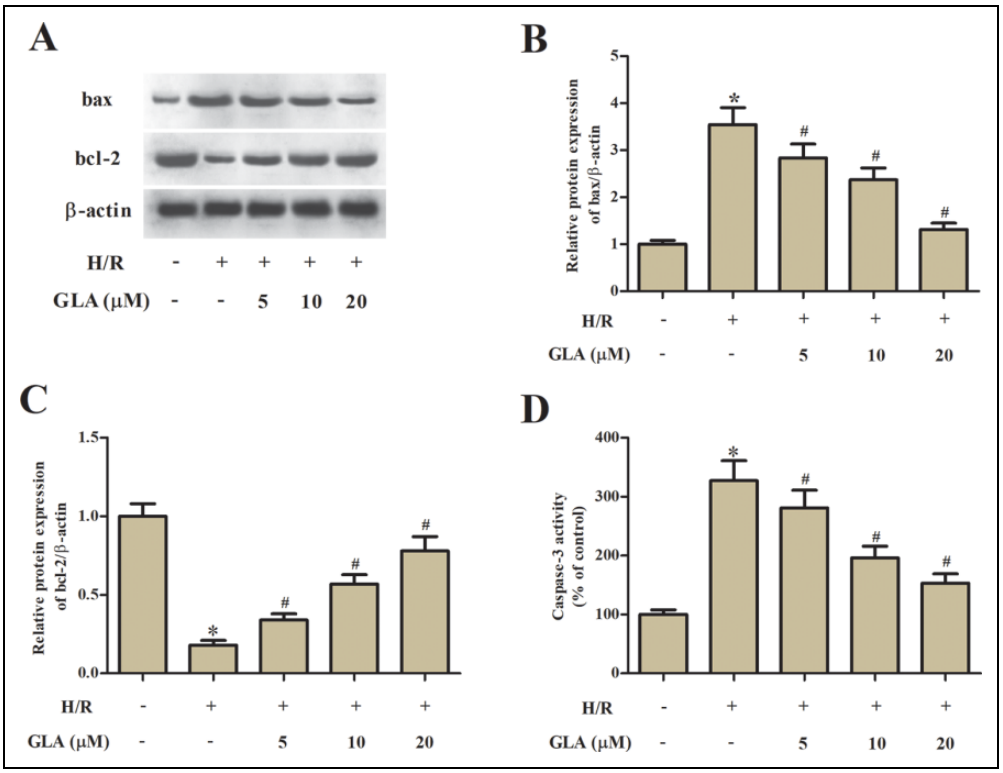

Fig. 3. Effect of GLA on apoptosis in H9c2 cells. H9c2 cells were preincubated with GLA (5, 10, and 20 μM) for 2 h, and then subjected to H/R stimulation. (A) Western blot analysis was performed to detect the expression levels of apoptosis-related proteins, including bax and bcl-2. (B, C) Quantification analysis of bax and bcl-2. (D) Colorimetric method was used to determine the caspase-3 activity with the substrate peptide Ac-DEVD-*p*NA. **P* < 0.05 indicates the significant difference compared with control H9c2 cells. #*P* < 0.05 indicates the significant difference compared with H/R-stimulated H9c2 cells. GLA: glaucocalyxin A; H/R: hypoxia/reoxygenation.

GLA (5, 10, and 20 μM) attenuated H/R-caused decreased cell viability of H9c2 cells (Fig. 1B).

GLA Represses Oxidative Stress in H9c2 Cells Exposed to H/R Treatment

As shown in Fig. 2A, the ROS level in H9c2 cells exposed to H/R treatment was markedly increased as compared with control H9c2 cells, while the induction of ROS production was mitigated by GLA. Moreover, H/R-caused decrease in SOD and GSH-Px activities, which were blocked by pretreatment with GLA (Fig. 2B, C).

GLA Suppresses Apoptosis in H9c2 Cells Exposed to H/R Treatment

Subsequently, cell apoptosis was assessed by detecting the expression levels of bax and bcl-2. H/R treatment caused a significant increase in bax expression and decrease in bcl-2 expression in H9c2 cells. The H/R-caused changes in the expression levels of bax and bcl-2 were prevented by GLA (Fig. 3A–C). In addition, the caspase-3 activity was significantly increased in H/R-stimulated H9c2 cells. However, the increased caspase-3 activity was decreased in H9c2 cells pretreated with GLA (Fig. 3D).

GLA Induces the Activation of Akt/Nrf2/HO-1 Signaling Pathway in H/R-Stimulated H9c2 Cells

It has been proven that the Akt/Nrf2/HO-1 signaling pathway is associated with the I/R-induced oxidative injury. To uncover the mechanism underlying the protective effect of GLA, the expression levels of nuclear Nrf2, HO-1, Akt, and p-Akt were detected using Western blot. As shown in Fig. 4, GLA treatment significantly induced the expression levels of p-Akt, nuclear Nrf2, and HO-1 in H/R-stimulated H9c2 cells.

Treatment with LY294002 Reserved the Effects of GLA on Cell Viability, Oxidative Stress, and Apoptosis in H/R-Stimulated H9c2 Cells

Then, the H9c2 cells were treated with LY294002 to block the activation of the Akt/Nrf2/HO-1 signaling pathway. Treatment with LY294002 caused a reduction in cell viability of H9c2 cells, as compared with the H9c2 cells pretreated with 20 μM GLA (Fig. 5A). The decreased ROS production caused by GLA was prevented by LY294002 (Fig. 5B). In addition, the GLA-caused decrease in caspase-3 activity was elevated after treatment with LY294002 (Fig. 5C).

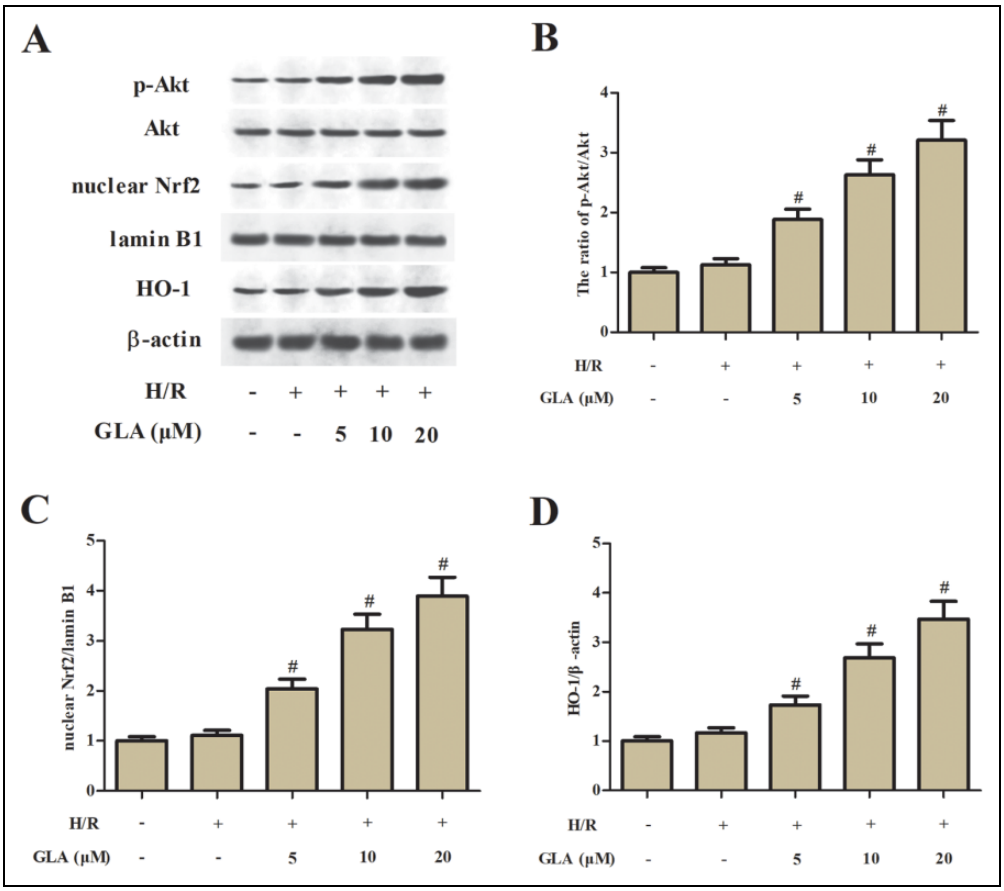

Fig. 4. Effect of GLA on the Akt/Nrf2/HO-1 signaling pathway in H9c2 cells. H9c2 cells were preincubated with GLA (5, 10, and 20 μM) for 2 h, and then subjected to H/R stimulation. (A) Western blot analysis was performed to detect the expression levels of p-Akt, Akt, nuclear Nrf2, and HO-1. (B) The ratio of p-Akt/Akt. (C) The ratio of nuclear Nrf2/lamin B1. (D) The ratio of HO-1/β-actin. $^{\#}P < 0.05$ indicates the significant difference compared with H/R-stimulated H9c2 cells. Akt: protein kinase B; GLA: glaucocalyxin A; HO-1: heme oxygenase-1; Nrf2: nuclear factor erythroid 2-related factor 2.

Discussion

In the current study, the results indicated that GLA protected H9c2 cells from H/R-stimulated oxidative damage, as evidenced by increased cell viability, decreased oxidative stress, and apoptosis. The protective effect of GLA was proved to be associated with the activation of the Akt/Nrf2/HO-1 signaling pathway.

GLA is a biologically active ent-kauranoid diterpenoid isolated from *Rabdosia japonica* var. *glaucocalyx*. GLA has been found to possess protective effect on myocardial I/R injury in mice. GLA administration significantly reduces infarct size, improves left ventricular ejection fraction, and left ventricular fractional shortening in mice subjected to myocardial I/R. The protective effect of GLA myocardial I/R injury is proposed to be mediated by the attenuation of microvascular thrombosis[15]. Based on the mechanism investigations, increased production of ROS and ROS-mediated oxidative damage during the I/R process play crucial roles in the development of I/R injury. Dong et al.[16] reported that pretreatment with GLA markedly suppresses transforming growth factor beta-1 (TGF-β1)-induced ROS level in hepatic stellate cells (HSCs), indicating that GLA may suppress the ROS production in response to TGF-β1 stimulation.

Thus, we aimed to explore the protective effect of GLA on ROS production and ROS-mediated oxidative damage during I/R injury. Our results showed that GLA treatment improved cell viability of H/R-stimulated H9c2 cells. GLA also suppressed the ROS production and elevated the activities of antioxidant enzymes, including SOD and GSH-Px. Additionally, GLA prevented H/R-stimulated cell apoptosis, as evidenced by increased bcl-2 expression and decreased bax expression and caspase-3 activity. These findings suggested that GLA protected H9c2 cells from H/R-stimulated oxidative damage. Our results demonstrated the mechanism underlying the protective effect of GLA on myocardial I/R injury, in addition to attenuation of microvascular thrombosis.

Nrf2 is known as an important transcription factor that plays an essential role in the cellular defense against inflammation and oxidative stress[17]. Under oxidative stress conditions, Nrf2 dissociates from Keap1 and translocates into the

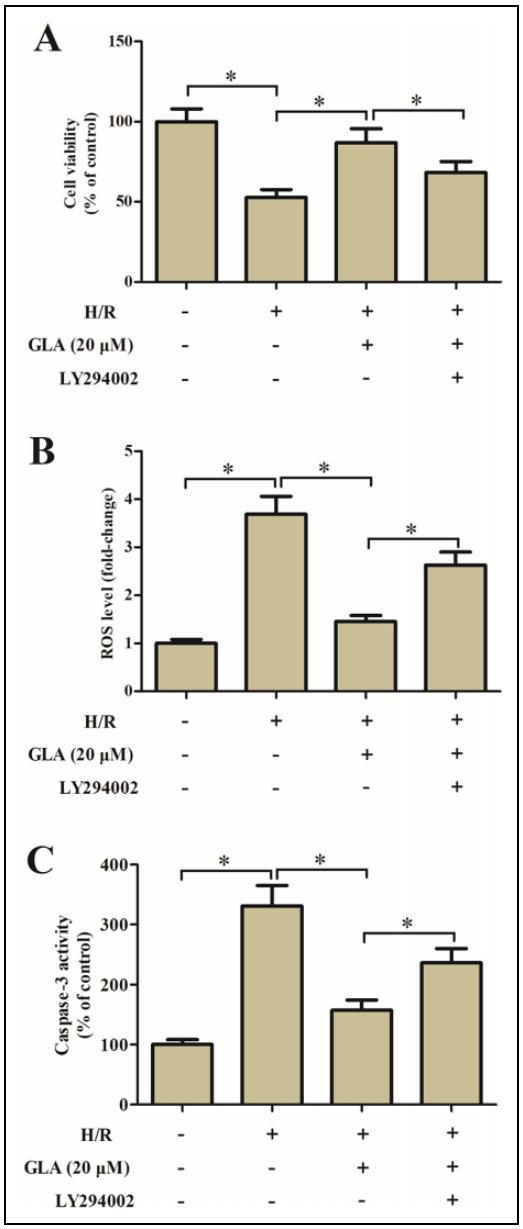

Fig. 5. Effect of LY294002 on cell viability, oxidative stress, and apoptosis in H9c2 cells. H9c2 cells were preincubated with GLA (20 μM) and/or LY294002 (10 μM) for 2 h and then subjected to H/R stimulation. (A) MTT assay was performed to assess cell viability. (B) Flow cytometry was performed to evaluate ROS production. (C) Colorimetric method was used to determine the caspase-3 activity. *$P < 0.05$. GLA: glaucocalyxin A; H/R: hypoxia/reoxygenation; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS: reactive oxygen species.

nucleus. The transcription factor then binds to the antioxidant response element and regulates the production of multiple antioxidative enzymes[18–20]. HO-1 is a well-known intracellular inducible phase II detoxifying enzyme that can be regulated byNrf2[21,22]. Nrf2/HO-1 signaling pathway is involved in cellular defense against various oxidative-inducing agents[22]. Increasing evidence has proven that the Nrf2/HO-1 signaling pathway represents a promising therapeutic intervention in preventing and attenuating I/R injury[23–25].

In addition to Nrf2 signaling, phosphatidylinositol 3-kinase (PI3K)/Akt is a key signaling pathway in governing the cellular defense system against oxidative injury[26]. Importantly, PI3K/Akt is upstream signaling of Nrf2 and associated with Nrf2 activation and HO-1 induction[27,28]. Indeed, the PI3K/Akt pathway has been observed to play a vital role in the Nrf2-mediated antioxidant response. Our results showed that GLA treatment significantly induced the expression levels of p-Akt, nuclear Nrf2, and HO-1 in H/R-stimulated H9c2 cells, implying that GLA induced the activation of Akt/Nrf2/HO-1 signaling pathway. Furthermore, inhibition of Akt signaling by LY294002 reserved the protective effects of GLA on H/R-stimulated oxidative injury in H9c2 cells. These findings suggested that the effects of GLA were mediated by Akt/Nrf2/HO-1 signaling pathway.

In conclusion, our findings demonstrated that GLA protects H9c2 cells against H/R-induced injury through the activation of the Akt/Nrf2/HO-1 pathway. Thus, GLA might be a promising therapeutic agent for the prevention and treatment of MI. Moreover, further *in vivo* investigations should be carried out in the future.

Ethical Approval

Ethical Approval is not applicable for this article.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

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