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Ginsenoside Rg1 protects cardiomyocytes from hypoxia-induced injury through the PI3K/AKT/mTOR pathway

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Aim: Myocardial ischemia (MI) is a leading cause of morbidity and mortality which makes the prevention and control of MI tremendously important. We aimed to explore the functional roles of ginsenoside (Gin) Rg1 in cardiomyocytes under hypoxia and to clarify underlying mechanisms. *Main methods*: Hypoxia-induced H9c2 cell injury was evaluated by alterations of cell viability, apoptosis and autophagy. Then, effects of Gin Rg1 on hypoxia-induced cell injury were measured. The activation of the phosphatidylinositol-3-kinase (PI3K)/AKT/mechanistic target of rapamycin (mTOR) pathways as well as expression of hypoxia-inducible factor 1α (HIF-1α) was determined with or without addition of PI3K or mTOR inhibitor. Finally, the effects of Gin Rg1 on rat ischemia/reperfusion (I/R) injury and underlying mechanism were studied. *Key findings*: First of all, hypoxia was identified to induce a decrease in cell viability and to increase cell apoptosis and autophagy. Then, these hypoxia-induced alterations were ameliorated by Gin Rg1, which had no effect on cell viability under normoxia. Subsequently, the phosphorylated levels of key kinases in the PI3K/AKT/mTOR pathways as well as expression of HIF-1α were all elevated by Gin Rg1. Activation of the PI3K/AKT/mTOR pathways and HIF-1α expression were inhibited by PI3K inhibitor, and activation of mTOR pathway and HIF-1α expression were inhibited by mTOR inhibitor. More *in vivo* experiments proved that Gin Rg1 ameliorated rat I/R injury through activating the PI3K/AKT/mTOR pathways. *Significance*: Gin Rg1 protected cardiomyocytes from hypoxia-induced cell injury by upregulating HIF-1α through activation of the PI3K/AKT/mTOR pathways.

1. Introduction

Myocardial ischemia (MI) is considered as a leading cause of morbidity and mortality that causes approximately 12 million deaths worldwide every year (Mozaffarian et al. 2015; Wang et al. 2017). Imbalance between oxygen supply (transported by coronary blood) and the myocardial metabolic demand is the widely acceptable reason of MI (Reshma et al. 2016). If the sudden occlusion of the coronary artery is prolonged more than 20 min, permanent damage to the myocardium can be observed, and after which, myocardium is substituted by fibrous scar tissues, resulting in heart failure (Ibanez et al. 2015). Although timely thrombolytic therapy or primary percutaneous coronary intervention ameliorates MI, the reperfusion itself may induce a secondary injury (Hausenloy and Yellon 2013). Arrhythmia, heart failure and myocardial infarction, which strongly threaten human life, are common consequences of MI, making the prevention and control of MI tremendously important.

Ginsenosides (Gins), extracted from ginseng, are triterpenoid saponins possessing four-ring steroidal structure, sugar moieties and an aliphatic side chain (Ahmed et al. 2016; Ardah et al. 2015). According to the difference of sugar moieties, Gins are categorized into three groups, including panaxadiol group, panaxatriol group and oleanolic acid group, and the most frequently investigated ones are Rg1, Rh2, Rb1, Rd, *etc*. (Kim 2013). The influence on ion channels and receptors underlies the neuroprotection of Gins, and Gins have been reported to possess therapeutic effects on multiple diseases, such as stroke, cancer, glaucoma and cardiovascular disease (Ahmed et al. 2016; Nah 2014). As a main member of Gins, Gin Rg1 has been reported to protect mice with Alzheimer's disease using a UPLC/MS-based metabolomics method (Li et al. 2016). Protective effects of Gin Rg1 on lipopolysaccharide-induced sepsis were reported (Su et al. 2015). The activity of Gin Rg1 in extension of blood clotting time has been verified by Li et al. (2013). Although Gin RK3 and Rb1 have been proved to protect H9c2 cells from hypoxia/reoxygenation-induced apoptosis (Ai et al. 2015; Sun et al. 2013), the functional roles of Gin Rg1 in H9c2 cells under hypoxia remain unclear.

Autophagy is a catabolic process, by which eukaryotic cells are self-digested through lysosome or vacuole (Levine and Klionsky 2017). Accumulating evidence has proved that autophagy is involved in the process of MI, and diverse factors are reported to alleviate MI through inhibiting autophagy (Li et al. 2017; Liu et al. 2016). In our study, the effects of Gin Rg1 (50-200 μM) on hypoxia-induced cell injury and autophagy of H9c2 cells as well as the underlying mechanism were explored, which may provide innovative therapeutic strategies for the treatment of MI. Moreover, the effects of Gin Rg1 on rats with ischemia/reperfusion (I/R) injury were also studied.

2. Investigations and results

2.1. Hypoxia induced cell injury of H9c2 cells

Cell viability of H9c2 cells under normoxia and hypoxia conditions were assessed. Along with the prolongation of the culture time, cell viability was significantly increased at 24 h and 48 h after incubation under normoxia compared with cells immediately after incubation (both $P < 0.05$, Fig. 1A). Conversely, cell viability was significantly reduced at 12 h ($P < 0.05$), 24 h ($P < 0.05$) and 48 h ($P < 0.01$) after incubation under hypoxia as compared to the cells immediately after incubation. The percentage of apoptotic cells was markedly increased at 12 h ($P < 0.05$), 24 h ($P < 0.05$) and 48 h ($P < 0.01$) under hypoxia compared with cells at 0 h (Fig. 1B). Likewise, with the increase of hypoxic exposure time, anti-apoptotic Bcl-2 was downregulated, whereas pro-apoptotic

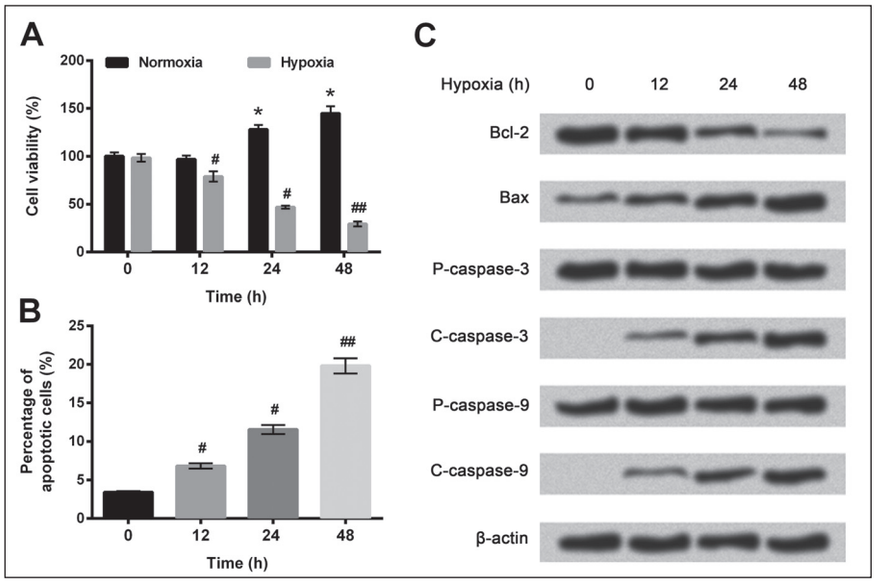

Fig. 1: Hypoxia induced decrease of cell viability and increase of cell apoptosis in H9c2 cells. Cells were cultured under normoxia or hypoxia for 48 h. A. Cell viability by a Cell Counting Kit-8 assay. B. Cell apoptosis by flow cytometry. C. Expression of apoptosis-associated proteins by Western blot analysis. Data presented are the mean ± SEM of at least three independent experiments. * indicates significance of difference compared with cells at 0 h post-incubation under normoxia. *, $P < 0.05$. # indicates significance of difference compared with cells at 0 h post-incubation under hypoxia. #, $P < 0.05$; ##, $P < 0.01$. Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein; C-, cleaved; P-, pro.

Bax, cleaved caspase-9 and cleaved caspase-3 were all upregulated, suggesting hypoxia induced cell apoptosis through mitochondrial- and caspase-dependent pathways (Fig. 1C). Results suggested that hypoxia could induce cell injury of H9c2 cells.

2.2. Hypoxia induced autophagy of H9c2 cells

To estimate the alteration of autophagy after hypoxia, expression of autophagy-related proteins was assessed. In Fig. 2A-B, ratio of LC3-II/LC3-I and expression of Beclin-1 were significantly upregulated ($P < 0.05$ or $P < 0.001$) whereas expression of p62 was remarkably downregulated ($P < 0.01$ or $P < 0.001$) as the increase of hypoxic exposure time when compared to cells without hypoxia treatment. Results suggested that autophagy of H9c2 cells was promoted by hypoxia treatment.

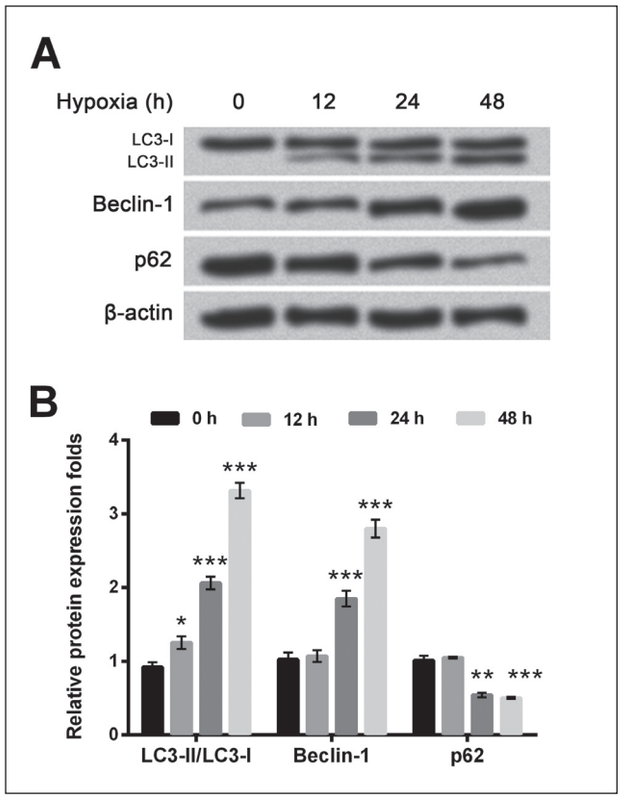

Fig. 2: Hypoxia induced autophagy in H9c2 cells. Cells were cultured under hypoxia for 48 h, and expressions of autophagy related proteins was assessed by Western blot analysis. A. Protein expression representative. B. Relative expression folds. Data presented are the mean ± SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. LC3, microtubule-associated protein 1 light chain-3; p62, p62/sequestosome 1.

2.3. Gin Rg1 ameliorated hypoxia-induced cell injury of H9c2 cells

The effects of Gin Rg1 on hypoxia-induced cell injury of H9c2 cells were determined. As shown in Fig. 3A, cell viability under normoxia remained unchanged after Gin Rg1 treatment. However, in cells which were maintained under hypoxia for 24 h, addition of 150 μM or 200 μM Gin Rg1 dramatically elevated cell viability ($P < 0.05$, Figure 3B) and significantly reduced cell apoptosis ($P < 0.05$, Fig. 3C) when compared to cells treated without Gin Rg1. Meanwhile, anti-apoptotic Bcl-2 was upregulated, whereas pro-apoptotic Bax, cleaved caspase-9 and cleaved caspase-3 were all downregulated by Gin Rg1 (100-200 μM) in cells under hypoxia (Fig. 3D). Results illustrated that hypoxia-induced cell injury could be ameliorated by Gin Rg1 treatments.

2.4. Gin Rg1 ameliorated hypoxia-induced autophagy of H9c2 cells

Alteration of autophagy after treatments of hypoxia and Gin Rg1 in H9c2 cells was further assessed. In Fig. 4A-B, when compared to hypoxia-treated cells, ratio of LC3-II/LC3-I was significantly decreased by Gin Rg1 (50-200 μM; $P < 0.001$) and expression of Beclin-1 was dramatically downregulated by Gin Rg1 (100-200 μM; $P < 0.001$). The expression of p62 was markedly upregulated by Gin Rg1 (150-200 μM; $P < 0.05$) compared with hypoxia-treated cells. Western blot analysis strongly stated that hypoxia-induced increase of autophagy was ameliorated by Gin Rg1 in H9c2 cells.

2.5. Gin Rg1 activated the PI3K/AKT/mTOR pathways under hypoxic condition

To reveal the underlying mechanisms of Gin Rg1, phosphorylated levels of key kinases involved in the PI3K/AKT/mTOR pathways were estimated. As shown in Fig. 5A-B, phosphorylated levels of PI3K, AKT and p70S6K were all significantly increased by Gin Rg1 (100-200 μM; $P < 0.05$ or $P < 0.001$). Meanwhile, phosphorylated level of mTOR was observably increased by Gin Rg1 (150-200 μM; $P < 0.001$). Immunoblotting results illustrated the PI3K/AKT/mTOR pathways were activated by Gin Rg1 in hypoxic H9c2 cells.

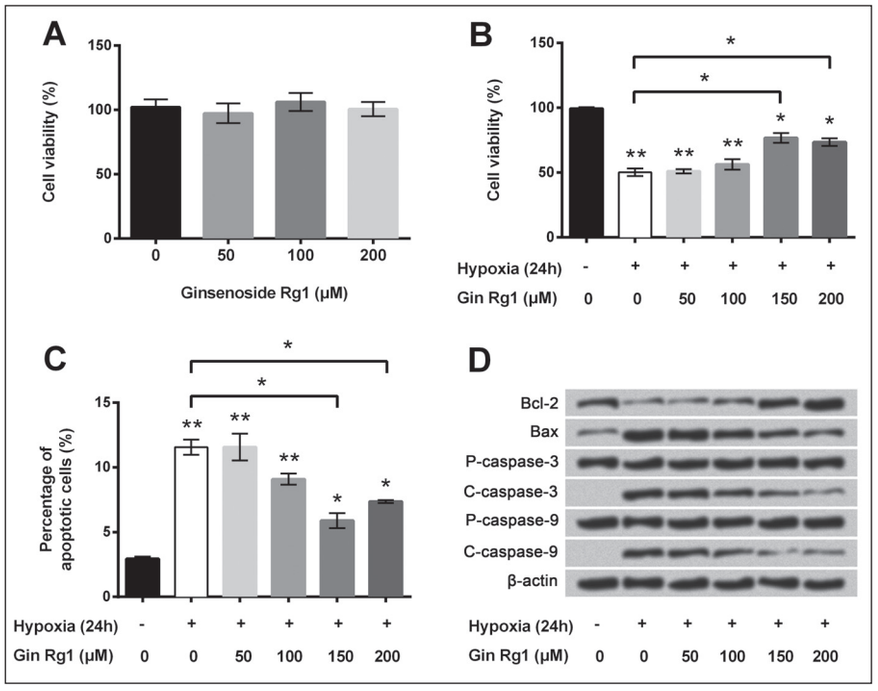

Fig. 3: Hypoxia-induced cell injury was ameliorated by ginsenoside (Gin) Rg1 in H9c2 cells. Cells were incubated with Gin Rg1 (0-200 μM) and cultured under hypoxia for 24 h. A-B. Cell viability by a Cell Counting Kit-8 assay. C. Cell apoptosis by flow cytometry. D. Expression of apoptosis-associated proteins by Western blot analysis. Data presented are the mean ± SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$. Bcl-2, B cell lymphoma-2; Bax, Bcl-2-associated X protein; C-, cleaved; P-, pro.

2.6. Gin Rg1 protected H9c2 cells from hypoxia-induced cell injury by upregulation of HIF-1α through activating the PI3K/AKT/mTOR pathways

Expression of HIF-1α in H9c2 cells treated with hypoxia and Gin Rg1 was tested. After hypoxia treatment, HIF-1α was markedly upregulated at both mRNA and protein levels ($P < 0.05$), which was further upregulated by addition of Gin Rg1 compared with the hypoxia group ($P < 0.05$, Fig. 6A-B). Moreover, p-PI3K, p-AKT, p-mTOR, p-p70S6K and HIF-1α were all downregulated by addition of LY294002 (a PI3K inhibitor) in H9c2 cells treated with hypoxia and 150 μM Gin Rg1 (Fig. 6C). Addition of rapamycin (an mTOR inhibitor) reduced expressions of p-mTOR, p-p70S6K and HIF-1α, and while expressions of p-PI3K and p-AKT were not affected. Synergistic effects of LY294002 and rapamycin were consistent with mono-addition of LY294002. Results declared that Gin Rg1 protected H9c2 cells from hypoxia-induced cell injury by upregulation of HIF-1α through activating the PI3K/AKT/mTOR pathways.

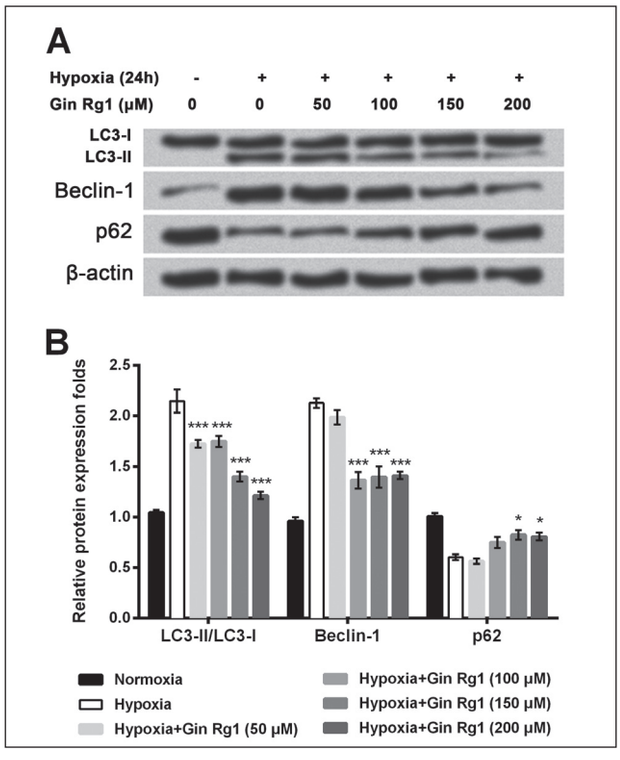

Fig. 4: Hypoxia-induced autophagy was ameliorated by ginsenoside (Gin) Rg1 in H9c2 cells. Cells were incubated with Gin Rg1 (0-200 μM) and cultured under hypoxia for 24 h. Expressions of autophagy-related proteins were assessed by Western blot analysis. A. Protein expression representative. B. Relative expression folds. Data presented are the mean ± SEM of at least three independent experiments. *, $P < 0.05$; ***, $P < 0.001$. LC3, microtubule-associated protein 1 light chain-3; p62, p62/sequestosome 1.

2.7. Gin Rg1 protected heart from I/R injury through activating the PI3K/AKT/mTOR pathways

Finally, the *in vivo* protective role of Gin Rg1 in rats was studied. Compared with the sham group, I/R caused significant increases of infarct size (Fig. 7A), CK activity ($P < 0.001$, Fig. 7B) and LDH activity ($P < 0.001$, Fig. 7C) and a remarkable decrease of NO level ($P < 0.01$, Fig. 7D). Western blot results showed that I/R downregulated phosphorylation of eNOS ($P < 0.01$) while upregulated iNOS expression ($P < 0.001$, Fig. 7E) relative to the sham group. Those alterations were all dramatically ameliorated by Gin Rg1 administration as compared to the I/R group ($P < 0.01$ or $P < 0.001$). Moreover, the effects of Gin Rg1 on those factors were all significantly reversed by LY294002 or rapamycin injection when compared to the I/R + Gin Rg1 group. Taken together, we concluded that Gin Rg1 could protect heart from I/R injury through activating the PI3K/AKT/mTOR pathways in rats.

3. Discussion

The mortality from MI remains high, thus, it is urgently needed to explore effective therapeutic methods. In our study, cell injury was evaluated through the decrease of cell viability and the increases of cell apoptosis and autophagy. These alterations induced by hypoxia could all be reversed by Gin Rg1 treatment, indicating a possible protective role of Gin Rg1 in hypoxic H9c2 cells. Phosphorylated levels of PI3K, AKT, mTOR and p70S6K were all increased by Gin Rg1 treatment, along with the upregulation of HIF-1α, which could be reversed by addition of PI3K or an mTOR inhibitor. More *in vivo* experiments consolidated that the activation of the PI3K/AKT/mTOR pathways were responsible for the protection of Gin Rg1 in myocardial I/R injury.

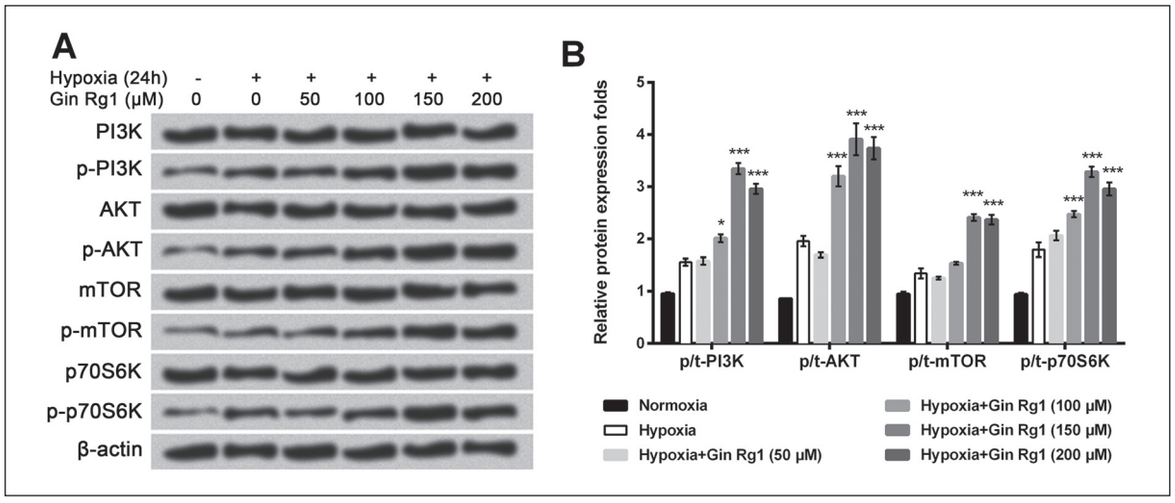

Fig. 5: The PI3K/AKT/mTOR signaling pathways were activated by ginsenoside (Gin) Rg1 in H9c2 cells. Cells were incubated with Gin Rg1 (0-200 μM) and cultured under hypoxia for 24 h. Expressions of key proteins in the PI3K/AKT/mTOR signaling pathways was assessed by Western blot analysis. A. Protein expression representative. B. Relative expression folds. Data presented are the mean ± SEM of at least three independent experiments. *, $P < 0.05$; ***, $P < 0.001$. PI3K, phosphatidylinositol-3-kinase; mTOR, mechanistic target of rapamycin; p-, phosphorylated.

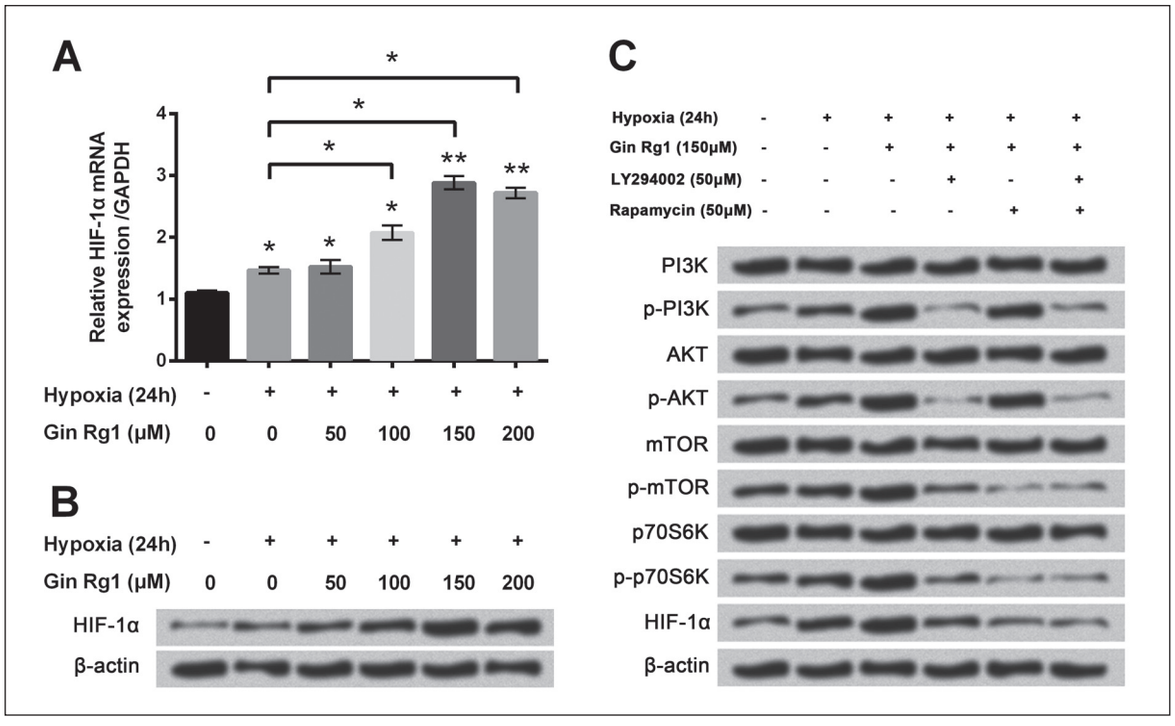

Fig. 6: Ginsenoside (Gin) Rg1 functioned through modulation of hypoxia-inducible factor 1α (HIF-1α) via the PI3K/AKT/mTOR signaling pathways in H9c2 cells. Cells were incubated with Gin Rg1 (0-200 μM) with or without PI3K inhibitor (LY294002) and mTOR inhibitor (Rapamycin), and cultured under hypoxia for 24 h. A. mRNA expression level of HIF-1α by quantitative reverse transcription PCR. B. Protein expression level of HIF-1α by Western blot analysis. C. Expression of key proteins in the PI3K/AKT/mTOR signaling pathways and HIF-1α after addition of PI3K or mTOR inhibitor. Data presented are the mean ± SEM of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$. PI3K, phosphatidylinositol-3-kinase; mTOR, mechanistic target of rapamycin; p-, phosphorylated.

Hypoxia contributes to cardiac dysfunction due to its effects on cell injury and cell apoptosis (Li 2015). Under hypoxia, mitochondrion is the rapid sensor, along with the permeabilization of the mitochondrial outer membrane, resulting in the release of pro-apoptotic proteins and the activation of downstream caspase cascade (Ullrich and Schildknecht 2014; Zheng et al. 2016). In our study, cell viability was significantly reduced while cell apoptosis was markedly increased, suggesting that hypoxia induced cell injury. In addition, with the increase of hypoxia exposure time, pro-apoptotic Bax was upregulated but anti-apoptotic Bcl-2 was downregulated, followed by upregulations of active caspase-9 and caspase-3, supporting that hypoxia induced cell injury through intrinsic apoptosis pathway. Subsequent experiments identified hypoxia-induced changes of cell viability and apoptosis were attenuated by Gin Rg1. Considering that Gin Rg1 had no effects on viability of cells under normoxia, we concluded that the hypoxia-induced cell injury was ameliorated by Gin Rg1 through modulating the intrinsic apoptosis pathway.

Autophagy is considered as the type 2 programmed cell death, which commits cells to undergo autophagic cell death (Bhutia et al. 2013). During autophagy, LC3-I is initially converted to LC3-II, which is located at the phagophore membrane (Martinez

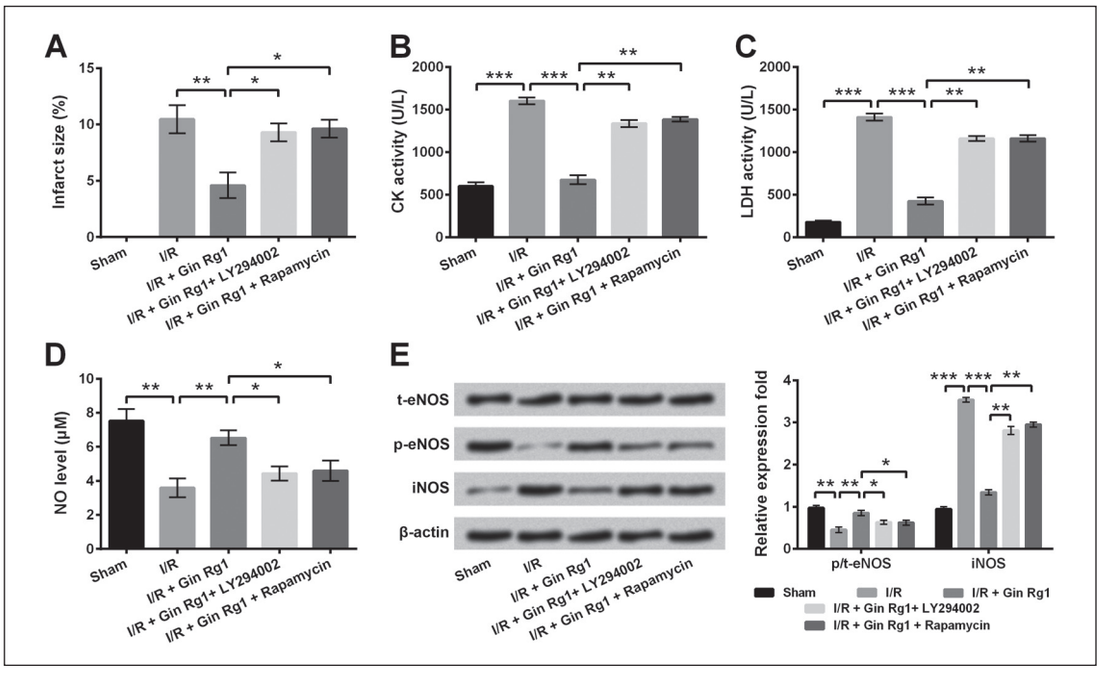

Fig. 7: Ginsenoside (Gin) Rg1 protected cardiocytes from ischemia/reperfusion (I/R)-induced injury through activating the PI3K/AKT/mTOR signaling pathways. Rats were randomly assigned into five groups. A. Infarct size by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Activities of creatine kinase (CK; B) and lactate dehydrogenase (LDH; C), and level of nitrate/nitrite (D) were determined by commercial assay kits. **E.** Protein expression of NO synthases by Western blot analysis. Data presented are the mean ± SEM of eight independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. t-eNOS, total endothelial NO synthase; p-eNOS, phospho-endothelial NO synthase; iNOS, inducible NO synthase.

et al. 2015). LC3-II interacts with adaptor molecules, such as p62 present on the protein and organelles which are ubiquitinated or damaged, and guides them into the lysosome, followed by degradation of proteins or organelles (Mukhopadhyay et al. 2014). Besides, Beclin-1, another marker of autophagy, is reported to be involved in the formation of autophagosomes (Fekadu and Rami 2016). In our study, the ratio of LC3-II/LC3-I as well as expression of Beclin-1 was increased but expression of p62 was downregulated after hypoxia, suggesting the inducible influence of hypoxia on autophagy. Subsequently, alterations of these proteins in hypoxic H9c2 cells were abolished by addition of Gin Rg1, which stated the inhibitory effects of Gin Rg1 on hypoxia-induced autophagy. The inhibitory effects of Gin Rg1 on apoptosis and autophagy of H9c2 cells were consistent with previous results (Zhang et al. 2012).

The involvement of signaling pathways is investigated to further explore the underlying mechanisms. The PI3K/AKT/mTOR pathways are essential for cellular functions, including cellular growth, proliferation and migration (Mabuchi et al. 2015). Recent studies found that microRNA-21 could protect H9c2 cells from hypoxia/reoxygenation injury through repressing autophagy via the PI3K/AKT/mTOR pathways (Huang et al. 2017). Another study also proved that jujuboside A protects H9c2 cells against cell injury *via* activating the PI3K/AKT/mTOR pathways (Han et al. 2016). In our study, we found that Gin Rg1 could activate the PI3K/AKT/mTOR pathways.

HIF-1α is a main factor that responses to hypoxia and it has been identified to regulate expressions of various target genes involved in cell proliferation, apoptosis and autophagy (Mucaj 2012). It was claimed that cell injury of mesenchymal stem cells under oxygen-glucose deprivation was ameliorated by HIF-1α, along with involvements of autophagy and activation of the PI3K/AKT/mTOR pathways (Lv et al. 2017). In our study, expression of HIF-1α was dramatically upregulated by addition of Gin Rg1 in hypoxic H9c2 cells, proving the involvements of HIF-1α in the modulation of Gin Rg1. Further studies showed activation of the PI3K/AKT/mTOR pathways, which was induced by Gin Rg1, was suppressed by a PI3K inhibitor, along with downregulation of HIF-1α. Likewise, activation of the mTOR pathway, which was induced by Gin Rg1, was suppressed by mTOR inhibitor, along with downregulation of HIF-1α. Accordingly, we might interestingly figure out that Gin Rg1 protected H9c2 cells from hypoxia-induced cell injury by up-regulating HIF-1α through activation of the PI3K/AKT/mTOR pathways.

The possible protective role of Gin Rg1 *in vivo* was verified in rats. In our study, Gin Rg1 exerted an effective cardioprotective effect, which was expressed as reduction in myocardial infarct size and activities of CK and LDH. NO is considered as a potent cardioprotective-signaling molecule, and increase of NO level leads to alleviation of myocardial I/R injury (Calvert and Lefer 2009). The catalytic action of NOS enzymes including eNOS and iNOS is closely associated with NO level (Lee 2017). In a previous study, activation of eNOS in myocardial tissues of rats underwent I/R was inhibited whereas iNOS level in I/R injured rat hearts was increased (Zhou et al. 2011). In our study, Gin Rg1 increased phosphorylation of eNOS and downregulated iNOS, consolidating the cardioprotective role of Gin Rg1. Moreover, the *in vivo* effects of Gin Rg1 on I/R injured rats were reversed by PI3K or mTOR inhibitor, verified that Gin Rg1 functioned through activating the PI3K/AKT/mTOR pathways.

In conclusion, Gin Rg1 could protect myocardial cells against hypoxia-induced injury both *in vitro* and *in vivo*. In hypoxic H9c2 cells, HIF-1α was further upregulated by Gin Rg1 through activating the PI3K/AKT/mTOR pathways, which might be responsible for the cardioprotection. This study identified that Gin Rg1 might be a potential drug for MI treatments.

4. Experimental

4.1. Cell culture and treatment

H9c2 cell line derived from rat embryonic ventricular cardiomyocytes was obtained from the American Type Culture Collection (Manassas, VA, USA). H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) and maintained in a humidified incubator filled with an atmosphere of 95 % air and 5 % CO_2 at 37 °C. Hypoxia treatments were performed in a hypoxia chamber (Thermo, Dreieich, Germany) which infused with a gas mixture of 94% N_2, 5% CO_2 and 1% O_2. For treatments of Gin Rg1, H9c2 cells were incubated in DMEM containing various

concentrations Gin Rg1 (0-200 μM). For inhibition of the PI3K/AKT and mTOR pathway, LY294002 (50 μM; Sigma-Aldrich, St. Louis, MO, USA) and rapamycin (50 μM; Sigma-Aldrich) were added into the culture medium, respectively.

4.2. Cell counting kit-8 (CCK-8) assay

Cell viability was assessed by using CCK-8 assay. Briefly, treated cells (5×10^3 cells/well) were plated in a 96-well plate and cultured at 37 °C. After treatments, 10 μl of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added into the medium of each well, and then the mixture was incubated at 37 °C. After 1 h of incubation, the absorbance of each well was read by a Microplate Reader (Bio-Rad, Hercules, CA, USA) at 450 nm.

4.3. Cell apoptosis assay

The percentage of apoptotic cells was determined by dual staining with fluorescein isothiocynate (FITC)-conjugated Annexin V and propidium iodide (PI). Briefly, after treatments, cells were washed in pre-cold phosphate buffered saline (PBS) and resuspended in binding buffer. Following addition of 5 μl Annexin V and 5 μl PI, both from the Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme, Jiangsu, China), the mixture was incubated for 10 min in the dark at room temperature. The apoptotic cells were identified by using a FACS can (Beckman Coulter, Fullerton, CA, USA), and the data were analyzed by FlowJo software (Tree Star, San Carlos, CA, USA).

4.4. Quantitative reverse transcription PCR (qRT-PCR)

The TRIzol reagent (Invitrogen) along with DNase I (Promega, Sunnyvale, CA, USA) was used for the isolation of total RNA according to the manufacturer's instructions. Then, RNA was reversely transcribed into cDNA by using the MultiScribe Reverse Transcriptase (Thermo Fisher Scientific Inc., Waltham, MA, USA) and random hexamer primers. The reverse transcription conditions were 10 min at 25 °C, 30 min at 48 °C, and a final step of 5 min at 95 °C. According to the instructions of supplier, 50 ng cDNA was utilized for qPCR using *Power* SYBR Green PCR Master Mix (Thermo Fisher Scientific Inc.) on a 7900HT Fast Real-Time System (Applied Biosystems). Primers, synthesized by Sangon Biotechnology Co., Ltd., (Shanghai, China), were HIF-α forward, 5'-ACAGC ACATT CACAG CTCCC CA-3', reverse 5'-TGTGG CTACC ATGTA CTGCT GGC-3'; β-actin forward, 5'-TCAGG TCATC ACTAT CGGCA AT-3', reverse 5'-AAAGA AAGGG TGTAA AACGC A-3' (Lin, Pan, Ruan et al. 2015). Relative expression fold of HIF-1α was calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) and β-actin was acted as the housekeeping gene.

4.5. Animals and groupings

Male adult Sprague-Dawley rats (n = 40) weighing 260-320 g were purchased from the Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). Four rats were housed in each cage with controlled conditions (12 h light/dark cycle, 22±2 °C, 40-60% humidity) and free access to food and water. Rats were randomly assigned to five groups (n = 8 per group), including sham, I/R, I/R + Gin Rg1, I/R + Gin Rg1 + LY294002 and I/R + Gin Rg1 + rapamycin groups. For those undergoing I/R, rats were given Gin Rg1 (10 mg/kg) or same volume of saline by oral gavage at 60 min before ischemia. For pharmaceutical blockages, rats were given LY294002 (0.25 mg/kg, s.c.) or Rapamycin (10 μg/kg, i.v.) prior to the oral administration of Gin Rg1 (10 mg/kg). All experimental procedures and protocols were approved by the Institutional Animal Care and Use Committee of Binzhou People's Hospital.

4.6. I/R protocol in rat heart

The I/R injury was constructed in rats as described previously (Wu et al. 2011). In brief, rats were anesthetized with pentobarbital sodium (Sigma-Aldrich, 40 mg/kg, i.p.). Then, the heart was exteriorized with a left thoracic incision, and a slipknot (6-0 silk) was passed around the left anterior descending (LAD) coronary artery. The slipknot was released after 30 min of ischemia, and the rats were received 180 min of reperfusion.

4.7. Measurements of infarct size

After reperfusion, the heart was quickly removed and stored at -80°C. Then, the heart was cut into small slices (1 mm) and stained by 1% 2,3,5-triphenyltetrazolium chloride (TTC) solution for 15 min. After that, the slices were placed in 4% formaldehyde solution overnight. The infarct area (white) and TTC stained area (red) were analyzed with a digital imaging system by computer. The infarct size was expressed as a percentage of infarct area (white) divided the total area (white + red).

4.8. Measurements of creatine kinase (CK) activity, lactate dehydrogenase (LDH) activity and nitrate/nitrite

After reperfusion, the blood from the carotid artery was collected. The activities of CK and LDH were determined by commercial assay kits (A032 and A020-2, respectively) from Nanjing Jiancheng Bioengineering Institute (Nanjing, China), according to the recommendation of suppliers. The concentration of nitrate/nitrite was assessed by using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) following the manufacturer's instructions. The absorbance at 540 nm was measured by a Microplate Reader.

4.9. Western blot analysis

Total soluble proteins of treated cells and myocardial tissues after reperfusion were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with 1 mM PMSF (Sigma-Aldrich). Following quantification with a BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA), equivalent proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Those PVDF membranes were blocked in 5% non-fat milk at room temperature for 1 h. Immunoblotting was performed by incubating the membranes overnight at 4 °C with primary antibody against B cell lymphoma-2 (Bcl-2, ab196495), Bcl-2-associated X protein (Bax, ab182733), pro caspase-3 (ab90437), cleaved caspase-3 (ab49822), microtubule-associated protein 1 light chain-3 (LC3; ab48394), Beclin-1 (ab62557), p62/sequestosome 1 (p62; ab155686), total (t)-phosphatidylinositol-3-kinase (t-PI3K; ab133595), phospho (p)-PI3K (ab182651), t-mechanistic target of rapamycin (t-mTOR, ab2732), p-mTOR (ab137133), inducible NO synthase (iNOS; ab204017), β-actin (ab8229, all Abcam, Cambridge, UK), pro caspase-9 (9508), cleaved caspase-9 (9507), t-AKT (9272), p-AKT (9271), t-p70S6K (9202), p-p70S6K (9205), hypoxia-inducible factor 1α (HIF-1α; 14179, all Cell Signaling Technology, Beverly, MA, USA), t-endothelial NO synthase (t-eNOS; SAB4502016) or p-eNOS (SAB4504393, both Sigma-Aldrich). Subsequently, PVDF membranes were incubated with secondary antibody marked by horseradish peroxidase at room temperature for 1 h. Then, proteins in the PVDF membranes were visualized by chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, New Jersey, USA). The signals were captured and the intensity of the bands was quantified using Image Lab™ software (Bio-Rad).

4.10. Statistical analysis

All experiments were repeated three times. The results were presented as the mean±standard error of the mean (SEM). Statistical analysis was performed using Graphpad Prism 5 software (GraphPad, San Diego, CA, USA). The *P*-values were calculated using the one-way analysis of variance (ANOVA). A $P < 0.05$ was considered as a significant difference.

Conflicts of interest: None declared.

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