



Ginsenoside Rg3 protects heart against isoproterenol-induced myocardial infarction by activating AMPK mediated autophagy

Gui-Zhi Sun¹, Fan-Ji Meng¹, Huai-Qiu Cai², Xue-Bo Diao³, Bo Zhang¹, Xiu-Ping Bai¹

¹Department of Cardiology, ²Department of Ultrasonography, The Fourth Affiliated Hospital of Harbin Medical University, Harbin 150001, China;

³Department of Gynaecology and Obstetrics, The First Affiliated Hospital of Harbin Medical University, Harbin 150001, China

Contributions: (I) Conception and design: B Zhang, GZ Sun; (II) Administrative support: XP Bai; (III) Provision of study materials or patients: FJ Meng, HQ Cai; (IV) Collection and assembly of data: XB Diao; (V) Data analysis and interpretation: B Zhang, GZ Sun; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Bo Zhang; Xiu-Ping Bai. Department of Cardiology, The Fourth Affiliated Hospital of Harbin Medical University, Harbin 150001, China; Email: zhangbo_zobe@163.com; baixiuping@medmail.com.cn.

Background: Panax ginseng is a well-known medicinal herb that is widely used in traditional Chinese medicine for treating various diseases. Ginsenoside Rg3 (Rg3) is thought to be one of the most important active ingredients of Panax ginseng. However, the molecular mechanism underlying the beneficial effects of Rg3 has been elusive.

Methods: In the mouse heart injury model induced by isoproterenol (ISO), we used brain natriuretic peptide (BNP), lactate dehydrogenase (LDH) and caspase-3 ELISA kits to test myocardium injury. To test whether Rg3 protects myocardial injury through AMPK mediated autophagy, we used specific AMPK inhibitor in combination with Rg3. NLRP3 inflammasome related molecules such as NLRP3, ASC and caspase-1 were measured by western-blot following Rg3 treatment.

Results: We found that Rg3 significantly reduced ISO induced myocardial injury indicated by the downregulation of serum BNP and LDH. In addition, we showed that the improvement of myocardial injury by Rg3 was associated with enhanced expression of autophagy related protein and activation of AMPK downstream signaling pathway.

Conclusions: We observed that inhibition of AMPK significantly reversed the myocardial protective effect of Rg3, which is associated with a decrease of Rg3 induced autophagy. These together suggested that Rg3 may improve myocardial injury during MI through AMPK mediated autophagy. Our study also provides important translational evidence for using Rg3 in treating myocardial infarction (MI).

Keywords: Ginsenoside Rg3; myocardial infarction (MI); autophagy; AMP-activated protein kinase (AMPK)

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Introduction

Myocardial infarction (MI) is a widespread medical condition that has been the leading cause of death across different countries (1). During infarction, myocardial cells are deprived of oxygen, which results in an irreversible damage to myocardial cells. Cardiac fibrosis (a form of tissue remodeling mechanisms) happened after MI has been viewed as a crucial risk factor of heart failure (HF)

(2,3). The myocardial injury during infarction is mediated by many cellular and molecular mechanisms including the impairment of mitochondria, endogenous antioxidants and lipid peroxidation (4).

Isoproterenol (ISO)-induced MI is a commonly animal model for MI (5). That can mimic many clinical and pathological features of MI in human. In this model, ISO can increase the workload of myocardial cells and raise the demand for O₂, which in turn leads to relative myocardial

hypoxia. On top of that, direct O₂-wasting effect of catecholamines or their autoxidation products may result in hypotension, which further intensify the infarction (6).

Autophagy is an important compensatory mechanism process in responses cellular stress, in which proteins and damaged cellular organelles are digested by lysosomes in order to provide essential nutrients for keeping cell alive (7). Autophagy is also actively involved in various heart diseases including MI (8). During MI, energy deprivation or hypoxia may lead to the activation of AMP-activated protein kinase (AMPK), a highly conserved protein serine/threonine kinase (9). AMPK can then facilitate autophagy through the regulation of metabolic pathways. Studies have shown that AMPK protects myocardiocyte from hypoxia induced injury both *in vivo* and *in vitro* (10-12).

Panax ginseng is an important traditional Chinese herbal medicine that is widely used in treating many diseases. Ginsenoside Rg3 (Rg3) is the active ingredients of *Panax ginseng*. In recent studies, it is reported that Rg3 can regulate mitochondrial turn-overs (13) and autophagy (14). In present study, we proposed that Rg3 can improve myocardial injury induced by ISO through AMPK mediated autophagy.

Methods

Animals and reagents

Balb/c mice (6–8 w, male) were purchased from the VLAT (Vital River Laboratory Animal Technology, Beijing, China). All procedures and protocols using animal were in compliance with the AMRC (Animal Management Rule of China, Ministry of Health, document no.55, 2001). Rg3 (Meilunbio, China) was diluted in DMSO for stocking solution and further diluted at indicated concentrations in culture medium. Isoproterenol (ISO) was purchased from Sigma-Aldrich (USA).

BNP and reactive oxygen species (ROS) ELISA kits and the kit for testing LDH activity were obtained from the Jiancheng Bioengineering Company (Nanjing, China). Polyclonal rabbit anti-Mouse p-AMPK (T172), p-ACC (AP0298), LC3B (A7198), Beclin1 (A7353), and p62 (A19700) antibodies were obtained from ABclonal (Wuhan, China). Polyclonal rabbit Anti-Bax, Bcl2, β -actin and secondary antibodies were purchased from Santa Cruz Biotechnology (CA, USA).

Experimental protocol for drug treatment in mice

Male Balb/c mice were housed under standard conditions and were given rodent chow and water freely. All of the experimental procedures were performed in accordance with the Guidelines for Animal Experiments from the Committee of Medical Ethics. We induced MI model using previously described experimental procedure (15). Mice were randomly divided into 4 groups: (I) control group (n=9): subcutaneous (s.c.) injection with DMSO (2 mL/kg/day, for 2 days); (II) ISO group (n=9): s.c. injection with ISO (20 mg/kg/day, for 2 days); (III) ISO plus Rg3 (n=9) and (IV) ISO plus Rg3 and compound C (n=9): s.c. injection with Rg3 (5 mg/kg, every 12 h) or compound C (10 μ mol/L, every 12 h) for 2 days before injection with ISO. Then, Serum was isolated from blood and used for testing BNP and LDH levels. All of the animals were sacrificed by exsanguinations.

Histopathology staining

Hearts were washed, immediately fixed with 10% NBF (neutral buffered formalin) and embedded in paraffin. We then performed H&E staining on these tissue sections. The images are captured by a light microscopic to monitor histopathological changes.

Western blotting

Western blotting was performed as previously described (16). We isolated protein from heart and resuspended the protein in sample buffer. The protein mixture was then denatured in boiling water for 10 min. The protein samples were loaded in 10% SDS-PAGE in running buffer. The proteins were then transferred to a nitrocellulose membrane for 2 hours at 4 °C at 200 mA with a transfer buffer. Nonspecific proteins were blocked by incubating the membrane with 5% non-fat dry milk in TBS-T for 1 hour at RT. Then, the nitrocellulose membrane was incubated with the primary antibodies overnight at 4–8 °C and then with secondary antibody for 1 hour at RT. The reaction was visualized by ECL. The films were scanned and then analyzed by a NIH imaging software. Protein contents were normalized to β -actin.

BNP, LDH and ROS in serum

BNP level and ROS release in serum were determined

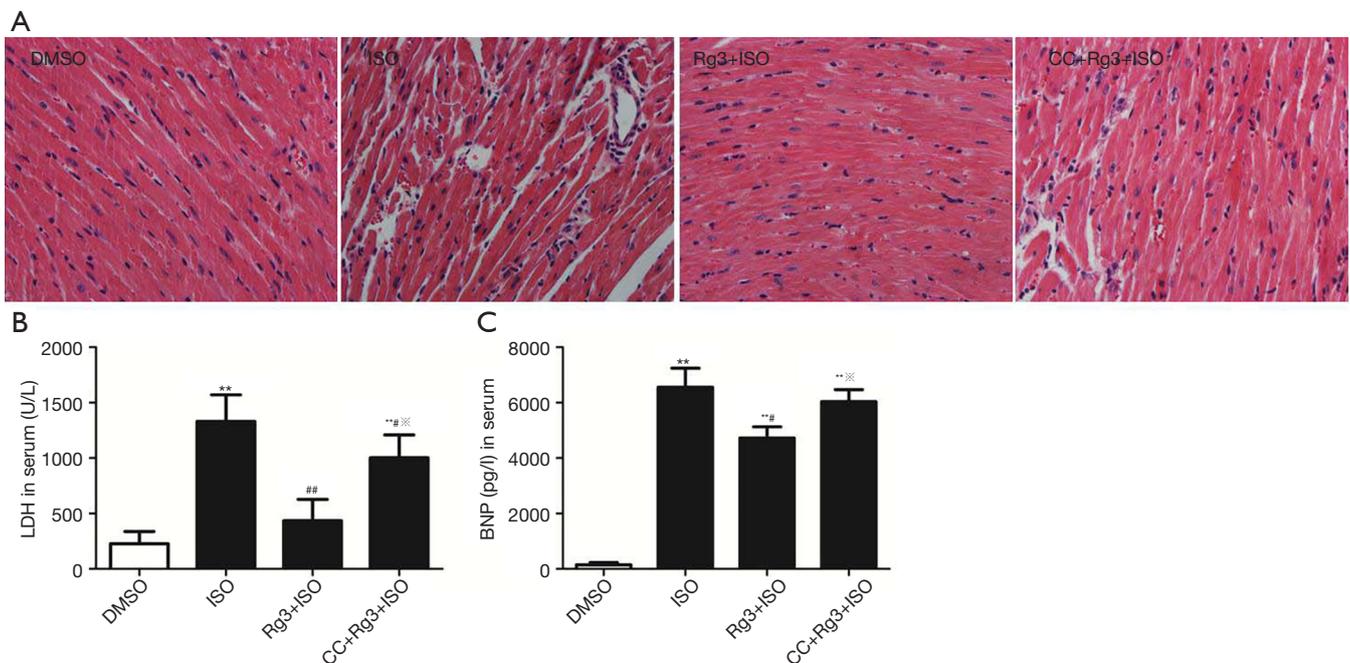


Figure 1 Rg3 ameliorated myocardial injury in mice with ISO-induced myocardial infarction. (A) HE staining of mice heart tissue with isoproterenol-induced myocardial infarction (400 \times); (B) serum LDH level; (C) BNP level; (D) ROS release. Data are mean \pm SD. n=9. *, P<0.05, **, P<0.01 vs. DMSO group; #, P<0.05, ##, P<0.01 vs. ISO group; *, P<0.05 vs. Rg3 group.

by commercially available ELISA kits. ELISA plates were coated with monoclonal antibody against BNP and ROS. Dilutions of cell-free supernatants were added in duplicate, and then a second horseradish peroxidase-conjugated goat polyclonal Ab against BNP and ROS were added. After washing to remove any unbound Ab-enzyme reagent, a substrate solution was added to the wells. The color development was stopped with 2N sulfuric acid, and the intensity of the color was measured at 540 nm on a spectrophotometer. The sensitivity of ELISA for BNP and ROS were 1 pg/mL. Measurement of LDH activity in serum was as described (15), level of LDH was expressed as unit per liter for serum.

Statistical analysis

The data are represented as means \pm SDs. One-way ANOVA was used to compare three or more groups, and Tukey's post-hoc test was used to test for comparing differences between individual groups. Graphpad Prism software, version 5, was used for data analysis and visualization. P<0.05 was considered statistically significant.

Results

Rg3 ameliorated myocardial injury in mice with isoproterenol-induced MI

H&E staining of the tissue section showed that in control mice the structure of the myocardium is homogenous and clear, with no signs of edema or any inflammation. On the other hand, mice injected with ISO had moderate-to-severe necrosis of myocardium, edema, as well as massive infiltration of immune cells including both myeloid cells and lymphocytes. Furthermore, the mice treated with Rg3, compared to ISO-challenged mice, showed significantly improved myocardial necrosis, a substantial decrease of edema and fewer infiltration of immune cells. Interestingly, the anti-myocardium injury effect of Rg3 was blocked by the administration of AMPK inhibitor compound C (Figure 1A).

To further investigate the effects of Rg3 on the heart injury *in vivo*, we tested the serum level of LDH and BNP (two important myocardial injury markers). Rg3 significantly attenuated LDH activity by 32.7% (Figure 1B) and decreased BNP level by 72.1% (Figure 1C). We also

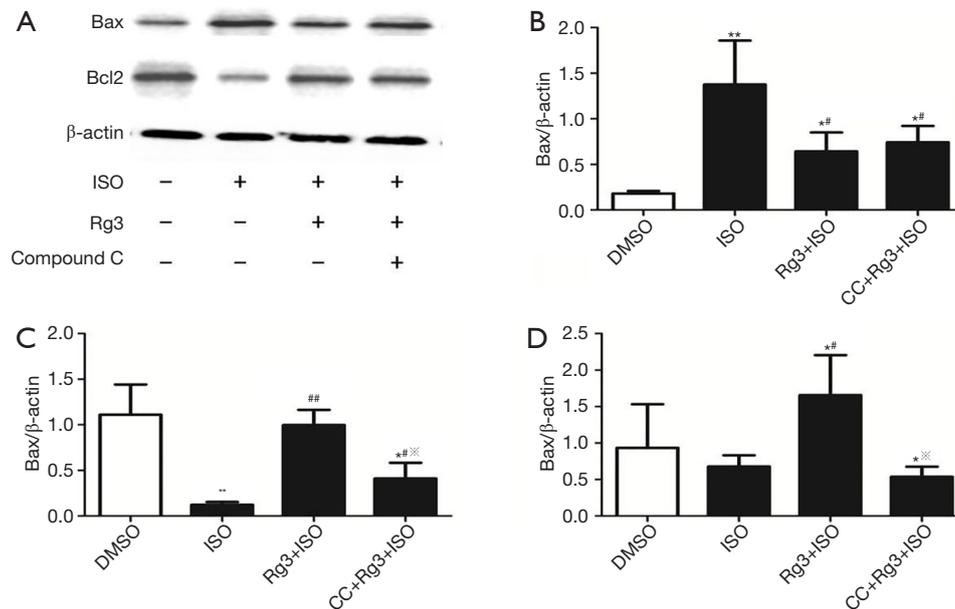


Figure 2 Rg3 attenuated myocardial apoptosis by activating AMPK. (A) Representative protein expression of Bcl-2, Bax and β -actin; (B) quantitative analysis of protein expression of Bax; (C) quantitative analysis of protein expression of Bcl-2; (D) the ratio of Bcl-2/Bax (n=3). *, P<0.05, **, P<0.01 vs. DMSO group; #, P<0.05, ##, P<0.01 vs. ISO group; *, P<0.05 vs. Rg3 group.

found Rg3 attenuated ROS release (Figure 1D).

Rg3 treatment attenuated ISO-induced myocardial apoptosis

We then examined the Bcl-2/Bax ratio at protein level to better understand the antiapoptotic action of Rg3 on myocardium. Interestingly, we found the decrease of myocardium apoptosis is associated with the reciprocal change pro-apoptotic molecule Bax and anti-apoptotic molecule Bcl-2, which has been shown to be important for myocardium survival during sepsis. These data together indicate that Rg3 can decrease myocardium apoptosis and improve cardiac function. Compared with ISO group, the ratio of Bcl-2/Bax strongly increased by 2.4-fold in group treated with Rg3+ISO (P<0.05, Figure 2). In addition, the cardioprotective effect of Rg3 was blocked by the administration of AMPK inhibitor compound C, indicating that Rg3 reduces ISO-induced cardiomyocyte apoptosis through the activation of AMPK (Figure 2).

Rg3 promotes autophagy in ISO-induced MI models via AMPK signal pathway activation

LC3B, p62, and Beclin-1 are commonly used markers to

monitor autophagy. Using these markers, we studied the effect of Rg3 on autophagy during ISO-induced MI. We identified that the LC3B and Beclin-1 levels were strongly increased in Rg3-treated group as compared to the diseased group (ISO only, 2.2 ± 0.29 vs. 1.26 ± 0.48 , P<0.01; 2.2 ± 1.01 vs. 0.69 ± 0.40 , P<0.01), while Rg3 treatment showed reverse effect on the p62 levels (Figure 3). These findings suggest that Rg3 could activate autophagy in ISO-induced MI models.

AMPK signaling pathway can enhance autophagy during multiple conditions. We also found the p-AMPK and p-ACC was increased after ISO-treated group. Pretreated with Rg3 abolished the increased level of p-AMPK and p-ACC. To further confirm the direct inhibitory effect of AMPK signal pathway on autophagy, we used an AMPK inhibitor, Compound C in the present study. Our results showed that ISO treatment decreased the levels of p-AMPK and p-ACC in myocardium, which indicates that ISO treatment reduces the activation of AMPK signal pathway. However, the Rg3 treatment reversed the inhibition effect brought by ISO (1.85 ± 0.27 vs. 0.33 ± 0.16 , P<0.05; 1.0 ± 0.12 vs. 0.32 ± 0.20 , P<0.05), further promoting the phosphorylation of the AMPK signal pathway related proteins (Figure 4). All these results indicate that the Rg3 exerts heart protective function via activation of AMPK signal pathway, further promotes myocardium autophagy in ISO-induced MI models.

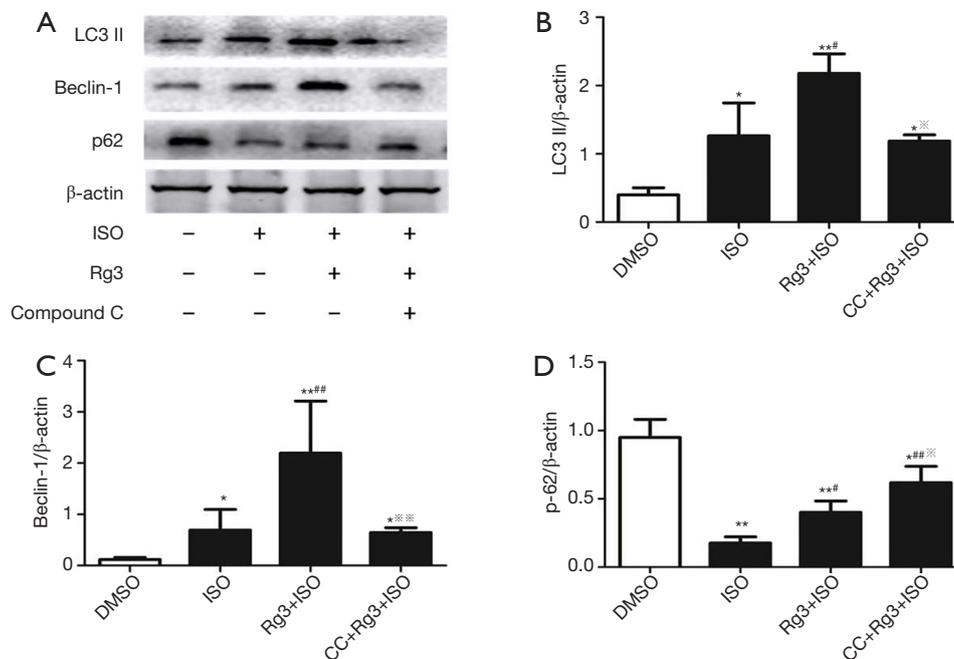


Figure 3 Rg3 promotes myocardial autophagy by activating AMPK. (A) Representative protein expression of LC3B, Beclin-1, p-62 and β -actin; (B) quantitative analysis of protein expression of LC3B; (C) quantitative analysis of protein expression of Beclin-1; (D) quantitative analysis of protein expression of p62 (n=3). *, P<0.05, **, P<0.01 vs. DMSO group; #, P<0.05, ##, P<0.01 vs. ISO group; *, P<0.05, **, P<0.01 vs. Rg3 group.

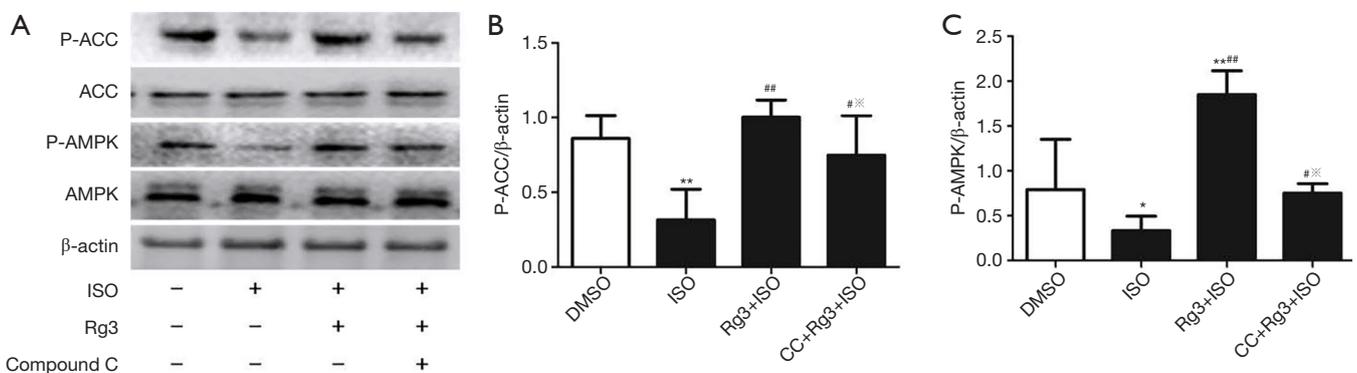


Figure 4 Rg3 activates AMPK signal pathway with isoproterenol-induced myocardial infarction. (A) Representative protein expression of p-ACC, p-AMPK and β -actin; (B) quantitative analysis of protein expression of p-ACC; (C) quantitative analysis of protein expression of p-AMPK (n=3). *, P<0.05, **, P<0.01 vs. DMSO group; #, P<0.05, ##, P<0.01 vs. ISO group; *, P<0.05 vs. Rg3 group.

Discussion

In this study, using mouse model of MI, we have demonstrated that Rg3 significantly attenuated ISO induced cardiac injury, as demonstrated by the reduced LDH and BNP levels in blood and reduced cardiomyocyte apoptosis. Our study also revealed that treatment of Rg3 upregulated mitochondrial autophagy, at least partially through AMPK signaling pathway.

MI, a serious CVD, is a prominent cause of death around the world (1). Cardiotoxicity caused by isoproterenol (ISO) at a higher dosage in an animal model exhibits a better discernment in myocardial damage and suggesting the involvement of oxidative stress. ISO is a classic tool agent in ischemic heart disease and HF investigations. ISO-induced MI animals show excessive generation of ROS followed by enhanced oxidative stress (17). Several preclinical studies have suggested that during MI, oxidative stress is significantly increased via ROS and involved in the pathogenesis of MI (18).

Rg3 has been demonstrated to have a broad range of beneficial effects for the cardiovascular system (19,20). Our study showed that Rg3 exerts potential cardioprotective effects during oxidative cardiac damage as indicated by reduced levels of cardiac injury markers and ameliorated histological changes in the heart. We also observed that serum BNP and LDH activities were significantly up-regulated in the ISO-treated mice, suggesting some leakage of LDH and BNP from dying/dead cardiac cells into the circulation. In our results, mice treated with Rg3 had decreased necrosis with edema and infiltration of myocardium cells. This finding presents a marked improvement in myocardium necrosis, possibly due to the antioxidant effects of Rg3. We also found Rg3 treatment to ISO-challenged mice drastically reduced ROS content in myocardium.

Autophagy is a self-degradative process and is actively involved in the pathogenesis of various heart diseases (8). Autophagy is important for the quality control of mitochondria (21). In the present study, Rg3 was also found to have the function to remove the dysfunctional mitochondria through increasing the mitophagy in ISO-induced myocardium. Mitophagy refers to selectively degrading mitochondria by autophagy, which can reduce the accumulation of mtDNA mutations (22). When the body undergoes negative stimulations from outside world,

the selective autophagy in mitochondria may occur (23). Our study found that Rg3 treatment has the function of inhibition of apoptosis of myocardium. Also, Rg3 can unregulated the autophagy-related proteins.

More interestingly, in the present study, Rg3 was found to promote the phosphorylation of AMPK. AMPK is an energy sensor of cells, regulating cellular energy metabolism, and its activation is conducive to rectifying metabolic disorders, making cell metabolism to be physically balanced (24). Activated AMPK can promote autophagy by inhibiting TORC1 complex (24). Therefore, we propose that Rg3 exerts mitochondrial protective function via activation of AMPK signal pathway, then further promotes mitochondrial autophagy in MI models. Furthermore, mitochondria are the key sites for fatty acid metabolism, while AMPK can affect the energy metabolism of mitochondria by influencing the activity of ACC (24,25). In the current study, we also showed that Rg3 promoted the phosphorylation of ACC. ACC can catalyze the carboxylation of acetyl CoA and thus convert it into malonyl CoA, which is the fatty acid synthesis precursor and meanwhile inhibits the oxidation of fatty acids in mitochondria (25). AMPK can phosphorylate ACC, resulting in its decrease in activity and thus reducing the level of malonyl CoA (24,25). We found Rg3 could significantly upregulate the phosphorylation of AMPK and ACC. In order to test the direct role of AMPK to ISO-induced cardiac dysfunction, we used AMPK inhibitor, compound C. We found that compound C pretreatment could block the effects of Rg3 on heart protection. Which suggests that, at least partially by AMPK-autophagy signaling pathway, Rg3 inhibits ISO-mediated myocardium injury.

Conclusions

Taken together, our study indicates that Rg3 pretreatment attenuated MI induced depressed cardiac function and upregulated autophagy process through the AMPK signaling pathway. For the first time, we show that Rg3 protects heart against MI, at least partially through upregulation of myocardial autophagy, which may provide a deeper insight into the mechanism underlying the wide cardiovascular protective actions of Rg3 and provide therapeutic basis for the clinical use of Rg3.

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Footnote

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/cdt.2020.01.02>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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