Ginsenoside Rg1 regulates autophagy and endoplasmic reticulum stress via the AMPK/mTOR and PERK/ATF4/CHOP pathways to alleviate alcohol-induced myocardial injury

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Abstract. It has been reported that ginsenoside Rg1 (G-Rg1) can alleviate alcoholic liver injury, cardiac hypertrophy and myocardial ischemia, as well as reperfusion injury. Therefore, the present study aimed to investigate the role of G-Rg1 in alcohol-induced myocardial injury, as well as to elucidate its underlying mechanisms of action. For this purpose, H9c2 cells were stimulated with ethanol. Subsequently, H9c2 cell viability and apoptosis were determined using a Cell Counting Kit-8 assay and flow cytometric analysis, respectively. The levels of lactate dehydrogenase and caspase-3 in the H9c2 cell culture supernatant were detected using corresponding assay kits. In addition, the expression of green fluorescent protein (GFP)-light chain 3 (LC3) and that of C/EBP homologous protein (CHOP) were evaluated using GFP-LC3 assay and immunofluorescence staining, respectively. The expression levels of apoptosis-, autophagy-, endoplasmic reticulum stress (ERS)- and adenosine 5'-monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway-related proteins were detected using western blot analysis. The results revealed that treatment with G-Rg1 enhanced the viability and suppressed the apoptosis of ethanol-stimulated H9c2 cells. G-Rg1 also attenuated autophagy and ERS in ethanol-stimulated H9c2 cells. In addition, the levels of phosphorylated (p)-protein kinase R (PKR)-like ER kinase (PERK), p-eukaryotic translation initiation factor 2a, activating transcription factor 4 (ATF4), CHOP, caspase-12 and p-AMPK were downregulated, while the p-mTOR level was upregulated in ethanol-stimulated H9c2 cells treated with G-Rg1. Furthermore, the co-treatment of G-Rg1-treated ethanol-stimulated H9c2 cells with AICAR, an AMPK agonist, or CCT020312, a PERK agonist, inhibited cell viability and promoted cell apoptosis, autophagy and ERS. Overall, the results of the present study suggest that G-Rg1 suppresses autophagy and ERS via inhibiting the AMPK/mTOR and PERK/ATF4/CHOP pathways to alleviate ethanol-induced H9c2 cell injury.

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Introduction

Epidemiological studies have confirmed that low-to-moderate alcohol consumption can reduce the incidence of cardiovascular diseases to a certain extent. However, excessive alcohol consumption or even alcoholism is considered a significant cause of cardiomyopathy (1). It has been reported that the daily consumption of 90-120 g alcohol for ~5-15 years can increase the risk of structural and functional changes in the heart (2,3). Non-ischemic dilated cardiomyopathy caused by long-term heavy alcohol intake is termed alcoholic cardiomyopathy (ACM) (4). ACM is characterized by myocardial hypertrophy and compensatory cardiac systolic disorder (5,6). In recent years, the incidence of ACM has gradually increased in China, with a mortality rate of 5.1 deaths/100,000 ACM cases (7). The onset and progression of alcohol-induced myopathic changes is caused by multiple factors, including mitochondrial damage, the toxicity of ethanol and its metabolites, and the accumulation of fatty acid ethyl esters, that appear to be the direct outcome of ethanol metabolism, which are common features of acute ethanol exposure (5,8). Therefore, identifying effective treatment approaches for ethanol-induced myocardial injury is of utmost importance.

Ginseng has been used as a tonic in China for >2,000 years. Ginsenoside Rg1 (G-Rg1), one of the most noteworthy components of ginseng, has been shown to exert several pharmacological effects, such as antitumor, anti-inflammatory, antioxidant and anti-diabetic effects (9-12). It has been reported that G-Rg1 can attenuate alcoholic liver injury. For example, a previous study demonstrated that G-Rg1 prevented reactive oxygen species production, mitochondrial

damage and hepatocyte apoptosis in a mouse model of chronic ethanol-induced hepatitis (13). Additionally, G-Rg1 has been found to attenuate inflammatory responses and to protect mice against alcohol-induced liver injury (14,15). G-Rg1 can also ameliorate heart injury. Therefore, previous research has suggested that G-Rg1 can attenuate myocardial ischemia and reperfusion injury in rats (16), while it can also alleviate cardiac hypertrophy and fibrosis to improve cardiac decompensation induced by abdominal aortic constriction in rats (17). However, to the best of our knowledge, the role of G-Rg1 in alcohol-induced myocardial injury has not been previously investigated.

Recently, the role of endoplasmic reticulum stress (ERS) and autophagy in the pathogenesis of ACM have received increasing attention. The study by Li and Ren (18) first demonstrated that ERS was activated in the heart tissues of mice exposed to ethanol, characterized by the increased expression of cardiac inositol-requiring transmembrane kinase/endoribonuclease 1α, eukaryotic translation initiation factor 2α (eIF2α) and C/EBP homologous protein (CHOP) (18), thus suggesting that ERS may be involved in the occurrence and development of ACM. Additionally, the ERS inhibitor, sodium 4-phenylbutyrate, has been shown to markedly attenuate ethanol-induced cardiomyocyte injury (19), while another study demonstrated that ERS induced by chronic alcohol intake was alleviated by aldehyde dehydrogenase 2 upregulation (20). Furthermore, another study revealed that ethanol-induced autophagy was suppressed by schisandrin B to prevent acute alcohol-induced heart injury (21). Additionally, hydrogen sulfide has been found to attenuate myocardial fibrosis in mice with ACM by attenuating autophagy (22). A previous study also indicated that G-Rg1 suppressed autophagy and ERS to reduce doxorubicin-induced cardiotoxicity in mice (23). It has also been shown that G-Rg1 attenuates aldosterone-induced autophagy in NRK-52E cells (24) and high-fat diet-induced ERS in obese rats (25). Therefore, it was hypothesized that G-Rg1 may also regulate autophagy and ERS to alleviate alcohol-induced myocardial cell injury.

The present study was performed to examine whether G-Rg1 can attenuate alcohol-induced autophagy and ERS in myocardial cells. In addition, the molecular mechanisms underlying alcohol-induced autophagy, ERS and G-Rg1-mediated therapy were also investigated.

Materials and methods

Cell culture and alcohol induction. H9c2 cells (cat. no. CL-0089; Procell Life Science & Technology) were cultured in DMEM (Procell Life Science & Technology) supplemented with 10% FBS (HyClone; Cytiva), 100 U/ml penicillin G (HyClone; Cytiva) and 100 U/ml streptomycin (HyClone; Cytiva) at 37˚C in a humidified incubator with 5% CO_2. Briefly, following treatment with various concentrations of G-Rg1 (0, 10, 20 and 40 μM; Shanghai Kanglang Biotechnology Co., Ltd.) for 2 h (26-28), the H9c2 cells were co-stimulated with 200 mmol/l ethanol (Sigma Technology China) for 24 h. When necessary, the cells were pre-incubated with 1 mM AICAR (Selleck, USA), an adenosine 5'-monophosphate-activated protein kinase (AMPK) agonist, for 1 h or 10 μM CCT020312 (Selleckchem), a protein kinase R (PKR)-like endoplasmic reticulum (ER) kinase (PERK) agonist, for 2 h.

Cell Counting Kit-8 (CCK-8) assay. The viability of the H9c2 cells was assessed using a CCK-8 assay kit (Dojindo Molecular Technologies, Inc.). Briefly, the H9c2 cells were seeded in 96-well plates at a density of $5x10^3$ cells/well and cultured for 24 h. After the indicated treatments, each well was supplemented with 10 μl CCK-8 solution, followed by incubation for an additional 2 h at 37˚C in an incubator with 5% CO_2. The absorbance at a wavelength of 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.).

Flow cytometric analysis. H9c2 cell apoptosis was measured using flow cytometry (Beckman Coulter, Inc.) with an Annexin V-fluorescein isothiocyanate/propidium iodide (Annexin V-FITC/PI) apoptosis detection kit (Beijing Biosea Biotechnology Co., Ltd.). The H9c2 cells were seeded in a six-well plate at a density of $1x10^5$ cells/well and were then treated as indicated. Subsequently, H9c2 cells were washed with ice-cold PBS (MilliporeSigma), re-suspended in binding buffer and mixed with 5 μl Annexin V-FITC and 10 μl PI. Finally, cell apoptosis was analyzed using a flow cytometer (Beckman Coulter, Inc.).

Detection of LDH and caspase-3. Following the indicated treatments, H9c2 cells were lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology), followed by centrifugation at 600 x g for 5 min at 4˚C to obtain the supernatant. The levels of LDH and caspase-3 in the culture supernatant were measured using the corresponding LDH (cat. no. ab102526) and caspase-3 (cat. no. ab39401) assay kits from Abcam based on the colorimetric method using the FLUOstar® Omega Microplate Reader (BMG Labtech GmbH).

Western blot analysis. Total proteins were extracted from the H9c2 cells using RIPA lysis buffer. The protein concentration was quantified using the BCA method. Equal amounts of protein extracts (30 μg) were subjected to 10% SDS-PAGE and were then transferred onto PVDF membranes. Subsequently, the membranes were blocked with 5% skimmed milk at room temperature for 1.5 h and were then incubated with primary antibodies against Bcl-2 associated X-protein (Bax; cat. no. ab32503; 1/1,000; Abcam), B-cell lymphoma 2 (Bcl-2; cat. no. ab196495; 1/1,000; Abcam), light chain 3 (LC3)-II/I (cat. no. ab192890; 1/1,000; Abcam), Beclin-1 (cat. no. ab207612; 1/1,000; Abcam), p62 (cat. no. ab109012; 1/10,000; Abcam), phosphorylated (p)-PERK (cat. no. #3179; 1/1,000; Cell Signaling Technology), PERK (cat. no. ab229912; 1/1,000; Abcam), p-eIF2α (cat. no. #3398; 1/1,000; Cell Signaling Technology), eIF2α (cat. no. #5324; 1/1,000; Cell Signaling Technology), activating transcription factor 4 (ATF4; cat. no. #11815; 1/1,000; Cell Signaling Technology), CHOP (cat. no. 15204-1-AP; 1/1,000; Proteintech Group, Inc.), caspase-12 (cat. no. ab62484; 1/1,000; Abcam), p-AMPK (cat. no. ab133448; 1/1,000; Abcam), AMPK (cat. no. ab207442; 1/1,000; Abcam), p-mammalian target of rapamycin (mTOR; cat. no. ab109268; 1/1,000; Abcam), mTOR (cat. no. ab134903; 1/10,000; Abcam) and GAPDH (cat. no. ab181602; 1/10,000; Abcam) at 4˚C overnight. Following washing three times

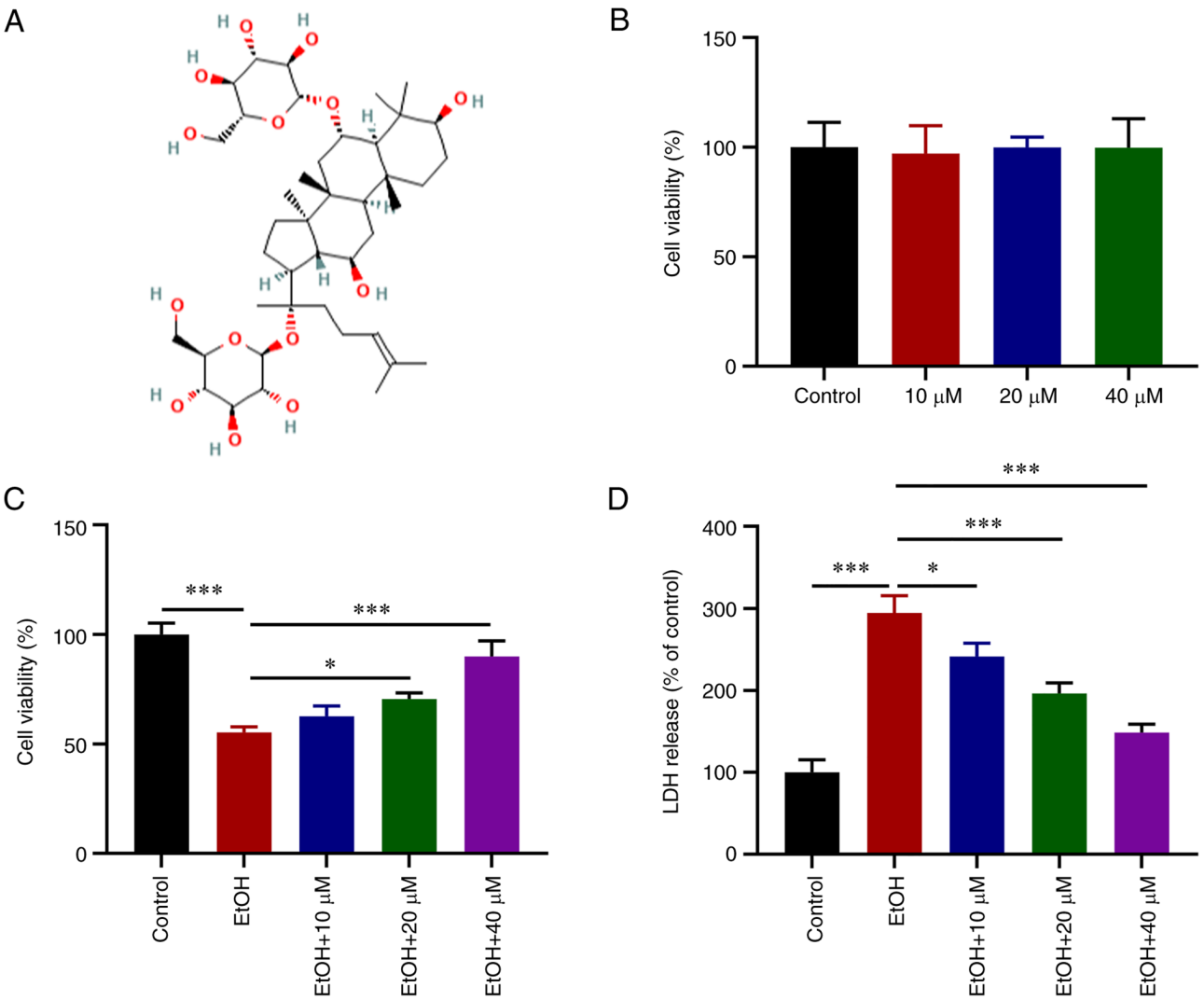

Figure 1. G-Rg1 enhances the viability of ethanol-stimulated H9c2 cells. (A) Chemical structure of G-Rg1. The effects of various concentrations of G-Rg1 on (B) H9c2 cells not simulated with ethanol and (C) ethanol-stimulated H9c2 cells was assessed using Cell Counting Kit-8 assay. (D) The levels of LDH in ethanol-stimulated H9c2 cells treated with various concentrations of G-Rg1 were detected using the corresponding kit. The results are expressed as the mean ± SD, The experiments were repeated three times. *P<0.05 and ***P<0.001. G-Rg1, ginsenoside Rg1; LDH, lactate dehydrogenase; EtOH, ethanol.

with PBS/Tween-20, the membranes were incubated with the corresponding anti-rabbit horseradish peroxidase-conjugated secondary antibody (cat. no. ab6721; 1/2,000; Abcam) at room temperature for 1 h. The bands were visualized using an ECL detection kit and their intensity was analyzed using ImageJ 1.8.0 software (National Institutes of Health).

Green fluorescent protein (GFP)-LC3 assay. Upon reaching 90% confluency, the cells were transfected with GFP-LC3 plasmid (Hanbio Biotechnology Co., Ltd.) or GFP control vector (Hanbio Biotechnology Co., Ltd.) using Lipofectamine® 2000, followed by incubation for 48 h at 37˚C. The volume of virus was 20 μl. Subsequent experiments were implemented 48 h post-transfection. Following treatment with 0, 10, 20 and 40 μM G-Rg1 and 200 mmol/l ethanol with the presence or absence of 1 mM AICAR or 10 μM CCT020312 and washing with ice-cold PBS, the H9c2 cells were fixed with 4% paraformaldehyde (Shanghai Macklin Biochemical Co., Ltd.) for 30 min at room temperature and counterstained with 4',6-diamidino-2-phenylindole (DAPI; MilliporeSigma) for 10 min at room temperature. Finally, the cells were observed under a fluorescence microscope (LSM 510 META, Zeiss GmbH).

Immunofluorescence staining. Following the indicated treatment with 0, 10, 20 and 40 μM G-Rg1 and 200 mmol/l ethanol with the presence or absence of 1 mM AICAR or 10 μM CCT020312, the H9c2 cells were seeded into a 24-well plate and fixed with 4% paraformaldehyde for 20 min at room temperature. Subsequently, the H9c2 cells were incubated with a primary antibody against CHOP (cat. no. 15204-1-AP; 1/500; Proteintech Group, Inc.) at 4˚C overnight. The following day, the H9c2 cells were incubated with the Cy3-labelled secondary antibody IgG (1:100; Sigma-Aldrich; Merck KGaA) in the dark for 1 h. Following staining with DAPI for 10 min at room temperature, CHOP-positive H9c2 cells were observed under a fluorescence microscope (Olympus Corporation).

Molecular docking analysis. The 3D structures of AMPK (PDB ID, 4ZHX) and PERK (PDB ID, 4G34) were downloaded

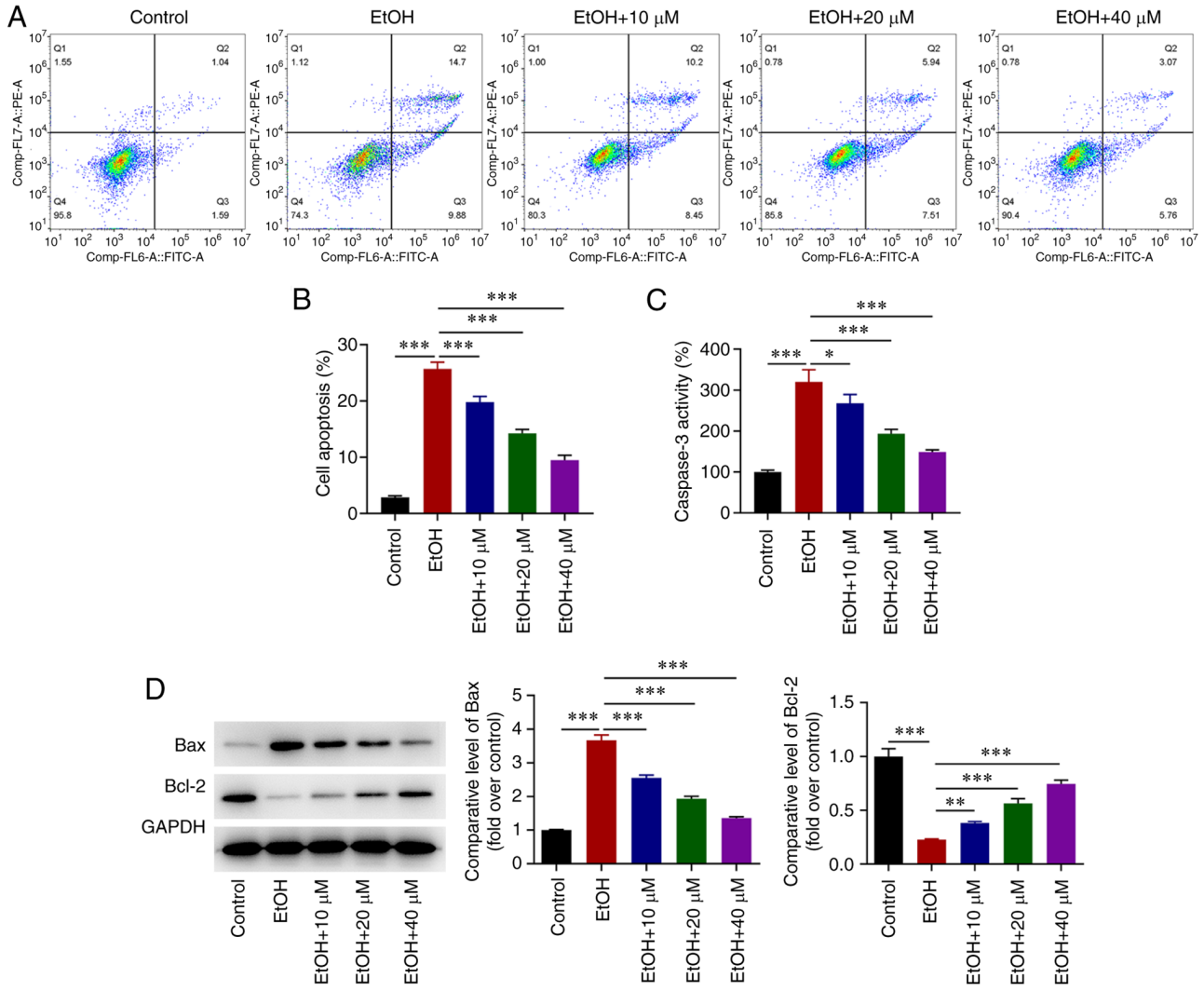

Figure 2. G-Rg1 inhibits the apoptosis of ethanol-stimulated H9c2 cells. (A and B) The apoptotic rate of ethanol-stimulated H9c2 cells treated with various concentrations of G-Rg1 was determined using flow cytometric analysis. (C) Caspase-3 activity in ethanol-stimulated H9c2 cells treated with various concentrations of G-Rg1 was measured using the corresponding kit. (D) The expression levels of Bax and Bcl-2 in ethanol-stimulated H9c2 cells treated with various concentrations of G-Rg1 were detected using western blot analysis. The results are expressed as the mean ± SD, The experiments were repeated three times. *P<0.05, **P<0.01 and ***P<0.001. G-Rg1, ginsenoside Rg1; EtOH, ethanol; Bax, Bcl-2 associated X-protein; Bcl-2, B-cell lymphoma 2.

from the Protein Data Bank (PDB) website (http://www.rcsb.org/) and saved in PDB format. Subsequently, the 3D structures in PDB format were converted into 'pdbqt' format using AutoDockTools 1.5.6. Finally, molecular docking was conducted using Autodock (version 4.2) and the results were visualized with Pymol 2.5.2 (https://pymol.org/2/) software.

Statistical analysis. All statistical analyses were performed using GraphPad Prism 8.0.1. (GraphPad Software, Inc.). The data were compared using one-way ANOVA followed by Tukey's post hoc test. All experiments were performed in triplicate. Data are expressed as the mean ± standard deviation (SD). P<0.05 was considered to indicate a statistically significant difference.

Results

G-Rg1 enhances the viability of ethanol-stimulated H9c2 cells. The chemical structural formula of G-Rg1 is presented in Fig. 1A. Following treatment of the H9c2 cells with various concentrations of G-Rg1, a CCK-8 assay was performed. The results revealed that G-Rg1 alone had no obvious effect on H9c2 cell viability (Fig. 1B). However, the viability of the H9c2 cells exposed to ethanol was increased following treatment with various concentrations of G-Rg1, and in particular at 40 μM (Fig. 1C). LDH production was also increased in the ethanol-stimulated H9c2 cells, which was then reduced following treatment with G-Rg1 (Fig. 1D).

G-Rg1 inhibits the apoptosis of ethanol-stimulated H9c2 cells. Flow cytometric analysis revealed that the apoptosis of H9c2 cells was enhanced by stimulation with ethanol. However, G-Rg1 gradually suppressed the apoptotic rate of the H9c2 cells in a concentration-dependent manner, and in particular, at the G-Rg1 concentrations between 10 and 40 μM (Fig. 2A and B). Consistent with these results, caspase-3 activity and Bax expression were increased, while Bcl-2 was downregulated in the ethanol-stimulated

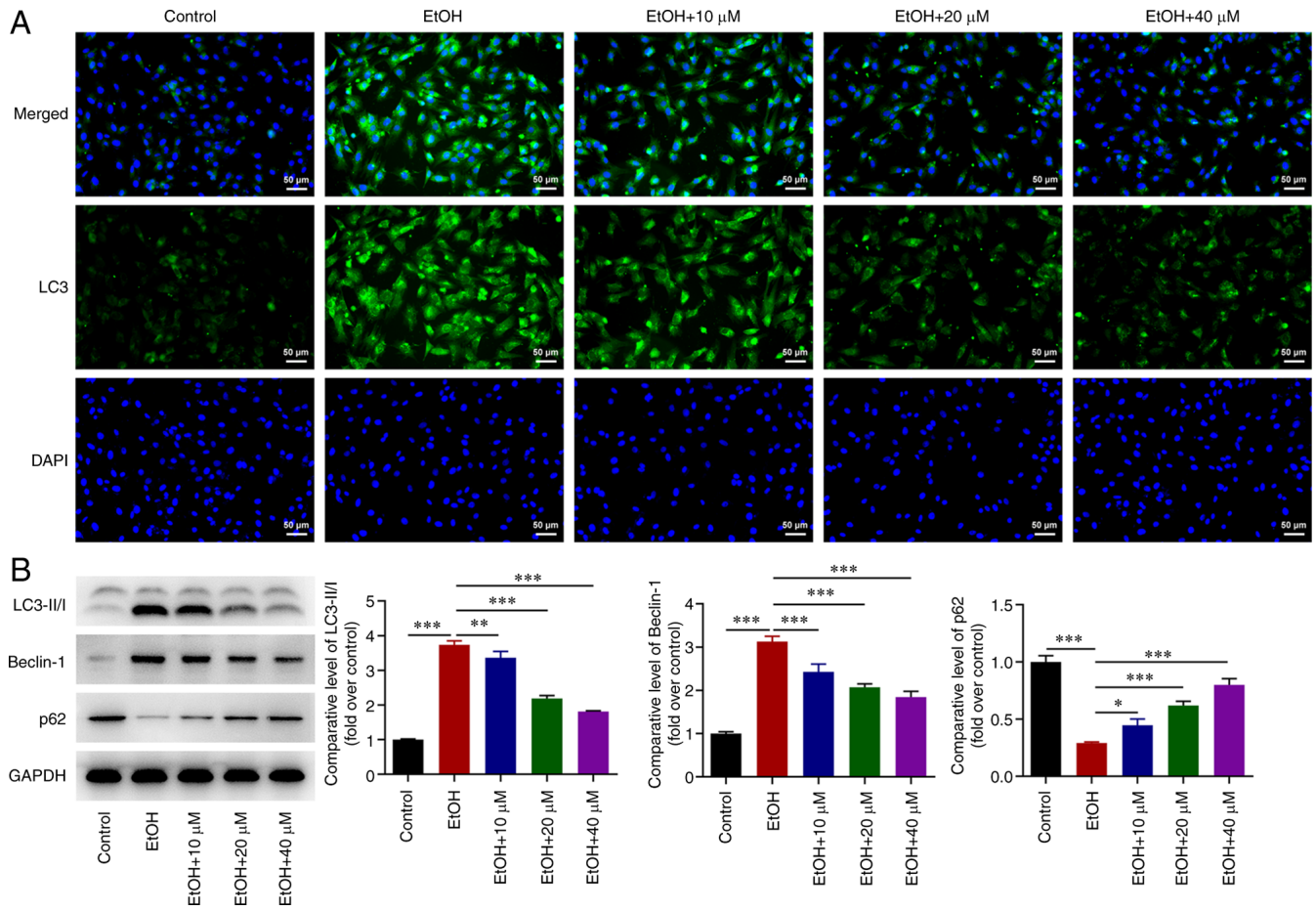

Figure 3. G-Rg1 inhibits autophagy in ethanol-stimulated H9c2 cells. (A) The expression levels of LC3 in ethanol-stimulated H9c2 cells treated with various concentrations of G-Rg1 were determined using a GFP-LC3 assay. (B) The expression levels of LC3-I, LC3-II, Beclin-1 and p62 were detected in ethanol-stimulated H9c2 cells treated with various concentrations of G-Rg1 using western blot analysis. The results are expressed as the mean ± SD. The experiments were repeated three times. *P<0.05, **P<0.01 and ***P<0.001. G-Rg1, ginsenoside Rg1; LC3, light chain 3; EtOH, ethanol.

H9c2 cells; these effects were reversed by G-Rg1 (Fig. 2C and D).

G-Rg1 inhibits the autophagy of ethanol-stimulated H9c2 cells. Further experiments demonstrated that LC3 expression was upregulated in ethanol-stimulated H9c2 cells. However, the effect of ethanol on LC3 upregulation was abrogated by G-Rg1 (Fig. 3A). Additionally, LC3-II/I and Beclin-1 expression levels were upregulated, while the expression of p62 was downregulated in ethanol-stimulated H9c2 cells. These effects were reversed following treatment of the ethanol-stimulated H9c2 cells with G-Rg1 (Fig. 3B).

G-Rg1 inhibits ERS and modulates AMPK/mTOR and PERK/ATF4/CHOP signaling in ethanol-stimulated H9c2 cells. Immunofluorescence staining demonstrated that the expression of CHOP was increased in H9c2 cells stimulated with ethanol, which was then inhibited by G-Rg1 treatment (Fig. 4A). The expression levels of p-PERK, p-eIF2α, ATF4, CHOP and caspase-12 were enhanced in the ethanol-stimulated H9c2 cells. However, G-Rg1 diminished the effects of ethanol on ERS in H9c2 cells (Fig. 4B). In addition, ethanol promoted the expression of p-AMPK and suppressed that of p-mTOR in H9c2 cells; these effects were also reversed by G-Rg1 (Fig. 4C).

Activation of AMPK/mTOR or PERK/ATF4/CHOP signaling inhibits the viability and promotes the apoptosis of ethanol-stimulated H9c2 cells treated with G-Rg1. The results of molecular docking analysis revealed that G-Rg1 exhibited strong affinity for AMPK, with a docking score of -7.2 kcal/mol. The amino acid binding sites were as follows: ASN-110, ASN-111, HIS-109, LYS-29 and LYS-31 (Fig. 5A). G-Rg1 also exhibited a strong affinity for PERK, with a docking score of -6.4 kcal/mol. In this case, the amino acid binding sites were as follows: LYS-938, LYS-987, GLY-997, GLY-601, GLY-604, ASP-936, ASP-954, LEU-957 and PHE-603 (Fig. 5B). In addition, the results demonstrated that G-Rg1 increased the viability of ethanol-stimulated H9c2 cells. This effect was abrogated following co-treatment of the cells with AICAR or CCT020312 (Fig. 6A). The production of LDH was decreased in ethanol-stimulated H9c2 cells treated with G-Rg1, an effect which was also reversed following co-treatment with AICAR or CCT020312 (Fig. 6B). In addition, the apoptosis of ethanol-stimulated H9c2 cells was inhibited by G-Rg1, and this effect was also reversed by AICAR or CCT020312 (Fig. 6C and D). Furthermore, caspase-3 activity and Bax expression were decreased, and Bcl-2 expression was increased in the H9c2 cells in the ethanol + G-Rg1 group compared with the ethanol group. This effect was diminished in the ethanol + G-Rg1 + AICAR or CCT020312 group (Fig. 6E and F).

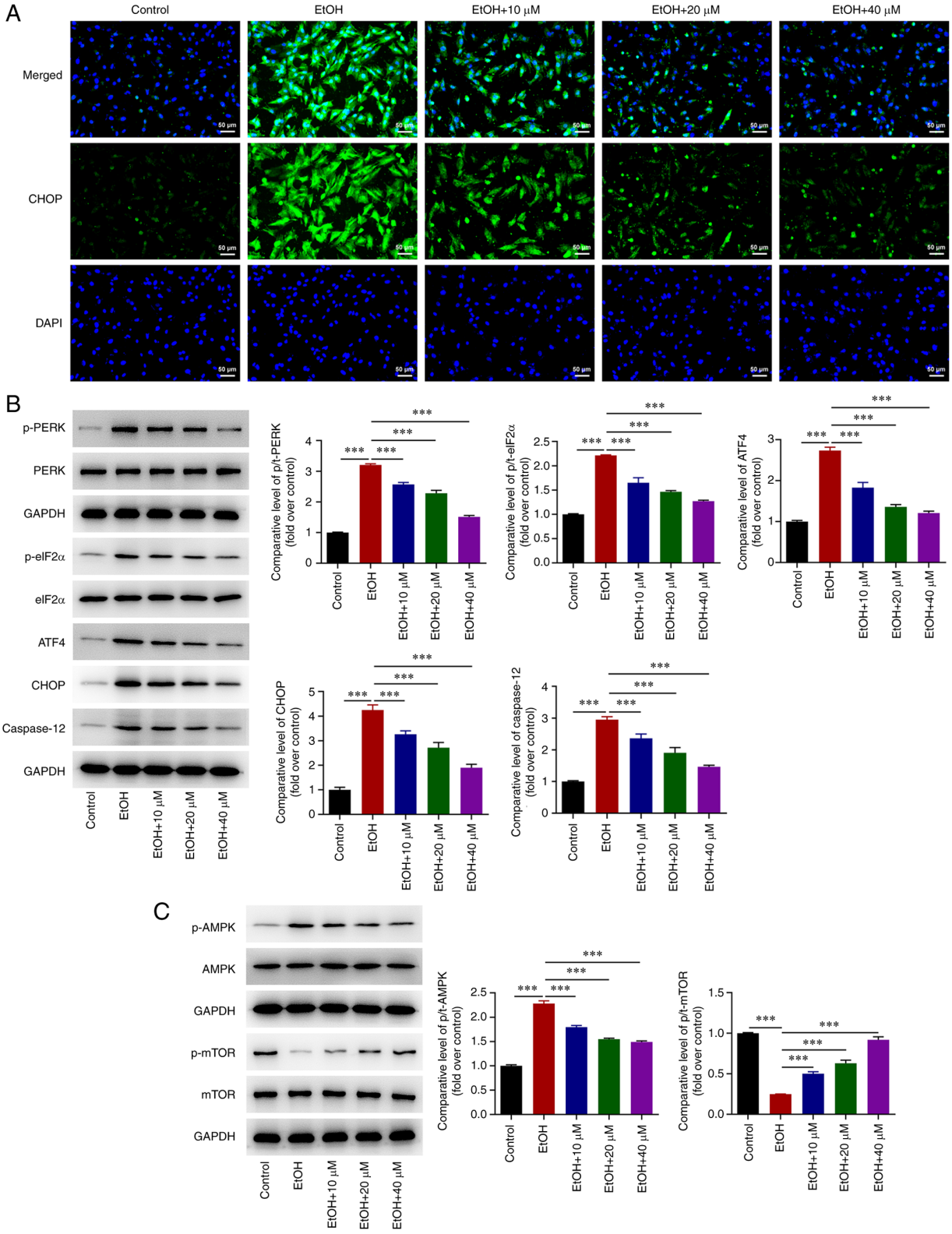

Figure 4. G-Rg1 inhibits ERS and modulates the AMPK/mTOR and PERK/ATF4/CHOP signaling pathways in ethanol-stimulated H9c2 cells. (A) The expression of CHOP in ethanol-stimulated H9c2 cells treated with various concentrations of G-Rg1 was determined using immunofluorescence staining. The expression levels of (B) PERK/ATF4/CHOP signaling pathway-related proteins and (C) AMPK/mTOR pathway-related proteins in ethanol-stimulated H9c2 cells treated with various concentrations of G-Rg1 were detected using western blot analysis. The results are expressed as the mean ± SD. The experiments were repeated three times. ***P<0.001. G-Rg1, ginsenoside Rg1; ERS, endoplasmic reticulum stress; CHOP, C/EBP homologous protein; AMPK, adenosine 5'-monophosphate-activated protein kinase; mTOR, mammalian target of rapamycin; PERK, protein kinase R (PKR)-like ER kinase; ATF4, activating transcription factor 4; eIF2α, eukaryotic translation initiation factor 2α; EtOH, ethanol.

Activation of the AMPK/mTOR or PERK/ATF4/CHOP signaling pathways promotes autophagy and ERS in ethanol-stimulated H9c2 cells treated with G-Rg1. Subsequently, the results revealed that G-Rg1 suppressed LC3

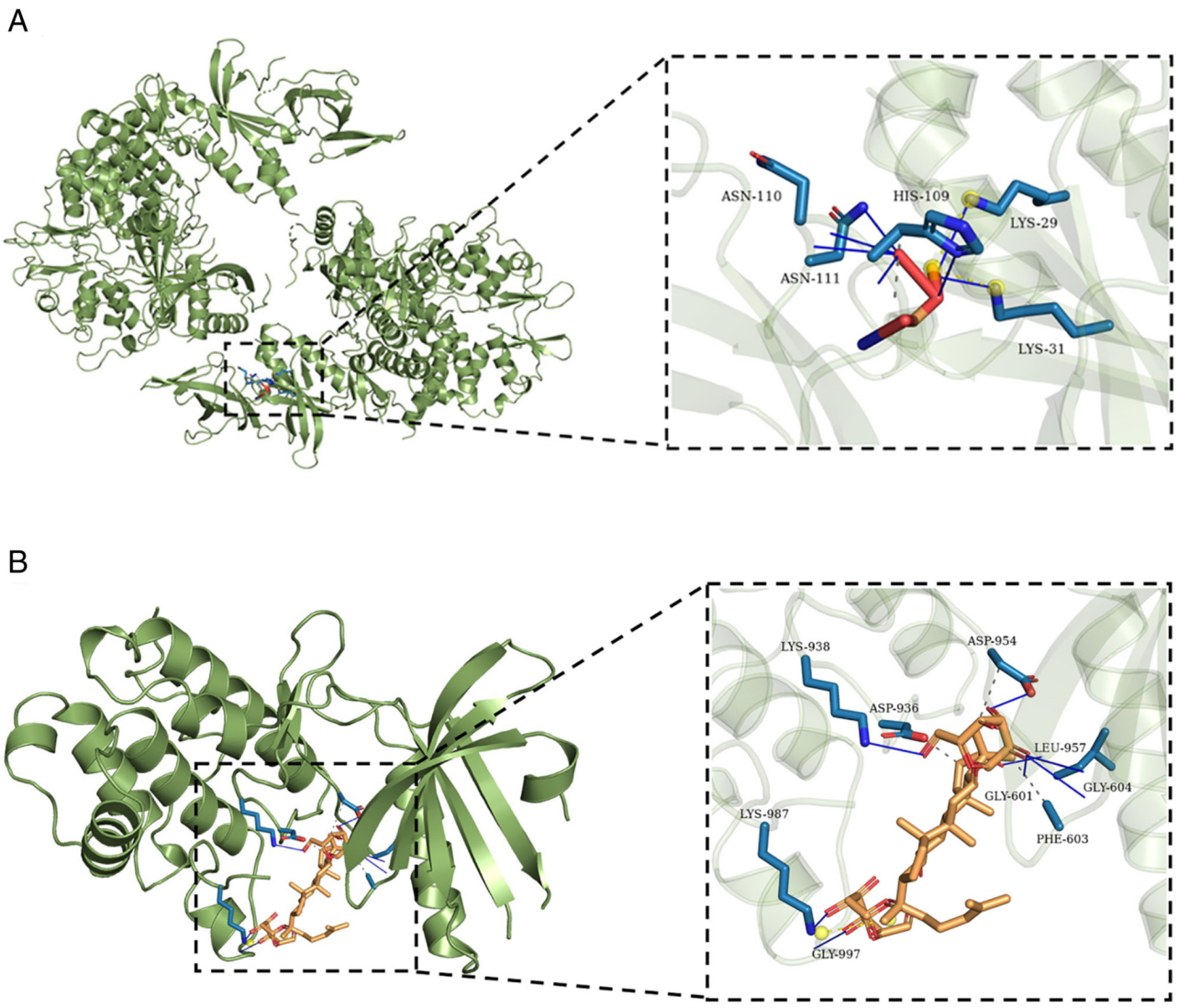

Figure 5. Molecular docking analysis of G-Rg1 and AMPK or PERK. G-Rg1 exhibited a strong affinity for (A) AMPK and (B) PERK. G-Rg1, ginsenoside Rg1; AMPK, adenosine 5'-monophosphate-activated protein kinase; PERK, protein kinase R (PKR)-like ER kinase.

expression in the ethanol-stimulated H9c2 cells, while LC3 expression was upregulated in the ethanol + G-Rg1 + AICAR group (Fig. 7A). Additionally, the LC3-II/I and Beclin-1 expression levels were downregulated, and p62 expression was upregulated in the ethanol-stimulated H9c2 cells treated with G-Rg1; these effects were reversed by AICAR treatment (Fig. 7B). Furthermore, the expression of CHOP was decreased in the ethanol + G-Rg1 group compared with the ethanol group. However, treatment of the H9c2 cells with CCT020312 promoted CHOP expression in the ethanol + G-Rg1 group compared with the ethanol group (Fig. 7C). Finally, the expression levels of p-PERK, p-eIF2α, ATF4, CHOP and caspase-12 were decreased in the ethanol + G-Rg1 group compared with the ethanol group. These effects were all reversed by CCT020312 (Fig. 7D).

Discussion

ACM, a cardiac disease caused by chronic alcohol consumption, is characterized by ventricular dilation and impaired cardiac function (29). Patients with ACM commonly have a history of excessive alcohol addiction for >10 years and are characterized by diverse clinical manifestations, such as cardiac insufficiency and arrhythmia, which can be self-alleviated or cured following alcohol withdrawal (30). Since uniform standards for the outcome of ACM are lacking, there is a lack of reliable treatment guidance (31). Therefore, the identification of agents capable of preventing and treating alcohol-induced myocardial injury is urgently required.

The present study using ethanol-stimulated H9c2 cells to investigate the roles of G-Rg1 in alcohol-induced myocardial injury; the same model has been used in previous studies (32-34). There is accumulating evidence to suggest that G-Rg1 protects against alcohol-induced liver injury (13-15). In addition, it has been reported that G-Rg1 exerts cardioprotective effects in several myocardial injury models, including ischemia/reperfusion injury (16), adriamycin (doxorubicin)-induced cardiotoxicity (23), diabetic cardiomyopathy (35) and sepsis-induced cardiac insufficiency (36). Consistent with the findings of these previous studies, the present study demonstrated that G-Rg1 protected against alcohol-induced myocardial injury, as evidenced by the

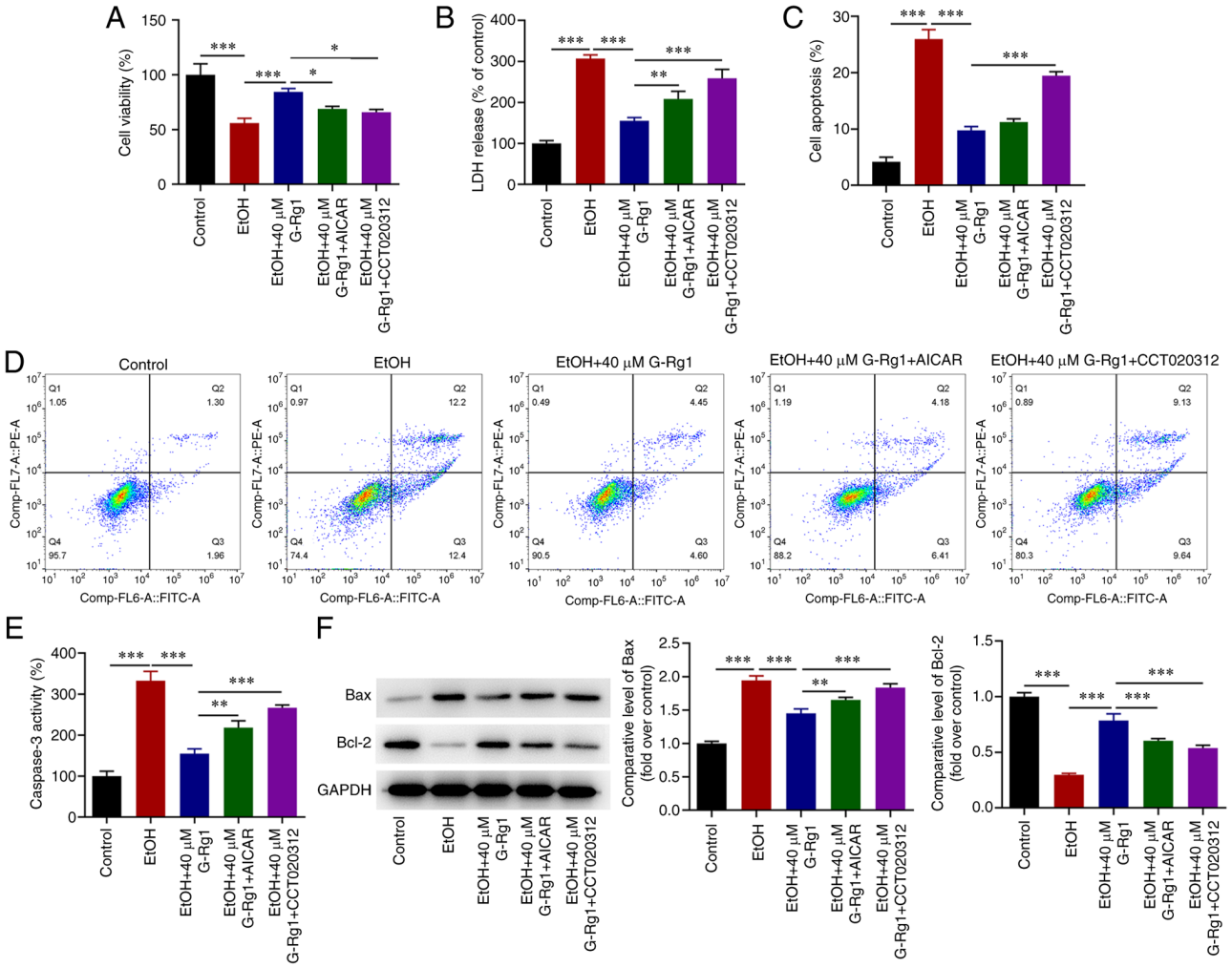

Figure 6. The activation of the AMPK/mTOR or PERK/ATF4/CHOP signaling pathways inhibits the viability and promotes the apoptosis of ethanol-stimulated H9c2 cells treated with G-Rg1. (A) The viability of ethanol-stimulated H9c2 cells co-treated with G-Rg1 and AICAR or CCT020312 was assessed using Cell Counting Kit-8 assay. (B) LDH levels were measured in ethanol-stimulated H9c2 cells co-treated with G-Rg1 and AICAR or CCT020312 using the corresponding LDH kit. (C and D) The apoptotic rate of ethanol-stimulated H9c2 cells co-treated with G-Rg1 and AICAR or CCT020312 was determined using flow cytometric analysis. (E) Caspase-3 activity in ethanol-stimulated H9c2 cells co-treated with G-Rg1 and AICAR or CCT020312 was detected using the corresponding kit. (F) The protein expression levels of Bax and Bcl-2 were measured in ethanol-stimulated H9c2 cells co-treated with G-Rg1 and AICAR or CCT020312 using western blot analysis. The results are expressed as the mean ± SD. The experiments were repeated for three times. *P<0.05, **P<0.01 and ***P<0.001. G-Rg1, ginsenoside Rg1; LDH, lactate dehydrogenase; AMPK, adenosine 5'-monophosphate-activated protein kinase; PERK, protein kinase R (PKR)-like ER kinase; mTOR, mammalian target of rapamycin; CHOP, C/EBP homologous protein; ATF4, activating transcription factor 4; LDH, lactate dehydrogenase; EtOH, ethanol; Bax, Bcl-2 associated X-protein; Bcl-2, B-cell lymphoma 2; EtOH, ethanol.

promotion of the viability and the inhibition of the apoptosis, autophagy and ERS of ethanol-stimulated H9c2 cells.

Long-term alcohol consumption can suppress cardiac protein synthesis and accelerate protein degradation, thus indicating that it can regulate catabolism and autophagy in alcoholic heart diseases (5,37). Of note, the expression levels of the autophagy-related markers, LC3II and autophagy related 7, have been found to be increased in the hearts of rats exposed to chronic alcohol, accompanied by a reduced mTOR activity, a negative regulator of autophagy (38). Another study demonstrated that 3-methyladenine suppressed autophagy to improve alcohol-induced myocardial injuries (39). It has been reported that G-Rg1 is involved in the regulation of cell autophagy. Therefore, G-Rg1 can suppress hypoxia/reoxygenation-induced autophagy in H9c2 cells by attenuating the activation of AMPKα, downregulating LC3II and Beclin-1, and promoting mTOR activation (40). In addition, G-Rg1 has been shown to markedly alleviate angiotensin II-induced podocyte autophagy via the inactivation of the AMPK/mTOR/PI3K pathway (41). Herein, the expression levels of the autophagy-related proteins, LC3-II and Beclin-1, were increased, while those of p62 were decreased in the ethanol-stimulated H9c2 cells compared with the control group. However, the expression levels of the aforementioned proteins were reversed following G-Rg1 treatment, thus suggesting that G-Rg1 attenuated autophagy, that was excessively activated in the ethanol-stimulated H9c2 cells. In addition, ethanol also upregulated p-AMPK and downregulated p-mTOR expression in H9c2 cells. This effect was also reversed by G-Rg1 treatment. Furthermore, molecular docking analysis indicated that G-Rg1 displayed a strong affinity for AMPK, thus suggesting that G-Rg1 suppressed the AMPK/mTOR pathway to protect H9c2 cells against ethanol-induced injury. To verify whether the cardioprotective effect of G-Rg1 against ethanol was triggered by the inhibition

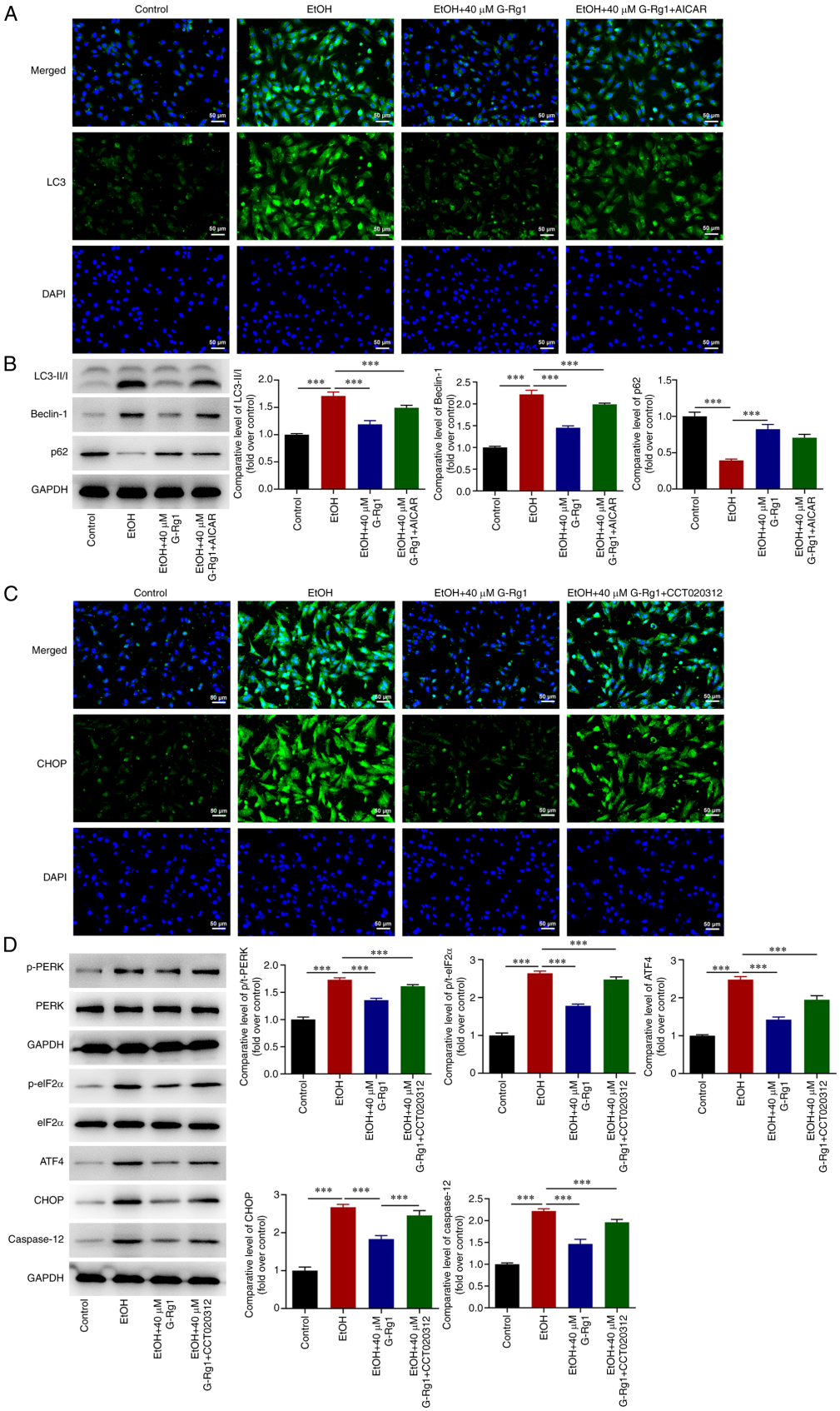

Figure 7. The activation of the AMPK/mTOR or PERK/ATF4/CHOP signaling pathways promotes autophagy and ERS in ethanol-stimulated H9c2 cells treated with G-Rg1. (A) The expression of LC3 in ethanol-stimulated H9c2 cells co-treated with G-Rg1 and AICAR was detected using GFP-LC3 assay. (B) The protein expression levels of LC3-I, LC3-II, Beclin-1 and p62 were measured in ethanol-stimulated H9c2 cells co-treated with G-Rg1 and AICAR using western blot analysis. (C) The expression of CHOP was evaluated in ethanol-stimulated H9c2 cells co-treated with G-Rg1 and CCT020312 using immunofluorescence staining. (D) The expression levels of ERS-related proteins in ethanol-stimulated H9c2 cells co-treated with G-Rg1 and CCT020312 were determined using western blot analysis. The results are expressed as the mean ± SD. The experiments were repeated three times. ***P<0.001. G-Rg1, ginsenoside Rg1; ERS, endoplasmic reticulum stress; AMPK, adenosine 5'-monophosphate-activated protein kinase; PERK, protein kinase R (PKR)-like ER kinase; mTOR, mammalian target of rapamycin; CHOP, C/EBP homologous protein; ATF4, activating transcription factor 4; LC3, light chain 3; EtOH, ethanol.

of autophagy, the cells were co-treated with AICAR, an AMPK agonist. Therefore, the anti-autophagy effect of G-Rg1 on ethanol-stimulated H9c2 cells was attenuated following co-treatment with AICAR.

ERS-induced apoptosis plays a critical role in the pathogenesis of several cardiovascular diseases, such as cardiac hypertrophy, heart failure and ischemic heart disease (42,43). To the best of your knowledge, the findings of ERS or ERS-related events have not yet been found in the tissues of humans with alcohol-induced myocardial injury. Previous studies have demonstrated that ERS is activated in the heart tissues of mice administered ethanol (18,20). However, whether ERS is involved in ethanol-induced cardiomyocyte apoptosis remains elusive. Herein, the results demonstrated that p-PERK, p-eIF2α, ATF4, CHOP and caspase-12 expression levels were upregulated in ethanol-stimulated H9c2 cells, thus suggesting that the PERK/ATF4/CHOP pathway was involved in ethanol-induced H9c2 cell injury. More importantly, treatment of the cells with G-Rg1 inhibited the expression of the aforementioned ERS- and apoptosis-related proteins, while molecular docking analysis revealed that G-Rg1 displayed a strong affinity for PERK. To further clarify whether the cardioprotective effect of G-Rg1 against ethanol-induced injury was mediated by ERS inhibition, the cells were co-treated with the PERK agonist, CCT020312. The results revealed that treatment with ethanol promoted the ERS and apoptosis of the H9c2 cells. However, co-treatment with CCT020312, reversed the anti-ERS effect of G-Rg1 on ethanol-stimulated H9c2 cells.

However, there are several limitations to the present study which should be mentioned. The present study only examined the effects of G-Rg1 on ethanol-induced myocardial injury in an *in vitro* model, which may not be as clinically relevant; thus, animal models need to be established in future studies. Additionally, the lack of PERK and AMPK inhibitors and other methods to detect the intracellular autophagy flow are also limitations of the present study. Further studies are required in the future to fully elucidate the effects and mechanisms of G-Rg1 on ethanol-induced myocardial injury.

In conclusion, the results of the present study suggested that G-Rg1 improved the viability and inhibited apoptosis, autophagy and ERS of ethanol-stimulated H9c2 cells via the inactivation of the AMPK/mTOR and PERK/ATF4/CHOP signaling pathways. Of note, AICAR or CCT020312 attenuated the cardioprotective effects of G-Rg1 on ethanol-stimulated H9c2 cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LZ designed and conceived the study. GT and JL conducted the experiments, and JL analyzed the experimental data. GT drafted the manuscript, which was reviewed and edited by LZ. All authors have read and approved the final version of the manuscript. GT and LZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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