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Original Paper

Compound K Inhibits Autophagy-Mediated Apoptosis Through Activation of the PI3K-Akt Signaling Pathway Thus Protecting Against Ischemia/Reperfusion Injury

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Key Words
Compound K • Autophagy • Apoptosis • Ischemic/reperfusion injury • PI3K-Akt pathway

Abstract
Background/Aims: A series of reports revealed that autophagy and apoptosis exerted detrimental effects on the pathology of cardiac ischemia/reperfusion (I/R) injury. Ginsenoside compound K (CK), a major intestinal metabolite underlying the pharmacological actions of orally administered ginseng, has a protective effect against myocardial I/R injury. However, the molecular mechanisms by which CK protects against I/R injury remain unclear. In this study, we hypothesized that the cardioprotective effects of CK against I/R injury are mediated by inhibiting autophagy/apoptosis-related signaling pathways in H9c2 cardiomyocyte cells. ***Methods:*** H9c2 cells were incubated with CK and exposed to I/R. Cell viability and damage was analyzed by MTT and lactate dehydrogenase assays. Reactive oxygen species (ROS) generation, mitochondrial damage, and cell apoptosis were analyzed by flow cytometry and TUNEL staining. The expression of autophagy, apoptosis, and related signaling proteins was analyzed by Western blotting and immunofluorescence staining. ***Results:*** CK pretreatment promoted cell viability and attenuated ROS accumulation and intracellular mitochondrial damage induced by I/R injury. Moreover, CK reduced autophagy by regulating the formation of phagocytic precursors to autophagosomes and also inhibited apoptosis through a mitochondrial-mediated pathway. Additionally, the cardioprotective effect of CK against I/R injury was mainly through the activation of the PI3K-Akt signaling pathway. ***Conclusions:*** CK pretreatment inhibits autophagy-mediated apoptosis induced by I/R injury through the

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activation of the PI3K-Akt signaling pathway, which reveals that CK may be one of the key bioactive ingredients of ginseng for the treatment of myocardial I/R injury.

Introduction

Myocardial infarction is a major cause of death and disability worldwide [1]. Therapeutic strategies, including thrombolytic primary angioplasty and primary percutaneous coronary intervention, are frequently used to reduce the size of a myocardial infarct and improve the outcome in routine clinical practice [2]. Nevertheless, reperfusion results in a worsening of myocardial ischemia/reperfusion (I/R) injury, which may inevitably reduce the beneficial effects of reperfusion therapy [3]. Therefore, it is necessary to explore novel therapeutic strategies to reduce reperfusion-associated tissue injury after I/R.

Panax ginseng C.A. Meyer, as a traditional medicinal plant, has been used for over 2000 years to heal all aspects of the body [4]. Ginseng is thought to possess beneficial effects for the prevention and treatment of cardiovascular, endocrine, nervous system, and immune system diseases [5, 6]. At present, ginseng is commonly used in individuals who have cardiovascular risk factors, such as hypertension and hypercholesterolemia [7]. Moreover, ginseng and its ginsenoside constituents as bioactive ingredients are widely used in the clinical setting for treating cardiovascular diseases, especially myocardial I/R injury [8]. Recent studies have reported that the ginsenosides Rb1 and Rb2 have cardioprotective effects in models of myocardial I/R *in vitro* and *in vivo* [9]. Indeed, the ginsenosides Rb1, Rb2, Rc, and Rd are transformed by intestinal bacteria to the major metabolite compound K (CK), that is, 20-O-β-(D-glucopyranosyl)-20(S)-protopanaxadiol (Fig. 1A), which is responsible for the pharmacological actions of orally administered ginseng [10]. It has been reported that CK has protective effects against I/R injury in a mouse model [11]. However, the mechanisms underlying the action of CK against I/R injury are unclear.

Autophagy is a cell survival mechanism that involves the degradation and recycling of cytoplasmic components [12]. If autophagic activity is insufficient, long-lived proteins and defective organelles accumulate to induce cell death [13]. In contrast, if autophagy destroys the cytosol and organelles beyond a certain threshold, autophagic cell apoptosis and death occur [14]. A variety of studies have suggested that autophagy during myocardial ischemia is cardiac protective and serves as a regenerative feedback mechanism, which suggests that enhancing autophagy could promote survival in response to mild forms of stress [15], but myocardial I/R-activated autophagy in the reperfusion period is detrimental and causes further myocardial damage [16]. The autophagy and apoptosis resulting from the reperfusion phase are accompanied by the activation of the PI3K-Akt signaling pathway and the up-regulation of Beclin-1 [17, 18]. After the activation of Beclin-1, autophagy-related gene (Atg) proteins, including Atg5, Atg7, and Atg8 (microtubule-associated protein 1 light chain 3, LC3 in mammals), are involved in the nucleation and elongation of autophagosomes. Similarly, as one of the key autophagy substrates, sequestosome 1 (SQSTM1, p62) interacts directly with LC3 and brings p62-containing protein aggregates to the autophagosomes [19, 20]. Thus, the prevention of excessive autophagy and apoptosis induced by I/R may reduce cardiomyocyte death and preserve cardiac function [21].

In this study, we aimed to investigate the protective effect of CK against I/R injury through the inhibition of autophagy and apoptosis via the PI3K-Akt pathway. To test this hypothesis, we used an autophagy inhibitor, 3-methyladenine (3-MA), and PI3K inhibitor, LY294002, to evaluate the potential mechanism of CK on autophagy and apoptosis in a model of I/R injury. Our results showed that autophagy-mediated apoptosis induced by I/R injury was significantly inhibited by CK through the activation of the PI3K-Akt signaling pathway. Our study reveals that CK may be one of the key bioactive ingredients of ginseng for the treatment of myocardial I/R injury.

Materials and Methods

Materials

CK, 3-MA, and LY294002 (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C before use. Rabbit primary antibodies against mTOR (monoclonal, 1:1000, #2983), phospho-mTOR (Ser2448, monoclonal, 1:1000, #5536), phospho-Beclin-1 (monoclonal, 1:1000, Ser15, #84966), Beclin-1 (monoclonal, 1:1000, #3495), Cleaved Caspase-3 (polyclonal, 1:1000, #9661), Bax (polyclonal, 1:1000, #2772), LC3A/B (polyclonal, 1:1000, #4108), Bcl-2 (monoclonal, 1:1000, #2870), SQSTM1/p62 (monoclonal, 1:1000, #8025), phospho-Akt (1:1000, Ser473, #4060), Akt (monoclonal, 1:1000, #4691), Atg7 (monoclonal, 1:1000, #8558), Atg5 (monoclonal, 1:1000, #12994), PARP (monoclonal, 1:1000, #9532), and mouse β-Actin (monoclonal, 1:2000, #3700) were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell culture and I/R modeling

H9c2 cardiomyocyte cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, NY, USA) with high glucose supplemented with 10% fetal bovine serum (FBS; CLARK Bioscience, Claymont, DE, USA), 100 U/mL penicillin (Biosharp, Hefei, China), and 100 μg/mL streptomycin (Biosharp) and incubated at 37°C in a water-saturated atmosphere of 5% CO_2. To establish the I/R injury model, H9c2 cells were seeded in a 96-well plate at a density of

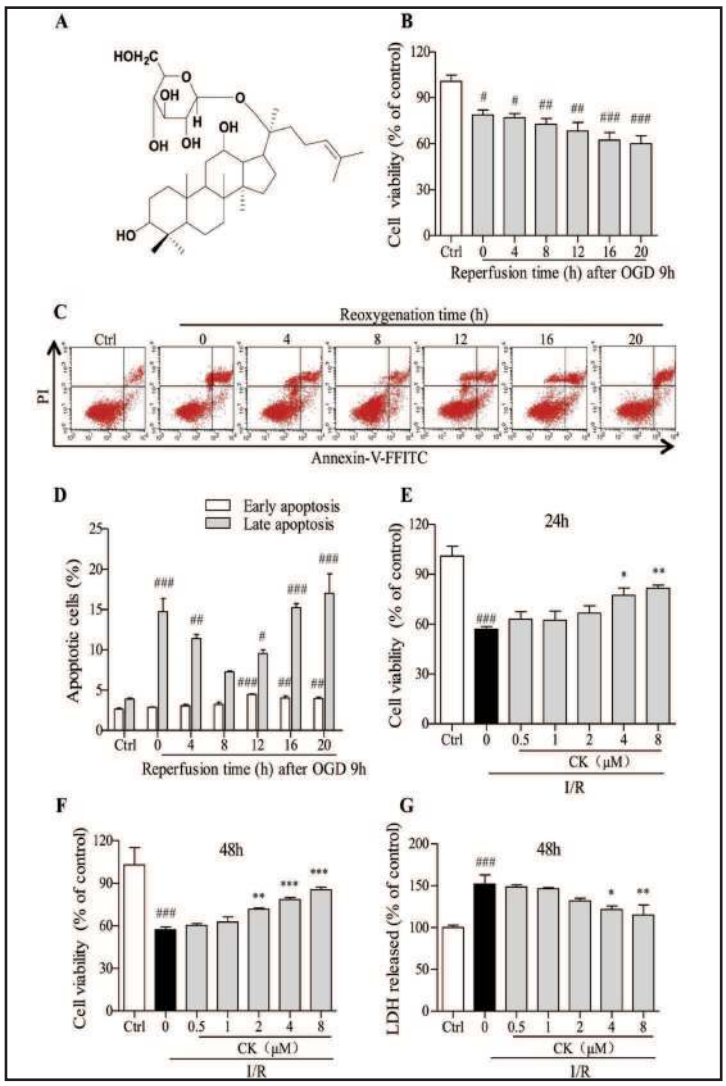

Fig. 1. CK pretreatment increases cell viability and inhibits cell damage induced by I/R. (A) Chemical structure of CK. (B) To investigate the protective effect of CK on I/R injury in H9c2 cardiomyocytes, the effect of reperfusion time after hypoxic incubation for 9 h on the viability of H9c2 cardiomyocytes was observed. (C) and (D) To investigate the protective effect of CK against I/R injury in H9c2 cardiomyocytes, the effect of reperfusion time on apoptosis after hypoxic incubation for 9 h was analyzed. (E) and (F) H9c2 cardiomyocyte viability following treatment with the indicated concentrations of CK for 24 and 48 h of I/R cell injury was assessed using an MTT assay. (G) Effect of CK for 48 h on the release of LDH in culture medium was determined at the end of reperfusion in H9c2 cardiomyocytes was measured using an LDH assay kit. Ctrl: Control group; H9c2 cells were incubated in high-glucose DMEM medium with 10% FBS under normoxia (37°C, 5% CO_2) in each experiment. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. the control group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. the I/R group.

1.0×10^4 cells/well and grown for 24 h. The medium was replaced with serum-free and glucose-free DMEM prior to culture under 95% N_2, 5% CO_2 by incubation for 3, 6, 9, and 12 h (oxygen and glucose deprivation, OGD) in a BioSpa automated incubator (BioTek, Winooski, VT, USA). During the process of reperfusion, the cells were subjected to reperfusion by changing DMEM with high glucose containing 10% FBS followed by incubation under normoxia for 4, 8, 12, 16, and 20 h [22].

MTT and lactate dehydrogenase (LDH) assays

The viability of H9c2 cardiomyocytes treated with different concentrations of CK for 48 h was determined using an MTT assay. After treatment, cells cultured in 96-well plates were incubated with MTT solution (0.5 mg/mL final concentration) at 37°C for 4 h. The formazan crystals were dissolved with DMSO, 100 μL/well, and absorbance was detected at 570 nm on a microplate reader (Infinite M200 Pro, Tecan, Männedorf, Switzerland). The medium from H9c2 cardiomyocytes treated with different concentrations of CK under different conditions was collected to measure LDH release using an LDH assay kit (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Measurement of reactive oxygen species generation

Cells treated with various concentrations of CK for 48 h prior to I/R injury were equilibrated in 10 μM carboxy-H2DCFDA (Beyotime Biotechnology, Shanghai, China) in DMEM for 20 min at 37°C and washed three times with DMEM. Analysis was performed by flow cytometry on a FACS-Calibur (BD Biosciences, San Jose, CA, USA).

Detection of mitochondrial membrane potential

A JC-1 fluorescent probe (Beyotime Biotechnology) was used to estimate the effect of CK on mitochondrial membrane potential (MMP). The cells were cultured in 6-well plates (1.0×10^5 cells/well) and pretreated with various concentrations of CK for 48 h prior to I/R. After I/R, the cells were stained with JC-1 in the dark at 37°C for 20 min and observed immediately under a fluorescence microscopy or analyzed by a FACScan flow cytometer (BD Biosciences).

Cell apoptosis by flow cytometry and TUNEL assay

After treatment and I/R incubation in 6-well plates, cell apoptosis was analyzed using Annexin V-propidium iodide (PI) and TUNEL kits (Beyotime Biotechnology), according to the manufacturer's instruction. Briefly, the cells were harvested, washed twice with cold phosphate-buffered saline (PBS), and then incubated with 5 μL Annexin V-FITC for 15 min and 10 μL PI for 5 min in the dark at room temperature, respectively. Cellular fluorescence was measured with a flow cytometer (FACS Calibur™; BD Biosciences). For TUNEL staining, cells after treatment with CK and I/R incubation were rinsed with PBS and fixed with 4% paraformaldehyde for 40 min. Then, the cells were permeabilized with 0.1% Triton X-100 for 10 min on ice and incubated in TUNEL reaction mixture for 60 min at 37°C in the dark. Finally, the cells were mounted with mounting medium, including DAPI (Beyotime Biotechnology), and covered with a glass coverslip. TUNEL-positive nuclei and the total number of nuclei were counted in at least 5 fields in three independent experiments.

Immunofluorescence staining by confocal microscopy

Briefly, H9c2 cells were placed in 12-well chamber slides (NEST Biotechnology Co., Ltd., Shanghai, China). After treatment with CK and I/R incubation, the cells were incubated with primary antibodies (1:200) and an Alexa Fluor 495-labeled goat anti-rabbit antibody (1:1000; BOSTER, Wuhan, China), and mounted with medium containing DAPI. A Nikon C2 confocal microscope with ZEN software (Nikon, Tokyo, Japan) was used to analyze the levels of p62, p-Akt, and β-Actin. Total laser intensity and photo multiplier gain were set constant for all groups and settings, and data were verified by two independent observers, who were blinded to the experimental group. A minimum of three coverslips were used for each experimental group, and at least three cell images were acquired from each coverslip.

Western blot analysis

Cell samples were lysed in RIPA buffer (Beyotime Biotechnology). Protein concentration was quantified with a BCA protein assay kit (Beyotime Biotechnology). Protein (30 μg) was subjected to 10% or

12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membranes, and probed with primary antibodies overnight at 4°C. β-Actin (1:1000) was used as a loading control. After incubation with appropriate secondary antibodies for 1 h at room temperature, protein bands were visualized using a chemiluminescent imaging system (FluorChem; ProteinSimple, San Jose, CA, USA).

Statistical analysis

Data from all experiments were analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Data are presented as the mean ± standard deviation for continuous variables. Student's two-tailed t test was used when there were only two groups for comparison, and one-way analysis of variance was performed to identify statistical significance in multiple-group comparisons. $P < 0.05$ was considered statistically significant.

Results

CK promotes cell survival and reduces cell damage in H9c2 cells after I/R injury

To investigate the protective effect of CK on I/R injury in H9c2 cardiomyocytes, the viability of H9c2 cells incubated in hypoxia and no glucose/FBS (OGD) for different periods of time and followed by reperfusion for different periods of time was evaluated to establish a model of I/R injury. Incubation under OGD for 9 h led to a 30% decrease in cell viability (Fig. 1B, $P < 0.05$). In the model of I/R injury, reperfusion for 4, 8, 12, 16, and 20 h after 9 h OGD caused a decrease of H9c2 cell viability in a time-dependent manner, with approximately 60% viability after 20 h reperfusion (Fig. 1B, $P < 0.001$). To confirm further reperfusion time after OGD, we examined the percentage of apoptotic cells at different times (0 to 20 h). As shown in Fig. 1C and 1D, OGD for 9 h

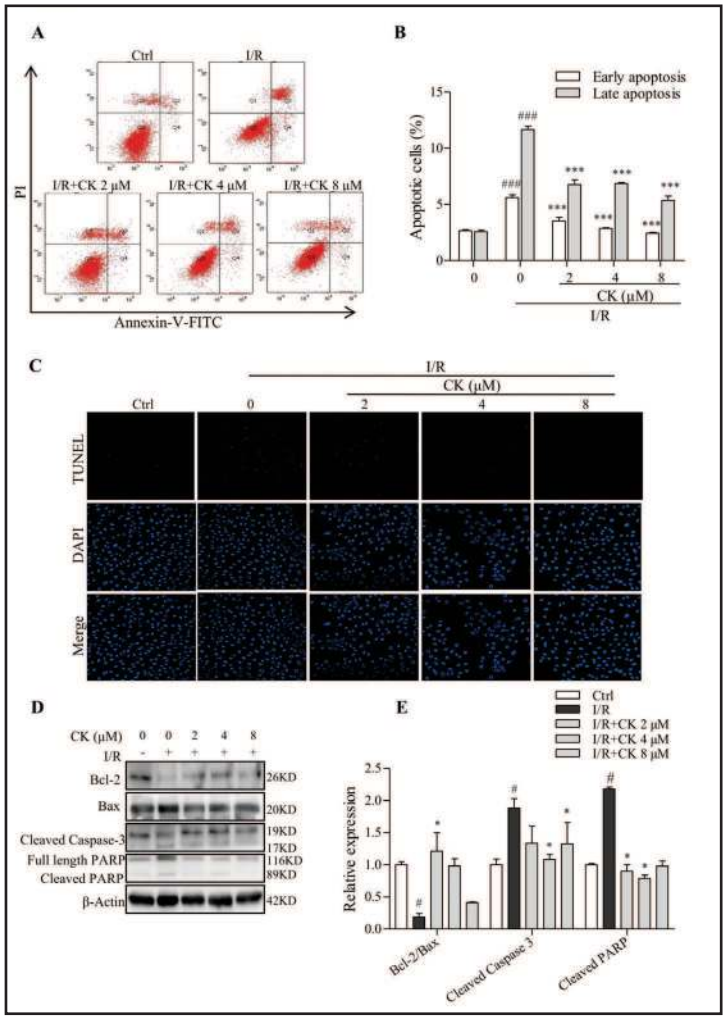

Fig. 2. CK protects H9c2 cardiomyocytes against I/R-induced apoptosis. (A) After treatment with different concentrations of CK (2, 4, or 8 μM) for 48 h, H9c2 cells were stained with Annexin V-FITC/PI to analyze the percentage of cells undergoing apoptosis by flow cytometry. (B) Bar diagram of early and late apoptotic cell rates from three separate experiments. (C) Representative images and bar graphs of TUNEL-positive nuclei (red fluorescence). Blue: DAPI staining. (D) Expression levels of Bcl-2, Bax, Cleaved Caspase-3, and PARP were detected by Western blot analysis. β-Actin was used as a loading control. (E) Relative levels of Bcl-2, Bax, Cleaved Caspase-3, and cleaved PARP to β-Actin were expressed using bar graphs from three separate experiments. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. the control group; $^{*}P<0.05$, $^{***}P<0.001$ vs. the I/R group.

caused late apoptosis (approximately 14.7%). Reperfusion for 12, 16, and 20 h after OGD significantly increased both early apoptosis and late apoptosis ($P < 0.01$, $P < 0.001$). On the basis of these findings, we confirmed the condition of I/R injury as 20 h of reperfusion after 9 h incubation with OGD for further study.

The potential cardioprotective effects of CK on H9c2 cardiomyocytes against I/R injury were estimated using MTT and LDH assays. Pretreatment with CK for 24 h (4 or 8 μM) or 48 h (2, 4, or 8 μM) effectively ameliorated cell viability under 9 h incubation of OGD and subsequent reperfusion for 20 h in a concentration- and time-dependent manner (Fig. 1E and 1F; $P < 0.05$, $P < 0.01$, $P < 0.001$). Moreover, LDH leakage, as a biomarker of cell death, was detected to evaluate the protective effect of CK on cell damage from I/R injury. As shown in Fig. 1G, CK treatment for 48 h significantly decreased the release of LDH induced by I/R injury ($P < 0.05$, $P < 0.01$). These results indicated that CK pretreatment increased cell viability and decreased cell damage to protect against injury from OGD for 9 h and reperfusion for 20 h.

CK decreases I/R-induced apoptosis in H9c2 cells

To observe the effect of CK on I/R-induced apoptosis, the cells were subjected to flow cytometry, TUNEL assay, and Western blot analysis. As shown in Fig. 2A and 2B, the early and late apoptosis induced by I/R injury was decreased after treatment with CK for 48 h ($P < 0.001$). Moreover, the TUNEL assay showed that CK pretreatment reduced the percentage of TUNEL-positive cells caused by I/R injury, which was consistent with the analysis from flow cytometry (Fig. 2C). To analyze further the mechanism of CK against I/R-induced apoptosis, the expression levels of apoptosis-related proteins, such as Bcl-2, Bax, Cleaved Caspase-3, and PARP, were examined by Western blot analysis. The ratio of the antiapoptotic protein Bcl-2 and proapoptotic protein Bax was increased by CK pretreatment for 48 h in H9c2 cells with I/R injury (Fig. 2D and 2E). Additionally, I/R injury

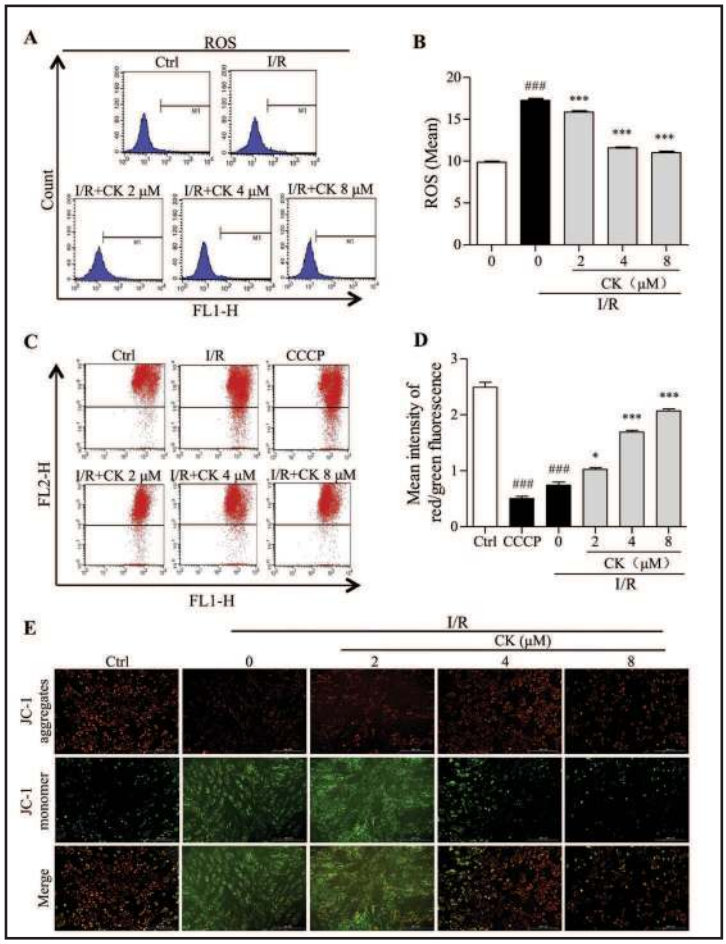

Fig. 3. CK pretreatment attenuates I/R-induced intracellular mitochondrial damage and ROS accumulation in H9c2 cardiomyocytes. (A) Intracellular ROS levels were evaluated by flow cytometry. (B) Bar diagram showing that CK decreased the I/R-induced accumulation of intracellular ROS from three separate experiments in H9c2 cardiomyocytes. (C) MMP was measured by flow cytometry. (D) Bar diagram showing that CK increased the ratio of red to green fluorescence intensity from three separate experiments in H9c2 cardiomyocytes. CCCP: positive control. (E) Representative images of JC-1 red/green cells. ###P<0.001 vs. the control group; *P<0.05, ***P<0.001 vs. the I/R group.

caused the Cleavage of Caspase-3 and PARP in H9c2 cells, which was greatly decreased by CK treatment (Fig. 2D and 2E, $P < 0.05$). Our results suggested that CK inhibited I/R-induced apoptosis in H9c2 cells through a mitochondrial-mediated apoptotic pathway.

CK protects H9c2 cardiomyocytes from intracellular reactive oxygen species accumulation and mitochondrial membrane depolarization

Oxidative damage mediated by free radicals and mitochondrial damage also contributes to I/R-induced injury in cardiomyocytes. Compared with the control, I/R injury increased the level of intracellular reactive oxygen species (ROS) in H9c2 cells by approximately 1.75-fold. CK preconditioning significantly inhibited the increase of ROS induced by I/R injury (Fig. 3A and 3B, $P < 0.001$). MMP was assessed in I/R-induced H9c2 cells treated with CK using JC-1 staining and flow cytometry. I/R resulted in a pronounced decrease in the ratio of red to green fluorescence intensity (Fig. 3C, 3D, and 3E, $P < 0.001$), which is a sign of early stage apoptosis. These results indicated that CK preconditioning could protect cardiomyocytes against damage from intracellular ROS accumulation and mitochondrial membrane depolarization induced by I/R injury.

CK decreases autophagy in H9c2 cardiomyocytes following I/R injury

Autophagy is activated in the initial phase of hypoxia and is excessively enhanced in the reperfusion period. Our findings showed that CK pretreatment decreased ROS generation and increased Bcl-2 expression, which are strong promoters of autophagy through the up-regulation of Beclin-1. To determine whether CK treatment decreased autophagy in H9c2 cells, we observed the effect of CK on the expression of autophagy-related proteins using Western blot analysis and immunofluorescence staining. Beclin-1 is a platform protein that controls the initiation of autophagocytosis and distinct phases in endocytosis [23]. Our

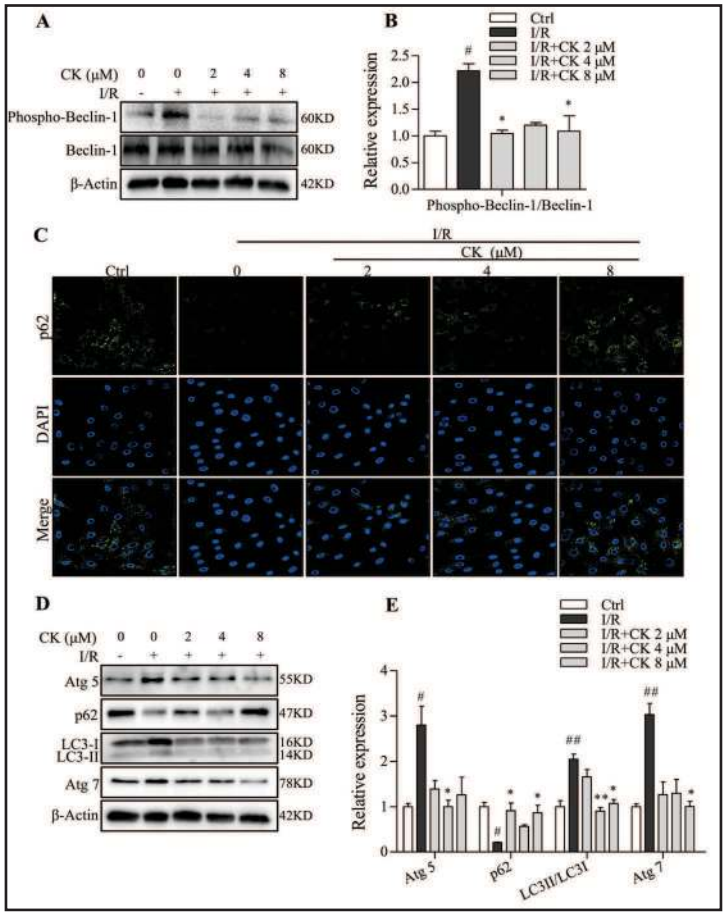

Fig. 4. CK decreases I/R-induced autophagy in H9c2 cardiomyocytes. (A) Expression levels of phospho-Beclin-1 and Beclin-1 in I/R-injured H9c2 cells treated with different concentrations of CK (2, 4, or 8 μM) for 48 h were detected by Western blot analysis. (B) Ratio of phospho-Beclin-1/Beclin-1 to β-Actin is shown in the bar graph. (C) Representative images of p62 staining in I/R-injured H9c2 cells treated with different concentrations of CK (2, 4, or 8 μM) for 48 h. (D) Expression levels of Atg5, p62, LC3-II/I, and Atg7 were detected by immunoblotting analysis. (E) Relative intensity of protein levels to β-Actin was determined using densitometry analysis and shown in the bar graphs. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. the control group; $^{*}P<0.05$, $^{**}P<0.01$ vs. the I/R group.

results showed that the ratio of phospho-Beclin-1 to Beclin-1 was increased in H9c2 cells exposed to I/R, which was greatly reduced by CK treatment (Fig. 4A and 4B, $P < 0.05$). Moreover, p62 binds directly to LC3 via a specific sequence motif and is itself degraded by autophagy to facilitate the degradation of protein aggregates [24]. Staining for p62 showed that I/R injury decreased the expression of p62 in H9c2 cells, which was reversed by CK pretreatment (Fig. 4C). In addition, Atg5 recruits phagocytic precursors to form phagophores, while the related Atg proteins, such as p62, LC3II/LC3I (Atg8), and Atg7, regulate phagophore-to-autophagosome formation [25]. We found that the expression of Atg5, LC3II/I, and Atg7 was increased in H9c2 cells exposed to I/R, which was greatly attenuated by different concentrations of CK (Fig. 4D and 4E, $P < 0.05$, $P < 0.01$). As expected, Western blot analysis showed that CK treatment increased the expression of p62 in H9c2 cells exposed to I/R (Fig. 4D and 4E, $P < 0.05$), which was similar to the finding shown in Fig. 4C. Taken together, these results suggest that CK reduced the autophagy induced by I/R injury by regulating the formation of autophagosomes by phagocytic precursors in H9c2 cells.

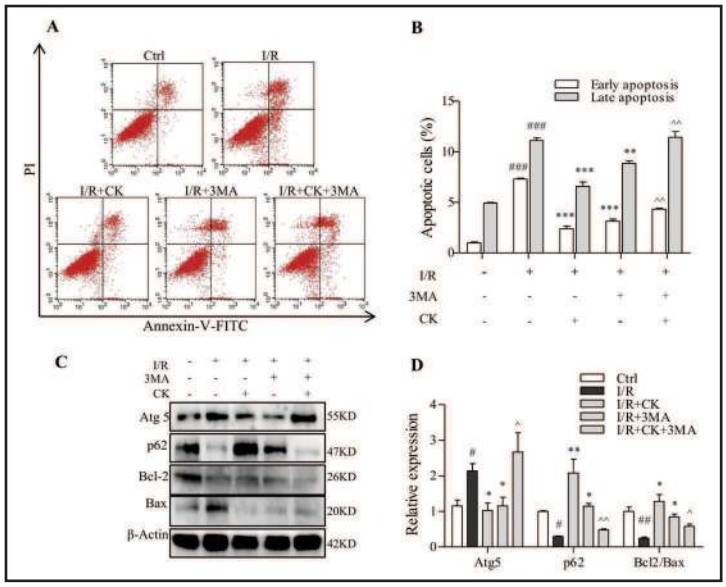

Fig. 5. CK protects cardiomyocytes by inhibiting autophagy-mediated apoptosis in H9c2 cells subjected to I/R. (A) H9c2 cells were treated with CK for 48 h, 3-MA, or their combination for 12 h, followed by I/R injury and harvested for Annexin V-FITC/PI staining to analyze apoptosis by flow cytometry. (B) Bar diagram of apoptotic cell rates (early and late apoptosis) from A in three separate experiments. (C) Expression levels of Atg5, p62, Bcl-2, and Bax were detected by Western blot analysis. (D) Relative intensity of protein levels (Atg5, p62, Bcl-2, and Bax) was determined using densitometric analysis. $^{\#}P<0.05$, $^{\#\#\#}P<0.001$ vs. the control group; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. the I/R group; $^{\wedge}P<0.05$, $^{\wedge\wedge}P<0.01$ vs. the I/R+CK group.

Autophagy-induced apoptosis is involved in the cardioprotective effects of CK in H9c2 cells

To further confirm our hypothesis that the inhibition of autophagy-induced apoptosis could be one of the mechanisms underlying the effect of CK on the recovery from I/R injury, we used a classical autophagy inhibitor, 3-MA, which interferes with the formation of autophagosomes in mammalian cells by inhibiting class III PI3K activity to clarify the relationship between apoptosis and autophagy inhibited by CK. As shown in Fig. 5A and 5B, the percentage of apoptotic cells induced by I/R was suppressed by CK or 3-MA alone treatment. The combination of 3-MA and CK significantly increased the percentage of cells undergoing early and late apoptosis in the I/R environment compared with CK alone, which was similar with the I/R group ($P < 0.01$ or $P < 0.001$). To verify further the effect of CK on alleviating apoptosis induced by I/R through autophagy-mediated pathways, we detected the expression of key autophagy and apoptosis proteins, such as Atg5, p62, Bcl-2, and Bax. Compared with the I/R+CK group, the combination of 3-MA with CK in the I/R group had no obvious effect on decreasing the expression of Atg5 and increasing the expression of p62. In addition, the ratio of Bcl-2 to Bax in the group with the combination of 3-MA and CK was lower than that in the I/R+CK group (Fig. 5C and 5D, $P < 0.05$ or $P < 0.01$). Overall, the results

demonstrated that the anti-apoptosis effect of CK in the I/R environment was attenuated by 3-MA, suggesting that CK alleviated apoptosis induced by I/R through autophagy-mediated pathways.

CK inhibits autophagy and apoptosis through the activation of the PI3K-Akt pathway in H9c2 cells subjected to I/R

The growth-stimulatory signaling axis of PI3K-Akt-mTOR pathways usually represses both autophagy and apoptosis, meaning that the activation of the PI3K-Akt pathway may serve as a potential mechanism for the treatment of cardiac I/R injury [26]. Moreover, AKT can phosphorylate mTOR to inhibit its pro-autophagic and pro-apoptotic functions, respectively [27]. To explore the molecular mechanisms by which CK protects against I/R injury, we used a PI3K inhibitor, LY294002, to evaluate the potential protective role of CK in autophagy-mediated apoptosis induced by I/R through the activation of PI3K signaling. As shown in Fig. 6A and 6B, the percentage of cells undergoing early and late apoptosis induced by I/R was suppressed by CK treatment ($P < 0.001$), which was attenuated by the combination of CK with LY294002 ($P < 0.05$ or $P < 0.01$). The ratio of Bcl-2 to Bax and p62 was higher in the I/R+CK group than with the combination of LY294002 and CK by Western blot analysis. In addition, CK treatment led to significant decreases in the expression of apoptosis markers, PARP cleavage, and autophagy markers, LC3II/I, compared to the I/R group, which were blocked by LY294002 (Fig. 6C and 6D, $P < 0.05$ or $P < 0.01$).

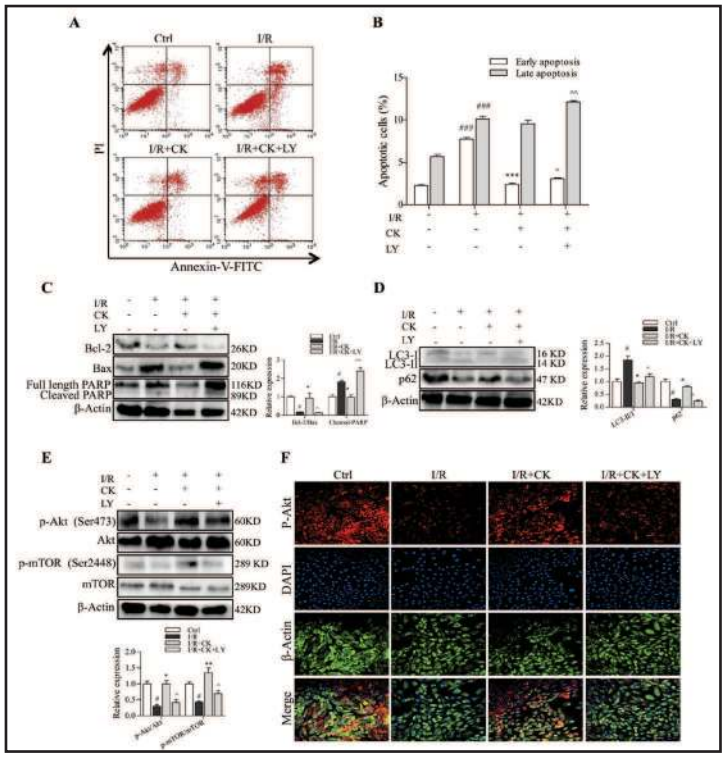

Fig. 6. CK inhibits autophagy and apoptosis in I/R-injured H9c2 cells by activating the PI3K-Akt pathway. (A) H9c2 cells were incubated in the presence of CK, LY294002 (25 μM), or their combination for 12 h and followed by I/R injury. The cells were stained with Annexin V-FITC/PI to analyze apoptosis. (B) Bar diagram of apoptotic cell rates from A in three separate experiments. (C) Cells were harvested and lysed for the detection of Bcl-2, Bax, and PARP and shown in bar graphs of relative protein levels to β-Actin. (D) Expression of LC3-II/I and p62 by Western blot analysis, and the relative intensity of protein levels (LC3-II/I and p62) to β-Actin is shown in the bar graphs. (E) Expression of phospho-Akt (Ser473), Akt, phospho-mTOR (Ser2448), and mTOR by Western blot analysis, and the relative intensity of the protein levels of phospho-Akt (Ser473)/Akt and phospho-mTOR (Ser2448)/mTOR to β-Actin is shown in the bar graphs. (F) Representative images of phospho-Akt (Ser473) and β-Actin were examined by laser scanning confocal microscopy (scale bar = 50 μm). LY: LY294002, PI3K inhibitor. #P<0.05, ##P<0.01, ###P<0.001 vs. the control group; *P<0.05, **P<0.01, ***P<0.001 vs. the I/R group; ^P< 0.05, ^^P<0.01 vs. the I/R+CK group.

To further confirm the effect of CK treatment on the PI3K-Akt pathway, we assessed related proteins from the PI3K-Akt pathway, namely, p-Akt (Ser473) and p-mTOR (Ser2448), by Western blot analysis in H9c2 cells. CK treatment resulted in a significant increase in the phosphorylation of Akt and mTOR compared with the I/R group, which was blocked

by LY294002 (Fig. 6E, $P < 0.05$ or $P < 0.01$). Immunofluorescence staining showed that I/R decreased the level of p-Akt (Ser473) in H9c2 cells, which was reversed by CK treatment. The combination of CK with LY294002 did not obviously increase the level of p-Akt in the I/R cell model compared to CK treatment (Fig. 6F). These data provide evidence that CK treatment attenuated autophagy and apoptosis through the activation of the PI3K-Akt pathway during I/R injury.

Discussion

Initial therapy for acute myocardial infarction is directed toward the restoration of blood supply as soon as possible to reduce damage to the myocardium [28]. However, reperfusion injury remains a major clinical problem that has failed to be improved by multiple pharmacologic approaches [29]. The inhibition of autophagy and apoptosis during the stress of I/R could provide a therapeutic target for cardiac protection. In the present study, we used a cardiomyocyte I/R model to demonstrate that CK, a key metabolite from ginseng after oral administration, exerts a protective effect against I/R-induced injury. Our findings identify a novel mechanism underlying the action of CK on the inhibition of autophagy and apoptosis and provide insights for the therapeutic use of ginseng for the treatment of cardiac disease.

After ischemia, a long period of secondary myocardial injury occurs following myocardial reperfusion, including oxidative stress, inflammation, apoptosis, and autophagy. During reperfusion, the reentry of oxygenated blood into ischemic tissue facilitates ATP restoration and results in the generation of ROS to exacerbate ischemic injury [30]. Being inhibited by low pH, the mitochondrial permeability transition pore (mPTP) is kept quiescent during ischemia [31]. Upon reperfusion, the huge increase of ROS induces the opening of the mPTP, which leads to cell and tissue damage [32]. Thus, antioxidant therapy could be effective in preventing oxidative stress-induced cell injury during I/R. We found that I/R-induced oxidative stress, MMP depolarization, and cell damage were effectively blocked by CK preconditioning in a dose-dependent manner.

Recent studies revealed that autophagy exerted detrimental effects in the pathology of cardiac I/R injury [33]. The process of autophagosome formation involves two major steps: nucleation and elongation of the isolation membrane. The UNC-51-like kinase/Atg1 kinase complex, class III PI3K complex, and their related proteins are important for the nucleation step, whereas the Atg12- and LC3/Atg8-conjugation systems are crucial for the elongation step [34]. Beclin-1 is required for autophagy vesicle nucleation, which is dramatically enhanced after I/R in the area at risk of myocardial infarction [35]. Moreover, LC3 plays a crucial role during the ensuing autophagosome elongation step, which serves as a widely used marker for autophagosomes [36]. Additionally, p62 is purported to act as an adaptor molecule linking ubiquitinated proteins to the autophagic machinery and interActing with LC3-II in the detection of different stages of autophagic vesicles [37]. In the present study, the phosphorylation of Beclin-1 and the ratio of LC3-II/LC3-I were increased after I/R injury, which were reversed significantly by CK. A specific inhibitor of autophagy, 3-MA, was used to explore whether CK played a protective role against I/R injury through autophagy. We found that the effect of CK on autophagy induced by I/R injury was abolished by 3-MA, which confirms that autophagy is a primary event during the secondary damage following reperfusion. To our knowledge, this is the first study indicating that CK protects cardiomyocytes against I/R injury through the inhibition of excessive autophagy.

The interplay between autophagy and apoptosis is highlighted by the fact that a regulator of apoptosis, namely, the Bcl-2 family, regulates autophagy. Conversely, certain autophagic proteins, such as Atg5 and Beclin-1, play important roles in apoptosis [38]. In myocardial I/R injury, autophagy may also promote cell death through the excessive self-digestion and degradation of essential cellular constituents or interaction with apoptotic cascade [39], which could be considered a potential target to decrease apoptosis. We chose several autophagy/apoptosis markers, such as Atg5, p62, and the Bcl-2/Bax ratio, using multiple assays to

assess better the effect of CK on autophagy-mediated apoptosis. Several lines of evidence demonstrated that the anti-apoptotic activity of CK through inhibiting autophagy was dramatically abolished by treatment with 3-MA.

The PI3K-Akt pathway, the main downstream signal activated by I/R injury, is particularly important for mediating myocardial survival under a wide variety of circumstances [20]. Akt phosphorylation can activate mTOR to inhibit autophagy and apoptosis during I/R injury [40]. It has been reported that the PI3K-Akt pathway partially mediates CK-mediated survival in a rat model of myocardial I/R injury [11]. Similarly, our data showed that the cardioprotective effect of CK was blocked by LY294002, suggesting that PI3K-Akt activation underlies the mechanism of CK on the inhibition of autophagy-mediated cell death against I/R injury.

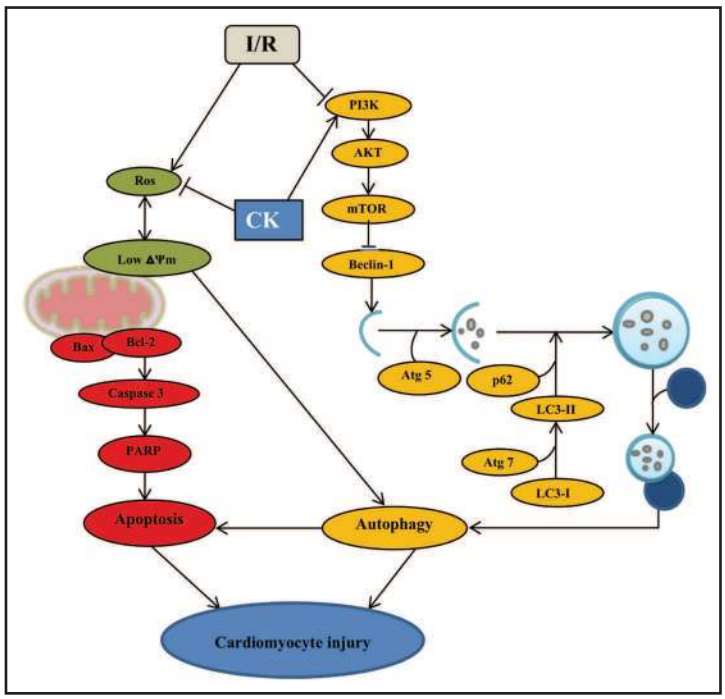

Fig. 7. Schematic illustration of the mechanisms underlying CK protection from I/R-induced autophagy and apoptosis in H9c2 cells. CK preconditioning significantly inhibited ROS generation and MMP induced by I/R injury. CK pretreatment reduced autophagy induced by I/R by regulating the formation of phagocytic precursors to autophagosomes. CK also inhibited I/R-induced apoptosis by reducing the levels of apoptosis-related proteins such as Bcl-2/Bax and increasing the cleavage of Caspase-3 and PARP, which was mediated by autophagy. Importantly, the protective effect of CK against autophagy and apoptosis induced by I/R was mainly through the activation of the PI3K-Akt signaling pathway.

Conclusion

In summary, we, for the first time, have demonstrated that CK provides a superior cardioprotective effect against I/R injury by reducing ROS, restoring MMP, and inhibiting myocardial autophagy-mediated apoptosis in H9c2 cardiomyocytes. Furthermore, our results illustrated that the cardioprotective effect of CK against I/R injury is mainly through the activation of the PI3K-Akt signaling pathway (Fig. 7). Our findings suggest that CK may be suitable for recovery from myocardial I/R injury as a key bioactive ingredient of ginseng.

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Disclosure Statement

The authors declare to have no competing interests.

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