Lycopene protects against apoptosis in hypoxia/reoxygenation-induced H9C2 myocardioblast cells through increased autophagy

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Abstract. Lycopene (Ly), the most common type of antioxidant in the majority of diet types, provides tolerance to ischemia/reperfusion injury. However, the underlying mechanism of the protective effects observed following Ly administration remains poorly investigated. The aim of the current study was to investigate whether Ly prevents damage to hypoxia/reoxygenation (HR)-induced H9C2 myocardioblasts in an autophagy-dependent manner. The levels of autophagic markers were detected using western blotting, the level of apoptosis was detected using western blotting and flow cytometry. The activation of autophagy was impaired via knockdown of the expression of 'microtubule-associated protein 1-light chain 3β (MAP1LC3B)' and 'Beclin 1'. After 16 h hypoxia, followed by 2 h reoxygenation, the expression levels of the microtubule-associated protein 1A/1B-light chain 3 (LC3) and Beclin 1 autophagic biomarkers, and cell viability were reduced, whereas the percentage of apoptotic cells, and the expression levels of the Bax/B-cell lymphoma 2 (Bcl-2) and active caspase-3 apoptotic biomarkers were increased. Pre-incubation of the cells with different Ly concentrations reversed the HR-induced inhibition of autophagy and cell viability, and the HR-induced elevation in apoptotic levels. The induction of autophagy was accompanied by reduced apoptosis, and decreased expression levels of Bax/Bcl-2 and active caspase-3. In addition, the impairment of autophagy by silencing the expression of *MAP1LC3B* and *Beclin 1* accelerated HR-induced H9C2 cell apoptosis and the Ly-mediated protective effects disappeared. Furthermore, Bax/Bcl-2 and active caspase-3 expression levels were increased. Moreover, Ly-induced autophagy was associated with increased adenosine monophosphate kinase (AMPK) phosphorylation. Suppressed AMPK phosphorylation using compound C terminates Ly-mediated cytoprotective effects. Ly treatment improves cell viability and reduces apoptosis as a result of the activation of the adaptive autophagic response on HR-induced H9C2 myocardioblasts. AMPK phosphorylation may be involved in the progression.

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Introduction

Ischemic heart disease is the primary cause of fatalities in developed countries, contributing to ~40% of total mortality (1). One type of ischemic heart disease condition, ischemia/reperfusion (IR) injury, induces myocardiocyte apoptosis and necrosis, and contributes to 25% of the total mortality in patients with acute myocardial infarction (2). In addition, IR injury results in the degradation of excess autophagic flux, and the recycling of misfolded proteins or dysfunctional organelles (3). With a 'housekeeper' function, autophagy is critical in maintaining cellular homeostasis and mediating resistance to apoptosis or senescence (4). Although the protective role of autophagy is widely known, the adaptive autophagic mechanism during myocardiocyte survival remains controversial (5). For example, the induction of autophagy through autophagy-related gene 7 (Atg7) overexpression was shown to be protective in the heart during ischemia (6). However, activated autophagy has been associated with aggravated cardiac hypertrophy in pressure overload-induced heart failure (7). Recently, adenosine and acetylcholine have been shown to provide cardioprotective effects through an underlying autophagy-dependent mechanism (8,9). Thus, autophagy may be required for myocardiocyte survival during ischemia.

Lycopene (Ly), the most common type of carotenoid in dietary intake, has been shown to confer protective effects against ageing, tissue damage, certain types of cancer, atherosclerosis and associated coronary artery disease (10). Possible

mechanisms of Ly-mediated effects are via antioxidant activity, increases in gap-junctional intercellular communication, cell growth control, the activation of the mevalonic acid pathway, suppressing the expression of the oncogene *ras* and the modulation of immune responses (11-14). However, the impact of Ly on IR injury induced by myocardiocyte apoptosis and necrosis has been poorly investigated. During IR, the balance between reactive oxygen species (ROS) generation and elimination is critical in determining cell survival (15). Furthermore, the deregulation of ROS directly induces autophagy in various stress conditions (16). Therefore, Ly may induce autophagy to switch from the cell death pathway to survival in hypoxia/reoxygenation (HR)-induced H9C2 myocardioblast cells.

The present study was conducted to investigate whether the autophagy induced by Ly is associated with HR-induced H9C2 myocardioblast cell damage and whether the proautophagic effect of Ly is involved in the cell protection mechanism.

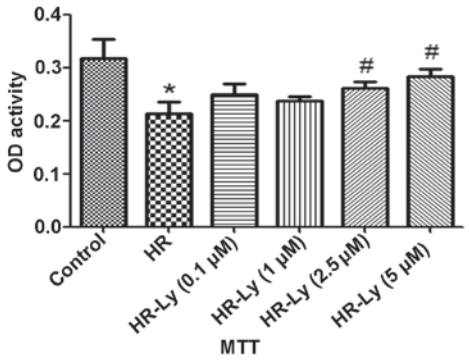

Figure 1. Effect of HR with or without Ly on H9C2 myocardioblast cell viability, as determined by MTT assay. Ly administered at 2.5 and 5 μM concentrations significantly promoted H9C2 cell survival during HR. *P<0.05 compared with control cells; #P<0.05 compared with HR cells. HR, hypoxia/reoxygenation; Ly, lycopene; OD, optical density.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Lipofectamine RNAiMax were purchased from Invitrogen (Carlsbad, CA, USA). Microtubule-associated protein 1-light chain 3β (*MAP1LC3B*), *BECN1* and negative control (*NC*) small interfering (si)RNA were synthesized by GenePharma (Shanghai, China). The chemical reagents compound C (CC), Ly, chloroquine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), were obtained from Sigma-Aldrich (St. Louis, MO, USA). The cell apoptosis detection kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Rabbit monoclonal antibodies specific to LC3 (2057-1; 1:5000), phospho-adenosine monophosphate kinase (p-AMPK, 2802-1, 1:1000), AMPK (1596-1, 1:10000), Bax (1063-1, 1:5000), B-cell lymphoma 2 (Bcl-2, 1017-1, 1:1000) and β-actin (5779-1, 1:10000) and the horseradish peroxidase (HRP) conjugated goat anti-rabbit immunoglobulin (Ig) G secondary antibody (3053-1, 1:10000) were obtained from Epitomics (Burlingame, CA, USA). Rabbit monoclonal antibodies specific to Beclin 1 (3738, 1:1000) and active-caspase-3 (9660, 1:1000) were purchased from Cell Signaling Technology (Denver, MA, USA).. The H9C2 cell line was donated by the Pharmacological Laboratory of Zhejiang University (Hangzhou, China).

Cell culture. The H9C2 cells were grown to 80% confluence in DMEM containing 10% FBS at 37˚C in a humidified 5% CO_2/95% air atmosphere. Subsequent to starvation in serum-free DMEM for 6 h in a normal atmosphere, the H9C2 cells were immediately incubated in serum- and glucose-free DMEM and were moved into a hypoxic atmosphere containing 94% N2, 5% CO2 and 1% O2 to mimic ischemia.. After 16 h of hypoxia, the cells were rapidly transferred into a normal atmosphere with DMEM containing 10% FBS and glucose for reoxygenation (2 h). Prior to the HR, the H9C2 cells were treated with *MAP1LC3B*, *BECN1* or *NC* siRNA, or Ly, with or without chloroquine, for 48 h, and 1 μM CC was added for 24 h prior to HR to suppress AMPK phosphorylation.

siRNA transfection. The following siRNA sequences were transfected into the cells: *MAP1LC3B* sense, 5'-CUCCCU AAGAGGAUCUUUATT-3'; *MAP1LC3B* antisense, 5'-UAA AGAUCCUCUUAGGGAGTT-3'; *BECN1* sense, 5'-GUGGAA UGGAAUGAGAUUATT-3'; *BECN1* antisense, 5'-UAAUCU CAUUCCAUUCCACTT-3'; *NC* sense, 5'-UUCUCCGAA CGUGUCACGUTT-3'; and *NC* antisense, 5'-ACGUGACAC GUUCGGAGAATT-3'. The H9C2 cells were transfected with the respective siRNAs according to the instructions provided by the manufacturers of Lipofectamine RNAiMax.

Cell viability assay. The H9C2 cells were seeded in 96-well plates. Following HR, 5 mg/ml MTT solution was added and the cells were incubated for 4 h at 37˚C. The supernatants were then removed and the cells were solubilized with 150 μl dimethyl sulfoxide (Shanghai Shengong Biotechnology Co., Shanghai, China). The absorbance at 490 nm was measured using an ELISA plate reader (Model 550; Bio-Rad, Hercules, CA, USA).

Detection of cell apoptosis. Following HR treatment, the cells were harvested and incubated with 5 μl Annexin V-fluorescein isothiocyanate and 2.5 μg/ml propidium iodide (PI) for 15 min, and then subjected to flow cytometric analysis (BD FACS; BD Biosciences, Franklin, NJ, USA).

Western blotting. Subsequent to HR, followed by careful washing, the H9C2 cells were harvested and lysed on ice for 45 min using moderate lysis buffer (Beyotime, Haimen, China). Equal quantities of cell proteins were separated via sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature and then probed with primary antibodies overnight at 4˚C, then washed with 1x TBST (Shanghai Shengong Biotechnology Co., Shanghai, China) for 3x10 min. Following expose to the horse radish peroxidase-conjugated secondary antibodies for 1 h at room temperature and then washed with 1x TBST for 3x10 min, immunoreactivity was detected using an enhanced chemiluminescence reagent (Bio-Max, Haemek, Israel).

Statistical analysis. The data are presented as the means ± standard error of the mean from a minimum of three experiments

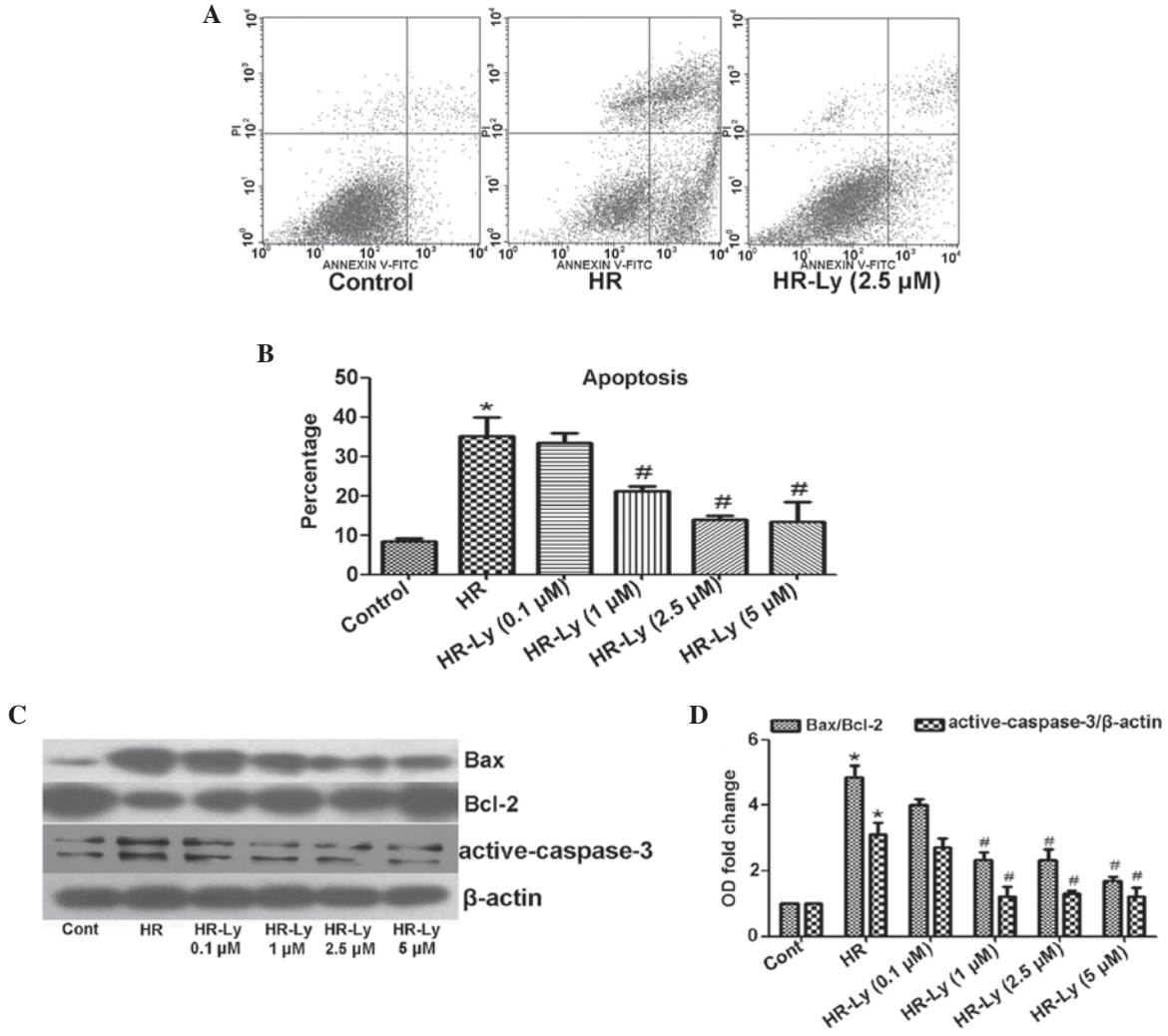

Figure 2. Effect of HR with and without lycopene treatment on H9C2 myocardioblast cell apoptosis. (A) Flow cytometric analysis of representative samples from control cells, HR cells and Ly-pre-incubated cells. Annexin V-positive, PI-positive and -negative cells were identified as apoptotic. (B) Quantitative analysis of the percentage of apoptotic cells during HR following Ly treatment. Ly efficiently attenuated HR-induced H9C2 cell apoptosis. (C) Effect of Ly co-treatment on apoptotic biomarker protein expression levels during HR, as assessed by immunoblot analysis (active-caspase-3, 19 and 17 kDa). (D) Quantitative analysis of the apoptotic biomarker protein expression levels. *P<0.05, as compared with control cells; #P<0.05, as compared with HR cells. HR, hypoxia/reoxygenation; Ly, lycopene; PI, propidium iodide; Bcl-2, B-cell lymphoma 2.

and analyzed with a one-way analysis of variance using SPSS 14.0 for Windows (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Ly improves HR-induced H9C2 cell viability. The MTT assay revealed that H9C2 cell viability was significantly reduced following 16/2 h H/R. Pre-incubation of the H9C2 cells with Ly resulted in the development of tolerance to HR in a dose-dependent manner (Fig. 1). These data indicate that Ly protected the H9C2 cells against HR-induced damage.

Ly reduces HR-induced H9C2 cell apoptosis. HR in H9C2 cells has been shown to result in an increase in the rate of apoptosis (9); therefore, whether Ly reduces the rate of H9C2 cell apoptosis following HR administration was investigated in the present study. As expected, HR treatment for 16/2 h significantly increased the percentage of apoptotic cells (Annexin V-positive, PI-positive and -negative cells) to 35.05%, as compared with that of the untreated cells (8.48%; P<0.05). Preincubation of HR-treated cells in Ly-enriched cell-medium significantly reduced the percentage of apoptotic cells to 21.22 (Ly, 1 μM), 13.9 (Ly, 2.5 μM) and 13.51% (Ly, 5 μM; all P<0.05; Fig. 2A and B), as compared with HR-treated cells maintained in control medium. To further examine the association between the inhibition of apoptosis and Ly treatment, the expression levels of the Bax/Bcl-2 and active-caspase-3 proteins, commonly used as apoptotic biomarkers, were detected. The data reveal significantly elevated levels of Bax/Bcl-2 (3.85-fold) and active-caspase-3 (2.12-fold) in the HR-treated cells (P<0.05); however, the Bax/Bcl-2 and active-caspase-3 protein expression levels in the HR-treated cells were reduced following the addition of Ly (Fig. 2C and D). These data indicate that Ly protected the H9C2 cells against HR-induced apoptosis.

Ly increases autophagy in HR-induced H9C2 cells. As autophagy mediates resistance to apoptosis in stress

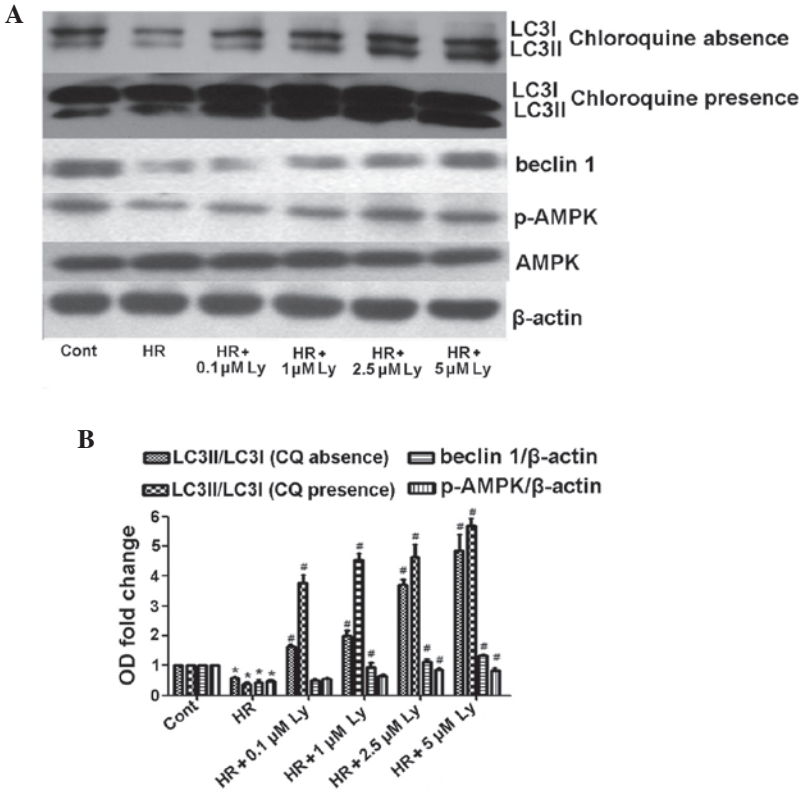

Figure 3. Effect of HR with or without Ly on H9C2 myocardioblast cell autophagy. (A) Effect of HR and Ly treatment on the levels of LC3 (LC3I, 18 kDa; LC3II, 16 kDa) and Beclin 1 protein expression, and AMPK phosphorylation. The presence of chloroquine (10 μM) was used to reflect precise autophagic flux in stress conditions. (B) Quantitative analysis of the autophagic biomarker protein expression levels and the levels of AMPK phosphorylation. *P<0.05 compared with control cells; $^{\#}$P<0.05 compared with HR cells. HR, hypoxia/reoxygenation; Ly, lycopene; LC3, light chain 3; p-AMPK, phospho-adenosine monophosphate kinase; CQ, chloroquine; OD, optical density.

conditions (4), the effect of Ly on autophagy during HR was investigated. Immunoblotting images revealed significantly lower levels of LC3II/LC3I (0.58-fold) and Beclin 1 (0.44-fold) in HR cells, as compared with control cells (P<0.05); however, the levels of these biomarkers in HR-induced cells were elevated by the addition of Ly in a concentration-dependent manner (Fig. 3A and B). An increase in LC3II/LC3I has been previously shown to reflect the impaired fusion of autophagosomes with lysosomes (4). In the present study, to accurately evaluate autophagic flux, chloroquine was used to inhibit autophagolysosomal degradation. Administration of chloroquine prior to HR treatment caused a further reduction in the LC3II/LC3I ratio, a 0.39-fold reduction from the control cell value, as compared with 0.58-fold reduction in HR-induced cells without chloroquine treatment (Fig. 3A and B). However, the addition of Ly with chloroquine increased the LC3II/LC3I ratio in HR-induced cells. These data indicate that Ly induces autophagic flux upregulation in HR-induced H9C2 cells.

MAP1LC3B or BECN1 knockdown enhances apoptosis in HR-induced H9C2 cells. Autophagy biogenesis is initiated by the activation of the Unc-51-like kinase 1 (ULK1) complex and the recruitment of the Beclin 1 complex, and the "isolation membrane" elongates under the LC3-phosphatidylethanolamine (PE) complex (17). To investigate the potential protective effect of autophagy on HR-induced H9C2 cells, *MCP1LC3B* or *BECN1* siRNA was used to inhibit autophagic flux. As expected, *MCP1LC3B* and *BECN1* siRNA effectively inhibited LC3 and Beclin 1 protein expression, respectively (Fig. 4A). Following incubation with *MCP1LC3B* or *BECN1* siRNA, the H9C2 cells were subjected to HR. When autophagy was activated with the addition of Ly, the percentage of apoptotic cells was significantly reduced, as compared with the HR treatment only cells (P<0.05; Fig. 4B and C). When autophagy was inhibited with *MCP1LC3B* or *BECN1* siRNA, the protective effect of Ly against apoptosis disappeared. To further analyze the cytoprotective effect of autophagy, Bax and active-caspase-3 protein expression levels were examined. Notably, autophagic deficiency abolished Ly treatment-mediated inhibition of Bax/Bcl-2 expression and also significantly increased the levels of active-caspase-3 during HR, as compared with those in cells treated with Ly only (Fig. 4D and E). These data indicate that autophagy protects H9C2 cells against apoptosis during HR following Ly administration.

Suppression of AMPK phosphorylation induces autophagic inhibition and cell apoptosis. AMPK is known to activate autophagy in a ULK1-dependent manner. AMPK phosphorylation initiates site specific phosphorylation of ULK1 and subsequently induces formation of the ULK1-complex (17). The data from the present study indicated significantly reduced AMPK phosphorylation in HR-treated H9C2 cells, as compared with control cells (P<0.05), although the levels of AMPK phosphorylation in the HR cells were higher following Ly administration (Fig. 3). To determine whether AMPK phosphorylation is responsible for Ly-induced autophagy, AMPK phosphorylation was suppressed using CC.

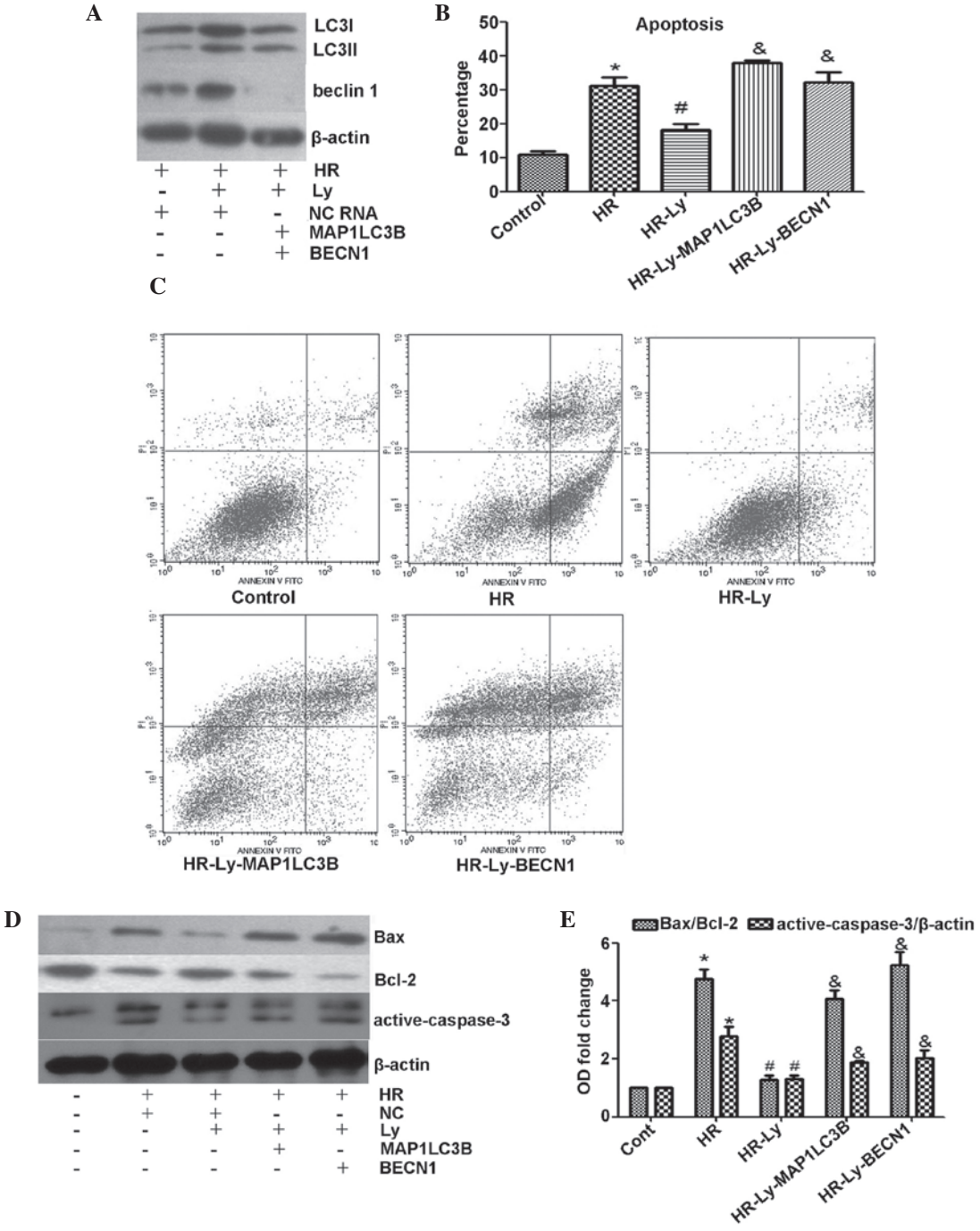

Figure 4. Effect of *MCP1LC3B* or *BECN1* siRNA on lycopene-induced cytoprotection. The administration of 2.5 μM Ly was previously demonstrated to provide maximum cytoprotection; therefore, 2.5 μM Ly was used during the subsequent experiments. (A) Knockdown efficiency of *MCP1LC3B* or *BECN1* siRNA was assessed by western blotting (LC3I, 18 kDa; LC3II, 16 kDa). (B) Quantitative analysis of the rate of apoptosis. Knockdown of LC3 or Beclin 1 abolished lycopene-mediated cytoprotection during HR. (C) Representative flow cytometric analysis images of control cells, HR cells, Ly-pre-incubated cells and siRNA-co-treated Ly cells. (D) Knockdown of LC3 or Beclin 1 exacerbated HR-induced apoptotic biomarker upregulation, as assessed by western blotting (active-caspase-3, 19 and 17 kDa). (E) Quantitative analysis of the apoptotic biomarker protein expression levels. *P<0.05 compared with control cells; $^{\#}$P<0.05 compared with HR cells; $^{\&}$P<0.05 compared with HR-Ly cells. MAP1LC3B, microtubule-associated protein 1-light chain 3β; BECN1, Beclin 1; siRNA, small interfering RNA; Ly, lycopene; HR, hypoxia/reoxygenation; LC3, light chain 3; NC, negative control RNA.

The CC treatment significantly reduced AMPK phosphorylation (P<0.05; Fig. 5A). In addition, as compared with control HR and Ly-treatment, the addition of CC significantly reduced the LC3II/LC3I ratio (P<0.05), induced significantly higher Bax/Bcl-2 and activate-caspase-3 expression levels (P<0.05) and significantly increased the apoptotic cell percentage (P<0.05) upon HR and Ly administration (Fig. 5A and B). The ULK1-complex and Beclin 1-complex exhibit different regulation mechanisms, as AMPK phosphorylation has been demonstrated to not affect Beclin 1 expression (17). In the present study, the Beclin 1 expression levels were not altered upon CC stress (Fig. 5A). These results indicate that AMPK phosphorylation-mediated autophagy exerted cytoprotective effects in HR-induced H9C2 cells following Ly treatment.

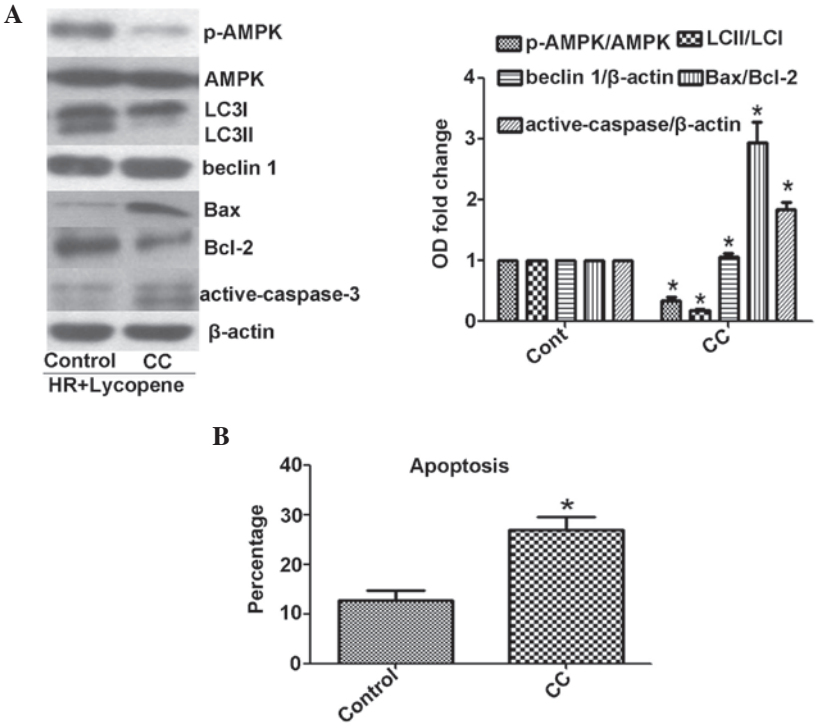

Figure 5. Effect of 1 μM CC on AMPK phosphoyrlation and lycopene-mediated autophagy and cytoprotection. Lycopene at 2.5 μM provides cytoprotection. (A) CC suppresses AMPK phosphorylation, and causes a reduction in LC3 protein expression levels (LC3I, 18 kDa; LC3II, 16 kDa) and an increase in Bax/Bcl-2 and active-caspase-3 expression levels (active-caspase-3, 19 and 17 kDa). (B) CC induces apoptosis upon HR-lycopene treatment. *P<0.05, as compared with control cells treated with HR and lycopene. CC, compound C; AMPK, adenosine monophosphate kinase; Bcl-2, B-cell lymphoma 2; HR, hypoxia/reoxygenation; p-AMPK, phospho-AMPK; LC3, light chain 3.

Discussion

In the present study, HR treatment was demonstrated to suppress autophagic flux and increase the rate of apoptosis in H9C2 cells. Nevertheless, preincubation with Ly ameliorated HR-induced H9C2 cell autophagic inhibition and, in turn, reduced cell apoptosis. The role of autophagic regulation in HR-induced H9C2 cell damage following Ly administration was further verified by *MCP1LC3B* or *BECN1* knockdown. In addition, the prevention of AMPK phosphorylation using CC reduced Ly-induced autophagy and increased H9C2 cell apoptosis upon HR stress. Therefore, autophagy exerted a protective effect on HR-induced H9C2 cells following Ly administration and AMPK phosphorylation may be involved in the regulation of this Ly-induced autophagy.

In response to stimuli, the ULK1 complex and the Beclin 1 complex produce phosphatidylinositol 3-phosphate, and a number of Atg proteins are recruited to initiate autophagy. Subsequently, Atg4 induces LC3 cleavage to LC3I, which becomes conjugated to PE to form LC3II, a protein critical for the elongation and closure of the phagophore. LC3II is then incorporated into the autophagosomal membranes. When the autophagosome fuses with lysosome and degenerates, LC3II reverts to LC3I, is released from the autophagolysosome and is available for future autophagosome formation (17,18). ULK1 site-specific phosphorylation is crucial for the assembly of the ULK1 complex and nucleation of the phagophore. AMPK is required for ULK1 phosphorylation and induction of autophagy. Knockdown of AMPK results in ULK1 dephosphorylation and autophagy impairment (16). As a required quality control mechanism, autophagy promotes cell homeostasis during stress conditions; however, inappropriate autophagy accelerates cell death (19). Therefore, whether autophagy exerts a protective effect in cardiovascular diseases remains controversial. Basal autophagy allows cells to constitutively clear damaged organelles and misfolded proteins. Upon exposure to stressors, such as IR injury and heart failure, rapidly upregulated autophagy is an adaptive response to prevent excessive ROS production, the consumption of ATP, the release of pro-apoptotic factors, the opening of mitochondrial permeability transition pores, and the induction of necrosis or apoptosis (20). Reduced survival and increased cardiac dysfunction have been observed in mice with hearts deficient in Parkin, a required factor for mitophagy, following myocardial infarction (21). In addition, the losses of Atg5 (22), a crucial autophagic initiation factor, and myeloid cell leukemia 1 (23), an analogue of Bcl-2, in adult heart have been shown to result in deficient autophagy and rapid heart failure. In clinical patients, lysosome-associated membrane protein 2 deficiency, a critical fusion factor between autophagosomes and lysosomes, results in a lethal cardiac hypertrophy (24). By contrast, autophagy-mediated maladaptive effects on cardiac diseases have been reported. During pressure overload, angiotensin II-mediated autophagy induces cardiac contractile dysfunction, pathologic remodeling and cardiac atrophy (25), and during the IR-injury phase, accumulating evidence has indicated that the induction of autophagy is harmful for cardiomyocyte survival, particularly under an AMPK-independent manner (26). AMPK-dependent autophagy also has been observed to be maladaptive during IR injury. Inhibiting autophagy by the downregulation of

ULK1 or AMPK reduces the size of myocardial infarction during the reperfusion phase (27). Notably, Beclin 1-dependent autophagy is usually considered to be harmful during the IR phase. Beclin 1 commonly binds to antiapoptotic proteins, such as Bcl-2 and Bcl-xL, to prevent its activation. Upon stress, Bcl-2 dissociation with Beclin 1 allows Beclin 1 activation and autophagic initiation. Matsui *et al* (28) and Ma *et al* (29) have reported that Beclin 1 reduction significantly attenuates the size of myocardial infarcts during the IR phase *in vivo*. Beclin 1-dependent autophagy also exerts protective effects on IR injury; Sala-Mercado *et al* (30) observed that increased expression levels of Beclin 1 and LC3 were associated with reduced infarct size. Furthermore, deficient Beclin 1 expression and the administration of 3-methyladenine have been shown to enhance ischemic injury (31). Increased Akt, Bcl-2, Beclin 1 and LC3B expression levels contribute to the resistance to IR injury in the immature heart, whereas a maturation-associated impairment in IR injury tolerance may be attributed to a reduction in Akt, Bcl-2, Beclin 1 and LC3B expression levels (32). *In vitro*, although Valentim *et al* (33) observed that Beclin 1 knockdown increases cardiomyocyte survival (33), the majority of studies reveal that Beclin 1-dependent autophagy is adaptive. Knockdown of Beclin 1 results in maladaptive autophagy and enhances cardiomyocytes impairment during IR injury (30,34). A possible explanation for this paradox is that different autophagic responses are generated during IR injury *in vivo* and *in vitro*. During IR injury, the autophagic flux elevates *in vivo* (35), whereas autophagic levels are reduced *in vitro* (9,34). In the present study, autophagy was demonstrated to be negatively correlated with the rate of apoptosis in H9C2 cells during HR, with and without Ly, and a positive correlation was detected between autophagy and H9C2 cell survival. Knockdown of LC3 or Beclin 1 suppressed AMPK phosphorylation, increased apoptosis and elevated the expression levels of apoptotic biomarkers, including Bax/Bcl-2 and active-caspase-3, which are the most widely investigated biomarkers of cardiovascular cell death (14). These data indicate that autophagy promotes H9C2 cell survival by providing resistance to apoptosis during HR.

Ly is the most prevalent type of antioxidant in the majority of diets; however, the association between Ly and heart disease remains controversial. An epidemiologic study has revealed that β-carotene intake has a modest but significant inverse correlation with the risk of coronary heart disease (CHD), although a significant correlation between Ly intake and CHD was not identified (11); this inefficiency may be due to a low intake of Ly. Sesso *et al* (36) observed that higher serum Ly concentrations reduced the risk of CHD by 50%. This association was further verified by the Kuopio Ischemic Heart Disease Risk Factor Study, which demonstrated a three-fold risk increase in acute coronary events and stroke in men with a low serum Ly concentration as compared with other men (37). Ly has also been reported to directly protect H9C2 cells and rat heart against ischemia/hypoxia-induced apoptosis (14,38). This protection depends on lipid peroxidation reduction, oxidative DNA damage alleviation, cholesterol synthesis inhibition, immune responses and intercellular communication modulation (14). Recently, an increased dietary intake of Ly has been reported to correlate with increased autophagy, reduced apoptosis, inactivated caspase-3 and improved cell survival in H_2O_2-induced H9C2 cells, as well as in iron-supplemented rats (13,14). In the present study, Ly treatment was negatively correlated with cell survival and apoptosis during HR, and this protective effect may occur through increased autophagy. Disruption of autophagy using *MCP1LC3B* or *BECN1* siRNA, or AMPK phosphorylation inhibition, counteracted Ly-induced cell survival and apoptosis reduction in H9C2 cells, and exacerbated cell injury during HR. Furthermore, active-caspase-3 and Bax/Bcl-2 protein expression levels were reduced following Ly treatment; however, knockdown of LC3 and Beclin 1 abolished active-caspase-3 and Bax/Bcl-2 inactivation by Ly. These data indicate that Ly provides a protective effect by upregulating autophagy against apoptosis to promote cell survival in H9C2 cells following treatment with HR.

In conclusion, autophagy may exert a protective effect against HR-induced H9C2 cell apoptosis. In the present study, Ly upregulated autophagy in H9C2 cells following HR, promoted cell survival and significantly prevented HR-induced apoptosis. Therefore, therapeutic Ly or autophagic induction may offer a novel method for CHD treatment. In addition, a novel mechanism for Ly activity in ischemic conditions has been described, although this mechanism requires further investigation.

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