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ORIGINAL PAPER

Elatoside C protects against hypoxia/reoxygenation-induced apoptosis in H9c2 cardiomyocytes through the reduction of endoplasmic reticulum stress partially depending on STAT3 activation

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Abstract Endoplasmic reticulum (ER) stress-induced apoptosis has been suggested to contribute to myocardial ischemia–reperfusion (I/R) injury. Elatoside C is one of the major triterpenoid compounds isolated from *Aralia elata* that is known to be cardioprotective. However, its effects on I/R injury to cardiac myocytes have not been clarified. This study aimed to investigate the possible protective effect of Elatoside C against hypoxia/reoxygenation (H/R)-induced H9c2 cardiomyocyte injury and its underlying mechanisms. H9c2 cardiomyocytes were subjected to H/R in the presence of Elatoside C. Our results showed that Elatoside C (25 μM) treatment provided significant protection against H/R-induced cell death, as evidenced by improved cell viability, maintained mitochondrial membrane potential, diminished mitochondrial ROS, and reduced apoptotic cardiomyocytes ($P < 0.05$). These changes were associated with the inhibition of ER stress-associated apoptosis markers (GRP78, CHOP, Caspase-12 and JNK), as well as the increased phosphorylation of STAT3 and an increased Bcl2/Bax ratio. Moreover, these effects of Elatoside C were prevented by the STAT3 inhibitor Stattic. Taken together, these results suggested that Elatoside C can alleviate H/R-induced cardiomyocyte apoptosis most likely by activating the STAT3 pathways and reducing ER stress-associated apoptosis.

Keywords Elatoside C · Cardiomyocyte apoptosis · Hypoxia/reoxygenation injury · ER stress · STAT3

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Introduction

Ischemia/reperfusion (I/R) injury is the most common cause of myocardial dysfunction and further cardiomyocyte death after cardiac surgery and myocardial infarctions [1]. Although several pharmacologic and mechanical strategies (such as ischemic pre- or post-conditioning) have been developed to limit myocardial I/R injury, there are still no effective therapeutic agents for preventing I/R injury of the heart due to its complicated and multi-factorial mechanisms [2, 3]. Recently, increasing studies have demonstrated that excessive endoplasmic reticulum stress (ERS) is one of the important pathological mechanisms of I/R injury [4]. Disordered Ca^{2+} homeostasis, ATP

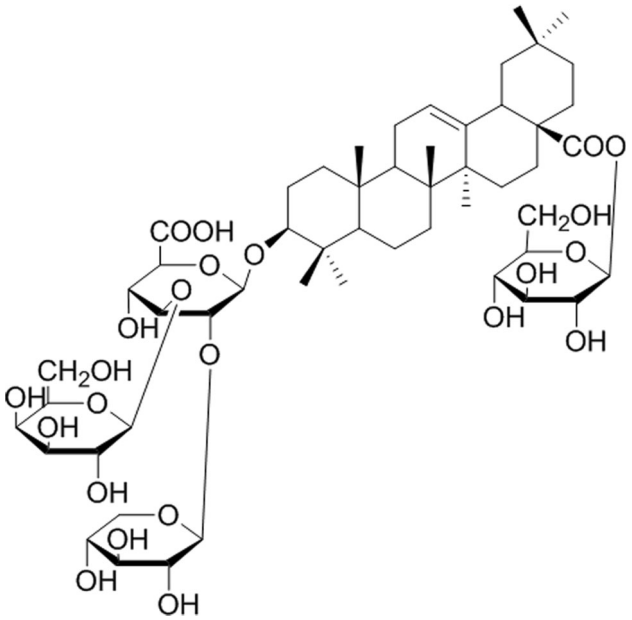

Fig. 1 The chemical structure of Elatoside C

depletion and oxidative stress during I/R injury can cause the accumulation of unfolded or misfolded proteins in the ER lumen, a condition termed ERS [5, 6]. Excessive and prolonged ER stress can trigger the activation of apoptosis through CCAAT/enhancer binding protein homologous protein (CHOP), Caspase-12, and JNK-dependent pathways, eventually leading to cell death [4, 6]. Therefore, the attenuation of ER stress-induced apoptosis may hold therapeutic promise for the treatment of I/R injury.

The signal transducer and activator of transcription 3 (STAT3) signaling pathway, which has been identified as a central point of the pro-survival "Survivor Activating Factor Enhancement (SAFE)" pathway [7], plays a vital role in mediating cardio-protection against I/R injury [1, 8]. The activation of STAT3 during I/R attenuates the extent of cell death and apoptosis, whereas the pharmacological inhibition of STAT3 activation or its genetic deletion has been shown to increase apoptosis and infarct size after I/R [8]. Both activation of the STAT3 pathways and suppression of ER stress are involved in the protection against I/R–induced cardiomyocyte apoptosis [9]. However, little is known of the relative importance of the two cellular signaling pathways during I/R.

Aralia elata (Miq) Seem is widely distributed in Northeastern China, Far East Russia, Japan, and Korea [10]. The bark and roots have been traditionally used as a tonic, antiarrhythmic, anti-arthritic, antihypertensive and anti-diabetic agent in traditional Chinese medicine [11]. Studies on the cardioprotective effects of the total saponins of *A. elata* (AS) have been carried out in our laboratory for many years. Previous studies confirmed that AS exhibits anti-myocardial ischemic and anti-hypoxic activities as well as anti-oxidative capacity [12, 13]. Various oleanane-type triterpene saponins were isolated and identified from AS [10, 14, 15]. However, there are few studies that have focused on the cardioprotective activity of these oleanane-type triterpene saponins. Elatoside C (Fig. 1) is one of the major triterpenoid compounds isolated from *A. elata* [14]. In the present study, we for the first time investigated whether Elatoside C can protect cardiomyocytes subjected to hypoxia/reoxygenation (H/R) injury. The roles of STAT3 as a cardioprotective component and the relationship between STAT3 phosphorylation and ER stress-associated apoptosis in cardioprotection with Elatoside C were also examined. Our results show that Elatoside C attenuates ER stress and cardiomyocyte apoptosis through the activation of the STAT3 pro-survival pathways, thereby reducing I/R injury in cardiomyocytes.

Materials and methods

Materials

Elatoside C was synthesised at the Institute of Medicinal Plant Development (Beijing, China), for details, see Supplementary material. Cell culture products were purchased from Gibco BRL (Grand island, NY, USA). MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and Stattic (a STAT3 blocker) were the products of Sigma Chemical Co. (St. Louis, MO, USA). The fluorescent dye JC-1 was purchased from Sigma-Aldrich (St. Louis, MO, USA). The Annexin V/propidium iodide (PI) apoptosis detection kit and MitoSOX^{TM} Red mitochondrial superoxide indicator were obtained from Invitrogen Corporation (Eugene, OR, USA). Primary antibodies against Bcl-2, Bax, GRP78, Caspase-12, CHOP, JNK, p-JNK and β-actin were obtained from Santa Cruz Biotechnology, Inc. (CA, USA). Primary antibodies against t-STAT3 and p-STAT3 were purchased from Abcam (Cambridge, UK). Horseradish peroxidase (HRP)-conjugated secondary antibodies were secured from CW Biotech (Beijing, China). All chemical reagents were of at least analytical grade.

Cell culture and hypoxia/reoxygenation

The H9c2 cardiomyocyte line was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured as previously described. Briefly, H9c2 cells were cultured in high glucose DMEM supplemented with 10 % (v/v) foetal bovine serum, 1 % penicillin/streptomycin (v/v), and 2 mM L-glutamine. The cells were maintained at 37 °C with 100 % relative humidity in a CO_2 incubator containing 5 % CO_2 at 37 °C.

The H/R procedures were modified from a previous study [16]. High glucose DMEM medium was changed to DMEM with no glucose to mimic ischemia. Then, the H9c2 cardiomyocytes were incubated at 37 °C in an anaerobic glove box (Coy Laboratory, USA), from which normal air was removed by a vacuum pump and replaced with 5 % CO_2, 5 % H_2, and 90 % N_2. The H9c2 cardiomyocytes were cultured under hypoxia for 6 h. Then, the cells were removed from the anaerobic glove box and the medium was replaced with high glucose medium and maintained in the regular incubator to mimic reperfusion. For all experiments, cells were plated at an appropriate density according to the experimental design and were grown for 24 h to reach 70 to 80 % confluence before experimentation.

Experimental protocols

The cultured H9c2 cardiomyocytes were randomly divided into different groups. In the control (Con) group, the H9c2 cardiomyocytes were incubated under normoxic conditions for equivalent durations with high glucose DMEM. The H/R group was conducted as described in the preceding section. In the Elatoside C-treated group (H/R + E), the H9c2 cardiomyocytes subjected to H/R were treated with Elatoside C during reoxygenation for various time periods, as indicated (4 or 12 h). Inhibitor-treated groups were processed the same as the H/R + E group, but the cells were co-incubated with 1 μM Stattic (H/R + S+E) for 1 h when they were treated with Elatoside C. Three experimental categories were included: the first includes the Con, H/R and H/R + E (12.5, 25, and 50 μM) groups; the second includes the Con, E (25 μM), H/R, and H/R + E (25 μM) groups; and the last includes the Con, Con + S, H/R, H/R + S, H/R + E (25 μM), and H/R + S+E(25 μM) groups.

Cell viability analysis

Cell viability was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium (MTT) assay as previously described [17]. Briefly, H9c2 cells were plated on 96-well plates at a density of 1×10^4 cells/well. After designated treatment, 20 μL MTT (5 mg/mL) was added to each well and incubated for 4 h. The medium was then removed, and the formazan crystals were dissolved with dimethyl sulphoxide (DMSO). Absorbance was read at 570 nm on a microplate reader (TECAN Infinite M1000, Austria).

Determination of mitochondrial transmembrane potential ($\Delta\Psi m$)

We used 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) (Sigma-Aldrich, St. Louis, USA) to analyse changes in the mitochondrial transmembrane potential as previously described [17]. After treatment, the cells were incubated with 2 μM JC-1 for 30 min in the dark and washed twice with PBS. The cells labelled with JC-1 were analysed by BD FACSCalibur flow cytometry using 488 nm excitation and green (525 nm) or orange-red (575 nm) emission wavelengths with CellQuest software.

Detection of mitochondria-derived ROS

The level of mitochondrial superoxide production was determined using MitoSOX Red (Invitrogen) according to the manufacturer's protocol. Briefly, following drug treatment, the H9c2 cells were incubated with MitoSOX Red (2.5 μM) in the dark for 20 min. After staining, cells were washed with Hanks' balanced salt solution. The mitochondrial ROS fluorescence intensity was determined using a microplate reader (TECAN Infinite M1000, Austria) at the excitation wavelength of 485 nm and the emission wavelength of 530 nm.

Flow cytometric detection of apoptosis

The percentages of early apoptosis and necrosis were measured using an Annexin V-FITC/PI apoptosis kit for flow cytometry according to the manufacturer's instructions (Invitrogen). After treatment, the cells were harvested and washed twice with cold PBS, and then incubated with 5 μL FITC-Annexin V and 1 μL PI working solution (100 μg/mL) for 15 min in the dark at room temperature. Cellular fluorescence was measured by flow cytometry analysis (FACS Calibur™, BD Biosciences, CA, USA).

Western blot analysis

Cell lysate preparation and western blot analysis were performed as previously described [17]. After treatment, H9c2 cells were harvested and lysed with cell lysis buffer containing 1 % phenylmethylsulfonyl fluoride. The lysate was centrifuged for 15 min at 12,000×*g* and 4 °C to remove insoluble materials. Protein concentration was measured by the bicinchoninic acid assay using a BCA kit (Pierce Corporation, Rockford, USA). Equal amounts of protein (20 μg) from each sample were separated by 12 % SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore Corporation, USA). Nonspecific sites were blocked by incubating the membranes (2 h at room temperature) in 5 % (w/v) non-fat milk powder in Tris-buffered saline containing 0.05 % (v/v) Tween-20 (TBS-T). Thereafter, the membranes were incubated overnight at 4 °C with appropriate primary antibodies. The membranes were washed with TBS-T and incubated with the appropriate secondary HRP-conjugated antibodies at 1:4,000

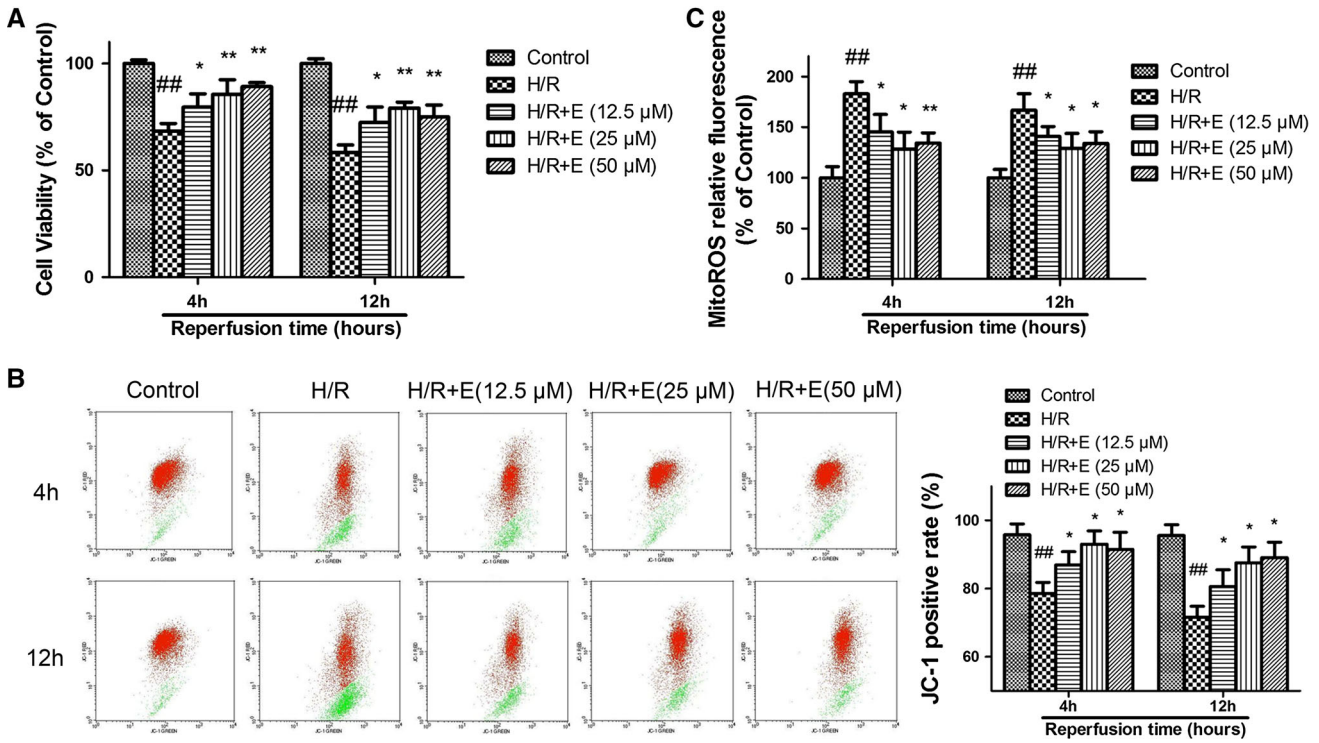

Fig. 2 Effects of Elatoside C on H/R-induced cell death and apoptosis in H9c2 cells. **a** Cell viability was determined by the MTT assay. **b** Flow cytometric analysis of mitochondrial membrane potential by JC-1 staining of H9c2 cells. **c** The mitochondrial ROS levels were measured via a fluorometric assay. *Con* Control; *H/R* Hypoxia/Reoxygenation; *E* Elatoside C. The data are expressed as mean ± SD from three independent experiments. ##$P < 0.01$ versus control; *$P < 0.05$ versus H/R-treated cells; **$P < 0.01$ versus H/R-treated cells

dilutions. Following a 30-min wash, the membranes were visualised by enhanced chemiluminescence using a Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA).

Statistical analyses

The results are expressed as mean ± SD. Comparisons were performed by Student's *t* test or one-way ANOVA followed by Tukey's multiple comparison test with Prism 5.00 software. Statistical significance was set at $P < 0.05$. All data are the result of at least three independent experiments.

Results

Elatoside C alleviated H/R injury

The MTT assay demonstrated that H/R significantly and time-dependently reduced cell viability compared with the Con group, while 12.5, 25, and 50 μM Elatoside C all protected the H9c2 cardiomyocytes against H/R injury (Fig. 2a). It is well known that mitochondria play an important role during I/R injury and that the disruption of mitochondrial membrane potential (△ψm) is an early event in the apoptotic cascade [18]. Mitochondrial depolarisation was indicated by a decrease in the red/green fluorescence intensity ratio of JC-1 staining. The H/R group exhibited an increase in green fluorescence intensity indicating △ψm dissipation (Fig. 2b), while the Elatoside C (12.5, 25, and 50 μM) treatment groups attenuated △ψm dissipation at the two time points (4 h and 12 h), indicating that Elatoside C can protect the cells against H/R-induced injury. Elatoside C treatment groups also eliminated H/R-induced mitochondrial ROS production as measured by MitoSOX staining (Fig. 2c). Furthermore, the different concentrations of Elatoside C (12.5, 25, and 50 μM) treatment increased the expression of anti-apoptosis-related proteins and decreased the expression of pro-apoptotic proteins (Supplementary material, Figure S1). In aggregate, these results indicate that Elatoside C protects H9c2 cells from H/R injury and that 25 μM Elatoside C has the most significant protective effect. Therefore, 25 μM Elatoside C was chosen for further experiments.

Elatoside C protected the cardiomyocytes against H/R injury partly through the attenuation of ER stress-dependent apoptosis activation

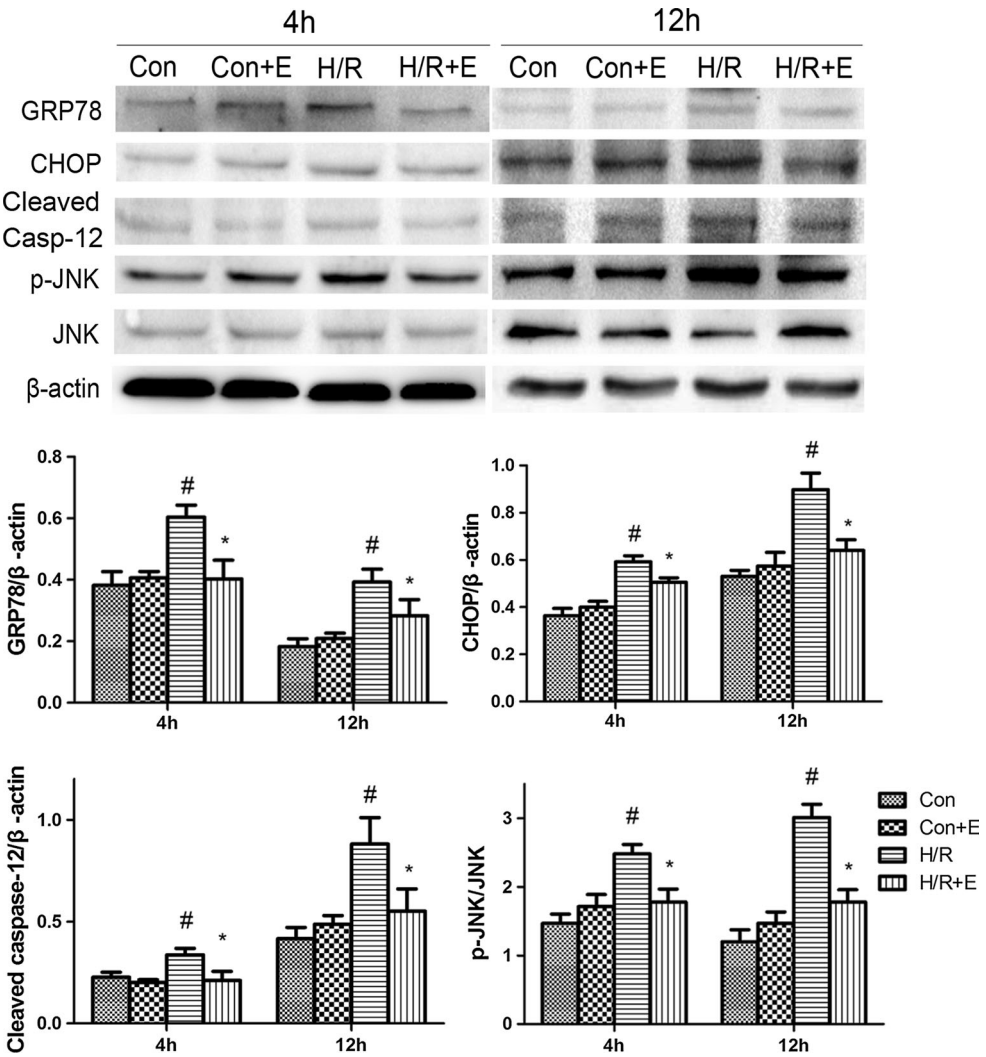

Fig. 3 Effects of Elatoside C on ER stress activation in H/R-treated cardiomyocytes. ER stress-related apoptosis signaling was quantified by measuring its protein markers, including GRP78, p-JNK, cleaved Caspase-12 and CHOP, with representative bands quantified in the corresponding *bar graph*. β-actin expression was examined as the protein loading control. The data are expressed as mean ± SD from three independent experiments. #$P < 0.05$ versus control; ##$P < 0.01$ versus control; *$P < 0.05$ versus H/R-treated cells; **$P < 0.01$ versus H/R-treated cells

We then tested whether 25 μM Elatoside C protects the cardiomyocytes via the modulation of ER stress-dependent apoptosis. As shown in Fig. 3, Elatoside C attenuated GRP78 expression in the H/R group at both 4 and 12 h of reoxygenation. Moreover, Elatoside C also significantly inhibited ER stress-related apoptotic signaling as evidenced by reduced p-JNK, cleaved Caspase-12 and CHOP expression levels, while having no effects in the controls.

Consistent with ER stress-dependent apoptosis activation, the ratio of Bcl2/Bax decreased in the H/R group, and this decrease was greatly minimised at the two measured time points (4 and 12 h) by Elatoside C treatment (Fig. 4a).

STAT3 signaling pathways

The activation of STAT3 was measured using the same samples obtained above. We showed that phospho-STAT3 was significantly increased by Elatoside C treatment at both 4 and 12 h of reoxygenation compared with the H/R group, although the p-STAT3 levels induced by Elatoside C were significantly lower at 12 h than at 4 h of reoxygenation (Fig. 4b). Thus, we suggest that Elatoside C can activate STAT3 pathways.

Inhibition of STAT3 abolished Elatoside C-induced cardioprotection

To determine whether the Elatoside C-induced STAT3 activation is responsible for its cell protective effect, the effect of blocking the STAT3 pathway on the ability of Elatoside C-induced cell protection was determined. As shown in Fig. 5, the STAT3 specific inhibitor Stattic reversed the protection against H/R injury. Increased cell apoptosis was detected as measured by the MTT assay (Fig. 5a) and the Annexin V/PI assay at both 4 and 12 h of reoxygenation (Figs. 5b, c). Meanwhile, in the presence of Stattic, the effect of Elatoside C treatment on the expression of p-STAT3 was also abolished (Fig. 6a). As

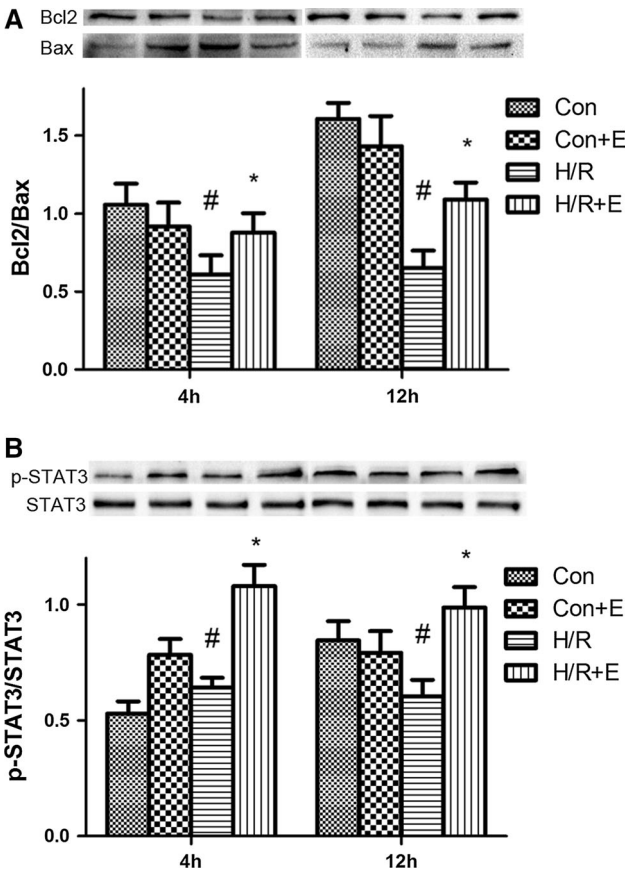

Fig. 4 Effects of Elatoside C on Bcl2, Bax, p-STAT3 and STAT3 expression levels in H/R-treated cardiomyocytes. Western blotting was performed for each group targeting the ratio of Bcl2/Bax (**a**), and the phosphorylation levels of STAT3 (**b**). The data are expressed as mean ± SD from three independent experiments. #$P < 0.05$ versus control; *$P < 0.05$ versus H/R-treated cells

expected, the Stattic + H/R group significantly decreased p-STAT3 expression compared to the H/R group. However, Stattic + H/R had little effect on the expression of total STAT3 compared with the H/R group. Therefore, the STAT3 pathway is involved in the anti-apoptotic effect of Elatoside C.

Roles for STAT3 pathways in modulating ER stress-dependent apoptosis activation

We next investigated whether the inhibition of STAT3 is involved in the protection against ER stress by Elatoside C. As shown in Fig. 6b, Stattic partially blocked the protection offered by Elatoside C against H/R-induced ER stress-dependent apoptosis signaling at 4 h of reoxygenation, as measured by GRP78, p-JNK, cleaved Caspase-12 and CHOP expression levels. At 12 h of reoxygenation, STAT3 inhibition by Stattic still significantly reduced this protection by Elatoside C.

The effect of STAT3 pathways in modulating ER stress-dependent apoptosis activation was also reflected by the ratio of Bcl2/Bax (Fig. 6c). STAT3 inhibition abolished the increased ratio of Bcl2/Bax by Elatoside C treatment against H/R injury at both 4 and 12 h of reoxygenation. Thus, the pharmacological inhibition of STAT3 activation mitigates ER stress-induced cell apoptosis.

Discussion

Aralia elata Xinmaitong capsules mainly composed of the total saponins of *A. elata* (Miq) Seem (AS), which we developed for the treatment of coronary heart disease, have obtained a Drug Clinical Trial Approval Document (Number 2003L01111) from the State Food and Drug Administration and successfully completed Phase III clinical trials in China. Elatoside C, a main oleanane type triterpenoid saponin isolated from *A. elata*, reportedly has strong pharmacologic activity [14]. However, its cardioprotective properties and underlying mechanisms are largely unknown. In this study, we demonstrated for the first time that Elatoside C can attenuate H/R-induced H9c2 cardiomyocyte injury. We further demonstrated that Elatoside C can significantly inhibit ER stress-dependent apoptosis activation, including the CHOP, Caspase-12, and JNK signaling pathways. Finally, we demonstrated that the cardioprotective effects of Elatoside C depend on the activation of STAT3 signal transduction pathways, which was shown to reduce ER stress and myocyte apoptosis in H/R injury.

Cardiomyocyte apoptosis is strongly associated with the pathogenesis of hypoxia and I/R injury [16]. To date, increasing studies suggest that ER stress plays a crucial role in I/R-induced cardiomyocyte apoptosis [19]. Studies verified that excessive ERS triggered by ischemia or H/R usually results in the upregulation of the ER stress response protein GRP78 [19–21]. In this study, we demonstrated that increased GRP78 expression during the H/R process was suppressed by Elatoside C. Meanwhile, we also observed that the ERS-induced activation of proapoptotic factors such as CHOP, JNK and Caspase-12 were significantly and time-dependently increased in H9c2 cells by H/R, which is in accordance with previous studies both in vivo and in vitro [20, 22]. Interestingly, Elatoside C markedly decreased the expression levels of CHOP, JNK, and Caspase-12 with similar time-dependent effects. However, it is noteworthy that the role of Caspase-12 in ER stress-induced apoptosis may differ among cell type and differentiation stage because of an absence of Caspase-12 in most humans [23]. Furthermore, the reversal of ER stress by Elatoside C was accompanied by reduced H/R-induced cell injury, as evidenced by improved cell viability,

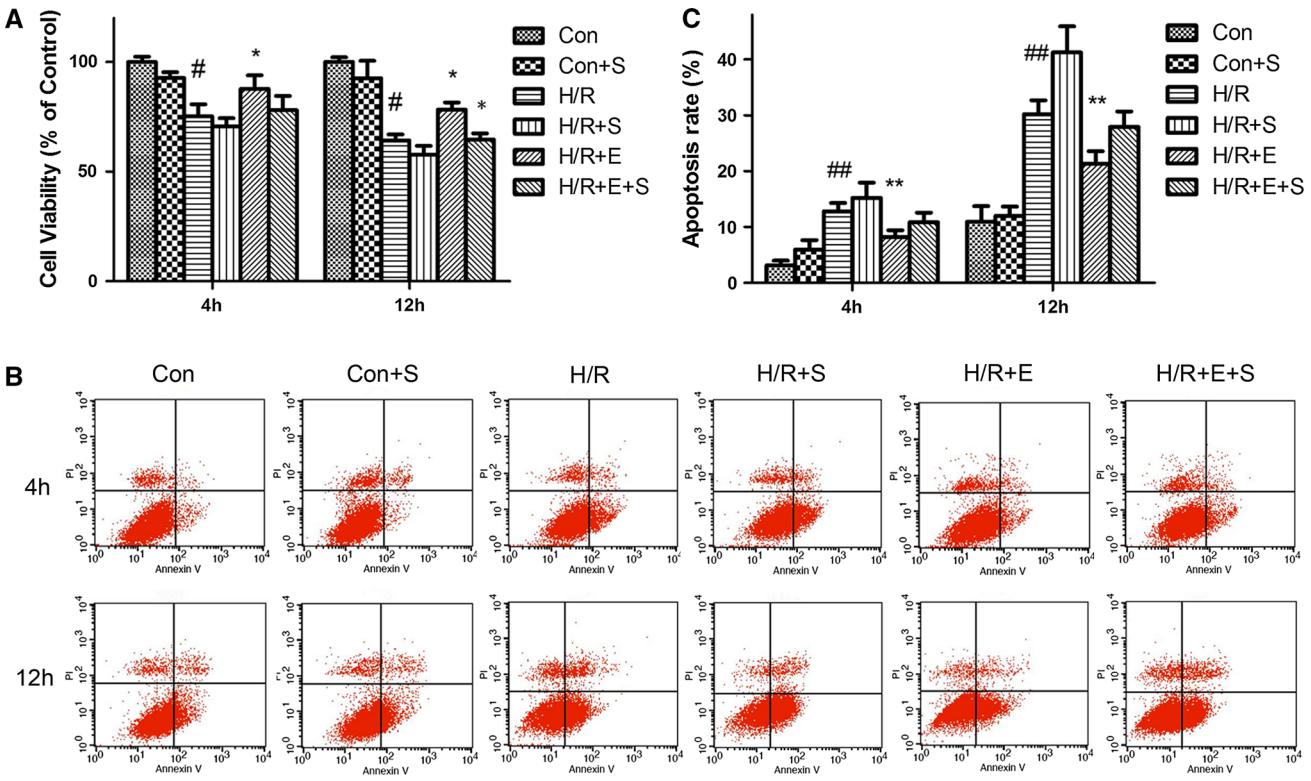

Fig. 5 Effects of Elatoside C and the STAT3 inhibitor Stattic on cell viability and cell apoptosis in H/R-treated cardiomyocytes. **a** Cardiomyocyte viability was assessed using the MTT assay. **b** The apoptotic ratio of H9c2 cardiomyocytes was detected by flow cytometry using Annexin V-FITC and PI staining. **c** Statistical analysis of the flow cytometry data. *Con* Control; *H/R* Hypoxia/Reoxygenation; *E* Elatoside C; *S* Stattic. The data are expressed as mean ± SD from three independent experiments. #$P < 0.05$ versus control; ##$P < 0.01$ versus control; *$P < 0.05$ versus H/R-treated cells; **$P < 0.01$ versus H/R-treated cells

maintained mitochondrial membrane potential, diminished mitochondrial ROS, an increased Bcl2/Bax ratio and reduced apoptotic cardiomyocytes. These results indicate that Elatoside C partially protects cardiomyocytes against H/R injury by attenuating excessive ERS associated - apoptosis.

Signal transducer and activator of transcription 3 (STAT3) has been shown to play an important role in the protection against I/R injury [8]. Previous studies have confirmed that some cardioprotective drugs such as Zoniporide, fasudil and rapamycin [9, 24, 25] can prevent myocardial injury during I/R through the regulation of JAK2/STAT3 signaling. Therefore, we focused on the potential role of the STAT3 pathway in Elaotoside-induced protection. Our results demonstrated that Elatoside C treatment significantly induced up-regulation of p-STAT3 expression compared with the H/R group. The inhibition of STAT3 by Stattic or S3I-201 (Supplementary material, Figure S2) abolished the Elatoside C-induced protection against apoptotic cell death following H/R injury, as shown by increased numbers of apoptotic cardiomyocytes and a reduced Bcl2/Bax ratio. Consistently, it has been reported that STAT3 activation is important in inducing anti-apoptotic signals, which are modulated by the upregulation of Bcl-2 or downregulation of Bax [26, 27]. Above all, we suggest that the STAT3 pathway is involved in the cardioprotection offered by Elatoside C and that Elatoside C-induced STAT3 signaling activation may promote anti-apoptotic signaling in cardiomyocytes during H/R injury.

In the present study, we found that STAT3 activation and ER stress are closely related during the I/R injury-induced apoptotic process. The ER stress in H/R-treated cardiomyocytes was associated with a reduction in phospho-STAT3. The importance of the STAT3 pathway in the mediation of the cytoprotective effect of Elatoside C against H/R-induced ER stress in cardiomyocytes was further confirmed by the use of the STAT3 inhibitor Stattic or S3I-201 (Supplementary material, Figure S2). We showed not only that the ER stress protein markers GRP78, CHOP and Caspase-12 expression levels were increased but also that the cardiomyocyte protection by Elatoside C was abolished when the STAT3 activation was inhibited. These findings indicate that STAT3 activation by Elatoside C can attenuate ER stress-induced cell apoptosis in the protection against H/R injury. However, the precise mechanisms linking the STAT3 pathway to ER stress are

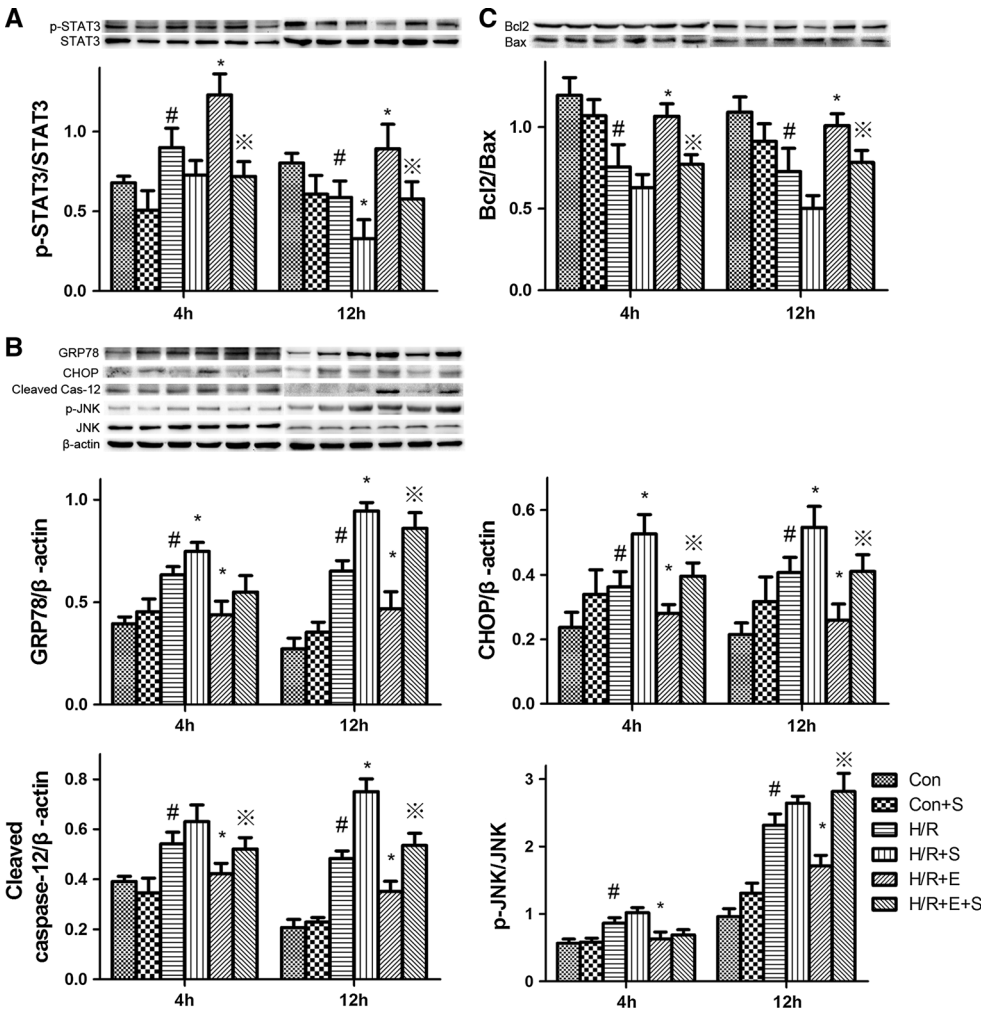

Fig. 6 Effects of Elatoside C and the STAT3 inhibitor Stattic on p-STAT3 (**a**), the ER stress markers (GRP78, CHOP, JNK, and cleaved Caspase-12) (**b**), and Bcl2 and Bax (**c**) expression levels in H/R-treated cardiomyocytes. β-actin expression was examined as the protein loading control. The data are expressed as mean ± SD from three independent experiments. #$P < 0.05$ versus control; *$P < 0.05$ versus H/R-treated cells; ※$P < 0.05$ versus H/R + Elatoside C-treated cells

still not fully understood. Further study is certainly warranted to confirm the efficacy and safety of Elatoside C in vivo and to elucidate the relationship between the STAT3 pathway and ER stress involved in the cardioprotective effects of Elatoside C by using transgenic animal models.

In summary, we found for the first time that Elatoside C possesses a profound cardioprotective effect against H/R injury for H9c2 cardiomyocytes. The underlying mechanisms of Elatoside C-mediated cardioprotection appear to be largely dependent on the activation of STAT3 signaling pathways, which leads to the upregulated expression of the pro-survival Bcl-2/Bax and the attenuation of ER stress-dependent apoptosis. These observations indicate that Elatoside C might act as a potential therapeutic candidate for the treatment of myocardial I/R injury.

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