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Astragaloside IV protects cardiomyocytes from hypoxic injury by regulating endoplasmic reticulum stress via eIF2α/CHOP signaling pathway

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Endoplasmic reticulum stress (ER stress) is suggested to promote cardiomyocyte apoptosis and ultimately lead to ischemic injury. Inhibition of ER stress-induced apoptosis may be a therapeutic strategy for MI injury. Astragaloside-IV (AST) from *Astragalus membranaceus* (Fisch) Bge, was reported to have cardioprotective properties. In this study, we investigated the protective effect of AST on cardiomyocytes against hypoxia injury by regulating ER stress and inhibiting apoptosis. H9c2 cardiomyocytes were divided into three groups, normal group, hypoxia group and AST group. Cell viability was determined by CCK-8 assay. Intracellular reactive oxygen species (ROS) production was detected by DCFH-DA (2,7- dichloro-dihydrofluorescein diacetate) florescent staining. The study showed that AST treatment could significantly increase the cell viability of H9c2 cells exposed to hypoxia. Furthermore, AST could restrain cell apoptosis and decrease the production of ROS. Compared with normal group, the protein levels of Bax, caspase-3, caspase-9, GRP78, p-eIF2α, and CHOP were enhanced in the hypoxia group, whereas the protein level of Bcl-2 was dramatically reduced. Compared with hypoxia group, AST markedly inhibited the phosphorylation of eIF2α and the expression of caspase-3, caspase-9 and CHOP, and promoted the protein expression of Bcl-2. Thus, AST can inhibit the ER stress-mediated apoptosis, partly through the eIF2α/CHOP pathway suppression to inhibit ER stress.

1. Introduction

In recent years, ischemic heart disease has become a global public health concern because of its increasing incidence and high morbidity and mortality (Benjamin et al. 2018). Progressive cardiomyocyte loss resulting from ischemic injury is a critical pathological process in the development of ventricular remodeling and heart failure. Apoptosis is a programmed cell death process (Lee and Gustafsson 2009), and apoptotic cardiomyocytes have been observed in animal models of cardiac tissues and in cardiac injury in patients with myocardial ischemia (MI), myocardial infarction, and heart failure (Narula et al. 1996; Qin et al. 2005). Recent studies have demonstrated that inhibition of cardiomyocyte apoptosis can reduce infarct size and improve ventricular systolic function after MI (Whelan et al. 2009). Consequently, reducing myocardial apoptosis is an essential strategy for the prevention and treatment of ischemic heart disease (Yang et al. 2013).

During ischemia, cardiomyocyte apoptosis may be caused by many factors, such as glucose and amino acid deprivation, oxidative stress, calcium overload, and inflammation (Elmore 2007). Increasing evidence suggests that endoplasmic reticulum (ER) stress occurs when cells are subjected to these environmental and physiological stimulus. In the early stage of ER stress, the unfolded protein response (UPR) signaling cascade is strongly triggered to reduce the aggregation of unfolded and misfolded proteins to maintain ER homeostasis. This adaptive process involves three ER stress sensors-inositol-requiring protein 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase-like ER kinase (PERK)-that cooperate to restore cellular homeostasis (Lin et al. 2008; Szegezdi et al. 2006c). However, when protein-folding stress is prolonged or overwhelming, the UPR begins to fail, and apoptosis is triggered. Therefore, inhibition of ER stress–related apoptosis may play a crucial role in the therapeutic strategy for myocardial ischemia injury (Wang et al. 2018). Excessive ER stress could increase PERK activation, which may result in the phosphorylation of eukaryotic initiation factor 2α (eIF2α) to increase the expression of the downstream target, C/RBP homologous proteins (CHOP), also known as growth inhibition and DNA damage-inducible gene (GADD) 153 (Boyce and Yuan 2006; Kasseckert et al. 2009). The CHOP pathway is a critical branch of the ER stress–mediated apoptosis pathways by regulating the expression of tribbles-related protein3 (TRB3), Bcl-2, GADD34, and death receptor 5 (McCullough et al. 2001).

In recent studies, astragaloside-IV (AST), an active substance found in *Astragalus membranaceus* (Fisch) Bge, has exhibited diverse pharmacological activities, including protective properties and anti-inflammatory, anti-hypertensive, and anti-diabetic activities both *in vitro* and *in vivo* (Gui et al. 2013; He et al. 2013; Luo et al. 1995; Zhang et al. 2011). Emerging investigations have demonstrated that AST can attenuate ER stress. AST has inhibited inflammation and reduced apoptosis through ER stress mechanisms, and AST participates in a protective effect against diabetic nephropathy (Chen et al. 2014; Wang et al. 2015; Zhao et al. 2015a). AST

Abbreviations
eIF2α, eukaryotic initiation factor 2α; CHOP, C/RBP homologous proteins; AST, Astragaloside-IV; ER, endoplasmic reticulum; CCK-8, Cell Counting Kit-8; ROS, reactive oxygen species; DCFH-DA, 2,7- dichloro-dihydrofluorescein diacetate; GRP78, glucose-regulated protein 78; UPR, unfolded protein response; MI, myocardial ischemia; IRE1, inositol-requiring protein 1, ATF6, activating transcription factor 6, PERK, protein kinase-like endoplasmic reticulum kinase.

has also reduced podocyte apoptosis and inhibited phosphorylated PERK (p-PERK) and phosphorylated eIF2α (p-eIF2α) protein levels in rats with streptozotocin-induced diabetes (Wang et al. 2015). In addition, AST has reduced the protein and mRNA levels of glucose-regulated protein 78 (GRP78) and ER-associated proteins (CHOP, TRB3), and AST could reverse the decreased Bcl-2/Bax ratio induced by ER stress. One review found that AST could inhibit caspase-3 to protect against ER stress–induced apoptosis, improve vascular endothelial function (Zhao et al. 2015b). Another study has shown that AST regulated neuronal apoptosis mediated by ER stress in a murine model of Parkinson's disease via a CHOP-related pathway (Ge et al. 2020).

We previously reported that AST could improve the survival of hypoxic cardiomyocytes and stabilize the mitochondrial membrane potential in H9c2 cells. However, the effect of AST on anoxic injury involving eIF2α/CHOP-induced ER stress–related apoptosis has not been extensively investigated, to our knowledge. Therefore, we investigated the protective effect of AST, mediated by the eIF2α/CHOP signaling pathway, on ER stress and cardiomyocyte apoptosis in hypoxia-induced H9c2 cells.

2. Investigations and results

2.1. AST Increased viability of H9c2 cells exposed to hypoxia-induced injury

To explore the myocardial protective effects of AST, we investigated cell proliferation in AST-treated H9c2 cells using CCK-8. H9c2 cells were incubated with different concentrations of AST (50, 10, 5, 1, and 0.5 μmol/l). As shown in Fig. 1, AST exposure induced significant upregulation of cell viability at the concentrations of 5 μmol/l and 10 μmol/l.

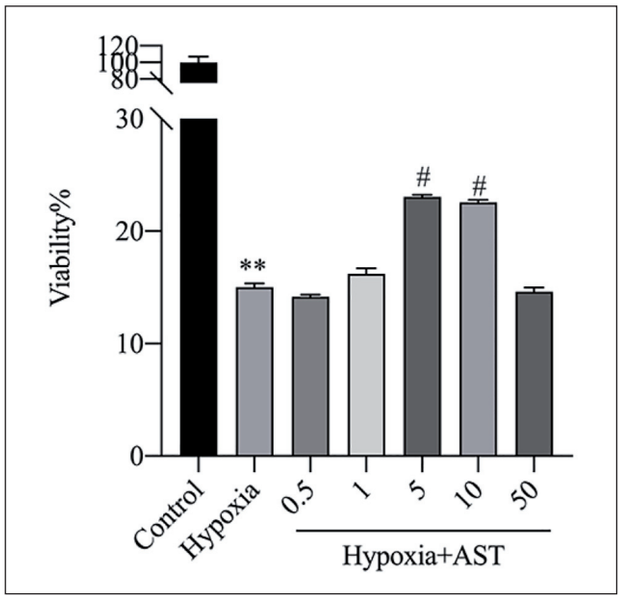

Fig. 1: Determination of H9c2 cell viability evaluated by Cell Counting Kit-8 (CCK-8). Proliferation of H9c2 cells treated with different concentrations (50, 10, 5, 1, and 0.5 μmol/l) of astragaloside IV. *$p < 0.05$ vs normal group; **$p < 0.01$ vs normal group; $^{\#}p < 0.05$ vs hypoxic group; $^{\#\#}p < 0.01$ vs hypoxic group. Statistics were calculated using results of three repetitions of each experiment.

2.2. AST Alleviated cell apoptosis

To study the effects of AST on apoptosis in the context of hypoxia, flow cytometry analysis was performed. The analysis revealed that the cell apoptosis rate (early apoptotic plus late apoptotic) was increased 24 h after induction of hypoxia (Fig. 2); however, AST resulted in a significant decrease in the ratio of apoptotic cells. Collectively, these results revealed that AST alleviated apoptosis in hypoxic H9c2 cells.

Western blot analysis revealed that the protein expressions of caspase-3 and Bax were increased under the hypoxic conditions but were decreased by AST treatment. Compared with the normal group, the hypoxia group had a decreased Bcl-2 level but the AST group had an increased Bcl-2 level (Fig. 3). These results indicated that the myocardial protective effect of AST against apoptosis induced by hypoxia was related to the suppression of caspase-3 and Bax and the activation of Bcl-2.

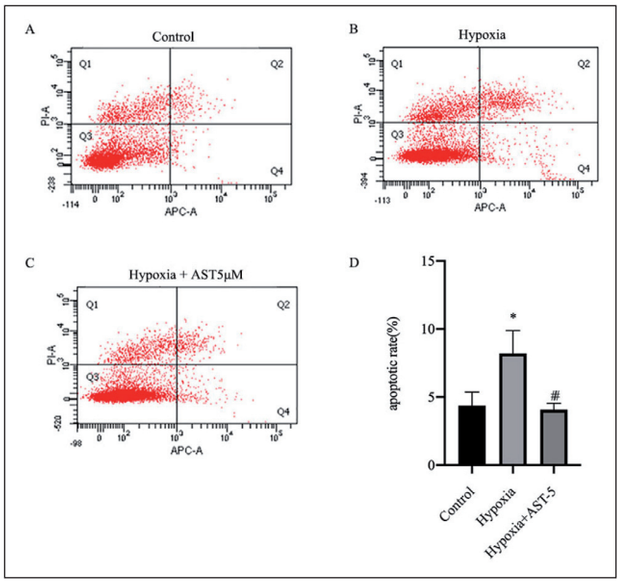

Fig. 2: Measurement of apoptosis analyzed by flow cytometry. *$p < 0.05$ vs normal group; **$p < 0.01$ vs normal group; $^{\#}p < 0.05$ vs hypoxic group; $^{\#\#}p < 0.01$ vs hypoxic group. Statistics were calculated using the results of three repetitions of each experiment.

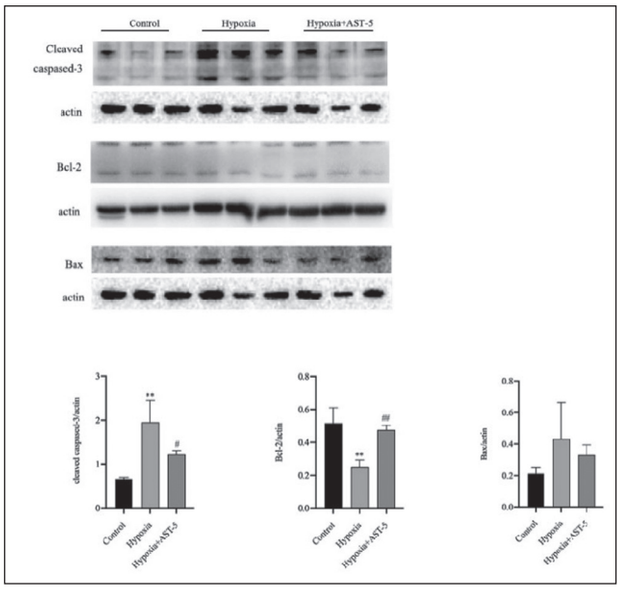

Fig. 3: Protein expression of Bcl-2, Bax, and caspase-3 in H9c2 cells measured by western blot analysis. *$p < 0.05$ vs normal group; **$p < 0.01$ vs normal group; $^{\#}p < 0.05$ vs hypoxic group; $^{\#\#}p < 0.01$ vs hypoxic group. Statistics were calculated using the results of three repetitions of each experiment.

2.3. AST attenuated oxidative damage induced by hypoxia in H9c2 cells

To observe the oxidative stress in cardiomyocytes under hypoxic conditions and the protective effect of AST on cells under hypoxia, DCFH was used to determine intracellular reactive oxygen levels. Experiments showed that the ROS level increased significantly in

the hypoxia group compared with the normal group ($p < 0.05$). The ROS level decreased significantly in the AST group compared with the hypoxia group ($p < 0.05$). These results indicated that AST had notable inhibitory effects on antioxidative stress.

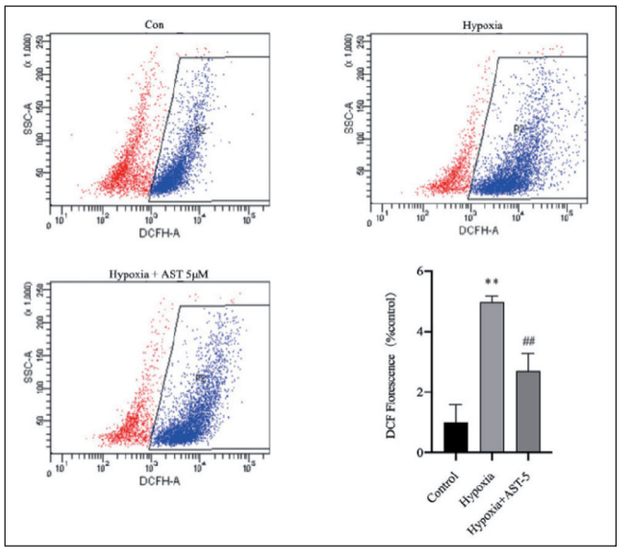

Fig. 4: The ROS level in the H9c2 cells as detected by flow cytometry. *$p < 0.05$ vs normal group; **$p < 0.01$ vs normal group; #$p < 0.05$ vs hypoxic group; ##$p < 0.01$ vs hypoxic group. Statistics were calculated using the results of three repetitions of each experiment.

2.4. *Effect of AST on ER-related apoptosis pathways in H9c2 cells*

Since ER stress plays an important role in cardiomyocyte apoptosis under hypoxia, we detected ER stress-related proteins and observed the effect of astragaloside IV on these proteins. The protein expression levels of GRP78, cleaved caspase-9, CHOP, eIF2α, and p-eIF2α, as determined by western blot analysis, are shown in Fig. 4. The result indicated that, after treatment of hypoxia, the intracellular protein expression levels of GRP78 cleaved caspase-9, CHOP, and p-eIF2α in the hypoxia group were all dramatically increased ($p < 0.01$). In contrast, when the H9c2 cells were treated with 5 μM of AST under hypoxia, the protein levels of cleaved caspase-9, CHOP, and p-eIF2α were significantly downregulated ($p < 0.01$).

3. Discussion

In this study, the protein expressions of p-eIF2α, CHOP, Bax, cleaved caspase-3, and cleaved caspase-9 in cardiomyocytes in hypoxic conditions was increased, and these increases, were blunted by AST administration. Moreover, AST inhibited ER stress–induced cardiomyocyte apoptosis and attenuated ROS generation. A possible mechanism of protection was related to the inhibition of the eIF2α/CHOP pathway.

MI injury is a complicated pathophysiological process involving multiple apoptosis-related signaling pathways (Johnson 2014). Previous research has focused on the function of the death receptor pathways and mitochondrial-mediated apoptosis. However, increasing evidence suggests that ER stress could promote cardiomyocyte apoptosis and ultimately lead to ischemic injury, which suggests that the inhibition of ER stress–related cardiomyocyte apoptosis could play a critical role in the therapeutic strategy for myocardial ischemia injury (Kaneko et al. 2017).

When ischemia develops, the blood supply to the heart is blocked, reducing the oxygen supply and causing a substantial accumulation of metabolic substances in the cardiomyocytes; these changes lead to various pathological events, such as calcium load alteration, oxidative stress, and inflammation. In response to these stresses, the UPR is activated to maintain homeostasis in the ER by

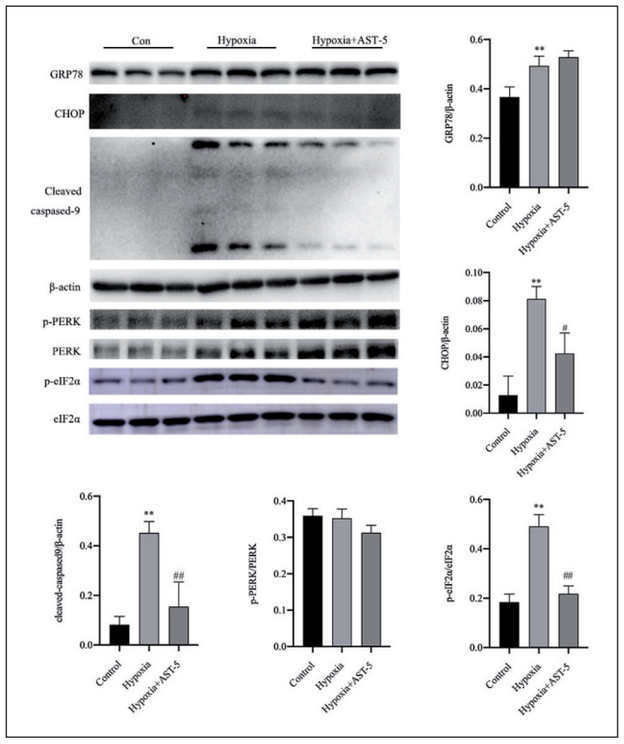

Fig. 5: Protein expression of cleaved caspase-9, C/RBP homologous proteins (CHOP), and glucose-regulated protein 78 (GRP78) in H9c2 cells under hypoxia, as measured by western blot analysis. *$p < 0.05$ vs normal group; **$p < 0.01$ vs normal group; #$p < 0.05$ vs hypoxic group; ##$p < 0.01$ vs hypoxic group. Statistics were calculated using the results of three repetitions of each experiment.

increasing the protein folding capacity, promoting the misfolded proteins, and suppressing protein synthesis. However, if protein aggregation is continuous and the ER stress is overwhelming, the signaling pathway shifts from pro-survival to pro-apoptotic mode (Azfer et al. 2006; Szegezdi et al. 2006a; Wang et al. 2018). Thus, inhibition of ER stress could be cardioprotective in the setting of MI-related injury.

In this study, the increased ROS level, the increased apoptosis rate, and the decreased cell viability in the hypoxia group revealed that severe injury was induced by hypoxia. Treatment with AST decreased the ROS level and apoptosis; these changes reflected the protective effect of AST against hypoxia in cardiomyocytes. AST exhibited a greater protective effect on cell viability at a concentration of 5 μM, compared with 1 μM treatment.

In this study, we identified apoptosis-related proteins and showed that the expressions of Bax and cleaved caspase-3 were significantly decreased, whereas Bcl-2 expression was increased in H9c2 cells by AST administration. Moreover, flow cytometry and TUNEL staining indicated that hypoxia-induced apoptosis in cardiomyocytes may be suppressed by AST. The Bcl-2 family of proteins includes pro- and antiapoptotic proteins that promote cytochrome c release and caspase activation that leads to apoptosis; these actions may be prevented by Bcl-2, an antiapoptotic protein (Edlich 2018). Activated caspase-3 is a crucial medium of apoptosis (Bratton et al. 2000). The observations from this study are in accordance with the previous findings in other cell types, which showed the antioxidant and antiapoptotic effects of AST (Chen et al. 2014; Ju et al. 2019).

To investigate how AST resists hypoxia-induced injury from ER stress and cardiomyocyte apoptosis, the expression levels of GRP78, PERK, eIF2α, and CHOP were determined. In response to ER stress, GRP78, a UPR regulator, plays a critical role in IRE1, PERK, and ATF6 activation (Szegezdi et al. 2006c). In the early stage of ER stress, the UPR is activated as an adaptive response process to inhibit protein translation, expand folding capacity, and begin protein degradation to relieve ER stress, particularly by

enhancing GRP78 levels; it also promotes cell survival by restoring normal ER function (Ardic et al. 2019). If ER stress is prolonged, UPR is insufficient to restore ER homeostasis; the UPR receptors may initiate a proapoptotic response characterized by increased transcriptional induction of CHOP (Zinszner et al. 1998). CHOP is a target downstream of the PERK/eIF2α pathway involved in UPR and plays a crucial role in ER stress–mediated apoptosis. The PERK pathway phosphorylates the substrate eIF2α during ischemia, which in turn could trigger the CHOP pathway (Fu et al. 2019). As a transcription factor, CHOP has regulated multiple apoptosis-associated genes, including *Bax* and *Bcl-2* (Zinszner et al. 1998).

In this study, the protein expressions of CHOP and p-eIF2α were increased in the hypoxia-exposed H9c2 cells compared with the normal controls, and this increase was significantly attenuated by AST administration. Szegezdi et al. (2006a,b) reported that the PERK substrate eIF2α was phosphorylated during ischemia; consistent with this report, we found that the expression of p-eIF2α was upregulated after hypoxic injury compared with the control group, whereas the H9c2 cells treated with AST showed notable attenuation of the p-eIF2α protein expression. If ER stress is prolonged, the UPR and cytoprotective effects switch to a proapoptotic response via upregulation of CHOP and activation of caspase-12 (Szegezdi et al. 2006b). The activation of PERK pathway protects cells not only *via* inhibition of protein synthesis during the early phase of ER stress (i.e., via the PERK-eIF2α pathway) but also during the later phase of the ER stress. In the later stage, activation increases CHOP expression and regulates apoptosis by activating caspase-12. Activated caspase-12 transfers to the cytoplasm, leading to cleavage of caspase-9 precursors and activation of caspase-9, which sequentially activates caspase-3, leading to apoptosis; this pathway is independent of mitochondria (Zhiming et al. 2018). In our study, we found an elevated expression of CHOP, caspase-9, and caspase-3 in H9c2 cells under hypoxic conditions, and these elevations were significantly reduced by AST treatment, as visualized by western blot.

Emerging investigations have demonstrated that AST can attenuate ER stress. Chen et al. (2014) reported that AST may inhibit podocyte apoptosis and the protein expression of p-PERK and p-eIF2α in streptozotocin-induced diabetes models. In addition, AST can reduce the level of ER stress–associated proteins (ATF4, CHOP, and TRB) and mRNA, reduce Bax levels, and increase the expression of Bcl-2. AST appears to inhibit podocyte apoptosis induced by ER stress, and its protective effect appears related to the PERK-ATF4-CHOP pathway. Wang et al. (2015) reported that AST can markedly decrease urinary albumin excretion rate, serum creatinine levels, and blood urea levels; can prevent glomerular mesangial dilation; and can inhibit the phosphorylation of PERK, eIF2α, and JNK in rats with streptozotocin-induced diabetes. In addition, AST can inhibit apoptosis of the podocytes induced by tunicamycin and can reduce the protein expression of CHOP and caspase-3. Hou (2017) reported that AST can significantly increase the expression of Bcl-2 protein and decrease the expression of caspase-3 and Bax protein, which can effectively reduce the apoptosis of endothelial cells caused by cerebral ischemia/reperfusion. Furthermore, the researcher demonstrated that AST can downregulate the expression of GRP78, CHOP, p-PERK, and p-eIF2α proteins and inhibit the ER stress in brain microvascular endothelial cells under oxygen-glucose deprivation/reoxygenation. However, the protective effect of AST on cardiomyocyte apoptosis and the potential mechanisms related to ER stress have not been extensively investigated. In this study, we demonstrated that AST could attenuate ER stress–associated apoptosis by inhibiting the eIF2α/CHOP pathway, thereby reducing hypoxia-induced cardiomyocyte injury.

In our research, we demonstrated that the protection of AST is related to its antioxidant and antiapoptotic effects. AST significantly reduced the elevated Bax and caspase-3 protein expression while increasing the Bcl-2 protein level reduced by hypoxic injury. Moreover, flow cytometry and annexin V/PI staining showed that hypoxia-induced apoptosis in cardiomyocytes may be inhibited by AST. We also investigated the regulation of AST on ER stress–related proteins, such as GRP78, PERK, CHOP, cleaved caspase-9, and eIF2α, and the results showed that ER stress induced the UPR activation and cardiomyocyte apoptosis, which was accompanied by an increase in GRP78, CHOP, eIF2α, and cleaved caspase-9 protein levels. CHOP, eIF2α, and cleaved caspase-9 protein changes were ameliorated by AST treatment. Therefore, we assume that CHOP is increased to activate the downstream signaling pathway to promote planned death/apoptosis. AST treatment may inhibit cell apoptosis induced by continuous ER stress directed mediated by CHOP.

In conclusion, this study suggests that activation of the eIF2α/CHOP signaling pathway is involved in ER stress-induced apoptosis of cardiomyocytes in H9c2 cells and that AST inhibited this apoptosis in part through suppression of the eIF2α/CHOP pathway to restore ER homeostasis. These novel findings support continued research into an alternative treatment of MI by targeting inhibition of ER stress.

The present research has several limitations. Firstly, the results of this study revealed that AST could attenuate ER-stress induced apoptosis by regulating the eIF2α/CHOP pathway, and all these experiments were carried out on H9c2 cell line. However, H9c2 cells do not contract autonomously, and it is not the most ideal experimental model. Thus, primary cardiomyocytes and animal model will be needed to further confirm our conclusion in the following research. Secondly, there should be more signaling pathways during ER stress-induced apoptosis in cardiac myocytes, and detailed molecular mechanisms need to be further investigated.

4. Experimental

4.1. Cell culture and treatment

H9c2 cardiomyocytes were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 4.5 g/l glucose.

H9c2 cells were plated at 5×10^3/well in 96-well plates after being cultured for 48 h in a 5% CO_2 incubator at 37 °C. Cells under hypoxic conditions were incubated in a hypoxic incubator filled with a gas mixture consisting of 94% N_2, 5% CO_2, and 1% O_2. Cells were incubated in DMEM containing 50, 10, 5, 1, and 0.5 μmol/l of AST (Chengdu Must Biotechnology Co., Ltd, Sichuan, China) for 24 h during the hypoxia treatments.

4.2. Cell viability assay

Cell viability was determined with a Cell Counting Kit-8 (CCK-8; Beyotime, Nanjing, China). H9c2 cells were exposed to 10 μl of CCK-8 reagent and incubated at 37 °C for another 2 h. Absorbance values were measured at 450 nm by an automated plate reader. The mean optical density was used to calculate the cell viability.

Cell viability was calculated as As/Ac×100%, in which As is the treated group and Ac is the normal group. The CCK-8 assay results were collected from replicate wells of three independent experiments and assayed by the Infinite M5 microplate reader (Tecan, PA, USA).

4.3. Western blotting analysis

The whole-cell lysates were obtained by lysis buffer (Beyotime) with 1 mM phenylmethylsulfonyl fluoride (PMSF, Beyotime). Equal amounts of samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) transferred to polyvinylidene fluoride membranes (Millipore, Boston, MA), and finally stained with the following primary antibodies: Bcl-2 (1:1000, Abcam, Cambridge, MA, USA), Bax (1:1000, Cell Signaling Technology CST, Danvers, MA, USA), cleaved caspase-3 (1:1000, CST), cleaved caspase-9 (1:1000, CST), p-PERK (1:1000, CST), PERK (1:1000, CST), GRP78 (1:1000, CST), CHOP (1:1000, CST), and β-actin (1:5000, KangChen Biotechnology, Shanghai, China). Primary antibodies were stained with horseradish peroxidase–conjugated secondary antibodies and visualized by a chemiluminescence ECL western blotting system (Millipore, Merck, Darmstadt, Germany). The bands were analyzed with Image Lab 6.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

4.4. Determination of intracellular reactive oxygen species

The reactive oxygen species (ROS), which accumulated in H9c2 cells, was detected using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) staining. Cell were seeded, washed, and stained with DCFH-DA (10 μmol/l) in a staining buffer for 20 min at 37 °C. Then, H9c2 cells were collected and observed by flow cytometry with a BD FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA).

4.5. Cell apoptosis assay

The apoptosis of cells was double stained using annexin V-FITC and PI (BD Biosciences). After the indicated treatment, cells were digested with trypsin, washed with cold phosphate-buffered saline, collected, and suspended in the annexin V binding buffer. After incubation, the cell suspensions were analyzed by flow cytometry with a BD FACS Count II flow cytometer (BD Biosciences).

4.6. Statistical analysis

One-way analysis of variance was performed with GraphPad Prism, version 5.0 for Mac (GraphPad Software, La Jolla, CA, USA) to calculate p values in repeated experiments. All values were expressed as the mean ± standard deviation. A value of $p < 0.05$ indicated significant statistical differences. Unless otherwise stated, all results were acquired from at least three independent experimental replications.

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Conflicts of interest: None declared.

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