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Ulinastatin inhibits high glucose-induced cardiomyocyte apoptosis through activating Akt signaling

Y. LONG[1], Y. ZHANG[1], S.-S. CAI[1], D.-M. SUN[1], Y.-H. LI[2]

[1]Department of Cardiovascular Medicine, Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Hospital), Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
[2]Department of Oncology, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Yuan Long and Yong Zhang contributed equally to this work

Abstract. – OBJECTIVE: Cardiomyocyte apoptosis is closely associated with the development of diabetic cardiomyopathy. Ulinastatin, a urinary trypsin inhibitor, exerts a protective effect on cardiac function. However, the molecular mechanism remains not fully clear. This study aims to explore the effect of ulinastatin on high glucose (HG)-induced cardiomyocyte apoptosis and the potential molecular mechanism.

MATERIALS AND METHODS: Neonatal rats cardiomyocytes were cultured and then treated with HG or/and ulinastatin. Cell viability was examined using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. Cell apoptosis was detected by flow cytometry. Mitochondrial membrane potential (MMP) was stained using a JC-1 probe and evaluated by fluorescence microscopy. Protein expressions of B-cell lymphoma 2 (Bcl-2) , BCL2-Associated X (Bax), cleaved caspase 3, p-Akt and Akt were determined by Western blot.

RESULTS: Ulinastatin increased the HG-induced reduction in cell viability and MMP expression. Ulinastatin also inhibited HG-induced apoptosis. Ulinastatin decreased the Bax/Bcl-2 ratio and cleaved caspase 3 expression in cardiomyocyte treated with HG. Further, ulinastatin increased the phosphorylation level of Akt in cardiomyocyte treated with HG. These effects of ulinastatin were abrogated by LY294002, an Akt inhibitor.

CONCLUSIONS: Ulinastatin inhibited HG-induced cardiomyocyte apoptosis through activating Akt signaling.

Key Words:
Ulinastatin, High glucose, Cardiomyocyte, Apoptosis, Akt.

Introduction

Diabetic cardiomyopathy (DCM) is the major cardiac complication and is the leading cause of morbidity and mortality in patients with diabetes mellitus (DM)[1]. The main features of DCM are left ventricular systolic and diastolic dysfunction in DM patients without hypertension and coronary artery disease, eventually leading to heart failure[2]. Current studies[3] have shown that DCM is an independent risk factor for heart failure. However, the molecular mechanism underlying the development of DCM has not yet been fully elucidated. Clinical treatment of DCM still lacks an effective strategy.

Mitochondria are the energy metabolism centers of cardiomyocytes. Many investigations[4,5] have shown that hyperglycemia can lead to mitochondria injury, oxidative stress and cardiomyocyte apoptosis. Akt pathway is one of the most important intracellular survival signaling pathways, which exerts a key role in cell survival and apoptosis[6]. Bcl-2 family proteins are the major downstream targets of Akt signaling that regulate mitochondria-mediated apoptosis[7]. Therefore, the activation of the Akt pathway and inhibition of mitochondria-mediated apoptosis may be considered as a potential approach to attenuate the development of DCM.

Ulinastatin, a urinary trypsin inhibitor, is widely used for the treatment of acute pancreatitis and sepsis-related multiple organ dysfunction[8]. Ulinastatin has been reported to exert a protec-

Corresponding Author: Yuhui Li, MM; e-mail: huihui090718@126.com

tive effect on cardiac function[9,10]. However, the effect of ulinastatin on DCM has not yet been fully confirmed. We hypothesized that ulinastatin may help to attenuate DCM. In this study, we cultured neonatal rat cardiomyocytes *in vitro* to investigate the effect of ulinastatin on high glucose-induced apoptosis and the potential molecular mechanism.

Materials and Methods

Ethics Statements

Neonatal Sprague-Dawley (SD) rats with 1-2 days old were provided by the Animal Experiment Center of Jiangsu Province (Jiangsu, China). All experimental protocols were conformed to the Guide for the Care and Use of Laboratory Animals (NIH). This study was approved by the Animal Ethics Committee of The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology Animal Center.

Cell Culture

Primary cultures of ventricular myocytes were prepared as previously described[11]. Briefly, after ventricles digestion, cells were pre-plated in a culture dish for 90 min at 37°C to remove non-cardiomyocytes. Cells were incubated in Dulbecco's modified eagle medium (DMEM, Gibco, Rockville, MD, USA) containing normal glucose concentration (5.5 mM), supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 1% penicillin and streptomycin, and 0.1 mM bromodeoxyuridine. After 2 days, cells were incubated in DMEM with 1% FBS overnight and then were divided into four groups: (1) the control group, cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) with 5.5 mM glucose; (2) the high-glucose (HG) group, cells were incubated in DMEM with 33.3 mM glucose for 48 h; (3) the HG + ulinastatin group, cells were incubated in DMEM with 33.3 mM glucose and 1×10^4U/L ulinastatin (Tianpu Biochemical Pharmaceutical, Guangzhou, China) for 48 h; (4) in the HG + ulinastatin+ LY294002 group, cells were incubated in DMEM with 33.3 mM glucose, 1×10^4U/L ulinastatin and 50 μM LY294002 (an Akt inhibitor) for 48 h.

MTT assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyl Tetrazolium Bromide)

Cell viability was measured using an MTT assay (Sigma-Aldrich, St. Louis, MO, USA). Briefly, cells were plated in a 96-well plate and then incubated with ulinastatin (0.5-8, $\times10^4$U/L) in the presence of HG for 48 h at 37°C. Next, cells were incubated with MTT solution (10 μL) for 4 h at 37°C. The optical density (OD) value at 490 nm was detected by microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The cell viability was calculated as a percentage.

Mitochondrial Membrane Potential Assay

The mitochondrial membrane potential (MMP) was detected using a fluorescent probe JC-1 (BD Company, Franklin Lakes, NJ, USA) and visualized under fluorescence microscopy (Nikon, Tokyo, Japan). JC-1 was aggregated and presented red fluorescence in mitochondria with normal MMP. However, JC-1 would become a monomer and emit green fluorescence under the circumstance of MMP depolarization. After cardiomyocytes were treated with HG or ulinastatin, they were incubated with JC-1 (10 μg/mL) for 20 min at 37°C and visualized under a fluorescence microscope. The ratio of red/green fluorescence was calculated.

Cell Apoptosis Assay

Cell apoptosis was measured using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining kit staining kit (Roche Applied Science, Penzberg, Germany). Briefly, cardiomyocytes in each group were harvested and washed with PBS. After cells were resuspended, they were stained with Annexin V-FITC (2 μL) and PI (2 μL) for 15 min at room temperature (RT) in the dark. The samples were analyzed using the flow cytometry. Annexin V^+/PI^- and Annexin V^+/PI^+ cells were considered as apoptotic cells in the early and late phase, respectively.

Western Blotting

Cytoplasmic proteins were isolated from cardiomyocytes using a cytoplasmic proteins extraction kit (Keygen Biotechnology, Nanjing, China) according to the protocol. Briefly, proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidine difluoride (PVDF) membrane. After blocking with 5% (v/v) non-fat dry milk, membranes were then incubated with primary antibodies of B-cell lymphoma 2 (Bcl-2), BCL2-Associated X (Bax), cleaved caspase 3, p-Akt and Akt (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After washing with Tris-buffered saline and

Tween 20 (TBST) (Beyotime, Shanghai, China), membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at RT for 1 h. The results were visualized by enhanced chemiluminescence.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (IBM, Armonk, NY, USA) were used for statistical analysis. All quantitative data were expressed as mean ± standard deviation. Comparison between groups was done using One-way ANOVA test followed by LSD (Least Significant Difference) Post-Hoc Test. The level of significance was set at $p<0.05$.

Results

Ulinastatin Protects Cardiomyocytes against HG-Induced Injury

We first determined the effect of ulinastatin on cell viability. Cells were treated with different doses of ulinastatin (0.5-8 $\times 10^4$U/L) for 48 h. There was no notable change in the viability of cardiomyocytes treated with ulinastatin (0.5-2 $\times 10^4$U/L) (Figure 1A).

We then evaluated the effect of ulinastatin on HG-induced cell injury. Cardiomyocytes were treated with HG (33.3 mM) for 48 h in the presence or absence of ulinastatin (0.5-2 $\times 10^4$U/L). Ulinastatin (0.5-2 $\times 10^4$U/L) significantly increased the viability of cardiomyocytes treated with HG. In particular, 1×10^4 U/L ulinastatin exerted maximal protection (Figure 1B).

Ulinastatin Prevents HG-Induced Reduction in the Mitochondrial Membrane Potential

As shown in Figure 2, the mitochondria in control cells emitted red fluorescence. However the green fluorescence was significantly increased in the HG group compared with the control group, indicating the dissipation of the MMP. Ulinastatin restored the MMP in cardiomyocytes treated with HG. However, the up-regulation of MMP by ulinastatin in cardiomyocytes treated with HG was abolished by LY294002.

Ulinastatin Inhibits HG-Induced Cardiomyocyte Apoptosis

Cardiomyocyte apoptosis was confirmed by flow cytometry. The cell apoptotic rate in HG group was significantly increased compared with that of control group. In contrast, ulinastatin substantially reduced cardiomyocyte apoptotic rate in cardiomyocytes treated with HG. However, the inhibition of HG-induced apoptosis by ulinastatin was abolished by LY294002 (Figure 3).

Ulinastatin Inhibits HG-Induced Mitochondria-Mediated Apoptosis Activation

As shown in Figure 4A, there was a significant increase in the Bax/Bcl-2 ratio and cleaved caspase 3 expression in the HG group compared with that of control group. Ulinastatin substantially decreased the Bax/Bcl-2 ratio and cleaved caspase 3 expression in cardiomyocytes treated with HG. However, these effects of ulinastatin in cardiomyocytes treated with HG were both abolished by LY294002.

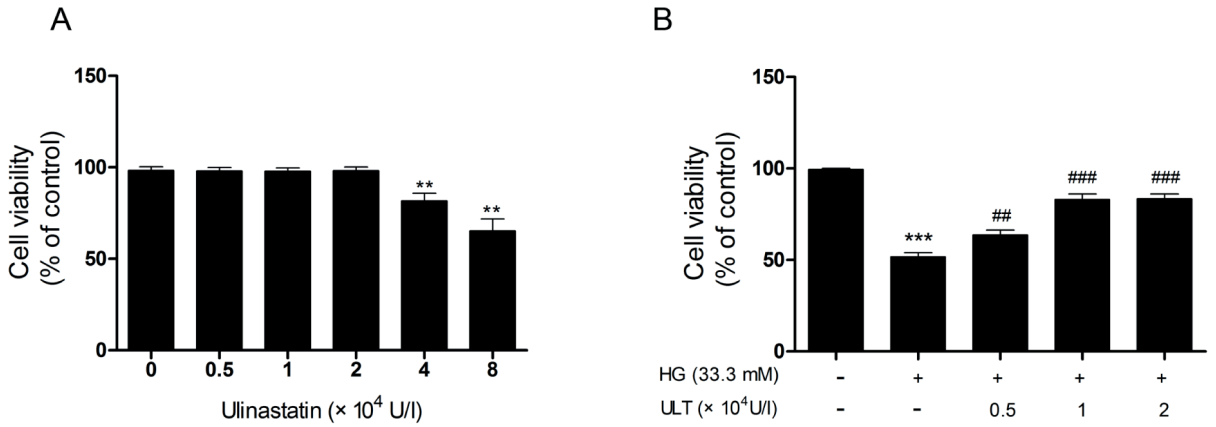

Figure 1. Protective effect of ulinastatin on HG-induced cytotoxicity. ***A,*** Cardiomyocytes were treated with various concentrations of ulinastatin alone for 48 h, and cell viability was determined by MTT. ***B,*** Cardiomyocytes were treated with HG (33.3 mM) for 48 h in the presence or absence of ulinastatin. HG: high glucose; ULT: ulinastatin. $^{**}p<0.01$ *vs.* control, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ *vs.* HG-treated cells.

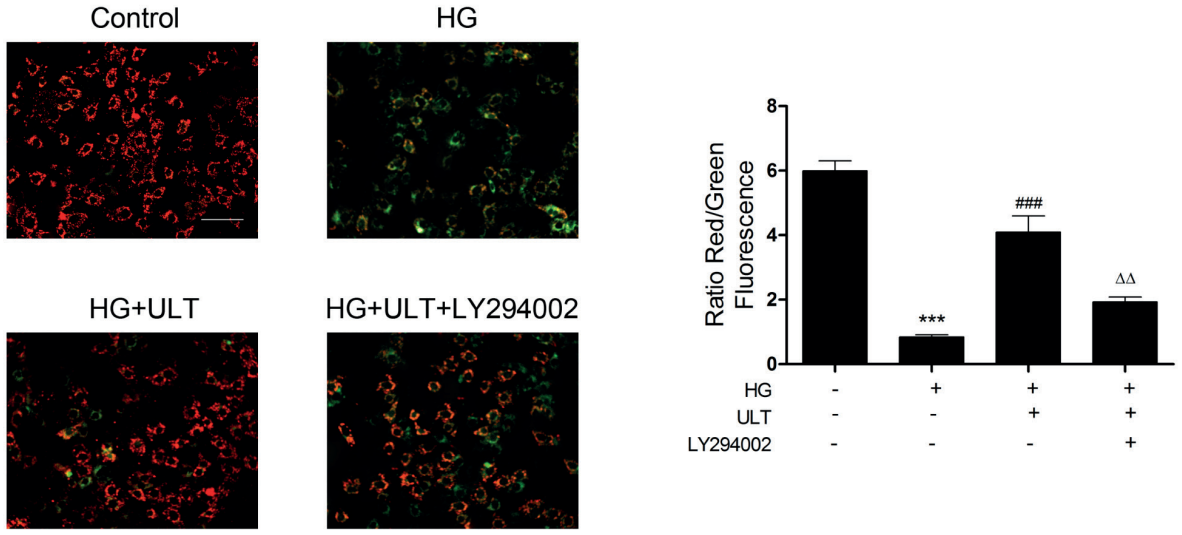

Figure 2. Ulinastatin prevents HG-induced reduction in the mitochondrial membrane potential (MMP). The MMP was visualized by fluorescence microscopy using a JC-1 probe. Red fluorescence represented cells with high MMP, whereas green fluorescence represented cells with low MMP. Scale bar = 100 μm. HG: high glucose; ULT: ulinastatin. ***p<0.001 *vs.* control, $^{###}p$<0.001 *vs.* HG group. ^{ΔΔΔ}p<0.001 *vs.* HG+ULT group.

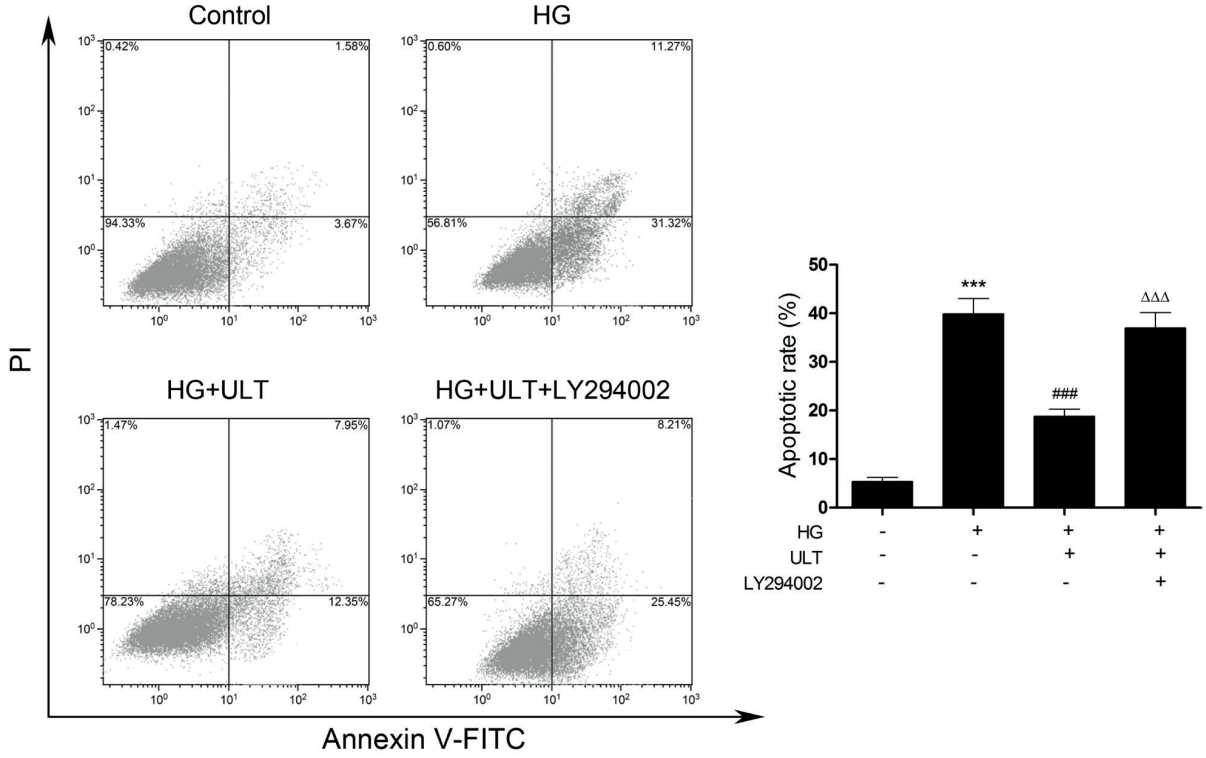

Figure 3. Ulinastatin protects cardiomyocytes against HG-induced apoptosis. Cardiomyocytes apoptosis were stained by Annexin V-FITC/PI and analyzed by flow cytometry. HG: high glucose; ULT: ulinastatin. ***p<0.001 *vs.* control, $^{###}p$<0.001 *vs.* HG group. ^{ΔΔΔ}p<0.001 *vs.* HG+ULT group.

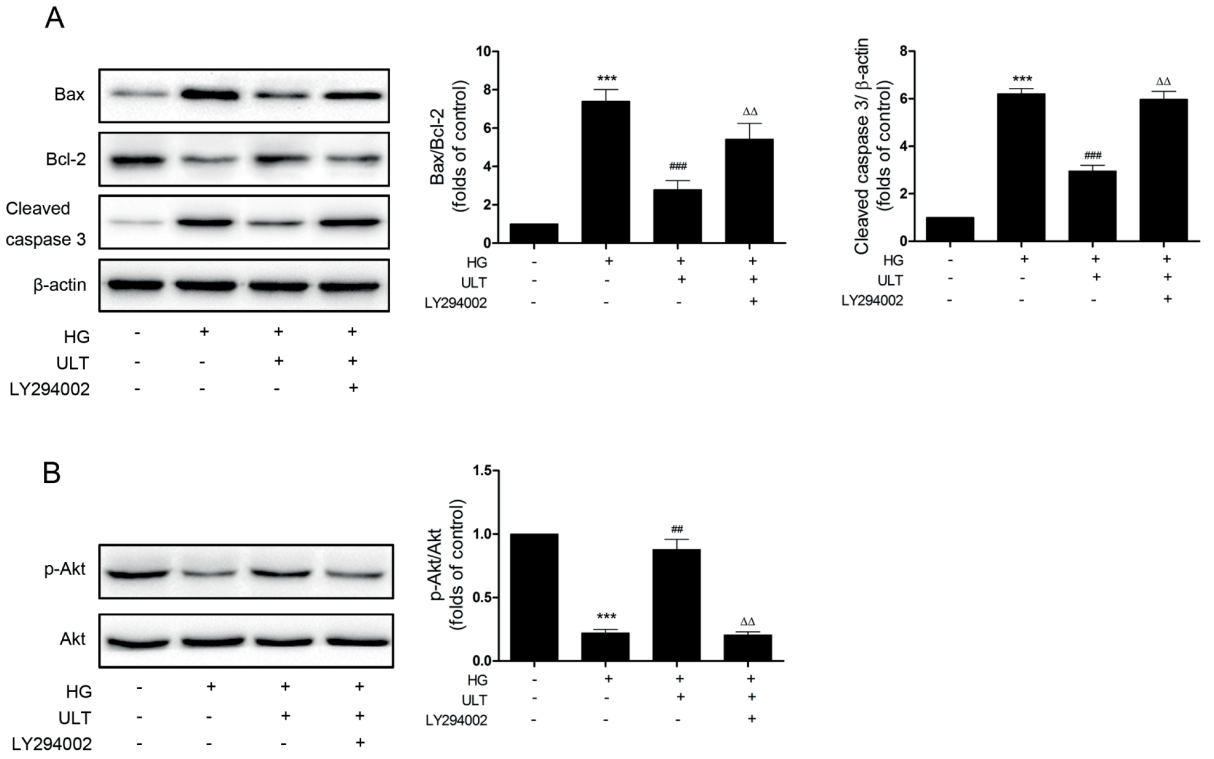

Figure 4. Ulinastatin inhibits mitochondria-mediated apoptosis and activates Akt signaling in HG-treated cells. ***A,*** The expressions of Bax, Bcl-2 and cleaved caspase-3. ***B,*** The expressions of p-Akt and Akt. HG: high glucose; ULT: ulinastatin. ***$p<0.001$ *vs.* control, $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ *vs.* HG group. $^{\Delta\Delta}p<0.01$ *vs.* HG+ULT group.

Ulinastatin Activates HG-Inhibited Akt Signaling

As shown in Figure 4B, the phosphorylation level of Akt was significantly decreased in the HG group compared with that of control group. Ulinastatin increased the phosphorylation level of Akt in cardiomyocytes treated with HG. However, the activation of Akt by ulinastatin in cardiomyocytes treated with HG was abolished by LY294002.

Discussion

Ulinastatin is a glycoprotein secreted by the liver and derived from urine[12]. It has been reported that the level of ulinastatin is enhanced in patients with DM. Ulinastatin level might reflect a systemic inflammatory response induced by hyperglycemia[13]. Recently, ulinastatin has been regarded as a cardio protective candidate. It has been revealed that ulinastatin is able to improve cardiac function after sepsis[10] and cardiac arrest[14] by inhibiting inflammatory responses and oxidative stress. Masuda et al[15] found that ulinastatin inhibited myocardial mitochondria dysfunction following hemorrhagic shock and reperfusion. In addition, Xiao et al[16] reported that ulinastatin could alleviate myocardial ischemia/reperfusion injury through regulating autophagy in a rat model. Moreover, Wang et al[17] recently found that ulinastatin could improve cardiac dysfunction and inhibit myocardial apoptosis in a rat DCM model. However, the protective mechanism of ulinastatin was not fully clear. In this study, we further explored the effect of ulinastatin on cardiomyocytes apoptosis induced by HG *in vitro*. Our results confirmed that ulinastatin could reduce HG-induced cardiomyocytes apoptosis *via* inhibiting mitochondria-mediated apoptosis pathway and activating Akt signaling.

It has been found that cardiomyocyte apoptosis is enhanced in the DCM patients and animal models, indicating a significant role in the development of DCM[18]. Apoptosis severely damages myocardial contractile tissue, thereby leading to cardiac hypertrophy, fibrosis and remodeling[19]. Mitochondria are considered as the central organelles of cardiomyocyte apoptosis. The dissipation of the mitochondrial membrane potential

(MMP) is the key step in the activation of mitochondrial-mediated apoptosis, which is predominantly modulated by the Bcl-2 family proteins. In Bcl-2 family, Bcl-2 exerts an anti-apoptotic effect and Bax exerts a pro-apoptotic effect. Increased ratio of Bax/Bcl-2 leads to the release of cytochrome c and activation of caspase-3, thereby resulting in apoptosis[20,21]. In the present study, flow cytometry analysis revealed that ulinastatin treatment significantly reduced cardiomyocyte apoptosis in HG condition. Ulinastatin treatment could restore the MMP, and decrease the Bax/Bcl-2 ratio and cleaved caspase 3 expression in HG-treated cardiomyocyte. Collectively, ulinastatin exerted an anti-apoptotic effect by inhibiting the activation of mitochondrial-mediated apoptosis pathway.

Akt is an important intracellular survival signaling and can be activated by various stimuli, including hypoxia, oxidative stress and mechanical stress[22]. It has been demonstrated that the activation of Akt signaling is sufficient to inhibit cardiomyocyte apoptosis and to preserve the function in surviving cardiomyocytes[23]. Su et al[24] recently reported that the activation of Akt signaling also inhibits cardiomyocyte apoptosis in response to HG stimulation. Such effect can be effectively blocked by LY294002, an Akt inhibitor. Bcl-2 family proteins are the major downstream effectors of Akt signaling in modulating the mitochondria-mediated apoptosis. Zhang et al[25] found that myricitrin could inhibit HG-induced cardiomyocytes apoptosis through activating Akt signaling, which was abolished by LY294002. We also found that ulinastatin could inhibit mitochondria-mediated apoptosis and promote Akt signaling activation in cardiomyocytes stimulated by HG. However, the protective effect of ulinastatin could be abolished by LY294002, indicating that the anti-apoptotic effect of ulinastatinin on HG-treated cardiomyocytes was associated with the activation of Akt signaling.

Conclusions

We identified that ulinastatin could inhibit HG-induced cardiomyocyte apoptosis. The molecular mechanism involved in the anti-apoptotic effect of ulinastatin was associated with the inhibition of mitochondria-mediated apoptosis pathway and the activation of Akt signaling. Our results suggest that ulinastatin can be applied as a potential treatment strategy for alleviating DCM.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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