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Ulinastatin protects rats with myocardial infarction by activating Nrf2/NOS pathway

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Abstract. – OBJECTIVE: The aim of the study was to explore the pharmacological role of ulinastatin in rats with myocardium infarction (MI) and its underlying mechanism.

MATERIALS AND METHODS: Rats were randomly assigned into sham group, MI group, and ulinastatin group, with 8 rats in each group. MI model in rats was constructed and specific drug administrations were performed in each group. Electrocardiogram and hemodynamics were detected before and after animal procedures. Myocardium function in rats was accessed by determining serum levels of creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH). Hematoxylin and eosin (HE) staining was performed to detect the infarct area in rat myocardium. Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) assay was conducted to calculate the ratio of apoptotic cells in rat myocardium. Subsequently, relative oxidative stress indicators in rats were accessed, including total antioxidant capacity (T-AOC), catalase (CAT), glutathione (GSH), Superoxide Dismutase (SOD), malondialdehyde (MDA), and reactive oxygen species (ROS). Western blot was conducted to detect protein levels of nuclear factor E2-related factor 2 (Nrf2), Nuclear Factor κB (NF-Kb), heme oxygenase-1 (HO-1), and NAD(P)H:quinone oxidoreductase 1 (NQO1) in rat myocardium.

RESULTS: No significant differences in heart rate, the voltage of the QRS wave and Q-T interval were observed among rats in sham group, MI group, and ulinastatin group prior to the animal procedures. However, at the end of the animal procedures, rats in ulinastatin group showed higher heart rate and voltage of QRS wave, as well as shorter Q-T interval than those in MI group. Rats in ulinastatin group presented lower serum levels of CK-MB and LDH compared with those in MI group, whereas they did not return to baseline. Rats in ulinastatin group showed higher levels of LVSP, dP/dtmax, LVEDP, and -dP/dtmax than those in MI group. Larger infarct area was observed in MI group compared with that of sham group, whereas ulinastatin treatment remarkably reduced the infarct area. HE staining showed remarkable pathological lesions in MI rats, whereas ulinastatin group showed milder lesions in rat myocardium. TUNEL assay showed fewer TUNEL-positive cells in ulinastatin group than those of MI group. Levels of T-AOC, CAT, GSH, and SOD were remarkably higher in myocardium homogenate of MI group than sham group, whereas ulinastatin treatment significantly decreased these levels. Ulinastatin group showed less ROS accumulation and decreased MDA level in rats than those of MI group. Ulinastatin treatment upregulated Nrf2, HO-1, and NQO1, whereas downregulated NF-κB expression.

CONCLUSIONS: Ulinastatin protects MI rats by inhibiting inflammatory response through activating Nrf2/NOS pathway.

Key Words:
Ulinastatin, Nrf2/NOS pathway, Acute MI.

Introduction

Coronary atherosclerotic heart diseases include acute myocardial infarction (MI), unstable angina pectoris, exertional angina pectoris, asymptomatic myocardial ischemia, etc.[1]. Among them, acute MI resulted from coronary artery stenosis, plaque rupture, and thrombosis, eventually leading to acute coronary occlusion, continuous reduction of myocardial blood supply, myocardial ischemia, and necrosis. Patients with acute MI are often complicated by ventricular tachycardia (VT) and serious cardiovascular events, such as malignant arrhythmia, ventricular septal perforation, papillary muscle dysfunction, chordae rupture,

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and cardiac rupture. Severe MI may even lead to sudden death[2-4]. According to the World Health Organization (WHO), acute MI has become the leading cause of human death[5,6]. Also, patients with acute MI who survive in the acute phase may progressively develop into chronic heart failure after experiencing a series of pathological lesions[4,5]. Hence, researches on MI treatment and improvement of myocardial remodeling are of great clinical significance.

The nuclear factor E2-related factor 2/Nitric Oxide Synthase (Nrf2/NOS) signaling system is the main mechanism for the body to resist internal and external oxidative stress. Studies[7,8] have reported that superoxide can induce transcriptional activity of Nrf2, thereby upregulating expressions of the downstream anti-oxidation and detoxification enzyme genes, such as heme oxygenase-1 (HO-1), glutathione S-transferase (GST), and NAD(P)H:quinone oxidoreductase 1 (NQO1). The Kelch-like ECH-binding protein 1 (Keap 1) structure contains a domain that binds to Nrf2[7]. Studies have shown that Keap1 anchors the inactivate-state cytoplasmic Nrf2. As a substrate conjugate, Keap1 promotes the degradation of Nrf2 by forming a complex with ubiquitin ligase substrate. Under stimulation of oxidative stress or ionization, Nrf2 dissociates with Keap1 and translocates into nucleus, thereafter promoting the transcription of downstream genes[9]. A great number of researches[10-12] have pointed out the potential role of endogenous activated Nrf2 and eNOS in MI-induced oxidative stress. In recent years, some natural or chemically synthesized antioxidants, such as resveratrol, sulforaphane, quercetin, fuel flavonoids, and their derivatives have been observed. They are found to be able to protect ischemia/hypoxic damage in neurons by upregulating Nrf2[13-15]. Therefore, the Nrf2/NOS antioxidant signaling pathway may be an important target for the prevention and treatment of MI.

MI causes fibrosis of cardiomyocytes, releases inflammatory mediators and leads to myocardial necrosis and apoptosis[16,17]. The ischemia-induced release of inflammatory factors exerts an essential role in myocardial remodeling[17,18]. Also, downregulated apoptosis-associated proteins inhibit apoptosis and improve cardiac function. Phosphorylation of protein kinase B (AKT) and endothelial nitric oxide synthase (eNOS) protect endothelial function[19,20]. Scholars[21,22] have shown that ulinastatin is capable of preventing oxidation, atherosclerosis, and inflammation. The potential role of ulinastatin in MI, however, remains to be unclear. This study aims to elucidate whether ulinastatin exerts a protective effect on MI rats.

Materials and Methods

Chemicals and Reagents

Ulinastatin was obtained from National Institutes for Food and Drug Control; pentobarbital sodium was obtained from Baomanbio (Shanghai, China); terminal deoxynucleotidyl transferase dUTP Nick-end labeling (TUNEL) apoptosis determination kit was obtained from Roche (Basel, Switzerland); paraformaldehyde was obtained from Sorlabio (Beijing, China); evans blue reagents were obtained from BioSharp (Shanghai, China); TTC (2,3,5-Triphenyltetrazoliumchloride) was obtained from Amresco (Solon, OH, USA); 4',6-diamidino-2-phenylindole (DAPI) was obtained from Sigma-Aldrich (St. Louis, MO, USA); hematoxylin and eosin (HE) and Masson reagents, and relative commercial kits, including creatine kinase-MB (CK-MB), lactate dehydrogenase (LDH), Total antioxidant capacity (T-AOC), catalase (CAT), glutathione (GSH), superoxide dismutase (SOD) and malondialdehyde (MDA), were obtained from Jiancheng (Nanjing, China). Other surgical instruments and disinfectants for establishing animal models were made in China.

The high-resolution ultrasound imaging system for small animals Vevo2100 was provided by Visualsonics (Toronto, Canada); small animal ventilator was provided by Alcbio (Shanghai, China); ordinary light microscope and fluorescent inverted microscope were provided by Nikon (Tokyo, Japan); computer installed with Image J software was provided by Lenovo (Beijing, China); paraffin-embedded microtome was provided by Hwotech (Shenzhen, China); electronic balance was provided by Mettler Toledo (Columbus, OH, USA); high-speed low-temperature desk centrifuge was provided by Heraeus (Hanau, Germany); digital gel imaging system was provided by Bio-Rad (Hercules, CA, USA).

Animal Procedures

24 male Sprague Dawley (SD) rats weighing 200±20 g (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were maintained in an environment with a 12 h/12 h light/dark cycle. Rats were given free access to food and water. According to the specific procedures and drug administrations, rats were randomly assigned into sham group, MI group, and ulinasta-

tin group, with 8 rats in each group. This study was approved by the Animal Ethics Committee of Zhengzhou University Animal Center.

Rats were anesthetized with intraperitoneal injection of 60 mg/kg pentobarbital sodium. Subsequently, rats were placed on the surgical table for performing a tracheotomy. Mechanical ventilation was conducted after connecting small animal ventilator and an electrocardiogram was performed for monitoring vital signs of rats. Left internal jugular vein catheterization was performed and arterial pressure was monitored through the right carotid artery cannula. Myocardial ischemia was induced by ligation of the ascending aorta. Pale myocardium and ST-segment elevation on electrocardiogram suggested the successful construction of MI model in rats. Rats in sham group received tracheotomy without ligation of the ascending aorta.

After construction of MI model in rats, they received intragastric administration of 80 mg/kg ulinastatin (ulinastatin group) or saline (sham group and MI group) for consecutive 3 weeks. Before animal procedures and on the 21st day after MI model construction, relative indexes were monitored by electrocardiogram, including LVEDD (left ventricular end-diastolic dimension), LVESD (left ventricular end-systolic dimension), LVEF (left ventricular ejection fraction), and LVFS (left ventricular fractional shortening). After collecting rat blood sample from an orbital vein, rats were sacrificed for the following experiments. Body weight and daily activities of each rat were regularly observed.

Measurement of Myocardial Infarction Area

On the postoperative 21st day, rats were anesthetized for exposure of abdominal aorta. 1 mL of Evans Blue (0.1 m/mL) was slowly injected into the abdominal aorta. Rat heart was harvested until blue staining about 1 minute later. Heart tissue was sliced into 4-5 pieces parallel to atrioventricular groove, with 1-2 mm in thickness. Slices were then incubated with TTC (pH 7.4) at 37°C for 30 min in the dark, followed by fixation in paraformaldehyde. Non-ischemic area and ischemic area were stained red and pale, respectively. The infarct size (%) was determined by Image J as the weight ratio of ischemic area and total area.

Hemodynamic Analyses

A 20-G heparin-filled catheter (Spacelabs Medical, Inc., Redmond, WA, USA) was inserted from the right carotid artery to the left ventricle. A pressure transducer (Biolab 420F, Taimeng, Chengdu, China) was connected for measurement of hemodynamic parameters. Left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), and maximum rate of increase/decrease in left ventricular pressure (±dP/dtmax) of each rat were recorded.

Histological Examination

Heart tissues were fixed with 10% paraformaldehyde and stained with hematoxylin and eosin. Histological changes were assessed by semi-quantitative detection of injury and necrosis of the myocardium. 5 randomly selected fields of each sample were evaluated for MI-induced pathological lesions.

Terminal Deoxynucleotidyl Transferase dUTP Nick-end Labeling (TUNEL) Assay

Cardiomyocyte apoptosis in heart tissue was detected by TUNEL assay according to the instructions of ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit. 4-μm paraffin section was counterstained with hematoxylin. TUNEL-positive cells (green) and DAPI-stained nucleus (blue) were counted in 5 randomly selected fields of each sample (magnification 200×).

Assessment of Cardiomyocyte Function

At the end of animal procedures, we extracted 2 mL of blood sample of each rat. Samples were centrifuged at 3500 g/min for collecting the supernatant. Serum levels of CK-MB (creatine kinase-MB) and LDH (lactate dehydrogenase) were detected using relative commercial kits.

Biochemical Measurements

After in situ perfusion with cold saline, rat myocardium was harvested until turning pale. Myocardium homogenate was then prepared for detecting levels of MDA, T-AOC, CAT, GSH, SOD, and ROS.

Western Blot

Protein samples were separated by 10% polyacrylamide gel electrophoresis. After transferring to PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA), the protein-contained membrane was blocked with 5% skim milk for 2 h. The corresponding primary antibody was added and incubated with proteins at 4°C overnight, followed by incubation of the secondary antibody for another 1 h. The ECL (enhanced

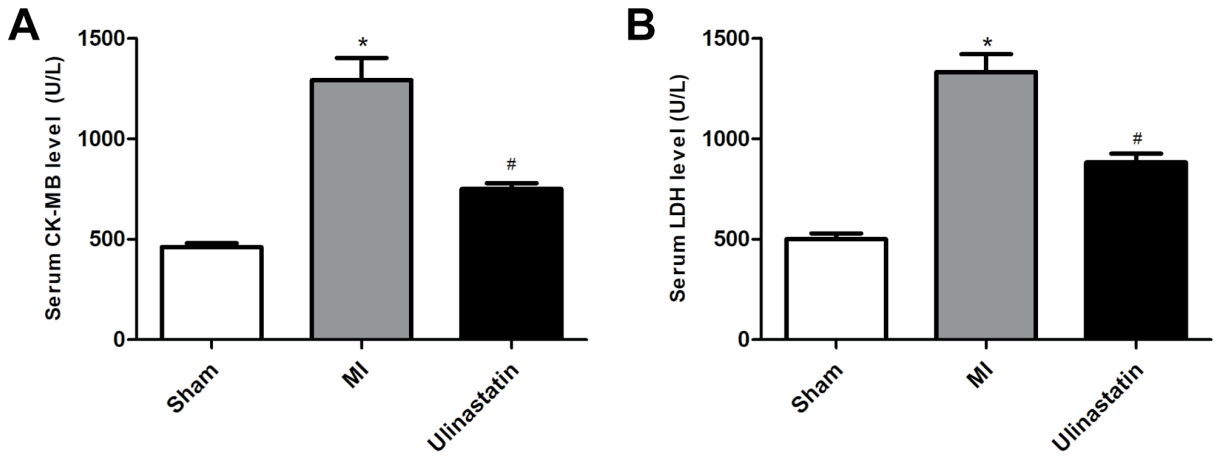

Figure 1. Ulinastatin protected myocardial function in MI rats. Rats were divided into sham group (n=8), MI group (n=8) and ulinastatin group (n=8). ***A,*** Serum level of CK-MB in rats of different groups. ***B,*** Serum level of LDH in rats of different groups. Data were expressed as mean±SD. *p<0.05 compared with sham group, $^{\#}p$<0.05 compared with MI group.

chemiluminescence) luminescent agent (Thermo Fisher Scientific, Waltham, MA, USA) was used to develop the protein imprint. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference here.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 22.0 statistical software (IBM, Armonk, NY, USA) was used for data processing and analysis. GraphPad Prism 5 was used for figure editing. The data were expressed as mean±SD. The *t*-test was used to compare the mean values of two independent samples. One-way ANOVA was used for comparing differences among groups, followed by Least Significant Difference (LSD) test. p<0.05 was considered statistically significant.

Results

Effect of Ulinastatin on the Electrocardiogram Results of MI Rats

No significant differences in heart rate, voltage of QRS wave, and Q-T interval were observed in rats among sham group, MI group, and ulinastatin group prior to the animal procedures. However, at the end of the animal procedures, rats in MI group presented lower heart rate and voltage of QRS wave compared with those of sham group (p<0.05). Some rats in MI even experienced arrhythmia typically shown in the electrocardiogram. By comparison, rats in ulinastatin group showed higher heart rate and voltage of QRS wave, as well as shorter Q-T interval than those in MI group (p<0.05, Table I).

Ulinastatin Improved Myocardial Function in MI Rats

After animal procedures, we collected blood samples of rats for detecting serum levels of CK-MB and LDH. The data elucidated higher levels of CK-MB and LDH in rats of MI group than those in sham group, suggesting the successful construction of MI model in rats. Moreover, rats in ulinastatin group presented lower levels of CK-MB and LDH compared with those in MI group, whereas they did not return to the baseline (p<0.05, Figure 1).

Ulinastatin Improved Hemodynamic Function in MI Rats

In Table II, the hemodynamic function in each group was listed in detail. Before the ani-

Table I. The effect of ulinastatin on the electrocardiogram of the model rats induced by acute myocardial infarction.

Group	Heart rate/min	QRS/mv	Q-T interval/ms
Sham	452.00±15.28	0.583±0.078	58.24±5.19
MI	367.00±38.42*	0.407±0.089*	72.15±5.45*
Ulinastatin	412.60±27.67$^{\#}$	0.570±0.076$^{\#}$	55.12±5.12$^{\#}$

Data are expressed as mean±SEM. n=8 in each group. *p<0.05 versus Sham, #p<0.05 versus MI.

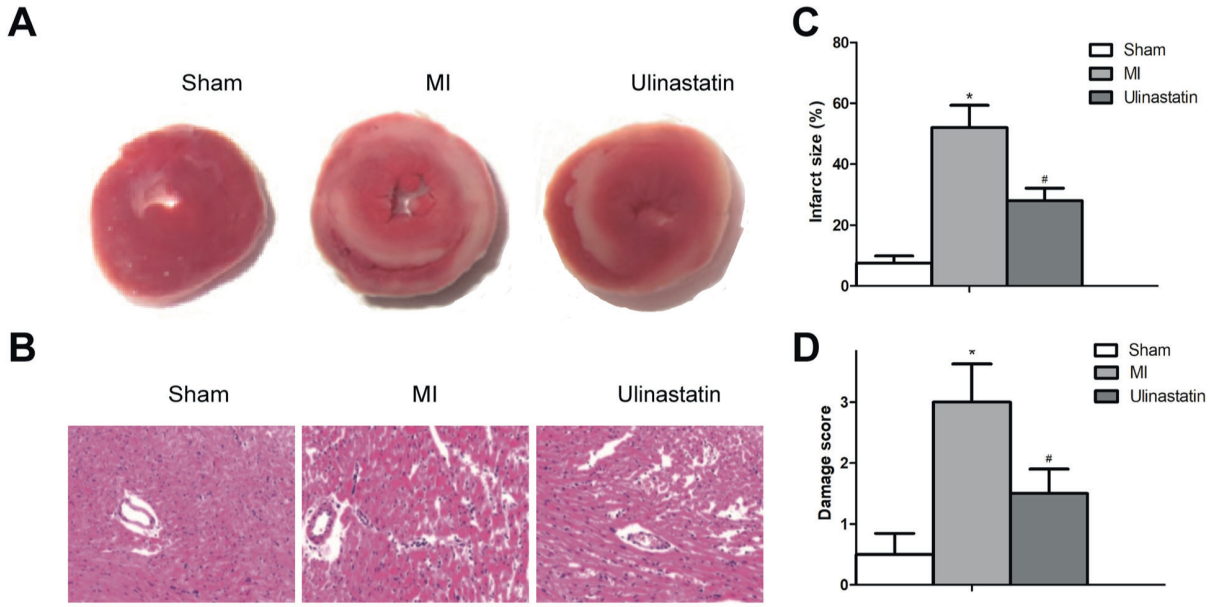

Figure 2. Ulinastatin prevented acute MI in rats. Rats were divided into sham group (n=8), MI group (n=8) and ulinastatin group (n=8). ***A,*** Infarct size in rats of different groups. Normal myocardium was stained blue and infarcted myocardium was stained pale. The infarct size was expressed as the weight ratio of ischemic area and total area. ***B,*** HE staining of rat myocardium (magnification 200×). ***C,*** Quantification of infarct size in different groups. ***D,*** The score of infarct lesion in different groups. Data were expressed as mean±SD. *$p<0.05$ compared with sham group, $^{\#}p<0.05$ compared with MI group.

mal procedures, we did not observe a significant difference in hemodynamic function of rats among the three group ($p>0.05$). At the end of the MI model construction, rats in MI showed a reduction in both systolic (lower LVSP and dP/dtmax) and diastolic functions (lower LVEDP and -dP/dtmax) compared with those in sham group ($p<0.05$). Ulinastatin treatment remarkably reversed hemodynamic function, manifesting as higher levels of LVSP, dP/dtmax, LVEDP, and -dP/dtmax than those in MI group ($p<0.05$).

Ulinastatin Preserved Myocardial Histologic Architecture and Mitigates Neutrophil Infiltration

Larger myocardial infarct area was observed in MI group compared with that of sham group, whereas ulinastatin treatment remarkably reduced the infarct area ($p<0.05$, Figure 2A and 2C). HE staining showed inflammatory infiltration, disordered myocardial cells, and pink protein mucus exudation in MI group. Ulinastatin group showed remarkably alleviated pathological lesions in cardiomyocytes, presenting only a small amount of inflammatory cells and milder cellular edema (Figure 2B and 2D).

Ulinastatin Decreased Apoptosis of Cardiomyocyte Tubular Cells After Acute MI

The TUNEL assay was conducted to access the apoptotic cells in ischemic myocardium of rats. As shown in Figure 3A and 3B, the amount of TUNEL-positive cells was higher in MI group than sham group ($p<0.05$). However, ulinastatin group showed fewer TUNEL-positive cells than those of MI group, suggesting the anti-apoptosis capacity of ulinastatin in MI rats.

Ulinastatin Decreased ROS Production and Tissue Impairment by Enhancing Antioxidant Capacity

To explore whether ulinastatin exerts anti-oxidation ability in MI rats, we detected relative indicators of oxidative stress using commercial kits. Ulinastatin group showed lower MDA level in rats (Figure 4A) and less ROS accumulation (Figure 4B) than those of MI group. It is demonstrated that levels of T-AOC, CAT, GSH, and SOD were remarkably higher in myocardium homogenate of MI group than sham group, whereas ulinastatin treatment significantly decreased these levels ($p<0.05$, Figure 4C-4F).

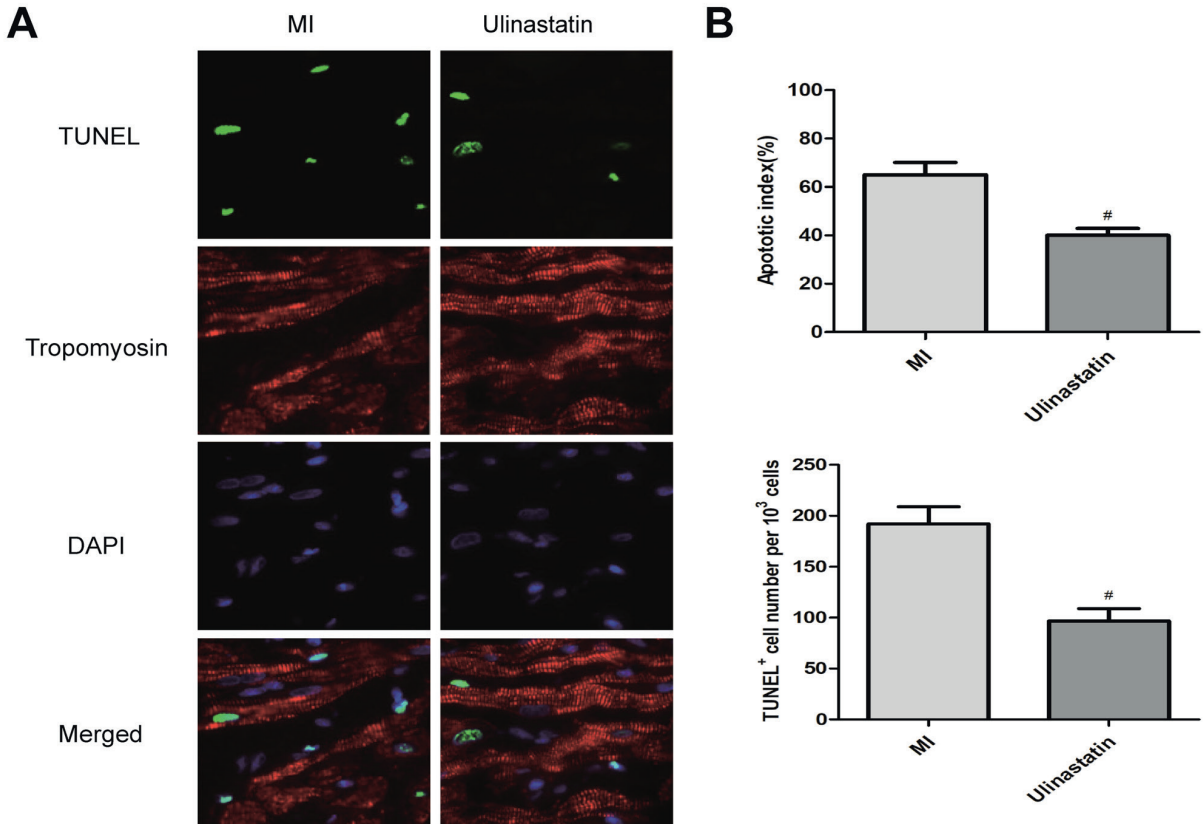

Figure 3. Ulinastatin decreased apoptosis in MI rats. Rats were divided into sham group (n=8), MI group (n=8) and ulinastatin group (n=8). ***A,*** TUNEL staining of rat myocardium in different groups (magnification 200×). ***B,*** TUNEL-positive cells per 10^3 germ cells in different groups. ***C,*** Apoptotic index in different groups. Data were expressed as mean±SD. *$p<0.05$ compared with sham group, $^{\#}p<0.05$ compared with MI group.

Ulinastatin Upregulated Expressions of Nrf2 and it Downstream Genes in MI Rats

To explore the potential protective mechanism of ulinastatin in MI, Western blot was performed to detect relative expressions of Nrf2 and Nuclear Factor κB (NF-κB) in the myocardium. The results showed that ulinastatin treatment upregulated Nrf2 expression, whereas downregulated NF-κB expression (Figure 5A-C). Subsequently, we harvested the cytoplasm of rat myocardium. Western blot results also revealed higher expressions of Nrf2 downstream genes HO-1 and NQO1 than those of MI group (Figure 5A, 5D and 5E).

Discussion

With the improvement of living standards in recent year, the incidence of coronary heart disease has increased year by year. Due to the younger onset and more complicated condition, coronary heart disease has been well concerned[1-3]. Acute coronary syndrome accounts for 30%-40% of the total cases of coronary heart diseases, which has been the leading cause of poor prognosis and sudden death in patients with coronary heart disease[2-4]. The basic pathological manifestations of coronary heart disease include coronary atherosclerosis, plaque formation, plaque instability and rupture, arterial thrombosis, vascular occlusion, myocardial ischemia, and even necrosis[1,5,7]. The vulnerable plaque and myocardium, as well as unstable blood flow eventually progress into acute coronary syndrome and even sudden cardiac death[23]. Studies[24,25] have shown that almost all plaque ruptures are associated with plaque instability, while coronary stenosis is rarely involved in. High levels of systemic inflammatory factors and accumulation of inflammatory cells in atherosclerotic plaques are associated with thinning of plaque fiber caps[26]. Identification and intervention of soft plaque as early as possible can greatly improve the prognosis of acute coronary syndrome[27].

At present, acute MI is clinically characterized by severe symptoms, high mortality, and high consumption of medical resources. Therefore, the molecular mechanism of acute MI is required for in-depth exploration. Effective drugs and molecular therapeutic targets are actively explored to prevent and alleviate MI[4-6]. So far, many basic researches on acute MI have revealed its causes, pathogenesis and clinical manifestations. Some beneficial prevention strategies have already been applied in clinical practice[3,5,6].

Under the normal circumstance, the oxidation and anti-oxidation system maintain in dynamic equilibrium. The imbalanced equilibrium resulted from internal or external stimuli would lead to accumulation of free radicals, thus leading to oxidative stress[28-30]. Accumulating evidence has suggested that oxidative stress is closely related to the occurrence and development of acute MI and its complications[31,32]. A complete antioxidant system includes enzymatic system (superoxide dismutase, peroxidase, glutathione peroxidase, etc.) and non-enzymatic system (vitamins, amino acids, metalloproteins, etc.)[33]. Free radicals generated in the body are eliminated through the antioxidant system, thereafter maintaining the normal function as much as possible[34]. In the present study, a decreased level of T-AOC, GSH, CAT, and SOD, as well as an increased level of MDA were observed in MI rats. It is suggested that oxidative stress is enhanced during the pathological process of MI.

Ulinastatin is a urinary trypsin inhibitor isolated from male urine. It is a glycoprotein with 143 amino acids and a molecular weight of about 67000 Dal. It is a typical Kunitz-type protease inhibitor. The sulfated chain on the O-terminal of ulinastatin could bind to calcium ions and TSG-6, thus stabilizing the lysosomal membrane and inhibiting the release of inflammatory cytokines. Besides, glucose in the N-terminal allows ulinastatin to bind to endothelial cells, monocytes, granulocytes, and lymphocytes. K region in the N-terminal of ulinastatin is capable of inhibiting the functions of cathepsin G and elastase, while K region in the C-terminal could inhibit multiple hydrolases[35-37]. Pharmacologically, ulinastatin could inhibit expressions of various proteases, glucose, lipolytic enzymes and myocardial inhibitory factor (MDF), improve microcirculation, stabilize lysosomal membranes and suppress the release of inflammatory mediators[37]. Ulinastatin was applied in the treatment of acute pancreatitis at first with confirmation of its safety and efficacy[38]. We

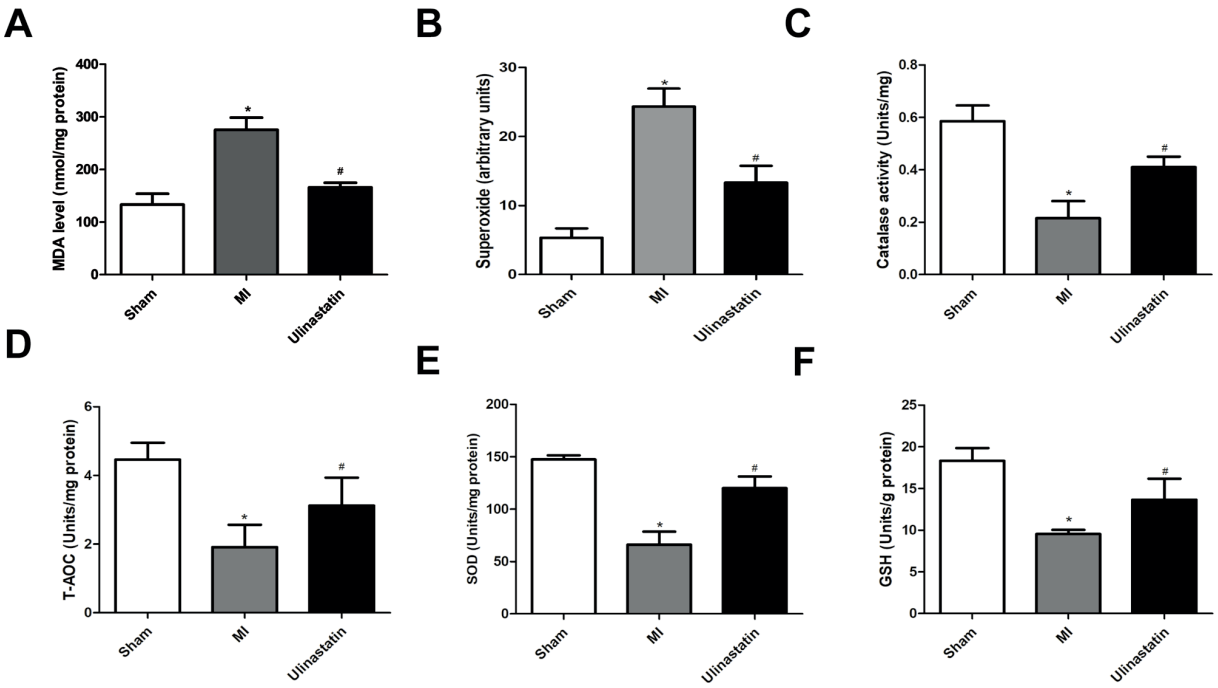

Figure 4. Ulinastatin alleviated oxidative stress in MI rats. Rats were divided into sham group (n=8), MI group (n=8) and ulinastatin group (n=8). ***A,*** MDA level of rat myocardium. ***B,*** ROS level of rat myocardium. ***C,*** CAT level of rat myocardium. ***D,*** T-AOC level of rat myocardium. ***E,*** SOD level of rat myocardium. ***F,*** GSH level of rat myocardium. Data were expressed as mean±**SD**. *p<0.05 compared with sham group, #p<0.05 compared with MI group.

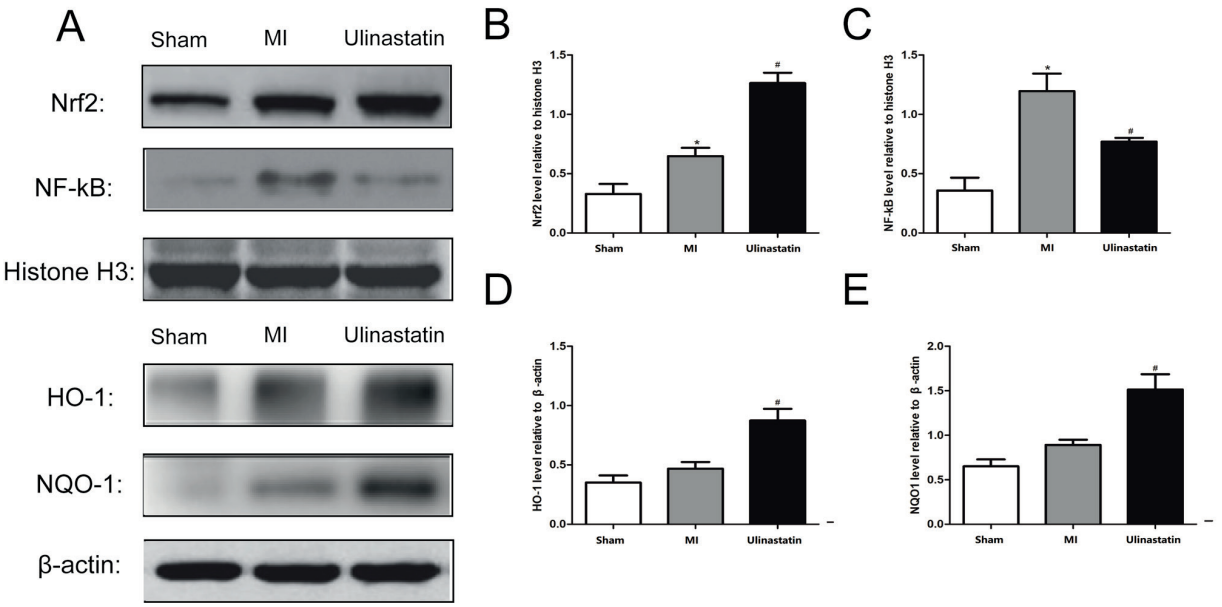

Figure 5. Ulinastatin upregulated expressions of Nrf2 and it downstream genes in MI rats. Rats were divided into sham group (n=8), MI group (n=8) and ulinastatin group (n=8). ***A,*** Protein expressions of Nrf2, NF-κB, HO-1 and NQO1 in different groups. ***B-E,*** Quantification of protein levels of Nrf2, NF-κB, HO-1 and NQO1 in different groups. Data were expressed as mean±SD. *p<0.05 compared with sham group, #p<0.05 compared with MI group.

believed that ulinastatin has a protective effect on ischemic tissues[21,22]. However, the effect of ulinastatin on MI has not been reported yet. In this study, rats in MI group and sham group suffered significant pathological changes of MI, specifically presenting higher oxidative stress levels and lower levels of antioxidant capacity. Evident oxidative stress remarkably leads to tissue and cell damage in the myocardium.

In the present study, NF-κB was highly expressed in the MI group compared with sham group and ulinastatin group. However, Nrf2 expression was lower in MI group than ulinastatin group. We considered that ulinastatin alleviates oxidative stress and inflammatory response in ischemic myocardium by activating Nrf2.

Conclusions

We revealed that ulinastatin could remarkably alleviate MI-induced pathological lesions in myocardium, oxidative stress, and inflammatory response. Ulinastatin protects MI rats by activating Nrf2.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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