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# Ulinastatin protects against myocardial ischemia/reperfusion injury in rats via Rho/ROCK signaling pathway

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**ABSTRACT**

We aimed to study the influences of ulinastatin on diseased myocardial tissues, cardiomyocyte apoptosis and inflammatory reaction in rats with myocardial ischemia/reperfusion injury (IRI) *via* the Ras homolog (Rho)/Rho-associated kinase (ROCK) signaling pathway and its mechanism. The rats were randomly divided into three groups: control group (C group), IR model group (IR group) and IR model + ulinastatin treatment group (UR group). The pathological changes in myocardial tissues were detected *via* HE staining, the markers of myocardial injury were examined using kits, and apoptosis was determined through TUNEL assay. Moreover, ELISA was applied to measure the expressions of TNF-α, interleukin-6 (IL-6) and IL-8 in cardiac tissues, and Western blotting was performed to detect the protein expression levels of RhoA, ROCK2 and MLCP. The myocardial infarction area in the IR group was markedly larger than that in the C group ($P<0.01$) but was significantly reduced after ulinastatin treatment ($P<0.05$), and the IR group had higher levels of AST, cTnI, CK-MB and LDH than C group, but the levels of those indexes were significantly reduced after ulinastatin treatment.The cardiomyocyte apoptosis was increased in the IR group compared with that in the C group, while it was decreased in the UR group in comparison with that in the IR group. Besides, the UR group exhibited lowered expression levels of the Rho/ROCK signaling pathway-related proteins compared with the IR group. Ulinastatin may ameliorate the prognosis of rats with myocardial IRI *via* the Rho/ROCK signaling pathway.

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## Introduction

Coronary heart disease (CHD), a major cause of death in the world, induced 7,254,000 cases of death (12.8% of all death cases) worldwide in 2008 according to the data from the World Health Organization (1). CHD is often attributed to acute myocardial ischemia-reperfusion injury (IRI) which generally occurs in patients with acute ST-segment elevation myocardial infarction (STEMI). Timely and effective thrombolytic therapy for myocardial reperfusion or primary percutaneous coronary intervention (PPCI) is the most efficacious therapeutic measure to reduce acute myocardial ischemic injury and limit myocardial infarction (MI) size. However, the myocardial reperfusion itself can further induce cardiomyocyte death, which is known as myocardial reperfusion injury (2).

Urinary trypsin inhibitor, also called ulinastatin, is a kind of protease inhibitor refined from fresh urine of healthy males (3). Ulinastatin reduces the release and represses the activity of elastase in leukocytes, especially polymorphonuclear neutrophils. Moreover, it is able to stabilize the lysosomal membrane and restrain the release of lysosomal enzymes (4,5). Although previous studies have manifested that ulinastatin exerts beneficial effects on IRI of the lung (6), liver (7) and kidney (8), the impact mechanism of ulinastatin on myocardial IRI still remains unclear.

Ras homolog (Rho)-associated kinase (ROCK) mediates various important cell functions, such as cell shape, secretion, proliferation and gene expression, and participates in the regulation of inflammation and oxidative stress (9). Currently, a variety of ROCK inhibitors undergoing clinical trials may serve as next-generation therapeutic agents for cardiovascular diseases.

This study aims to investigate the influences of ulinastatin on diseased myocardial tissues, cardiomyocyte apoptosis and inflammatory reaction in rats with myocardial IRI and its mechanism.

## Materials and Methods

### Materials

Laboratory animals: Specific Pathogen Free (SPF) male Sprague-Dawley (SD) rats aged 8 weeks old and weighing 180-200 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Reagents: Ulinastatin injection was bought from Guangdong Triplex International Biosciences (Guangzhou, China) Co., Ltd., kits of cardiac troponin I (cTnI), creatine kinase-MB (CK-MB) and lactate dehydrogenase (LDH) from Sigma (St. Louis, MO, USA), enzyme-linked immunosorbent assay (ELISA) kits of interleukin-6 (IL-6), IL-8, IL-10 and tumor necrosis factor-alpha (TNF-α) from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China), antibodies of RhoA, Rock2, myosin

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light chain phosphatase (MLCP) and GAPDH from Abcam (Cambridge, MA, USA), and horseradish peroxidase-labeled goat anti-rabbit/mouse secondary antibodies from Applygen Technologies Inc. (Beijing, China).

**Grouping of laboratory animals and modeling of myocardial IRI**

Animal grouping: The SD rats were randomly divided into a control group (C group, n=6), the myocardial IR model group (IR group, n=6) and the myocardial IR model + ulinastatin treatment group (UR group, n=6). On the next day, the branches of the left coronary artery of the rats in the IR group were obstructed for 30 min and then released for reperfusion for 24 h. In the UR group, the branches of the left coronary artery were obstructed for 30 min, released and reperfused for 24 h on the next day, followed by drug administration for 3 consecutive days. However, the branches of the left coronary artery were subjected to surgery, without blocking. This study was approved by the Animal Ethics Committee of the Animal Center of The First Hospital of Hebei Medical University.

**Measurement of the content of serum aspartate aminotransferase (AST), cTnI, CK-MB and LDH**

After reperfusion for 48 h, 1 mL of blood sample was collected, placed at room temperature for 1 h and centrifuged at 4°C and 1,000 g for 10 min. Later, the supernatant was collected and stored in a refrigerator at -20°C. Finally, the content of AST, cTnI, CK-MB and LDH in the serum was detected using a biochemical analyzer (Olympus Corporation, Tokyo, Japan).

**Hematoxylin-eosin (HE) staining**

The rats were sacrificed at 72 h after reperfusion, and the cardiac tissues were fixed in 4% paraformaldehyde for 72 h. Next, the fixed cardiac tissues were dehydrated, embedded in paraffin and sliced into 5 μm-thick sections, followed by deparaffinization in xylene, elution in different concentrations of alcohol and washing with purified water. After deparaffinization, the sections were stained with hematoxylin which was washed away using clear water later, and then the sections were put into a differentiation medium. After that, the sections were light blue. Subsequently, the sections were washed under running water until they turned sky blue. Then the sections were immersed in an eosin solution which was washed away by clear water. The water on the slide was absorbed, and the sections were completely dehydrated in ethanol and then transparentized in xylene. Later, the cleared sections were added with chewing gum drops, sealed by a cover glass and dried, followed by observation of pathological changes in cardiac tissues under a light microscope (×400).

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) apoptosis assay**

At 48h after reperfusion, the rats were killed according to the operations in the TUNEL apoptosis assay kit. Then paraffin-embedded cardiac sections were prepared through fixation, flushing and penetration with 0.1% Trition X-100. Later, the sealed sections were labeled using a phosphor developer, and the TUNEL-positive cells were observed under a fluorescence microscope. Finally, the percentage of TUNEL-positive cells was calculated in ten fields of vision.

**Detection of TNF-α, IL-6 and IL-8**

The heart was homogenized and centrifuged at 4°C and 1000 g for 10 min. Next, the supernatant was taken, standard substances and experimental samples were added into a microtiter plate in accordance with the usage of ELISA kits, and the reaction wells were sealed, followed by incubation at room temperature and shaking on a horizontal shaker for 2 h. After that, the plate was rinsed using washing solution, enzymes were added into each well to detect the antibodies after the washing solution was removed, and the reaction wells were sealed, followed by incubation at room temperature on the horizontal shaker for 2 h. Then the plate was washed again, and chromogenic substrates were added into each well and incubated at room temperature for 30 min. Subsequently, the suspension was added to each well. Finally, the content of TNF-α, IL-6 and IL-8 was measured via ELISA.

**Western blotting assay**

The total protein was extracted from the cardiac tissues, and its concentration was determined. The protein prepared was mixed with sodium dodecyl sulphate (SDS) loading buffer and boiled at 95°C for 3 min. Next, an equal volume of protein was separated by means of 8-10% polyacrylamide gel electrophoresis (Applygen Technologies Inc., Beijing, China) and then transferred onto a nitrocellulose membrane. After sealing in 10% skim milk, the proteins were incubated with primary antibodies in an oscillator at 4°C overnight. Following the first washing 3 times, the proteins were incubated on another oscillator at room temperature for 1 h. Finally, an electrochemiluminescence (ECL) solution was added to expose and develop the image after washing 3 times, and the relative expression of target proteins was analyzed using ImageJ software (NIH, Bethesda, MD, USA).

**Statistical analysis**

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was employed for statistical analysis, and the measurement data were presented as mean ± standard deviation. Differences between the two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using a One-way ANOVA test followed by Post Hoc Test (Least Significant Difference), and $P<0.05$ suggested that the difference was statistically significant.

## Results

**Influences of ulinastatin on cardiac injury markers in IRI rats**

Compared with those in the C group, the content of serum AST, cTnI and CK-MB was increased ($P<0.05$), and that of LDH rose markedly ($P<0.01$) in the IR group. However, reduced content of AST, cTnI, CK-MB and LDH in the serum was detected in the UR group in comparison with that in the IR group (Table 1).

**Influences of ulinastatin on diseased cardiac tissues in IRI rats**

C group had regularly arranged cardiomyocytes, continuous and unbroken muscle fibers and no apparent cardiomyocyte infiltration. In the IR group, however, the cardiac tissue structure was disturbed, the muscle fibers were

**Table 1.** Comparisons of AST, cTnI, CK-MB and LDH levels in cardiac tissues among groups (n=6, χ±s).

| Group | AST (U/L) | cTnI (ng/mL) | CK-MB (U/L) | LDH (U/L) |
|---|---|---|---|---|
| C group | 97.7±10.87 | 0.28±0.07 | 21.87±2.74 | 618.4±58.9 |
| IR group | 339.8±41.8[a] | 5.84±0.46[a] | 92.44±9.51[a] | 1631.8±110.8[b] |
| UR group | 146.7±15.3[c] | 2.38±0.25[c] | 57.19±5.94[c] | 978.4±104.8[c] |

Note: [a]P<0.05 & [b]P<0.01 vs. C group, [c]P<0.05 vs. IR group.

discontinuous, and inflammatory cell infiltration occurred. Furthermore, the above pathological changes were ameliorated in the UR group (Figure 1).

**Influences of ulinastatin on inflammatory factors in cardiac tissues of IRI rats**

The levels of IL-8, IL-6 and TNF-α in cardiac tissues were higher in the IR group than those in the C group (P<0.05), while they were lower in the UR group than those in the IR group (P<0.05) (Table 2).

**Influence of ulinastatin on apoptosis in cardiac tissues of IRI rats**

IR group exhibited enhanced apoptosis in cardiac tissues compared with the C group (P<0.05), while the UR group displayed weakened apoptosis in cardiac tissues in comparison with the IR group (P<0.05) (Figure 2).

**Influence of ulinastatin on activation of Rho/ROCK signaling pathway in cardiac tissues of IRI rats**

The expressions of RhoA, ROCK2 and MLCP were up-regulated in the IR group compared with those in the C group (P<0.05 or P<0.01), while they were down-regulated in the UR group in comparison with those in the IR group (P<0.05) (Figure 3).

**Discussion**

The intervention opportunity for STEMI patients who are suffering from acute myocardial ischemia is only limited to the time of myocardial ischemia attack or myocardial reperfusion (PPCI). The myocardial reperfusion process under PPCI will be continuously improved through early reperfusion, advancement of PCI technique and

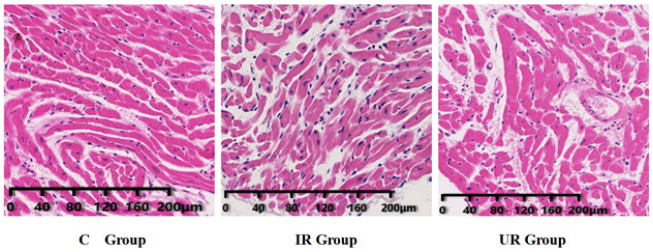


**Figure 1.** Manifestations of pathological changes in cardiac tissues in each group (HE, magnification: 200×).

**Table 2.** Comparisons of TNF-α, IL-6 and IL-8 levels in cardiac tissues among groups (pg/mg, n=6, $\bar{\chi}$±s).

| Group | TNF-α | IL-6 | IL-8 |
|---|---|---|---|
| C group | 18.74±1.84 | 39.84±3.81 | 51.09±3.94 |
| IR group | 38.67±2.97[a] | 81.14±9.11[a] | 97.24±8.61[a] |
| UR group | 20.34±2.17[b] | 57.64±4.91[b] | 69.95±5.93[b] |

Note: [a]P<0.05 & [b]P<0.01 vs. C group, [c]P<0.05 vs. IR group.

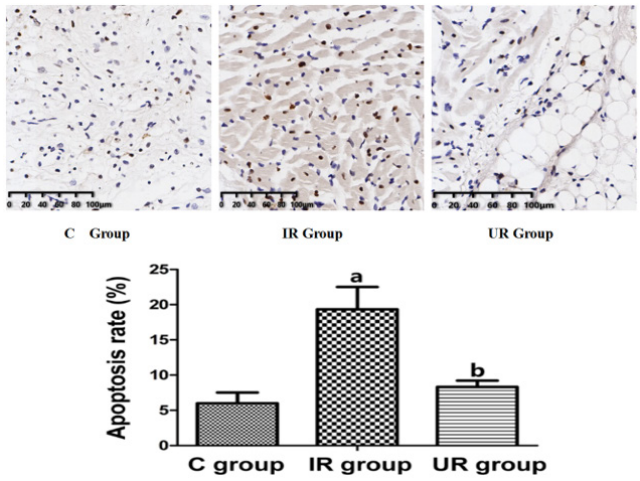


**Figure 2.** Comparison of apoptosis rate in cardiac tissues among groups (TIUNEL, magnification: 400×). Note: [a]P<0.05 vs. C group, [b]P<0.05 vs. IR group.

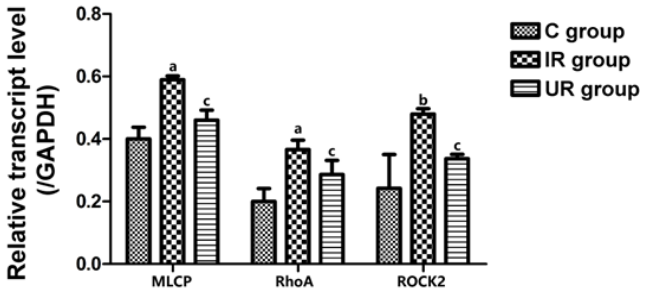


**Figure 3.** Comparison of absorbance of protein bands related to Rho/ROCK signaling pathway in cardiac tissues in each group. Note: [a]P<0.05 & [b]P<0.01 vs. C group, [c]P<0.05 vs. IR group.

introduction of more effective antiplatelet and antithrombotic agents to maintain the patency of coronary artery. However, there is still no efficacious therapeutic agent that can prevent fatal myocardial reperfusion injury in STEMI patients undergoing PPCI. Therapeutic hyperoxia (10) and hypothermic treatment (11) are two mechanical intervention measures beneficial to myocardial IRI reported in animal studies. Existing pharmacological modulators include adenosine, atrial natriuretic peptide, atorvastatin, erythropoietin and exenatide, while other known reagents such as cyclosporin A and sodium nitrite can maintain the mitochondrial functions during acute IRI.

Almost all the small G protein family (e.g. Rho and Ras) is involved in intracellular signal transduction (12). The Rho family members regulate not only cytoskeleton reorganization but also gene expression. Similar to other Rho guanosine triphosphatases (GTPases), Rho acts as a molecular switch and circulates between active guanosine triphosphate (GTP)-bound state and ineffective guanosine diphosphate (GDP)-bound state (10). The switching between active state and inactive state is controlled by

several regulatory proteins. Rho mainly exists in the cytoplasm of unstimulated cells in an inactive GDP-bound form, and Rho guanine nucleotide dissociation inhibitor conjugates with Rho-GDP and is extracted from the membrane into the cytoplasm. When the cells are stimulated by some agonists, Rho-GDP is transformed into Rho-GTP under the action of Rho-GEF, then Rho-GTP targets the cell membrane and interacts with specific targets, and Rho GTPase activator protein inactivates Rho by phosphorylating GTP into GDP (13). ROCK, the most typical downstream effector of Rho, mediates various cell functions. It was defined as one of the downstream effectors of Rho in the middle 1990s and possesses two isotypes, namely ROCK1 and ROCK2, which are extensively expressed in human. ROCK2 is highly expressed in the brain and heart, while ROCK1 is preferentially expressed in the lungs, liver, spleen, kidneys and testis (14).

Previous studies have revealed that ulinastatin can decrease the release of elastins from neutrophilic granulocytes and repress the accumulation of neutrophilic granulocytes in diverse IRI models (15,16), meaning that the protective effect of ulinastatin is attributable to its anti-inflammatory response. It has been discovered in some studies that ulinastatin improves myocardial contractility, reduces the area of MI and lowers the levels of serum CK and cTnI after regional IRI (17). It is known that such protection appears to be stimulated by inhibition of TNF-α and myeloperoxidase (MPO) activity which serves as an index of neutrophil infiltration and activation in tissues. MI causes neutrophil infiltration into the infarct region and increases the production of pro-inflammatory cytokines such as TNF-α (18-26). Moreover, as a manifestation of acute inflammation, neutrophil accumulation triggers myocardial injury, so it is held that ulinastatin probably exerts myocardial protective effects by suppressing the inflammatory process. The above findings coincide with the results of this study. The MI area in the IR group was markedly larger than that in the C group ($P<0.01$) but was significantly reduced after ulinastatin treatment, and the IR group manifested higher levels of AST, cTnI, CK-MB and LDH than the C group. However, the levels of those indexes were significantly reduced after ulinastatin treatment. The cardiomyocyte apoptosis was increased in the IR group compared with that in the C group, while it was decreased in the UR group in comparison with that in the IR group. Besides, the UR group exhibited lowered expression levels of the Rho/ROCK signaling pathway-related proteins compared with the IR group.

It was revealed in the present study that ulinastatin may ameliorate the prognosis of rats with myocardial IRI through the Rho/ROCK signaling pathway, providing new diagnosis and treatment thoughts for myocardial IRI and laying a theoretical basis for further clinical application of ulinastatin.

## Conflict of interest

The authors declared no conflict of interest.

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