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Effect of ulinastatin on myocardial ischemia reperfusion injury through ERK signaling pathway

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Abstract. – OBJECTIVE: To study the effect of ulinastatin (UTI) on myocardial ischemia-reperfusion injury (MIRI) through the extracellular signal-regulated kinase (ERK) signaling pathway.

MATERIALS AND METHODS: A total of 24 Sprague-Dawley rats were randomly divided into sham group (n=8), I/R group (n=8), and UTI group (n=8), and the rat model of MIRI was established. The changes in the content of serum biochemical indexes, including superoxide dismutase (SOD) and malondialdehyde (MDA), were detected using the kits, and the changes in the expressions of serum inflammatory factors, including interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), were detected using the quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) and enzyme-linked immunosorbent assay (ELISA) kits. Moreover, the ERK phosphorylation level in myocardial tissues was detected using the immunofluorescence method, and the ERK phosphorylation level and cleaved caspase-3 expression were detected *via* qRT-PCR and Western blotting.

RESULTS: Compared with those in sham group, the serum SOD content significantly declined, while the MDA content was significantly increased in I/R group, and they were significantly improved in UTI group ($p<0.01$). The results of detection using qRT-PCR and ELISA kits revealed that the inflammatory factors (IL-6 and TNF-α) in UTI group were significantly improved ($p<0.01$). The immunofluorescence results showed that the ERK phosphorylation level in myocardial tissues was significantly increased in UTI group. The results of qRT-PCR and Western blotting manifested that both ERK phosphorylation level and cleaved caspase-3 expression were significantly improved in UTI group ($p<0.01$).

CONCLUSIONS: UTI can play a protective role in MIRI through up-regulating the ERK signaling pathway.

Key Words:
Ulinastatin, ERK, Myocardial ischemia-reperfusion injury, Rats.

Introduction

Acute myocardial infarction ranks first in the cause of death in patients in China. The area and severity of myocardial infarction seriously affect the prognosis of patients. Although early reperfusion therapy is the most direct and effective means to reduce the area of myocardial infarction, the dysfunction and structural damage of ischemic myocardium cannot be repaired in the first time or even become worse after reperfusion, which is known as ischemia-reperfusion injury[1], seriously hindering the greatest efficacy of reperfusion therapy. Therefore, finding new treatment means to protect ischemic myocardium has become a problem urgently to be solved in the reperfusion therapy of acute myocardial infarction[2].

This ischemia-reperfusion injury is a very common pathophysiological phenomenon in ischemic heart diseases and during the perioperative period of cardiac surgery, so simulating such reperfusion injury in basic research is of great importance in directly observing the effect of drugs on cells and further exploring the mechanism deeply[3]. A large number of studies have demonstrated that extracellular signal-regulated kinase (ERK) protein is lowly expressed in normal myocardial cells, while it will be significantly activated and highly expressed in the case of myocardial ischemia-reperfusion injury (MIRI)[4]. According to many studies, the ERK signal transduction pathway plays an important role in MIRI[5]. The main pharmacological activity of ulinastatin (UTI) is the protease inhibitor, which has a significant inhibitory effect on such serine proteases as trypsin, plasmin, and chymotrypsin, and the anti-inflammatory and anti-shock biological functions of UTI have been confirmed[6,7].

In the present study, UTI was used to intervene in the rat model of MIRI, and the protective effect of UTI in injured myocardial tissues through the ERK signaling pathway and its possible mechanism were preliminarily investigated.

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Materials and Methods

Animals

A total of 30 clean-grade male Sprague-Dawley rats weighing (200±20) g were provided by the Capital Medical University Laboratory Animal Center They were fed in strict accordance with the standards of animal laboratory and had free access to food and water. This study was approved by the Animal Ethics Committee of Capital Medical University Animal Center. After the rats were anesthetized with pentobarbital sodium (20 mg/kg), the left anterior descending coronary artery was ligated at 0.5 mm using the surgical suture to block the blood flow, causing ischemia. Then, it was found that the distal myocardial tissues of the left anterior descending coronary artery gradually turned dark purple. The surgical suture was loosened to simulate the reperfusion process and, then, the distal myocardial tissues of the left anterior descending coronary artery gradually returned to be bright red. At 30 min before the operation, UTI (2500 U/kg) was administered intravenously in UTI group, while an equal amount of normal saline was given in sham group and I/R group.

Reagents and Instruments

UTI (Sigma-Aldrich, St. Louis, MO, USA), immunohistochemical staining kit SP-9001 (Beijing Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China), ribonucleic acid (RNA) extraction kit (Invitrogen Life Technologies, Carlsbad, CA, USA), quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) kit (Tiangen, Beijing, China), ERK, cleaved caspase-3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology Inc., Shanghai, China), bicinchoninic acid (BCA) protein quantification kit and cell lysis buffer (Hanbio, Shanghai, China), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kits (Hanbio, Shanghai, China).

Detection of Biochemical Indexes SOD and MDA

After the blood was drawn and centrifuged, the serum was carefully separated to detect the content of SOD (xanthine oxidase colorimetry) and MDA (thiobarbituric acid method) according to the instructions.

Detection of Changes in Serum IL-6 and TNF-α Content via ELISA

The myocardial tissues of rats cryopreserved at -80°C were taken, placed into Eppendorf (EP) tubes, added with an appropriate amount of tissue lysis buffer, thoroughly ground with a grinder, and placed on ice for 15 min, followed by centrifugation at 12000 g and 4°C for 5 min. Then, the supernatant was taken into another new batch of EP tubes, and the protein concentration was determined using the BCA method and quantified. The expression levels of inflammatory factors TNF-α and IL-6 in myocardial tissues of rats were detected according to the instructions of the ELISA kits.

Immunofluorescence Detection of p-ERK Expression Level in Myocardial Tissues

First, the myocardial tissues fixed were embedded in paraffin, sliced, and deparaffinized. The endogenous peroxidase was inactivated with 3% H_2O_2, followed by microwave antigen retrieval. The tissues were sealed with serum at an appropriate concentration, marked with the immunohistochemical pen, and added with the primary antibody for incubation in a wet box at 4°C overnight. On the next day, the tissues were washed with PBS for 3 times, dropwise added with the fluorescence secondary antibody for incubation for 30 min, and washed again with PBS for 3 times, followed by color development using diaminobenzidine (DAB). Finally, the resin adhesive was dropwise added and the sections were covered with the cover glass, followed by photography under a microscope (TE2000-U, Nikon, Tokyo, Japan).

Detection of p-ERK and Cleaved Caspase-3 mRNA Expression Levels Via qRT-PCR

After 50 mg myocardial tissues of rats cryopreserved at -80°C were taken into the EP tube, 200 μL TRIzol (Invitrogen, Carlsbad, CA, USA) was added into each tube, the tissues were ground using the grinder on ice, and then, TRIzol was added until the total volume was 1 mL. After standing for 5 min, chloroform was added, shaken violently for 15 s, and placed for 15 min. Then, the water-phase layer was taken into another new batch of EP tubes, added with isopropanol and turned upside down for several times, followed by centrifugation. The white precipitate at the bottom of the tube was RNA. After RNA was washed and dissolved in water, its concentration and purity were detected. The qualified RNA (A_{260}/A_{280} =1.8-2.0) was reversely

Table I. qRT-PCR primer sequences.

Gene	Primer	Primer sequence
ERK	Forward primer	5'-CTCATCGCAGATGCCTGGAA-3'
	Reverse primer	5'-TTCAGGTAATAGGCACCCTTGAAGA-3'
Caspase-3	Forward primer	5'-ACGCACGACGTCTTCCAGTA-3'
	Reverse primer	5'-CCACCTGGTTCAACTCACTCC-3'
GAPDH	Forward primer	5'-GCTTCGGCAGCACATATACTAAAAT-3'
	Reverse primer	5'-CGCTTCACGAATTTGCGTGTCAT-3'

transcribed into complementary deoxyribose nucleic acid (cDNA), and the mRNA expression level was detected *via* qRT-PCR. The primer sequences are shown in Table I. Reaction conditions are as follows: 94°C for 5 min, amplification at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s for a total of 40 cycles, and 72°C for 5 min. The data were processed using Microsoft Excel, and the relative expression level of the target gene was calculated using the $2^{-\Delta\Delta Ct}$ method according to the following formula, with GAPDH as a control gene: ΔCt (target gene) = Ct (target gene) – Ct (control gene), $\Delta\Delta Ct$ = ΔCt (target gene) – ΔCt (standard value).

Detection of p-ERK and Cleaved Caspase-3 Protein Expression Levels Via Western Blotting

After 50 mg myocardial tissues of rats cryopreserved at -80°C were taken into the EP tube, 200 μL radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China) was added into each tube, and the tissues were ground with the grinder on ice and shaken once every 5 min. After 20 min when the tissues were fully lysed, they were centrifuged at 12000 rpm and 4°C for 10 min. Then, the supernatant was collected into another new batch of EP tubes. The concentration of the protein extracted was measured using the BCA kit, and the protein was quantified. 30 μg proteins were taken and detected *via* Western blotting. After electrophoretic separation, the proteins were electrically transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) in electrophoresis buffer, sealed with 5% skim milk at room temperature for 2 h, washed on a shaker and incubated in an incubator containing the primary antibody (1:1000) at 4°C overnight. After the membrane was fully washed with TTBS, the secondary antibody (1:5000) was added for incubation at room temperature for 1 h, followed by image development with enhanced chemiluminescence (ECL) in a dark room. The images were scanned and recorded using a gel imager (Bio-Rad Laboratories, Hercules, CA, USA). Finally, the gray scale was analyzed and compared, with GAPDH as an internal reference.

Statistical Analysis

The data were expressed as mean ± standard deviation and processed using Statistical Product and Service Solutions (SPSS) 17.0 software (International Business Machines Corporation, Armonk, NY, USA). The comparison between groups was done using the One-way ANOVA test followed by the Post-Hoc Test (Least Significant Difference). $p<0.05$ suggested that the difference was statistically significant.

Results

Effects of UTI on SOD and MDA Content

Compared with those in sham group, the SOD content significantly declined ($p<0.01$), while the MDA content was significantly increased in I/R group after the operation ($p<0.01$), and they were significantly improved in UTI group ($p<0.01$) (Figure 1).

Effects of UTI on IL-6 and TNF-α Content Detected Via ELISA

Compared with that in sham group, the IL-6 and TNF-α content was significantly increased in I/R group after the operation ($p<0.01$), while it significantly declined in UTI group ($p<0.01$) (Figure 2), indicating that UTI can significantly improve the levels of inflammatory factors.

Effects of UTI on IL-6 and TNF-α mRNA Expressions Detected Via qRT-PCR

Compared with those in sham group, the IL-6 and TNF-α mRNA expression levels were significantly increased in I/R group after the operation ($p<0.01$), while they significantly declined in UTI group ($p<0.01$) (Figure 3), indicating that UTI can significantly down-regulate the mRNA expression levels of inflammatory factors.

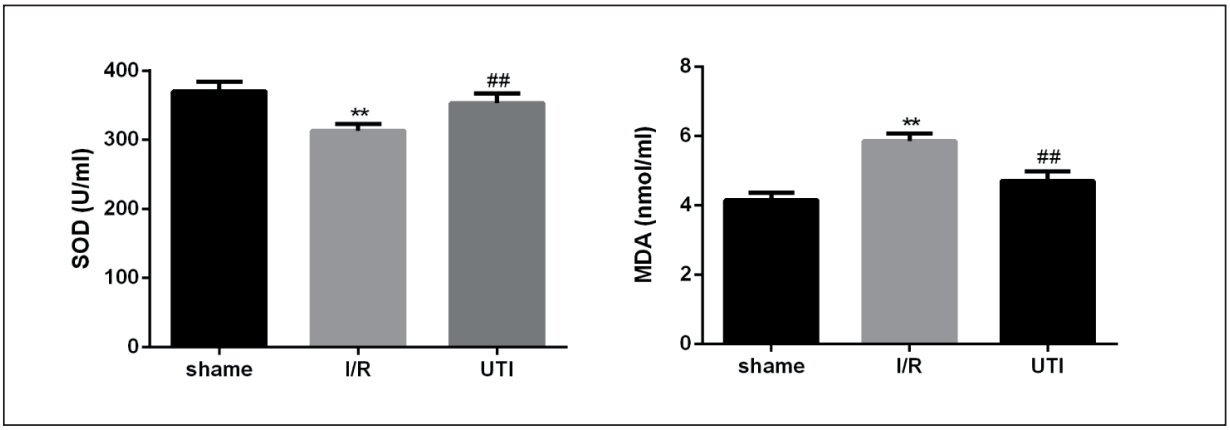

Figure 1. Effect of UTI on SOD and MDA content. **p<0.01 *vs.* sham group, $^{\#\#}p$<0.01 *vs.* I/R group.

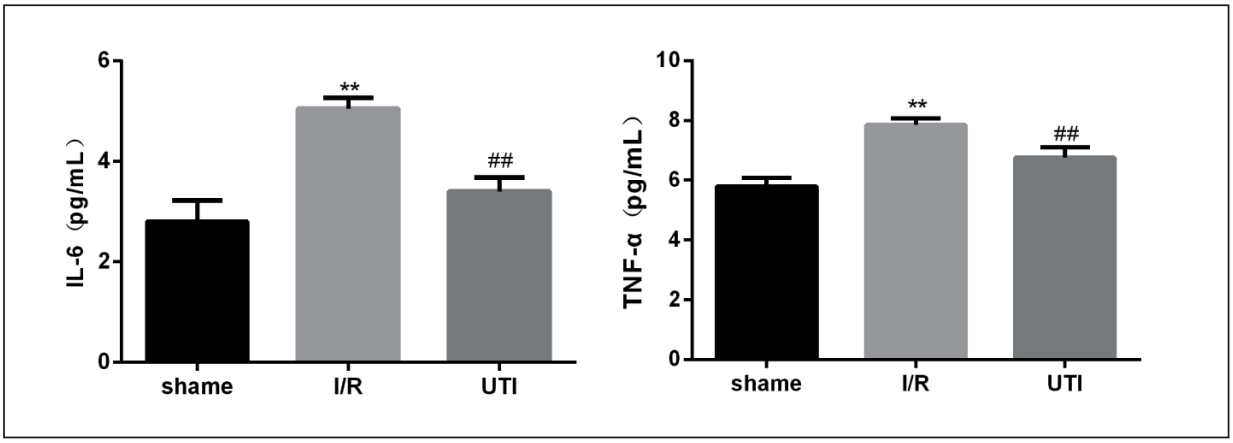

Figure 2. Effect of UTI on IL-6 and TNF-α content detected via ELISA. **p<0.01 *vs.* sham group, $^{\#\#}p$<0.01 vs. I/R group.

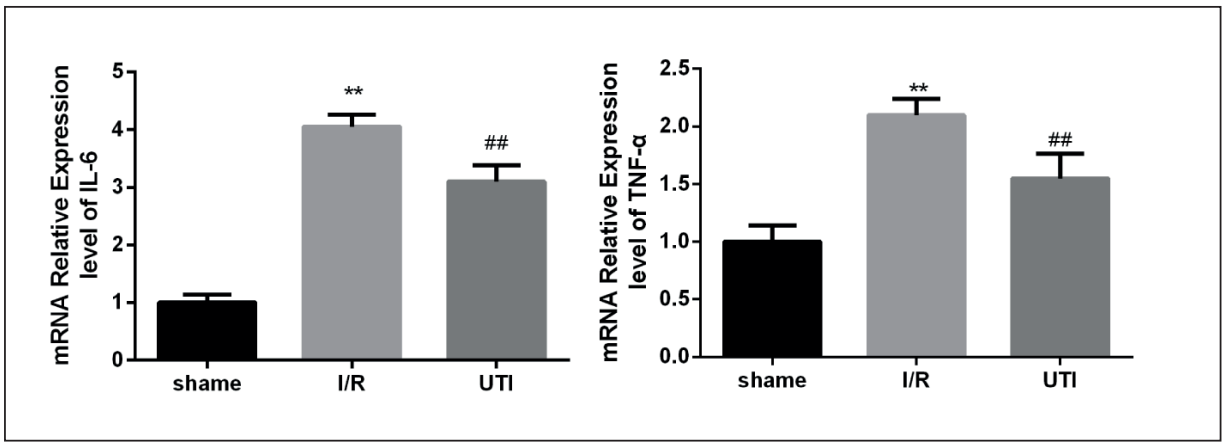

Figure 3. Effect of UTI on IL-6 and TNF-α mRNA expressions detected via qRT-PCR. **p<0.01 vs. sham group, $^{\#\#}p$<0.01 *vs.* I/R group.

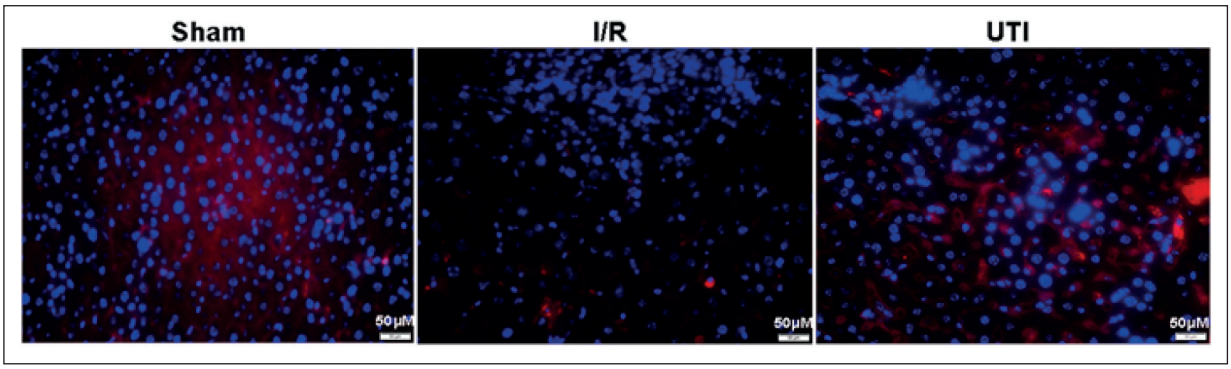

Figure 4. Effect of UTI on p-ERK expression in myocardial tissues detected via immunofluorescence (magnification: 40×).

Effects of UTI on p-ERK Expression in Myocardial Tissues Detected Via Immunofluorescence

As shown in Figure 4, compared with sham group, I/R group had increased number of apoptotic cells and decreased expression level of p-ERK, while UTI group had decreased number of apoptotic cells and increased expression level of p-ERK.

Effects of UTI on p-ERK and Cleaved Caspase-3 mRNA Expressions in Myocardial Tissues Detected Via qRT-PCR

As shown in Figure 5, the mRNA expression levels of p-ERK and cleaved caspase-3 after the operation were significantly increased in I/R group compared with those in sham group (p<0.01), and the cleaved caspase-3 mRNA expression level significantly declined in UTI group compared with that in I/R group (p<0.01).

Effects of UTI on p-ERK and Cleaved Caspase-3 Protein Expressions in Myocardial Tissue Detected Via Western Blotting

As shown in Figure 6, the p-ERK and cleaved caspase-3 protein expression levels after the operation were remarkably increased in I/R group compared with those in sham group (p<0.01), and the p-ERK protein expression level was also remarkably increased in UTI group compared with that in I/R group (p<0.01), suggesting that UTI can significantly activate the expression of p-ERK and inhibit the myocardial apoptosis.

Discussion

When the injury caused by myocardial ischemia-reperfusion accounts for 1/2 or more of the final area of myocardial infarction, it will eventually develop into congestive heart failure if

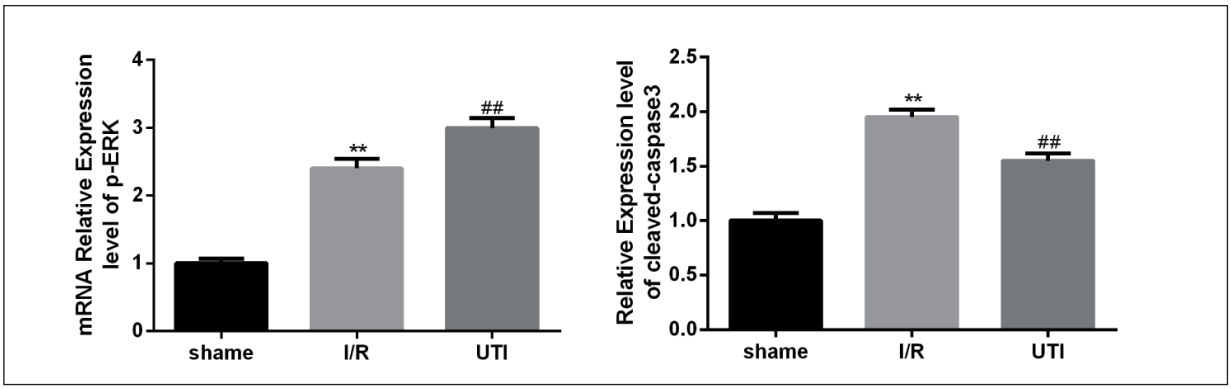

Figure 5. Effect of UTI on p-ERK and cleaved caspase-3 mRNA expressions in myocardial tissues detected via qRT-PCR. **p<0.01 *vs.* sham group, $^{\#\#}p$<0.01 *vs.* I/R group.

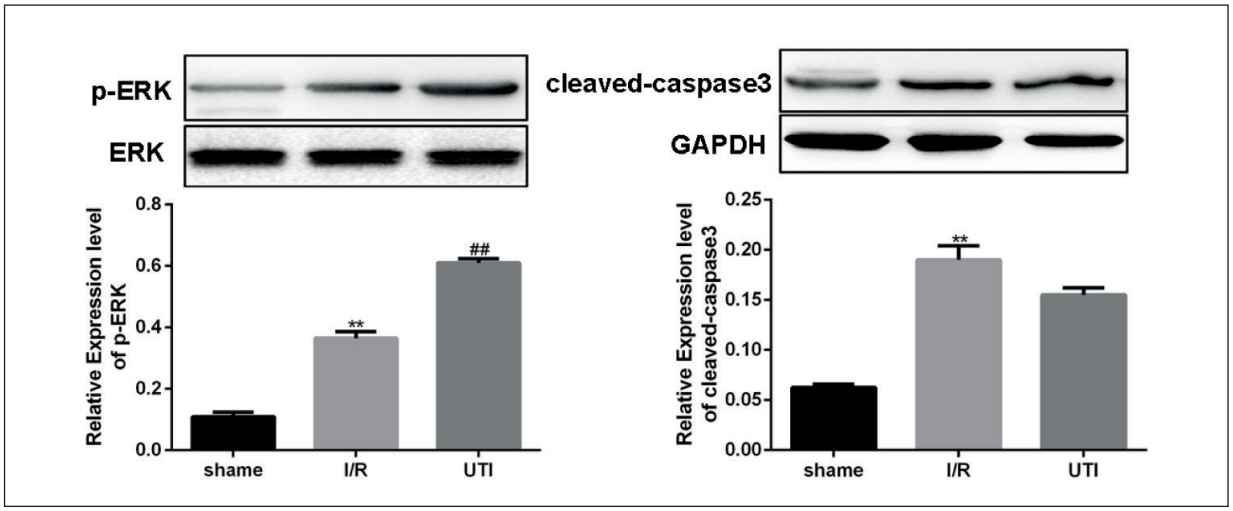

Figure 6. Effect of UTI on p-ERK and cleaved caspase-3 protein expressions in myocardial tissue detected via Western blotting. ***p*<0.01 *vs.* sham group, ##*p*<0.01 *vs.* I/R group.

the remaining healthy myocardium cannot compensate for the damaged myocardium to exert the normal contractile force. Such a conclusion has been widely recognized[7,8]. At present, the clinical treatment means, including stem cell transplantation and gene therapy, obtain no significant effects. Therefore, it is of great significance to actively explore the more effective therapeutic drugs or the new mechanisms of existing drugs, and find new therapeutic targets to reduce the injury caused by myocardial ischemia-reperfusion.

The ERK signaling pathway plays an important protective role in the injury caused by myocardial ischemia reperfusion[9], whose main mechanism includes the activation of anti-apoptotic signaling pathways and inhibition on pro-apoptotic signaling pathways[10,11]. After the phosphorylation of ERK, the physiological activity of Bcl-2 family members can be positively regulated. A large number of studies[12,13] have demonstrated that Bcl-2 is able to antagonize the expression of the pro-apoptotic protein Bax, inhibiting the release of cytochrome C and the activation of caspase-3, ultimately keeping cell homeostasis and inhibiting apoptosis. Also, Bcl-2 can inhibit the normal function of membrane phosphatidylserine and synergize with p53 to further inhibit apoptosis. Moreover, ERK can also activate ribosomal S6 protein kinase in a non-transcription-dependent manner to promote cell survival, and it can also promote the phosphorylation of the pro-apoptotic protein Bax to inhibit apoptosis[14-16]. Besides, the activation of ERK can also inhibit the activation of pro-apoptotic factors, such as caspase-8 and caspase-3. The pharmacological activity of UTI is a typical inhibitor of a variety of proteases to simultaneously suppress the activity of various proteases. It can also inhibit the release of some inflammatory factors, such as IL-6 and TNF-α, block the vicious circle between these inflammatory factors and inflammatory cells, and alleviate the damage of proteases that cause an inflammatory response to the function of tissues and organs[17,18].

In the present work, the rat model of MIRI was established. The results revealed that compared with those in sham group, the serum SOD content significantly declined, while the MDA content was significantly increased in I/R group, and they were significantly improved in UTI group. The results of detection using qRT-PCR and ELISA kits showed that the inflammatory factors (IL-6 and TNF-α) in UTI group were significantly improved. The immunofluorescence results showed that the ERK phosphorylation level in myocardial tissues was significantly increased in UTI group. The results of qRT-PCR and Western blotting manifested that both ERK phosphorylation level and cleaved caspase-3 expression were significantly improved in UTI group. Research reports have indicated that a large amount of TNF-α and IL-6 and some chemotactic factors are released during myocardial ischemia-reperfusion, thereby recruiting some inflammatory cells in the ischemic region, aggravating the inflammatory response and damaging the myocardial cells[19]. At the same time, studies have shown that the activated ERK can induce different growth factors to quickly exert a protective effect in the damaged myocardium[20].

Conclusions

We found that UTI can play a protective role in MIRI through up-regulating the ERK signaling pathway.

Conflict of Interests

The authors declare that they have no conflict of interest.

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