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Ulinastatin reduces myocardial injury induced by doxorubicin in SD rats

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Abstract. – OBJECTIVE: The aim of this study was to observe the protective effect of Ulinastatin on myocardial injury induced by doxorubicin (DOX) in rats.

MATERIALS AND METHODS: 30 male Sprague Dawley (SD) rats were divided into control group, DOX group, and Ulinastatin group by random number table method. The control group was intraperitoneally injected with saline, while the DOX group and the Ulinastatin group were injected intraperitoneally with DOX (2 mg/kg) once every other day to establish an acute myocardial injury (AMI) model. In the Ulinastatin group, Ulinastatin (1500 IU/100 mg) was injected intraperitoneally once a day for 2 weeks after the model was established. The changes in cardiac structure were observed with a light microscope, the changes in cardiac function in rats were detected with biochemical kits, and expression of oxidative stress and inflammatory response-related factors were detected by Western blotting, enzyme-linked immunosorbent assay (ELISA), and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR).

RESULTS: Myocardial tissues in the control group were neatly arranged and dense, with complete and clear structure. The myocardial tissues in the DOX group were disorderly arranged, the interstitial fibrosis was evident, and the myocardial transverse striations broke and disappeared. The structure of tissues in Ulinastatin group was dramatically relieved compared with DOX group. The serum SOD and GSH-Px levels of the DOX groups were significantly lower than those of the control group, while the levels of MDA and ROS were dramatically higher than those of the control group. The serum SOD and GSH-Px level of Ulinastatin group were higher than that of DOX group, and the levels of MDA and ROS were lower than those of DOX group. LDH, AST, ALT, and CK levels were dramatically higher than those in the control group, while the above-mentioned serum myocardial zymogram levels in the Ulinastatin group were decreased. The expressions of IL-1β, IL-6, TNF-α, and iNOS in the DOX and Ulinastatin groups were dramatically higher than those in the control group, while the expressions of the above inflammatory factors in the Ulinastatin group were all inhibited.

CONCLUSIONS: Ulinastatin intervention can reduce myocardial injury in rats with DOX. The protective effect may be due to the elimination of oxygen free radicals, enhanced antioxidant enzyme activity, reduced lipid peroxidation and inflammatory responses, and thus repaired myocardial injury.

Key Words:
Ulinastatin, Doxorubicin, Oxidative Stress, Inflammation.

Introduction

Doxorubicin (DOX) is one of the most effective and widely used antitumor drugs in clinical practice. It is often used to treat various malignant tumors and leukemias. However, the cardiotoxic effects of DOX have greatly limited their antitumor effects to a certain extent[1]. With the increase of the dosage, the clinical symptoms of myocardial injury gradually increase, and there is no effective prevention and treatment method. Cardiotoxicity caused by DOX can be divided into acute injury and chronic injury. The probability of acute injury is very low. It usually occurs in patients who take a large dose of DOX. The clinical manifestations are acute tachyarrhythmia and acute heart failure (AHF)[2]. Chronic myocardial injury is characterized as dose dependent. The main clinical manifestations are myocardial damage and cardiomyopathy several years after the last application of DOX[3]. No matter it is AMI or chronic myocardial injury, it can cause abnormal heart function, cardiomyopathy, and finally develop into severe heart failure (HF) or even death. Children and adolescent tumor patients treated with DOX are more likely to develop myocardial damage than

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adult patients[4]. In recent decades, people have been working on the mechanism of myocardial injury induced by DOX and achieved promising results. The mechanisms of DOX-induced myocardial injury have been increased the levels of oxidative stress (OS), such as increased levels of ROS and lipid peroxides, and corresponding reductions in antioxidants and thiols[5]. However, the above-mentioned cellular events eventually lead to myocardial cell death. Since cardiomyocytes are terminally differentiated cells, they do not have the ability to regenerate. Accumulation of myocardial cell death caused by DOX eventually exceeds the maximum limit that can be tolerated by normal compensation of the heart, thus causing a series of ventricular remodeling processes similar to those caused by other myocardial injuries: cardiac dysfunction, cardiomyopathy, and HF[6]. Exploring the mechanism of myocardial injury induced by DOX and developing corresponding treatment strategies is the most urgent task at present.

Ulinastatin is a glycoprotein extracted from fresh urine of healthy young men and has inhibitory effects on a variety of proteases. Zhang[7] has found that Ulinastatin can play a therapeutic role in some inflammation or OS-related diseases, such as ulcerative colitis and acute glomerulonephritis. However, whether it has protective effects on DOX-induced cardiomyopathy induced by multiple mechanisms including inflammatory mechanisms and OS responses remains to be further studied.

The inflammatory response is a component of the body's innate immunity. When the pathogen invades, the body will respond quickly to clear the pathogen and protect the body from harm[8]. Inflammatory regulators are produced by innate immune cells, such as monocytes and Toll-like receptors (TLRs). The TLRs family participates in the body's adaptive immunity and innate immunity as inflammatory regulators, and is widely expressed in a variety of cells, such as epithelial cells, endothelial cells, mesothelial cells, fibroblasts, neutrophils, T cells, NK cells, macrophages, and so on[9]. Among the TLRs family, TLR4 is the first TLR discovered by humans. It has been reported that the activation of TLR4, Nuclear factor-kappa B (NF-kB) and Myeloid differentiation factor 88 (MyD88) is involved in the pathophysiology of myocardial injury[10]. The related mechanism of TLR4/MyD88/NF-kB pathway in myocardial injury has been a research hotspot.

Materials and Methods

Experimental Animal

Thirty adult healthy male Sprague Dawley (SD) rats (ALF Biotechnology, Suzhou, China), 6-8 weeks of age, have been managed by the animal room, fed with standard feed, and pure water. They were fed in an environment of 60% air humidity and 21-26°C room temperature, 12 hours/12 hours light/dark, for 5-7 days before the experiment. This study was approved by the Animal Ethics Committee of Taizhou People's Hospital Animal Center.

Experimental Grouping

Thirty SD rats were randomly divided into 3 groups: the control group (intraperitoneal injection of physiological saline), the DOX group [intraperitoneal injection of DOX (Tianpu Biochemical Pharmaceutical, Guangzhou, China] 2 mg/kg every other day for 1 week), and the Ulinastatin group [Ulinastatin; Tianpu Biochemical Pharmaceutical, Guangzhou, China) 1500 IU/100 mg was injected intraperitoneally daily for 2 weeks after the DOX injection].

Collection and Processing of Specimens

Rats were injected intraperitoneally with 10% chloral hydrate (Sigma-Aldrich, St. Louis, MO, USA). After the rats were under anesthesia, they were fixed, and the skin was prepared on the chest. The echocardiography was performed on the rats in each group to observe the changes in the cardiac structure and cardiac function of the rats. After the examination, the blood was taken from the abdominal aorta. The blood was left to stand for about 1 hour, put into a centrifuge, 3000 rpm, and centrifuged at 4°C for 15 minutes at low temperature. After separation, the supernatant was taken and stored in a refrigerator at -40°C. After the rats were sacrificed, the heart tissues were quickly taken out, rinsed in normal saline, and then, placed on a filter paper to suck out the surface moisture. We put the heart tissues in 4% paraformaldehyde solution, fixed it for future use, and performed the next staining.

Echocardiography

After anesthesia, the rats were fixed on a thermostatic plate, and the chest was depilated with 8% Na_2S for skin preparation. Then, the rats in each group were subjected to transthoracic echocardiography (Philips, Eindhoven, The Netherlands) using cardiac ultrasonic detector to observe the changes in cardiac structure and function.

Table I. Real time PCR primers.

Gene name	Forward (5′>3′)	Reverse (5′>3′)
SOD1	GGTGAACCAGTTGTGTTGTC	CCGTCCTTTCCAGCAGTC
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGAGATTGTT
GPX1	ATCATATGTGTGCTGCTCGGCTAGC	TACTCGAGGGCACAGCTGGGCCCTTGAG
GPX2	GCCTCAAGTATGTCCGACCTG	GGAGAACGGGTCATCATAAGGG
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
TLR4	TGGAAGTTGAACGAATGGAATGTG	ACCAGAACTGCTACAACAGATACT
MyD88	TCATGTTCTCCATACCCTTGGT	AAACTGCGAGTGGGGTCAG
P65	ACTGCCGGGATGGCTACTAT	TCTGGATTCGCTGGCTAATGG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RNA Isolation

50 mg heart tissues were taken in an enzyme-free Eppendorf (EP; Hamburg, Germany) tube, we added 1 mL of TRIzol (Thermo Fisher Scientific, Waltham, MA, USA), grinded the tissue, mixed it upside down for 10 times, and left it on ice for 10 minutes. We added 0.2 mL chloroform, and then, we shook the test tube vigorously for 15 seconds to mix thoroughly, let it stand on ice for 10 minutes, and then, centrifuged them at 12000 rpm for 10 minutes. We transferred the upper aqueous phase to a new 1.5 mL EP tube (about 400-500 μL), added 0.5 mL isopropanol, mixed well, placed at -20°C for 1 hour, and then, centrifuged at 12000 rpm for 10 minutes. We discarded the supernatant, added 1 mL of fresh 75% ethanol (pre-chilled) and washed once, then, they were centrifuged at 7500 rpm for 5 minutes. Then, we added 20 μL ribonuclease free water (Thermo Fisher Scientific, Waltham, MA, USA) to the tube to dissolve, and finally measured the RNA concentration on the machine.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The conditions of reverse transcription cycle were: 55°C for 15 minutes, 85°C for 15 s. Based on the mRNA sequence found on GenBank, the software was used to design primers. The following reagents (cDNA 1.0 μL, front primer, 0.2 μL, back primer 0.2 μL, 2×Mix Taq 5 μL, ribonuclease free water 3.6 μL) were mixed in a 0.2 mL in the PCR tube (TaKaRa, Otsu, Shiga, Japan). The relative expression of mRNA was calculated using $2^{-\Delta\Delta}$CT method. Primers used were shown in Table I.

Enzyme-Linked Immunosorbent Assay (ELISA)

The 5 standard concentration gradient wells were set, each concentration setting corresponds to parallel wells, we added the sample dilution solution (Elabscience, Wuhan, China) and sample to the sample wells. Then, we sealed the plate with a sealing plate and incubated the cells for 30 minutes. We carefully peeled off the sealing plate membrane, added the washing solution to each well after discarding the liquid, and discarded it. We repeated these passages 5 times. Finally, a developer was added to each well, mixed with gentle shaking, and terminated after 15 minutes of color development at 37°C, and measured the absorbance (OD value) of each well in order at 450 nm.

Biochemical Indicator Detection

According to the kit's instructions (Jiancheng, Nanjing, China), we took an appropriate amount of serum or homogenized tissue supernatant, and tested the corresponding biochemical indicators according to the instructions.

Hematoxylin-Eosin (HE) Staining

The heart tissues were paraffin sectioned, dewaxed to water, stained with hematoxylin (Sinopharm Chemical Reagent, Shanghai, China) for 10 minutes, and differentiated with 0.7% hydrochloric acid ethanol for several seconds, and rinsed them immediately with running water until the sections turned blue. Gradient ethanol and xylene were used for dehydration. Finally, we observed the tissue section under a light microscope (Olympus, Tokyo, Japan).

Immunohistochemistry

We followed the steps of the immunohistochemistry kit. By using diaminobenzidine (DAB) (Jiancheng, Nanjing, China) method, the heart tissues were paraffin sectioned and dewaxed. Goat serum was used to heat repair antigens, block nonspecific antigens, and we added the diluted

antibody Caspase-3 (Abcam, Cambridge, MA, USA, Rabbit, 1:1000) at 4°C overnight, while the next day, the secondary antibody and DAB complex were added dropwise. After DAB color development, the tissue sections were dehydrated and sealed. The appearance of the brown particles under the light microscope (Olympus, Tokyo, Japan) was a positive signal.

Western Blotting Technology

We took an appropriate amount of tissues and washed them 3 times with pre-chilled phosphate buffered saline (PBS). The tissues were transferred into a 1.5 mL EP tube, then, a mixture of radioimmunoprecipitation assay (RIPA; Thermo Fisher Scientific, Waltham, MA, USA) and phenylmethylsulfonyl fluoride (PMSF; Thermo Fisher Scientific, Waltham, MA, USA) were added to the tube, and placed on ice for 10 min. The suspension was placed in a low-temperature ultracentrifuge at 14000 rpm for 10 minutes, the supernatant was transferred to another new EP tube, and we used the bicinchoninic acid (BCA) protein concentration measurement kit (Jiancheng, Nanjing, China) to detect the protein concentration. After the protein was boiled and denatured, the protein sample was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Then, the separated protein was transferred to a polyvinylidene difluoride (PVDF; Thermo Fisher Scientific, Waltham, MA, USA) membrane. After the membrane was blocked with 5% skim milk, the above PVDF membranes were incubated with primary antibodies (Collagen-I, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; SOD1, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; SOD2, Abcam, Cambridge, MA, USA, Rabbit, 1:3000; IL-1β, Abcam, Cambridge, MA, USA, Rabbit, 1:5000; iNOS, Abcam, Cambridge, MA, USA, Rabbit, 1:2000; TLR4, Abcam, Cambridge, MA, USA, Rabbit, 1: 2000; MyD88, Abcam, Cambridge, MA, USA, Rabbit, 1: 2000; p65, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; p-p65, Abcam, Cambridge, MA, USA, Rabbit, 1:1000; GAPDH, Proteintech, Rosemont, IL, USA, 1:2000) at 4°C overnight. After TBST rinsing, the PVDF membrane was incubated with the corresponding secondary antibody (goat anti-rabbit IgG antibody, Yifei Xue Biotechnology, Nanjing, China, 1:2000) at room temperature for 2 h. The enhanced chemiluminescence (ECL) technology (Thermo Fisher Scientific, Waltham, MA, USA) was used to develop Western blot bands.

Reactive Oxygen Species (ROS) Levels

An appropriate amount of heart tissues was taken, and centrifuged after homogenization. The supernatant was taken in a flow tube. 1 mL diluted DCFH-DA was added to the tube according to the kit instructions (Jiancheng, Nanjing, China), and then, incubated for 20 minutes. After discarding the supernatant and washing it with PBS, the OD value was measured under the microplate reader (Roche, Basel, Switzerland).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM Corp., Armonk, NY, USA) was used to analyze the data. The differences between the two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Data are expressed as mean ± standard deviation (SD), and $p<0.05$ indicates that the difference is statistically significant.

Result

Ulinastatin Improves DOX-Induced Myocardial Structural and Functional Changes

In order to observe the changes of myocardial structure by DOX and Ulinastatin, we observed through HE staining that the myocardial cells in the control group were neatly arranged, dense, complete, and clear, with uniform intercellular space and less extracellular matrix. However, in the DOX group, the myocardial cells were disordered, the fibrosis of the interstitial space was evident, the nucleus was condensed or dissolved, the interstitial space was widened, and the myocardial striations were broken and disappeared. However, Ulinastatin intervention can dramatically relieve myocardial structural disorders and interstitial fibrosis. At the same time, we used immunohistochemical staining to detect the expression of Caspase-3 in the myocardial tissue of each group. The results showed that DOX caused an increase in the expression of Caspase-3 in myocardial tissue, and Ulinastatin intervention could markedly reduce the increase of Caspase-3 (Figure 1A). Then, we detected Collagen-I by Western blot and found that the expression of Collagen-I in the DOX groups was dramatically higher than that in the control group, while the expression of Collagen-I in the Ulinastatin group was markedly low-

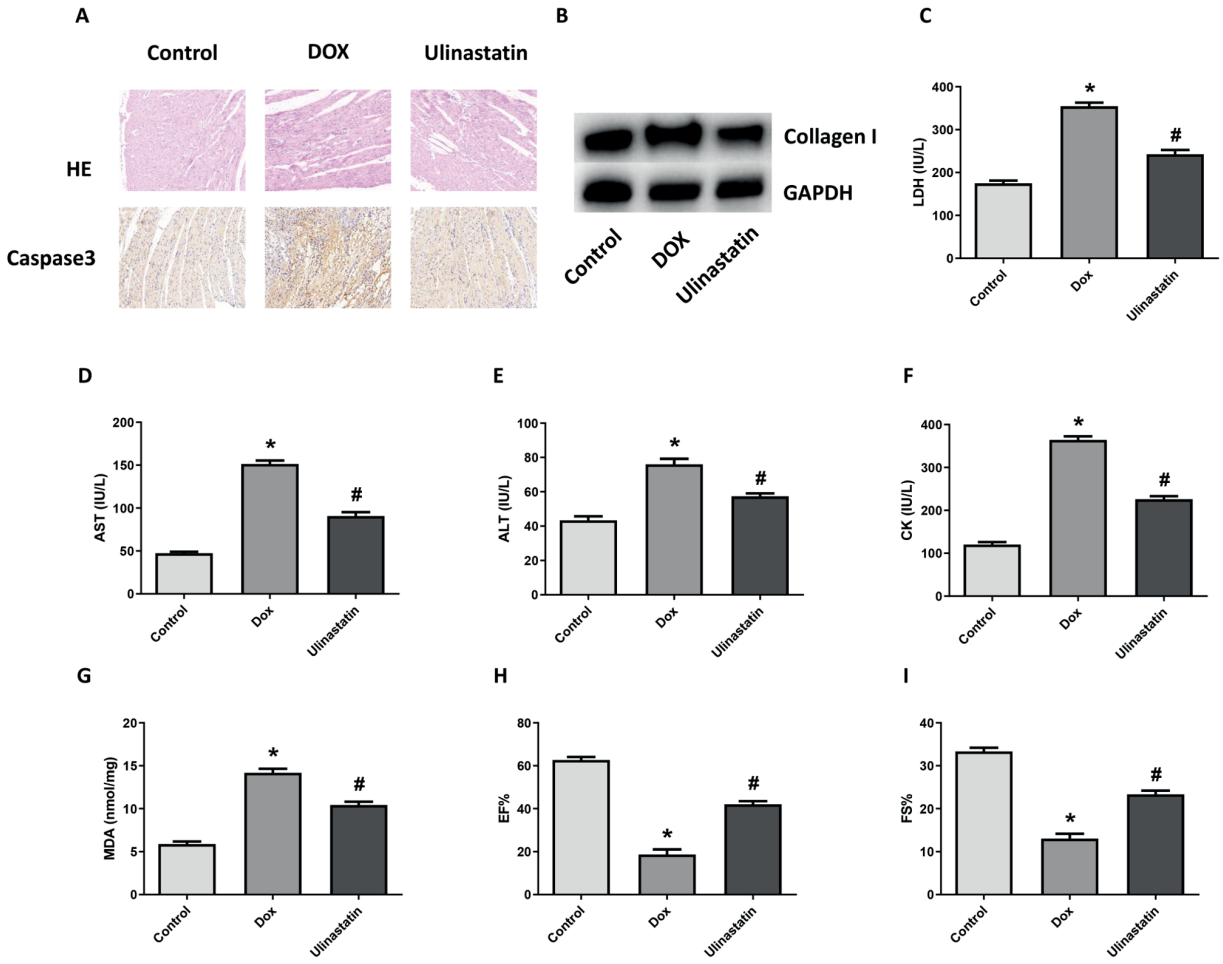

Figure 1. Ulinastatin improves DOX-induced myocardial structural and functional changes. **A,** HE staining and Immunohistochemistry (magnification: 200×). **B,** Collagen I was detected by Western blot. **C-F,** Detection of biochemical indicators. **G,** MDA content detection. **H,** and **I,** Heart function test. ("*" indicates statistical difference from the control group $p<0.05$ and "#" indicates statistical difference from the DOX group $p<0.05$).

er than that in the DOX group (Figure 1B). These results confirm that Ulinastatin can improve the structural changes of the heart caused by DOX. In addition, we found that the serum myocardial enzymes (LDH, AST, ALT, CK) and MDA levels of rats in the DOX group and the Ulinastatin group were dramatically higher than those in the control group, but the serum myocardial enzymes and MDA levels in the Ulinastatin group were lower than those in the DOX group (Figure 1C-1G). Through analysis of echocardiography, we found that Ulinastatin can alleviate the decrease of EF and FS caused by DOX, thereby enhancing the blood supply capacity of the heart (Figure 1H and 1I). The above results also indicate that Ulinastatin has a protective effect on DOX-induced myocardial injury.

Ulinastatin Inhibits DOX-Induced Cardiomyocyte Oxidative Stress

We tested the ROS level in myocardial tissue through the ROS kit and found that DOX can dramatically promote ROS elevation, while Ulinastatin can effectively inhibit ROS elevation (Figure 2A). In addition, we detected by qRT-PCR and Western blotting that SOD1 mRNA, SOD2 mRNA, GPX1 mRNA, and GPX2 mRNA were dramatically reduced in the DOX group (Figure 2B-2E). Similar results were obtained for SOD1 and SOD2 protein expression (Figure 2F). In addition, SOD activity and GSH-Px activity also decreased after DOX treatment, and when we treated with Ulinastatin, we found that it can promote the above indicators to reduce the redox imbalance caused by DOX (Figure 2G and 2H).

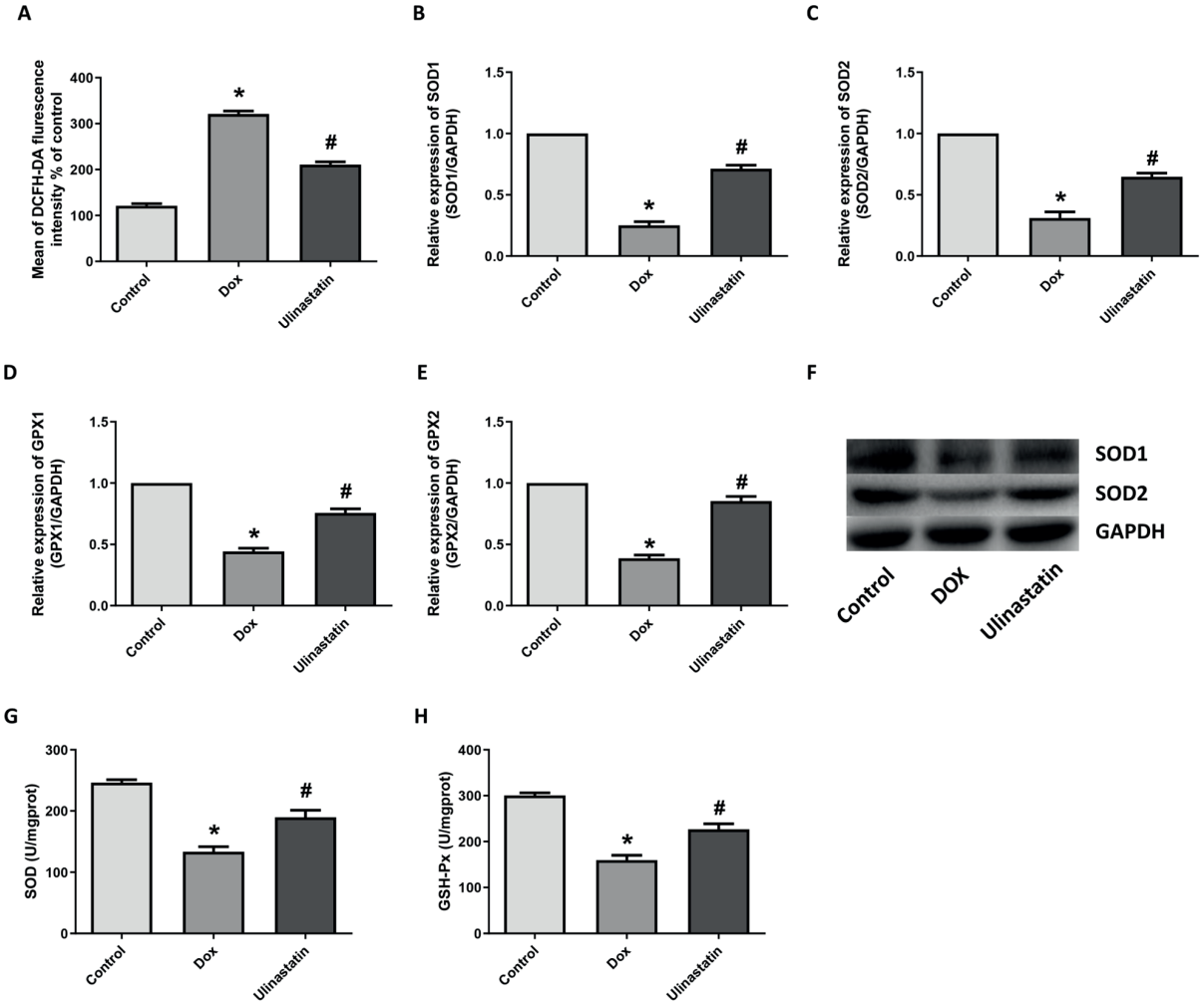

Figure 2. Ulinastatin inhibits DOX-induced cardiomyocyte oxidative stress. **A,** Tissue ROS level detection. **B-E,** qRT-PCR detected the expression of SOD1, SOD2, GPX1 and GPX2. **F,** Western blot was used to detect the expression of SOD1 and SOD2. **G,** and **H,** Detection of SOD and GSH-Px activity. ("*" indicates statistical difference from the control group $p<0.05$ and "#" indicates statistical difference from the DOX group $p<0.05$).

Ulinastatin inhibits DOX-induced cardiomyocyte inflammation

In order to determine the degree of cardiomyocyte inflammation induced by DOX, we examined the inflammatory factors by ELISA method. DOX treatment can markedly increase the expression of IL-1β, IL-6 and TNF-α in cardiomyocytes (Figure 3A-3C). At the same time, we detected by Western blot and qRT-PCR technology that iNOS and IL-1β protein expressions were dramatically increased (Figure 3D). IL-1β mRNA, IL-6 Mrna, and TNF-α mRNA were also dramatically increased (Figure 3E-G). Therefore, we showed that DOX can induce inflammatory responses in cardiomyocytes. At the same time, Ulinastatin treatment can effectively inhibit the inflammatory response, thereby alleviating the inflammation damage caused by DOX.

Ulinastatin May Participate in TLR4/MyD88/NF-kB Signaling Pathway

In order to clarify the pathogenesis of DOX cardiomyopathy and its prevention and treatment measures. We detected the TLR4/MyD88/NF-kB signaling pathway by Western blotting. It was found that TLR4, MyD88, and p65 were dramatically increased in myocardium of rats after 1 week of DOX treatment. At the same time, we found that p-p65 increased dramatically after DOX treatment, indicating that p65 entered the nucleus after DOX treatment, thereby promoting downstream factor expression (Figure 4A). Simi-

lar results were obtained with qRT-PCR. Ulinastatin dramatically inhibited DOX-induced increase in TLR4 mRNA, MyD88 mRNA and p65 mRNA expression (Figure 4B-4D). The above results confirm that DOX can activate the TLR4/MyD88/NF-kB signaling pathway. The Ulinastatin treatment can dramatically inhibit the increase of the above-mentioned expression, which indicates that Ulinastatin can inhibit the excessive activation of the TLR4/MyD88/NF-kB signaling pathway, thereby inhibiting downstream inflammation and OS responses.

Discussion

DOX is a widely used chemotherapeutic drug in the clinic, but due to its high affinity for the myocardium, it will cause severe cardiotoxicity to the body after administration. Early manifestations include myocarditis and arrhythmia. We dilated cardiomyopathy or congestive HF may appear even in advanced stages[11]. The exact mechanism of DOX cardiotoxicity is still unclear. Previous studies have focused on free radical damage, mitochondrial damage, abnormal energy metabolism, calcium overload, apoptosis, and cell atrophy[12].

It has been reported that the application of DOX caused cardiomyopathy that occurred about 34 days after the last application of DOX, and the occurrence was about 1.7%, and the mortality rate was as high as 50%[13]. Cardiac toxicity caused by cymbals is cumulative and dose dependent. Studies have shown that 4% of patients with cumulative DOX doses of 500-550 mg/m^2 have cardiomy-

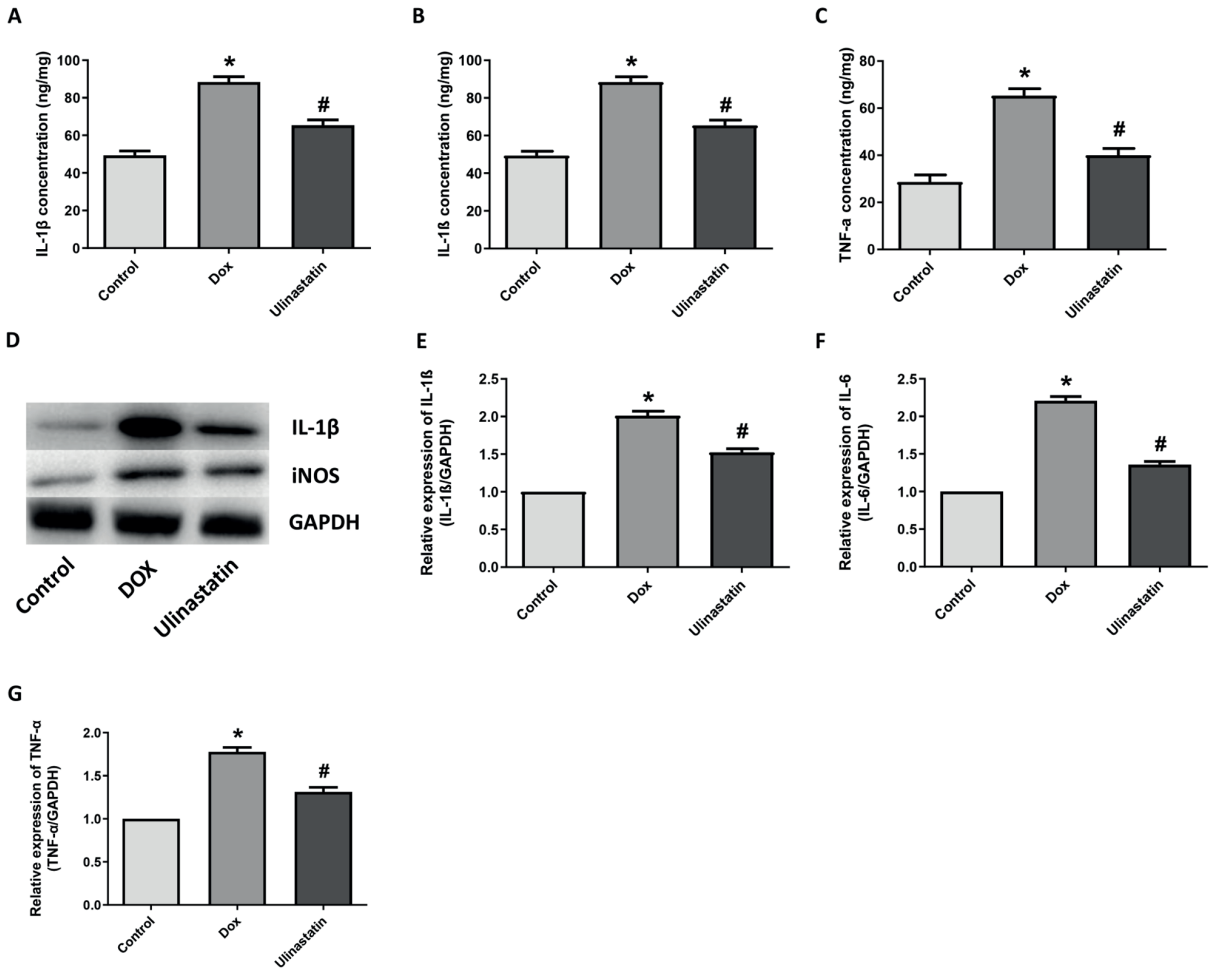

Figure 3. Ulinastatin inhibits DOX-induced cardiomyocyte inflammation. **A-C,** Elisa detected IL-1β, IL-6 and TNF-α expression. **D,** Western blot was used to detect IL-1β and iNOS expression. **E-G,** qRT-PCR detected the expression of IL-1β, IL-6 and TNF-α. ("*" indicates statistical difference from the control group $p<0.05$ and "#" indicates statistical difference from the DOX group $p<0.05$).

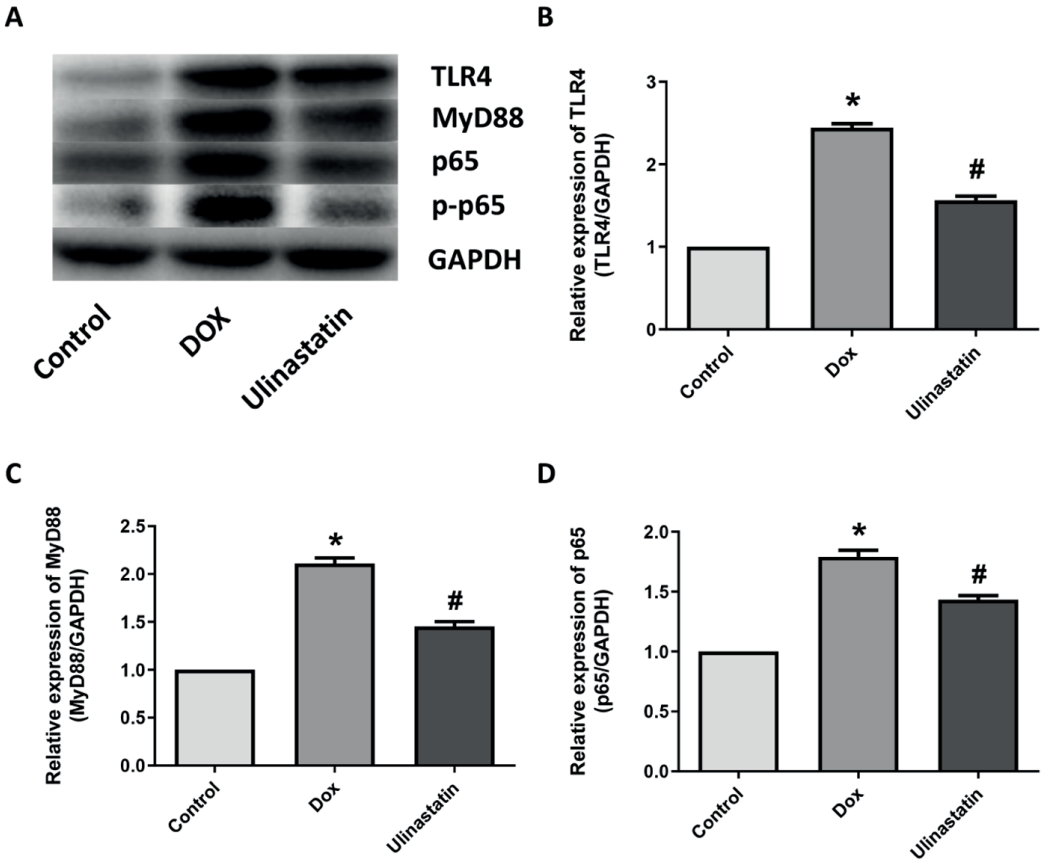

Figure 4. Ulinastatin may participate in TLR4/MyD88/NF-kB signaling pathway. **A,** TLR4, MyD88, p65 and p-p65 expression were detected by Western blot. **B-D,** qRT-PCR was used to detect TLR4, MyD88 and p65 expression. ("*" indicates statistical difference from the control group $p<0.05$ and "#" indicates statistical difference from the DOX group $p<0.05$).

opathy or HF. The occurrence of cardiomyopathy increased to 18% when it reached 551-660 mg/m^2, and it increased to 36% when the cumulative dose was >600 mg/m^2. The prognosis of cancer patients with myocardial damage is generally poor, especially in patients who develop disease within 4 weeks after the last application of DOX and die within 2 weeks after the diagnosis of cardiomyopathy[14]. To date, a large number of studies have shown that inflammatory response and OS play a role in promoting disease progression in myocardial cell death, cardiomyopathy and HF caused by DOX[15]. Thus, this study simulates the internal environment of myocardial injury induced by DOX in tumor patients *in vitro.*

Ulinstatin is a typical Kuniz protease inhibitor. Structurally, the chondroitin sulfate sugar chain at the O-terminal can combine with cells and calcium ions, stabilize lysosomal membrane, and inhibit the release of inflammatory cytokines. In addition, animal experiments have proved that it can play an organ protection role by reducing MDA level, upregulating SOD activity, reducing oxygen free radical damage and other mechanisms[16]. Since the half-life of Ulinstatin *in vivo* is very short[17], we tried to verify the protective effect of continuous Ulinstatin therapy on DOX-induced AMI 2 weeks after the preparation of the AMI model.

TLR4-MyD88 complex can activate inflammatory factors and recruit NF-kB, and NF-kB is involved in regulating the expression of a large number of proteins related to inflammatory responses, such as mitogen-activated protein kinases (MAPKs), and further involved in the mechanism of myocardial inflammation injury response[18]. NF-kB is a transcription factor involved in regulating signaling pathways related to inflammation, and it plays an important role in inflammatory response and cell survival[19]. Our results also showed that DOX promotes NF-kB expression and upregulates the expression of inflammatory factor. When we

used Ulinastatin to treat rats, we found that Ulinastatin markedly inhibited the DOX-induced inflammatory response. At the same time, by examining the ROS levels in the tissue, we also found that the ROS level in the myocardial cells of the DOX group was dramatically increased, and the antioxidant enzyme activity was dramatically reduced, resulting in the excess ROS being unable to be removed and the accumulation of oxygen free radicals in the tissue, which promoted membrane, mitochondria, and DNA damage increased. In addition, our results also showed that MDA levels in the DOX group are significantly higher than those in the control group, and MDA is an indicator that can directly respond to tissue oxidative damage[20]. The antioxidant activity of the DOX group was dramatically reduced, which further increased the ROS in the tissue. After the rats were treated with Ulinastatin, we found that Ulinastatin can reduce MyD88 and TLR4 expression, thereby inhibiting NF-kB-induced inflammatory response and OS, alleviating tissue damage and fibrosis, and reducing myocardial tissue damage induced by DOX.

Conclusions

Altogether, our results have found that Ulinastatin can reduce myocardial cell damage caused by DOX by inhibiting cardiomyocyte inflammatory response and OS. TLR4/MyD88/NF-kB signaling pathway at least partially mediates Ulinastatin's protection against DOX-induced cardiomyocyte damage effect. This also provides a basis for the prevention and treatment of DOX cardiomyopathy.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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References

1) Koleini N, Kardami E. Autophagy and mitophagy in the context of doxorubicin-induced cardiotoxicity. Oncotarget 2017; 8: 46663-46680.
2) Ahmad S, Panda BP, Kohli K, Fahim M, Dubey K. Folic acid ameliorates celecoxib cardiotoxicity in a doxorubicin heart failure rat model. Pharm Biol 2017; 55: 1295-1303.
3) Swain SM, Whaley FS, Ewer MS. Congestive heart failure in patients treated with doxorubicin: a retrospective analysis of three trials. Cancer-Am Cancer Soc 2003; 97: 2869-2879.
4) Biancaniello T, Meyer RA, Wong KY, Sager C, Kaplan S. Doxorubicin cardiotoxicity in children. J Pediatr 1980; 97: 45-50.
5) Cappetta D, De Angelis A, Sapio L, Prezioso L, Illiano M, Quaini F, Rossi F, Berrino L, Naviglio S, Urbanek K. Oxidative stress and cellular response to doxorubicin: a common factor in the complex milieu of anthracycline cardiotoxicity. Oxid Med Cell Longev 2017; 2017: 1521020.
6) Kharin S, Krandycheva V, Tsvetkova A, Strelkova M, Shmakov D. Remodeling of ventricular repolarization in a chronic doxorubicin cardiotoxicity rat model. Fundam Clin Pharmacol 2013; 27: 364-372.
7) Zhang QF. Ulinastatin inhibits renal tubular epithelial apoptosis and interstitial fibrosis in rats with unilateral ureteral obstruction. Mol Med Rep 2017; 16: 8916-8922.
8) Hatakeyama N, Matsuda N. Alert cell strategy: mechanisms of inflammatory response and organ protection. Curr Pharm Des 2014; 20: 5766-5778.
9) Zhang L, Wang CC. Inflammatory response of macrophages in infection. Hepatobiliary Pancreat Dis Int 2014; 13: 138-152.
10) Su Q, Li L, Sun Y, Yang H, Ye Z, Zhao J. Effects of the TLR4/Myd88/NF-kappaB signaling pathway on NLRP3 inflammasome in coronary microembolization-induced myocardial injury. Cell Physiol Biochem 2018; 47: 1497-1508.
11) Sun FD, Wang PC, Luan RL, Zou SH, Du X. MicroRNA-574 enhances doxorubicin resistance through down-regulating SMAD4 in breast cancer cells. Eur Rev Med Pharmacol Sci 2018; 22: 1342-1350.
12) Renu K, V GA, P BT, Arunachalam S. Molecular mechanism of doxorubicin-induced cardiomyopathy - An update. Eur J Pharmacol 2018; 818: 241-253.
13) Takemura G, Fujiwara H. Doxorubicin-induced cardiomyopathy from the cardiotoxic mechanisms to management. Prog Cardiovasc Dis 2007; 49: 330-352.
14) Saltiel E, McGuire W. Doxorubicin (adriamycin) cardiomyopathy. West J Med 1983; 139: 332-341.
15) Akolkar G, Da SDD, Ayyappan P, Bagchi AK, Jassal DS, Salemi V, Irigoyen MC, De Angelis K, Singal PK. Vitamin C mitigates oxidative/nitrosative stress and inflammation in doxorubicin-induced cardiomyopathy. Am J Physiol Heart Circ Physiol 2017; 313: H795-H809.
16) Xu CE, Zhang MY, Zou CW, Guo L. Evaluation of the pharmacological function of ulinastatin in experimental animals. Molecules 2012; 17: 9070-9080.
17) Ohzawa N, Takahashi Y, Ogihara T, Nakai Y, Ishiguro J. Metabolic fate of ulinastatin (2); Pharmacokinetics in rabbits following intra-articular administration. Biol Pharm Bull 1997; 20: 732-73
18) Guo X, Jiang H, Chen J, Zhang BF, Hu Q, Yang S, Yang J, Zhang J. RP105 ameliorates hypoxia

reoxygenation injury in cardiac microvascular endothelial cells by suppressing TLR4MAPKsNF-kappaB signaling. Int J Mol Med 2018; 42: 505-513.

19) Raish M. Momordica charantia polysaccharides ameliorate oxidative stress, hyperlipidemia, inflammation, and apoptosis during myocardial infarction by inhibiting the NF-kappaB signaling pathway. Int J Biol Macromol 2017; 97: 544-551.

20) Gokce CB, Yurtdas M, Keskin GS, Gunes HN, Atac UC, Aytac B, Durak ZE, Yoldas TK, Durak I, Cubukcu HC. Serum glutathione peroxidase, xanthine oxidase, and superoxide dismutase activities and malondialdehyde levels in patients with Parkinson's disease. Neurol Sci 2017; 38: 425-431.