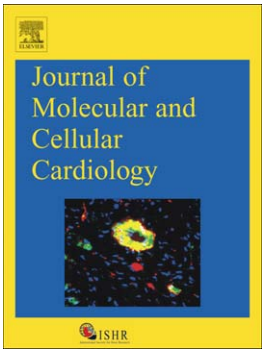

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PII: S0022-2828(16)30234-6
DOI: doi: 10.1016/j.yjmcc.2016.08.004
Reference: YJMCC 8430

To appear in: *Journal of Molecular and Cellular Cardiology*

Received date: 1 December 2015
Revised date: 17 July 2016
Accepted date: 4 August 2016

Please cite this article as: Wang Yi-Hsin, Chen Ke-Min, Chiu Ping-Sung, Lai Shih-Chan, Su Hsing-Hui, Jan Ming-Shiou, Lin Chia-Wei, Lu Dah-Yuu, Fu Yuan-Tsung, Liao Jiuan-Miaw, Chang Jinghua Tsai, Huang Shiang-Suo, Lumbrokinase attenuates myocardial ischemia-reperfusion injury by inhibiting TLR4 signaling, *Journal of Molecular and Cellular Cardiology* (2016), doi: 10.1016/j.yjmcc.2016.08.004

Lumbrokinase attenuates myocardial ischemia-reperfusion injury by inhibiting TLR4 signaling

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Running head: Lumbrokinase Attenuates Myocardial I-R injury

Abstract

Lumbrokinase, a novel antithrombotic agent, purified from the earthworm *Lumbricus rubellus*, has been clinically used to treat stroke and cardiovascular diseases. However, inflammatory responses associated with the cardioprotective effect of lumbrokinase remain unknown. In this study, the signaling pathways involved in lumbrokinase-inhibited expressions of inflammation mediators were investigated in rats subjected to myocardial ischemia-reperfusion (I-R) injury. The left main coronary artery of anesthetized rats was subjected to 1 h occlusion and 3 h reperfusion. The animals were treated with/without lumbrokinase and the severities of I-R-induced arrhythmias and infarction were compared. Lumbrokinase inhibited I-R-induced arrhythmias and reduced mortality, as well as decreased the lactate dehydrogenase levels in carotid blood. Lumbrokinase also inhibited the enhancement of I-R induced expressions of cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and matrix metalloproteinase (MMP)-9 through toll-like receptor 4 (TLR4) signaling pathway. Moreover, our results demonstrated that stimulation with lumbrokinase decreases the phosphorylation of JNK, IκB, and NF-κB. These findings suggested that lumbrokinase is a potent cardioprotective drug in rats with I-R injury. The cardioprotective effects of lumbrokinase may be correlated with its inhibitory effect on the I-R-induced expressions of COX-2, iNOS and MMP-9, mediated by TLR4 signaling through JNK and NF-κB pathways.

Abbreviations

I-R, ischemia-reperfusion; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; MMP-9, matrix metalloproteinase-9; TLR4, toll-like receptor 4; e-PA, extrinsic plasminogen activator; t-PA, tissue plasminogen activator; HR, heart rate; BP, arterial blood pressure; VPC, ventricular premature complexes; VT, ventricular tachycardia; VF, ventricular fibrillation; LDH, lactate dehydrogenase; MAPKs, mitogen-activated protein kinases

Keywords

Lumbrokinase; Cardioprotection; Ischemia; Reperfusion; TLR4

1. Introduction

Coronary artery and associated ischemic heart diseases are prevalent worldwide. Myocardial ischemia-reperfusion (I-R) injury, which is one of the world's fastest growing conditions, is a major contributor to the morbidity and mortality associated with coronary artery diseases [1, 2]. Myocardial ischemia is a heart condition resulting from lack of coronary blood supply caused by thrombosis or acute alterations of coronary atherosclerotic plaques [1]. During myocardial ischemia, a characteristic pattern of metabolic and ultrastructural changes leads to irreversible injury. Early restoration of blood flow to the ischemic myocardium is a common treatment strategy to limit myocardial infarct size and to reduce mortality [3]. However, the return of blood flow can cause additional cardiac damage and complications that can paradoxically increase infarct size, a condition referred to as myocardial I-R injury [4, 5].

Myocardial I-R injury is a pathological condition characterized by an initial restriction of blood supply to the myocardium, subsequent restoration of perfusion, and concomitant reoxygenation. Over the past two decades, studies on myocardial I-R injury have focused on the role of molecular, rather than cellular, targets such as complement receptors [6, 7], toll-like receptors (TLRs) [8] and proinflammatory cytokines [7]. Myocardial I-R injury is characterized by a rapid increase in cytokines and chemokines and an influx of leukocytes into the vulnerable region bordering the infarcted site. This inflammatory response may not only lead to cardiomyocyte apoptosis, but also compromise myocardial function. Therefore, limiting I-R-induced myocardial inflammation may lower mortality and reduce myocardial I-R-induced ventricular arrhythmia and myocardial infarction [9].

Lumbrokinase, an extract of *Lumbricus rubellus*, was identified more than two decades ago [10]. Lumbrokinase is a group of bioactive proteolytic enzymes with molecular weights ranging from 25 kDa to 32 kDa [11]. Lumbrokinase includes plasminogen activator and plasmin [12]. The extrinsic plasminogen activator (e-PA) in lumbrokinase is similar to the tissue plasminogen activator (t-PA) found in other species. However, lumbrokinase dissolves fibrin clots by converting plasminogen to plasmin, while exhibiting relatively good heat stability and broad optimal pH range [13]. Lumbrokinase enzymes demonstrate thrombolytic activity only in the presence of fibrin. Therefore, lumbrokinase does not cause hemorrhage, as opposed to streptokinase or urokinase, due to hyperfibrinolysis during treatment [12]. Lumbrokinase has been clinically applied to the treatment of strokes and cardiovascular diseases [14]. In China, lumbrokinase has been marketed for more than 10 years mainly for the treatment of acute-phase ischemic stroke [14], prevention of secondary ischemic stroke [15], and improvement of myocardial perfusion in patients with stable angina [16]. However, few studies have reported the pharmacological mechanisms of the effects of lumbrokinase on myocardial I-R injury in rats.

Evidence has shown that TLRs play a central role in I-R injury [8, 17, 18]. The results of the present study demonstrated that lumbrokinase treatment significantly attenuates the myocardial I-R-induced expression of the TLR4 protein when compared with vehicle-treated group. It is well known that TLR4 signaling plays a dominant role in myocardial I-R injury [8]. Mice lacking TLR4 sustain smaller infarct size and exhibit less inflammation when compared with wild-type mice after myocardial I-R injury [18]. Therefore, modulation of TLR4 signaling may be a potential therapeutic strategy in myocardial I-R injury [19]. The present study aimed to explore the possible anti-inflammatory mechanisms (with specific focus on the TLR4 signaling pathway) of lumbrokinase for ameliorating myocardial I-R injury in rats.

2. Methods

Study protocols conformed with those published in the Guide for the Care and Use of Laboratory Animals by the National Research Council of the National Academies (NIH publication, revised 2011). Every effort was made to minimize animal suffering and to reduce the number of sacrificed animals.

2.1. Animals

Six-week old male Sprague-Dawley rats (National Laboratory Animal Center) weighing 250-300 g were housed in the Animal Center of Chung Shan Medical University at an ambient temperature of 25 ± 1 °C, under a 12 h light-dark cycle. The animals were fed normal chow and given water ad libitum. All surgical procedures for myocardial I-R were reviewed and approved by the Institutional Animal Care and Use Committee of Chung Shan Medical University.

2.2. Experimental protocol

Myocardial I-R injury was induced by temporary occlusion of the left main coronary artery as previously described [20]. Briefly, the rats were anesthetized with a single intraperitoneal injection of thiobutabarbital sodium salt (100 mg/kg) and urethane (400 mg/kg) and then placed on a heated, small animal operating table. Rectal temperature was maintained at 38 ± 0.5 °C. After tracheotomy, the intubated animals were ventilated with room air through a respirator for small rodents (Model 131, NEMI, USA) with stroke volume of 15 ml/kg body weight and rate of 60 strokes/min to maintain normal P_{O2}, P_{CO2} and pH parameters (blood gas analyzer, GEM-5300 IL, CO, USA). The jugular vein was cannulated to administer drugs and Evans blue at the end of the experiment. Lumbrokinase (1 or 10

μg/kg, Canada RNA Biochemical Inc., Richmond, BC, Canada) or vehicle (normal saline) was infused via the jugular vein 15 min before coronary artery occlusion. The rats injected with vehicle were used as controls. The vehicle had no effects on myocardial I-R-induced arrhythmia or infarction. The animals were randomly allocated to drug treatment or vehicle administration groups. Polyethylene catheters (PE-50) were inserted into the common carotid artery with a Statham P23 XL transducer to continuously monitor heart rate (HR) and arterial blood pressure (BP), as well as adequacy of the anesthesia. Data were acquired with a Gould RS-3400 physiological recorder (Gould, Cleveland, OH, USA). Standard lead-1 ECG was recorded via silver electrodes attached to the extremities.

The heart was exposed via left thoracotomy and the fourth and fifth ribs were sectioned approximately 2 mm to the left of the sternum. The heart was rapidly externalized and inverted. Then, a 6/0 silk ligature was placed around the left main coronary artery. The heart was repositioned in the chest and the animal was allowed to recover for 15 min. Animals that developed arrhythmia or sustained a decrease in mean BP to less than 70 mmHg during the procedure were not included in the study. A small plastic snare from a Portex P-270 cannula was threaded through the ligature and placed in contact with the heart. The left coronary artery was then occluded by tightening the ligature and reperfusion was achieved by releasing the tension applied to the ligature (operated groups). Successful ligation of the coronary artery was validated by decreased arterial pressure and ECG changes (increase in R wave and ST segment elevation), which are indicators of ischemia. Sham-operated animals underwent the same surgical procedure, except that the silk ligature that was placed around the left coronary artery was not tied.

Changes in HR, BP, and ECG were simultaneously recorded using a personal computer with waveform analysis software (AcqKnowledge, Biopac System, Goleta, CA, USA) before and during the ischemia and reperfusion periods. To evaluate antiarrhythmic effects of lumbrokinase during myocardial I-R injury, we occluded the coronary artery for 1 h and then subjected it to reperfusion for 3 h. The ventricular ectopic activity was evaluated according to the diagnostic criteria advocated by the Lambeth Conventions [21]. Incidence and duration of ventricular tachyarrhythmias, including ventricular tachycardia (VT) and ventricular fibrillation (VF), were determined in the surviving rats and in the rats that eventually died. In rats with irreversible VF, the duration of VF was recorded until mean BP was < 15 mmHg.

2.3. Determination of infarct size and collection of myocardium samples

Evaluation of the infarct zone and collection of myocardium samples for further analyses were carried out in rats that survived 1 h of ischemia and 3 h of reperfusion. To reduce the number of animals used in this study, an effective dose of 10 μg/kg was applied to investigate whether lumbrokinase ameliorates myocardial I-R injury and to uncover the

underlying mechanism involved in the cardioprotective effect of lumbrokinase. The sizes of the occluded and infarct zones in the hearts were determined using a previously described procedure [22]. At the end of the experiment, the coronary artery was reoccluded, and 2.0 ml of 3% Evans blue solution were intravenously injected to stain non-ischemic myocardium and determine the area at risk. The heart was then excised and the atria were removed. Ventricular tissues were sliced into 1 mm sections and incubated in tetrazolium dye [2% 2,3,5-triphenyltetrazolium chloride (Sigma, USA) in normal saline] at 37 °C for 40 min in darkness. The sections were placed in a solution of 10% formaldehyde in saline for 1 day before excision of infarcted (white) tissues. The weight of the infarcted tissues is expressed as a percentage of the total ventricle or the area at risk.

2.4. Determination of myocardial damage

Myocardial cellular damage was estimated by measuring lactate dehydrogenase (LDH) leakage into plasma. Arterial blood was collected from the carotid catheter at the end of myocardial I-R injury for LDH measurements. LDH activity was determined using a commercially available assay kit (Sigma, St Louis, MO, USA).

2.5. Protein extraction and western blot analysis

Heart tissues were homogenized with a tissue protein extraction reagent (Thermo Scientific, USA) containing protease inhibitor cocktail (Sigma, USA). The tissue homogenates were centrifuged at 12,000 g for 10 min at 4 °C. Protein concentration was determined with protein assay kits (BioRad, USA) using bovine serum albumin as the standard. The samples were mixed with an equal volume of loading buffer [62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% SDS, 5% (v/v) 2-mercaptoethanol, and 0.05% (w/v) bromophenol blue] and heated at 95 °C for 5 min.

The protein samples were subjected to SDS-PAGE and electrophoretically transferred onto PVDF protein sequencing membranes for 90 min. The membrane was blocked with 5% non-fat milk in PBS-buffered saline with 0.1% (v/v) Tween-20 (PBST) at room temperature for 1 h. The membrane was then washed and blotted with the antibodies of matrix metalloproteinase (MMP)-9, MMP-2 (R&D Systems, USA), COX-2 (Cayman Chemiacal, USA), TLR4, TLR2, c-fos (Santa Cruz, USA), interleukin-1 receptor-associated kinase (IRAK)4, phospho-IRAK4, phospho-JNK, JNK, phospho-NF-κB, NF-κB, phospho-IκB-α, IκB-α, iNOS, eNOS, nNOS, phospho-ERK 1/2, ERK 1/2, phospho-p38, and p38 (Cell Signaling, USA), MPO (Spring Bioscience, USA). β-actin (Abcam, UK) served as internal loading control. The membrane was incubated with HRP-conjugated secondary antibody

(Jackson ImmunoResearch Laboratories, USA) prior to chemiluminescence detection (Pierce, USA).

2.6. Gelatin zymography

Myocardial I-R-induced proteinase in rat hearts was analyzed by gelatin zymography as previously described [23] with slight modification. Briefly, the heart homogenates were loaded onto SDS-PAGE (7.5%) polyacrylamide gel, which was co-polymerized with 0.1% (w/v) gelatin (Sigma, USA). The stacking gels were comprised of 4% (w/v) polyacrylamide without gelatin substrate. Electrophoresis was performed in running buffer (25 mM Tris, 250 mM glycine, 1% SDS) at room temperature. The gel was washed twice in double-distilled water containing 2.5% Triton X-100 for 30 min each time, then incubated in reaction buffer [50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$, 0.02% (w/v) Brij® -35, 0.01% (w/v) NaN_3] at 37 °C for 18 h. Subsequently, the gel was stained with 0.25% (w/v) Coomassie brilliant blue R-250 (Sigma, USA) for 1 h and destained with 15% (v/v) methanol and 7.5% (v/v) acetic acid. Gelatinase activity was detected as unstained bands on a blue background. Quantitative analysis was performed with a computer-assisted imaging densitometer system (UN-SCAN-IT™ gel version 5.1, Silk Scientific, UT).

2.7. Immunohistochemical staining

Rat hearts were fixed separately in 10% neutral-buffered formalin for 24 h. They were then dehydrated in a graded ethanol series (50%, 75%, 95% and 100%), cleared in xylene, and embedded in paraffin at 55 °C for 24 h. For the immunohistochemical analysis, relatively thick (5 μm) serial sections of wax-embedded heart were cut, mounted on glass slides, dewaxed, and then rehydrated in the same manner as for histological analysis. Subsequently, the sections were treated with 3% H_2O_2 in methanol for 10 min to inactivate any endogenous peroxidase and washed thrice with PBS for 5 min each time. Afterward, the sections were blocked with 3% BSA at room temperature for 1 h and then incubated at 37 °C for 1 h with a 1:50 dilution of the rabbit anti-human MPO polyclonal antibody and a 1:100 dilution of the rabbit anti-human TLR4 polyclonal antibody in 1% BSA. Following another three washes in PBS, the sections were incubated at 37 °C for 1 h with a 1:100 dilution of the HRP-conjugated rabbit anti-goat IgG in 1% BSA and then washed three more times in PBS. Finally, the sections were incubated at room temperature for 3 min with 3,3′-diaminobenzidine (0.3 mg/mL) in 100 mM Tris (pH 7.5) containing 0.3 μL H_2O_2/mL. After washing thrice in PBS, the sections were mounted in 50% glycerol in PBS and examined under light microscope.

2.8. Flow cytometry analysis

The peripheral blood mononuclear cells (PBMCs) were washed twice with 3 ml of FACScan medium (RPMI-1640 medium with 2% FBS and 0.1% NaN_3). An aliquot of 1 x 10^5 cells was used for each FACScan tube (FALCON 352052). After double labeling with either a mixture of anti-TLR4-PE (Novus Biologicals, USA) and anti-CD3 FITC (BD Biosciences, USA) or a mixture of anti-TLR4-PE and anti-CD19-FITC (Abcam, UK) for 45 minutes on ice in the dark, cells were washed twice and resuspended in FACScan medium. The florescence intensity was detected with flow cytometer (FACScalibur; Becton Dickinson, USA) and analyzed with Flowing Software (2.5.1). The expressions of TLR4 in T lymphocytes and B lymphocytes are presented as the values of geometric mean fluorescence intensity (gMFI).

2.9. Blood-clot lysis activity assay

An in vitro blood-clot lysis activity assay was performed as described by Prasad et al. Fresh rat blood was drawn from the carotid artery. Then, 600 µl of blood were transferred to pre-weighed Eppendorf tubes and incubated at 37°C for 45 minutes. Serum was completely removed after clot formation, and the tubes containing the clots were weighed again. Clot weight was determined by subtracting the weight of each tube (ie., clot weight = weight of clot-containing tube – weight of empty tube). Then, 200 µl of lumbrokinase at varying concentrations (10, 100, 96000 units), dissolved in PBS, were added to the tubes containing clots. The tubes were then incubated at 37°C for 90 minutes and observed for clot lysis. Lysed fluid was completely absorbed from each tube with filter paper and the tubes were then re-weighed. Weight differences of each tube before and after incubation were calculated for treated and control samples.

2.10. Statistical analysis

Data are expressed as mean ± standard error of mean (SEM). Statistical analyses of differences were carried out using one-way analysis of variance (ANOVA) for combined data followed by Bonferroni's tests. The differences in the percentage incidences of VT, VF, and mortality were compared using $\chi 2$ test. Spearman rank correlation was applied to test the correlations between MMP-9 activity and MPO expression in rats subjected to myocardial I-R injury. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Hemodynamics

There were no statistically significant differences in pre-ischemic hemodynamic parameters among the groups of rats. The injection of lumbrokinase into the jugular vein did not affect the mean BP or HR of rats. Moreover, hemodynamic parameters did not significantly differ between the vehicle- and lumbrokinase-treated rats during myocardial I-R injury (Figure 1).

3.2. Myocardial I-R-induced mortality and rhythm disturbances

The effects of lumbrokinase on myocardial I-R-induced mortality and arrhythmia in rats are shown in Table 1. In the vehicle-treated group, the I-R-induced mortality was 53.3%. However, administration of 10 μg/kg lumbrokinase significantly reduced the I-R-induced mortality to 10%.

Jugular vein injection of the vehicle or lumbrokinase did not elicit arrhythmia in rats. The number of ventricular premature complexes (VPC) was 53.8 ± 28.4, and the durations of VT and VF were 19.0 ± 4.8 and 82.9 ± 14.9 s, respectively, in the vehicle-treated group during the I-R period. However, administration of 10 μg/kg lumbrokinase significantly decreased the number of VPC to 16.7 ± 7.0 and the durations of VT and VF to 3.0 ± 0.8 and 2.3 ± 1.3 s, respectively.

3.3. Myocardial damage and infarct size

There were no significant differences in the size of the area at risk between the vehicle- and lumbrokinase-treated rats, indicating that similar amounts of myocardial tissues were at risk from occlusion of the left coronary artery in each group. In the vehicle-treated group, the infarct size was 47.4 ± 1.8% of the area at risk. However, administration of 10 μg/kg lumbrokinase significantly reduced the infarct size to 19.6 ± 1.4% of the area at risk. Furthermore, in comparison with vehicle-treated rats, administration of 10 μg/kg lumbrokinase significantly decreased LDH activity, a marker of cell damage, after myocardial I-R injury. The resulting LDH levels were consisted with infarct size data (Table 2).

3.4. Effects of lumbrokinase on the protein expressions of TLR2 and TLR4 in myocardial tissues

TLRs play a central role in I-R injury [8, 17, 18] and may be involved in the cardioprotective effects of lumbrokinase (Figure 2). As shown in Figure 2A and 2B, TLR2 expression did not significantly change due to vehicle or 10 μg/kg lumbrokinase treatment in

rats subjected to myocardial I-R injury. However, the protein expression of TLR4 significantly increased in the vehicle-treated group when compared with the sham-operated group. In contrast, lumbrokinase treatment significantly attenuated the myocardical I-R-induced expression of the TLR4 protein in comparison with the vehicle-treated group.

Immunohistochemical staining for TLR4 was performed on the tissues acquired from the area at risk. Lumbrokinase treatment reduced TLR4 staining in the myocardium obtained from rats subjected to myocardial I-R (Figure 2C-2G) when compared with the vehicle group. This is consistent with our finding that pretreatment with lumbrokinase decreases the protein expression of TLR4 in myocardial tissues after I-R injury. As TLRs are also expressed in immune cells [24], we conducted experiments using flow cytometry to determine TLR4 expression in lymphocytes following myocardial I-R injury. Supplementary Figure 1 shows similar levels of expressions of TLR4 in T lymphocytes between lumbrokinase-treated and vehicle-treated groups. The expression of TLR4 was lower in B lymphocytes in the lumbrokinase-treated group when compared with the vehicle-treated group, however the difference did not reach statistical significance. Fallach et al. reported that cardiac function of TLR4-knockout mice and chimeric mice expressing TLR4 in the immunohematopoietic system, but not in the heart, is resistant to LPS and reduced cardiac depression following MI [24]. In addition, Avlas et al. observed that cardiac function is significantly less depressed following coronary artery ligation in chimeric mice deficient in TLR4 in the heart, but not in the immunohematopoietic system, similar to TLR4-knockout mice [25]. These findings suggest that TLR4 expressed by the cardiomyocytes themselves plays a key role in acute myocardial dysfunction caused by septic shock and myocardial ischemia [24, 25]. Moreover, TLR4/NF-κB signal pathway is known to be of importance in I-R injury and to be markedly upregulated in ischemic myocardium [26]. As shown in Figure 2E and F, intense TLR4 staining was observed in the cardiomyocytes of vehicle-treated group. However, TLR4 positive staining was rarely observed in the cardiomyocytes of lumbrokinase-treated group. These results are consistent with the findings of previous reports.

3.5. Effects of lumbrokinase on TLR4 signaling pathway

Figure 3 shows the effects of lumbrokinase on TLR4 signaling pathway in rats subjected to myocardial I-R injury. In parallel with the TLR4 expression, IRAK4 protein expression and phosphorylation level were also examined. The expression of the IRAK4 protein was not altered by vehicle or lumbrokinase treatment in rats subjected to myocardial I-R injury. However, the phosphorylated IRAK4 (p-IRAK4) protein level significantly increased in the vehicle-treated group. This increase was alleviated by treatment with lumbrokinase (Figure 3A and 3B).

We studied the signaling pathways involved in the cardioprotective effect of

lumbrokinase. The protein expressions of p38, ERK 1/2 and JNK and their phosphorylation levels and c-fos were determined. As shown in Figure 3C-G, treatment with vehicle or lumbrokinase did not affect the expressions of p38, phosphorylated p38 (p-p38), ERK 1/2, phosphorylated ERK 1/2 (p-ERK 1/2), or JNK. However, phosphorylated JNK (p-JNK) and c-fos expressions were higher in the vehicle-treated group. Lumbrokinase treatment diminished the increases in p-JNK and c-fos expressions. Additionally, the protein expressions of phosphorylated IκB (p-IκB), NF-κB, and phosphorylated NF-κB (p-NF-κB) significantly increased in the vehicle-treated group when compared with the sham-operated group. In contrast, lumbrokinase treatment significantly attenuated the myocardial I-R-induced increases in expressions of p-IκB, NF-κB, and p-NF-κB when compared with the vehicle-treated group (Figure 3H-J).

3.6. Effects of lumbrokinase on the protein expressions of NOSs, COX-2, and MMPs activity in myocardial tissues

The effects of lumbrokinase on the protein expressions of iNOS, eONS, and nNOS in rats subjected to myocardial I-R injury are shown in Figure 4A. The protein expressions of eNOS and nNOS were not altered by vehicle or lumbrokinase treatment in rats subjected to myocardial I-R injury. However, the protein expression of iNOS in the vehicle-treated group significantly increased when compared with the sham-operated group. In contrast, lumbrokinase treatment significantly attenuated the myocardial I-R-induced increase in expression of iNOS protein when compared with the vehicle-treated group. In addition, the expression of COX-2 significantly increased in the vehicle-treated group when compared with the sham-operated group. However, marked increase in COX-2 expression in rats with myocardial I-R was prevented by treatment with lumbrokinase (Figure 4B).

Zymography was performed to measure the changes in the activities of MMP-2 and MMP-9, which are the characteristic MMPs of the myocardium [27]. The effects of lumbrokinase on the activities of MMP-2 and MMP-9 in rats subjected to myocardial I-R injury are shown in Figure 4C. MMP-2 activity was not altered following vehicle or lumbrokinase treatment. However, MMP-9 activity significantly increased in the vehicle-treated group when compared with the sham-operated group. Administration of lumbrokinase significantly decreased the activity of MMP-9 in rats after myocardial I-R injury.

3.7. Effects of lumbrokinase on myocardial inflammation

Myocardial TLR4 and MMP-9 are critical to the myocardial expressions of chemoattractant proteins that mediate neutrophil infiltration [28]. We further examined

whether smaller infarction in the lumbrokinase-treated group corresponds to less myocardial inflammation as demonstrated by neutrophil infiltration. The protein expression of MPO, a marker enzyme of neutrophils, significantly increased in the vehicle-treated group when compared with the sham-operated group. In contrast, lumbrokinase treatment significantly attenuated the myocardial I-R-induced increase in expression of the MPO protein when compared with the vehicle-treated group (Figure 5A and 5B). Immunohistochemical staining for MPO was performed on the tissues obtained from the area at risk. Hearts from the vehicle-treated group exhibited more neutrophil infiltration (arrow). Administration of lumbrokinase significantly reduced the I-R induced neutrophil infiltration into heart tissues when compared with the vehicle group (Figure 5C-G). Therefore, we suggest that lumbrokinase decreases TLR4 protein expression and MMP-9 activity, which in turn reduces inflammation of myocardial tissues after I-R injury.

4. Discussion & Conclusions

The cardioprotective effect of lumbrokinase in rats subjected to myocardial I-R injury was examined. Administration of 10 μg/kg lumbrokinase significantly suppressed the incidence and number of VPCs, as well as the incidences and durations of VT and VF. This treatment also reduced mortalityand size of cardiac infarct zone in rats subjected to 1 h of coronary artery occlusion and 3 h of reperfusion. These results are consistent with our finding that pretreatment with lumbrokinase leads to decreased carotid blood LDH levels during I-R injury. LDH level is an indicator of cellular damage.In addition, we demonstrated that the cardioprotective effect of lumbrokinase in rats after myocardial I-R injury via attenuated TLR4 regulates signaling pathway. Lumbrokinase exhibited inhibitory effects on myocardial I-R-activated TLR4 and activation of the corresponding downstream signaling molecules such as IRAK4, JNK, c-fos, IκB, and NF-κB. Subsequently, myocardial I-R-induced upregulation of MMP-9 activation and iNOS and COX-2 protein expressions were reduced, thus inhibiting myocardial I-R injury-induced inflammation.

TLR4 signaling plays a dominant role in myocardial I-R injury. TLR4 expression and infiltrating leukocytes within the myocardium significantly increase after I-R injury [8]. Accumulating evidence supports the notion that activation of the TLR4 pathway directly impairs the contractility of isolated cardiomyocytes [24, 25, 26, 29]. Furthermore, mice lacking TLR4 sustain smaller infarct size and exhibit less inflammation when compared with wild-type mice after myocardial I-R injury [18]. Therefore, modulation of TLR4 signaling may be a potential therapeutic target in myocardial I-R injury [19]. Myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adaptor protein inducing interferon-β–mediated transcription-factor (Trif) are critical to TLR4 signaling and mediation of activation of NF-κB. However, previous studies have strongly suggested that TLR4

activation confers potent cardiac protection against I-R injury *via* an MyD88-dependent, Trif-independent signaling mechanism [30, 31]. Recent studies have reported that TLR4-mediated NF-κB signaling contributes to myocardial I-R injury while TLR4-mediated MyD88-dependent signaling pathway is essential for NF-κB activation [32]. Activated MyD88 recruits IRAK4 and IRAK1, leading to the phosphorylated degradation of IκB and NF-κB activation [33]. The present study demonstrated that myocardial I-R injury activates IRAK4 through TLR4 signaling. Importantly, lumbrokinase inhibited the myocardial I-R-induced increase in TLR4 expression and markedly reduced IRAK4 activation. After TLR4 activation, the downstream signaling molecules, including transcription factor NF-κB and mitogen-activated protein kinases (MAPKs), can be activated [34]. In this study, lumbrokinase effectively inhibited myocardial I-R-induced IκB degradation and NF-κB activation. We further examined the involvement of MAPK signaling pathway in the cardioprotective effect of lumbrokinase in rats after myocardial I-R injury. Lumbrokinase inhibited myocardial I-R-induced phosphorylation/activation of JNK and c-fos expressions. The JNK/c-fos pathway has been implicated in the signal transduction pathways responsible for the regulation of inflammatory response following myocardial I-R injury [35, 36]. Although studies have shown that ERK1/2 and p38 MAPK are also involved in the production of inflammatory mediators, lumbrokinase did not reduce their production or phosphorylation in rats subjected to myocardial I-R injury in the present study. These observations indicated that JNK/c-fos, but not ERK1/2 and p38, MAPK-dependent pathways, are involved in the cardioprotective effect of lumbrokinase in rats after myocardial I-R injury.

The activation of transcription factor NF-κB and MAPKs results in the induction of COX-2 and iNOS expressions. COX-2 and iNOS are co-existing, key inflammatory proteins that play fundamental roles under similar pathophysiological conditions [37]. In this study, lumbrokinase reduced the protein expressions of COX-2 and iNOS in rats after myocardial I-R injury. Lumbrokinase inhibited myocardial I-R-induced activation of the NF-κB signaling cascade and phosphorylation of JNK, rather than p38 MAPK and ERK1/2. This suggested that the NF-κB and JNK pathways modulate the suppression of the myocardial I-R-induced protein expressions of COX-2 and iNOS by lumbrokinase.

In addition, we examined inflammatory cell infiltration into the ischemic zone of the I-R heart tissues. The results of the present study demonstrated that lumbrokinase reduces neutrophil infiltration into the injured myocardial tissues after I-R. Accumulating evidence has demonstrated that infiltrating neutrophils are the major source of MMP-9 and may localize MMP-9 activation in the I-R injured myocardium [38, 39]. A significant positive correlation was found between the relative intensity of MMP-9 and number of MPO positive cells in myocardium ($r = 0.7921$, $p < 0.05$), as shown in Supplementary Figure 3. In addition, previous studies have demonstrated increases in the expression and activity of myocardial MMP-9 in experimental myocardial I-R injury. Thus, MMP-9 may serve as a target for

prevention or treatment of acute myocardial I-R injury [38, 39, 40]. The results also showed that lumbrokinase reduces inflammation during myocardial I-R, thus limiting the infiltration of neutrophils into the injured tissues. Moreover, MMP-9 activity significantly decreased in the lumbrokinase-treated group when compared with the vehicle-treated group after myocardial I-R injury.

Lumbrokinase has been used in oral dosage to prevent and treat clotting, such as that related to myocardial infarction and cerebral thrombus [41]. Oral lumbrokinase (300,000 units) administered three times daily for 30 consecutive days improves regional myocardial perfusion in patients with stable angina [16]. In this study, we conducted experiments to elucidate the effectiveness of various doses of lumbrokinase. We found that 10 μg/kg (100 units) lumbrokinase protects the heart against I-R injury in rats. In addition, we demonstrated that 10 μg/kg lumbrokinase significantly decreases myocardial I-R-induced number of VPC and durations of VT and VF in rats. The effective dose for ameliorating myocardial I-R injury in rats is lower than that used in the clinical treatment of cardiovascular diseases.

Varda-Bloom et al. reported that immune-mediated injury from ischemia indicates that lymphocyte proliferation increases in the second week following myocardial infarction [42]. Activation of lymphocytes has been reported in later stages. In this study, we occluded the coronary artery for 1 h and then subjected it to reperfusion for 3 h. We found a significant difference in TLR4 expression in the peri-infarct area and no difference in the blood at 3 hours following reperfusion. Fallach et al. also reported that they did not detect a significant infiltration of leukocytes into the heart in the acute phase (4 hours), which corresponded to maximal depression of heart contraction and absence of leukocyte infiltration in TLR4-ko mice at longer time points after the LPS challenge and MI [24]. However, 24 hours following MI massive leukocyte infiltration was detected. Ao et al. using isolated hearts, demonstrated that myocardial tissue TLR4, rather than neutrophil TLR4, is the determinant of myocardial neutrophil infiltration after global I-R [28]. Therefore, we suggest that TLR4 is expressed in cardiomyocytes, but not in lymphocytes, following acute myocardial I-R injury.

We also evaluated the blood-clot lysis activity of lumbrokinase. Supplementary Figure 2 shows that treatment with 96000 units of lumbrokinase significantly lyses blood clots in vitro, which is consistent with the findings of a previous study [43]. However, the blood-clot lysis effect of lumbrokinase at a dose of 100 units was similar to that of vehicle. At the effective dose of lumbrokinase in rats subjected to myocardial I-R injury in this study blood-clot lysis was rarely observed. Therefore, the cardioprotective effect of lower dose lumbrokinase may be through anti-inflammatory mechanisms that protect the heart against I-R injury.

In conclusion, this study is the first to report that lumbrokinase is a potent cardioprotective drug in rats with I-R injury. Lumbrokinase decreases the number of VPC and the durations of VT and VF following I-R. We provide evidence that lumbrokinase significantly improves ventricular arrhythmias and reduces infarct volume from myocardial

I-R injury. This may correlate with the inhibitory effect of the myocardial I-R-induced expressions of COX-2, iNOS, and activation of MMP-9, mediated by TLR4 signaling through JNK and NF-κB pathways. As lumbrokinase has recently become available in tablet form and has been recommended as a dietary supplement, it is important to determine the novel signaling mechanism involved in the cardioprotective effect of lumbrokinase pretreatment. This mechanism controls innate immune response with anti-inflammatory action during myocardial I-R injury. We further conducted experiments to test the post-treatment effects of lumbrokinase in rats subjected to myocardial I-R injury. We found that 10 μg/kg lumbrokinase significantly reduces ventricular arrhythmias and myocardial infarction in rats after myocardial I-R injury. Further studies to determine the underlying mechanisms of post-treatment lumbrokinase in the amelioration of myocardial I-R injury in rats will be undertaken. Lumbrokinase exhibits significant potential as a cardioprotective agent. Lumbrokinase may also be beneficial for future use as a novel agent for cardiovascular disorders, such as myocardial infarction. Elucidating the mechanism by which lumbrokinase modulates the expressions of inflammatory mediators would provide a molecular basis for developing therapeutic compounds to ameliorate myocardial I-R injury.

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Acknowledgements

This work was supported financially by research grants from Chung Shan Medical University (CSMU-INT-102-06), and the Ministry of Science and Technology (MOST 103-2320-B-040-001-MY3) of Taiwan to Dr. Shiang-Suo Huang.

Conflict of Interest

None of the authors have any competing financial interests to declare.

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Figure Legend

Figure 1. (A) Mean BP and (B) heart rate (C) ECG were recorded during myocardial I-R injury. Each experimental group contains ten rats. Results are expressed as mean ± SEM. No significant differences were recorded between the vehicle and two doses of lumbrokinase (LK-1: 1 μg/kg; LK-10: 10 μg/kg) treated rats. L indicates coronary ligation. R indicated reperfusion.

Figure 2. Effects of lumbrokinase on TLRs protein expression in rats subjected to myocardial I-R injury. Rats were treated with vehicle or 10 μg/kg lumbrokinase (LK-10). (A) Representative images of the Western blot results TLR2 and TLR4 expression in heart tissue samples from rats after myocardial I-R injury. (B) Quantitative densitometric analysis of TLR2 and TLR4 protein expression with β-actin as an internal standard. Data were normalized with sham and presented as percentage rates. Each value represents the mean of three individual experiments. Results are expressed as mean ± SEM (n = 3). *p<0.05 compared with sham. $^{\#}p$<0.05 compared with vehicle. (C-F) Representative areas of TLR4 immunohistochemical (IHC) staining slides of rats subjected to myocardial I-R injury. Panels C and D original magnification, panels E and F are higher magnification of small box in panels C and D. The dark brown color indicates expression of TLR4. (G) Quantitative densitometric analysis of TLR4 staining slides of rats subjected to myocardial I-R injury. Data were normalized with vehicle and presented as percentage rates. Results are expressed as mean ± SEM (n = 3). *p<0.05 compared with vehicle.

Figure 3. Effects of lumbrokinase on TLRs signaling pathway in rats subjected to myocardial I-R injury. Rats were treated with vehicle or 10 μg/kg lumbrokinase (LK-10). (A) Representative images of the Western blot results. (B) Quantitative densitometric analysis of IRAK4 and p-IRAK4. (C) Representative images of the Western blot results. Quantitative densitometric analysis of (D) p38 and p-p38 (E) ERK1/2 and p-ERK1/2 (F) JNK and p-JNK (G) c-fos (H) Representative images of the Western blot results. Quantitative densitometric analysis of (I) IκB and p-IκB (J) NF-κB and p-NF-κB protein expression in heart tissue samples from rats after myocardial I-R injury. Data were normalized with sham and presented as percentage rates. Each value represents the mean of -three individual experiments. Results are expressed as mean ± SEM (n = 3). *p<0.05 compared with sham. $^{\#}p$<0.05 compared with vehicle.

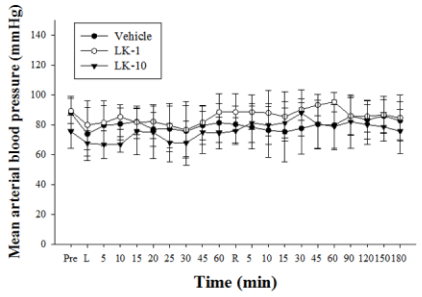
Figure 4. Effects of lumbrokinase on the protein expression of NOSs and COX-2 and MMP-9 activity in rats subjected to myocardial I-R injury. Western blot analysis for (A) NOSs (B) COX-2 expression, and gelatin zymography analysis for (C) MMP-2 and MMP-9

activity in heart tissue samples from rats after myocardial I-R injury. Rats were treated with vehicle or 10 μg/kg lumbrokinase (LK-10). Data were normalized with sham and presented as percentage rates. Each value represents the mean of three individual experiments. Results are expressed as mean ± SEM (n = 3). *p<0.05 compared with sham. $^{\#}p$<0.05 compared with vehicle.

Figure 5. Effects of lumbrokinase on MPO expression in rats subjected to myocardial I-R injury. Rats were treated with vehicle or 10 μg/kg lumbrokinase (LK-10). (A) Representative images of the Western blot results MPO expression in heart tissue samples from rats after myocardial I-R injury. (B) Quantitative densitometric analysis of MPO protein with β-actin as an internal standard. Data were normalized with sham and presented as percentage rates. Each value represents the mean of three individual experiments. Results are expressed as mean ± SEM (n = 3). *p<0.05 compared with sham. $^{\#}p$<0.05 compared with vehicle. (C-F) Representative areas of MPO immunohistochemical (IHC) staining slides of rats subjected to myocardial I-R injury. Panels C and D are original magnification and panels E and F are higher magnification of small box in panels C and D. The red arrow indicates the infiltration of neutrophils. (G) Quantitative analysis of MPO positive cells of rat heart slides which subjected to myocardial I-R injury. Results are expressed as mean ± SEM (n = 3). *p<0.05 compared with vehicle.

Figure 1

A



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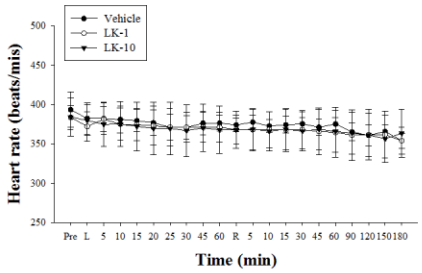

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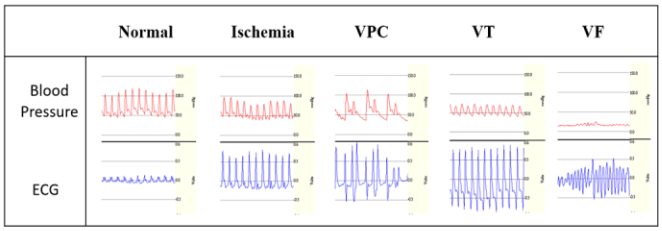


Figure 2

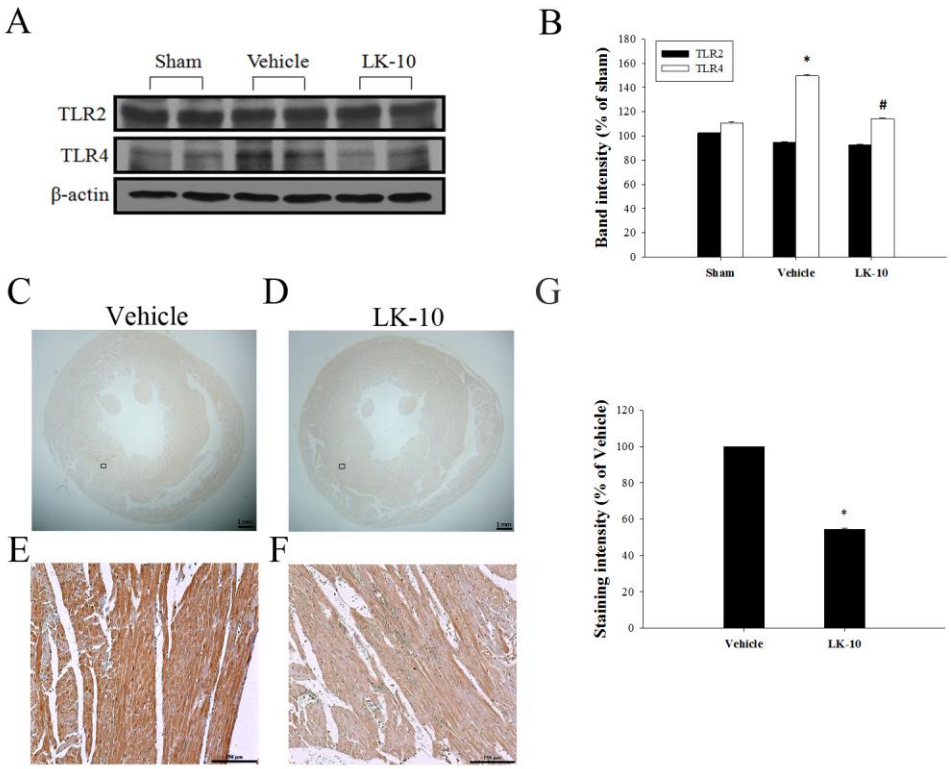


Figure 3

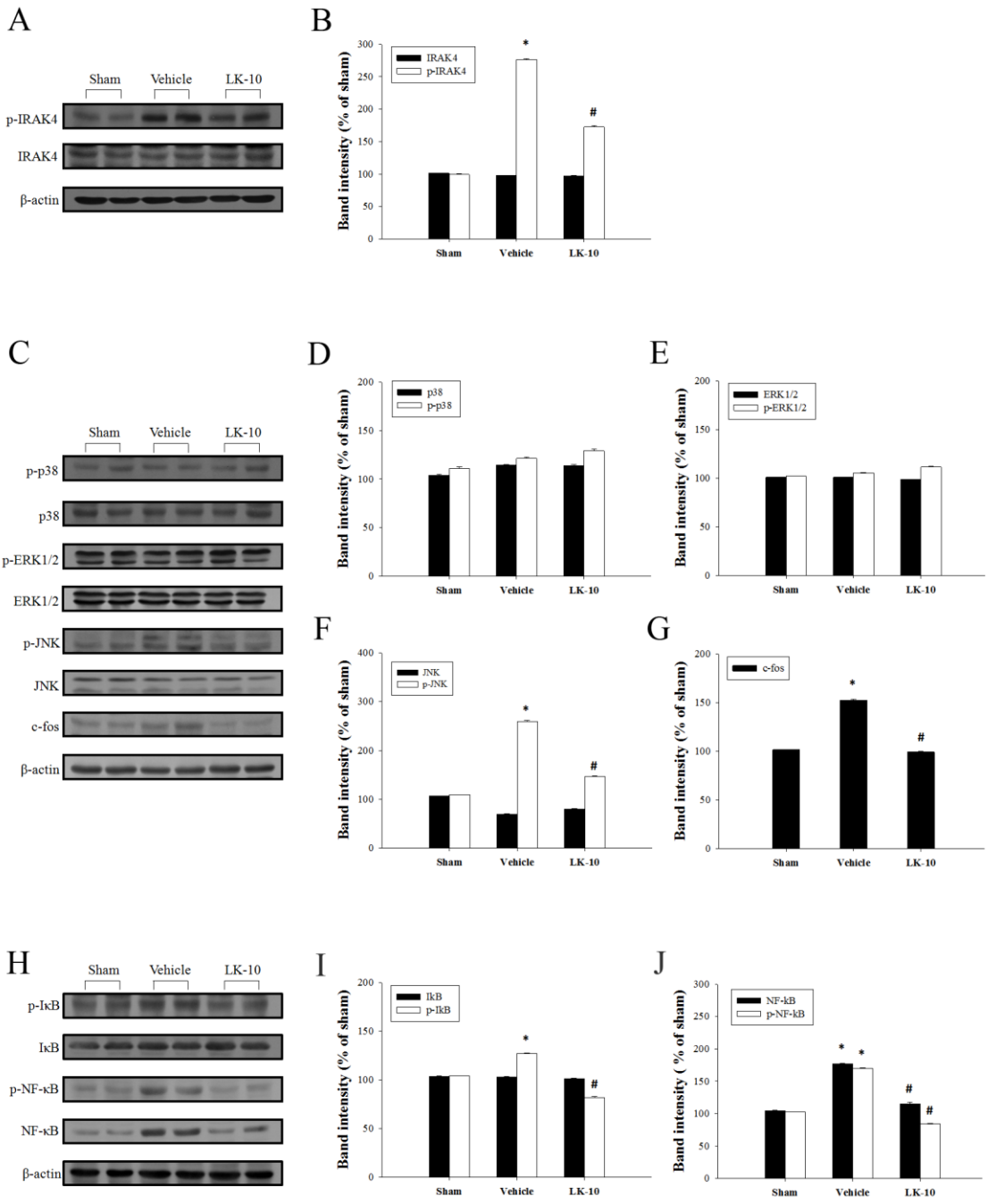


Figure 4

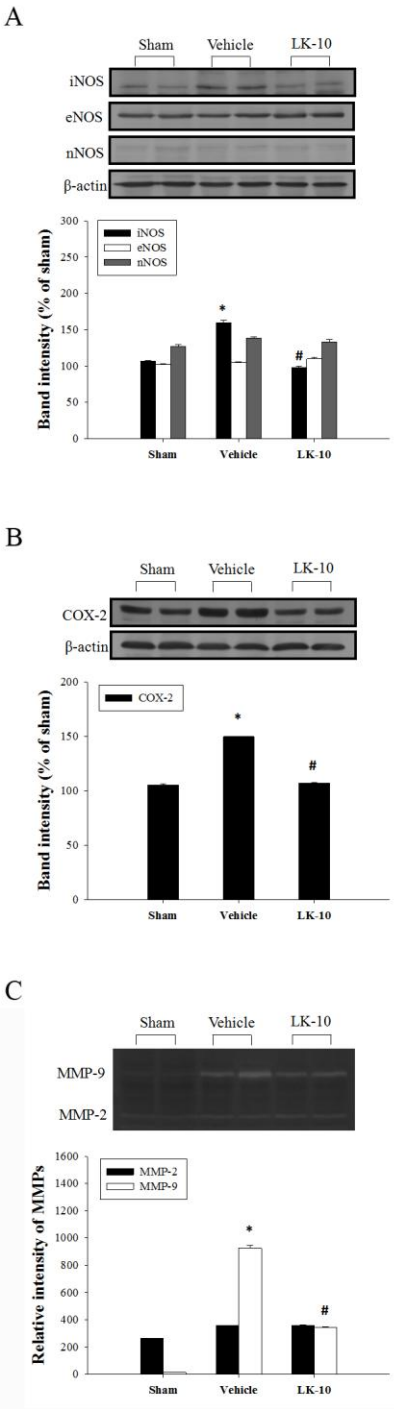


Figure 5

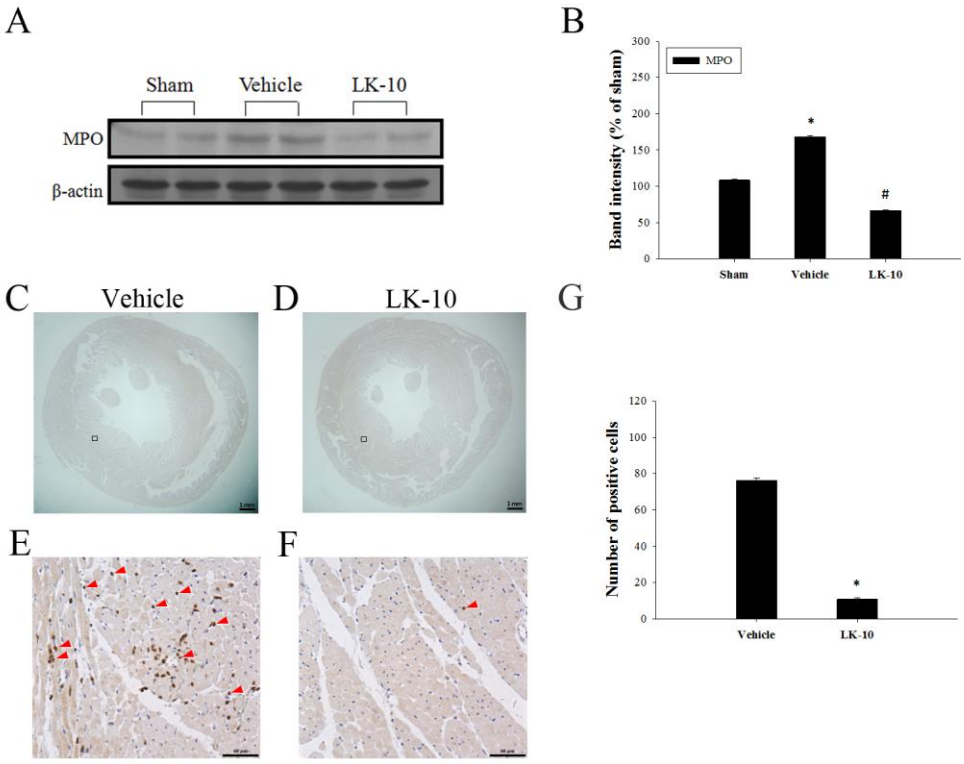


Table 1. Effect of Lumbrokinase on Arrhythmias Induced by myocardial I/R injury in Anesthetized Rats

	n	VPC		VT		VF		Mortality (%)
		Incidence (%)	Count (number)	Incidence (%)	Duration (sec)	Incidence (%)	Duration (sec)	
Sham								
Vehicle	5	-	-	-	-	-	-	-
LK-10	5	-	-	-	-	-	-	-
Operated (ligated)								
Vehicle	15	80	53.8±28.4	67	19.0±4.8	47	82.9±14.9	53.3
LK-1	10	50	34.6±9.5	40	6.2±1.1*	10*	4.7±2.1*	20
LK-10	10	30*	16.7±7.0*	20*	3.0±0.8*	10*	2.3±1.3*	10*

VPC, ventricular premature complexes; VT, ventricular tachycardia; VF, ventricular fibrillation.

Vehicle is normal saline. n, number of experiments; values for duration of VT and VF are shown as the mean ± SEM.

*Statistical difference at the level of $P<0.05$ as compared with vehicle.

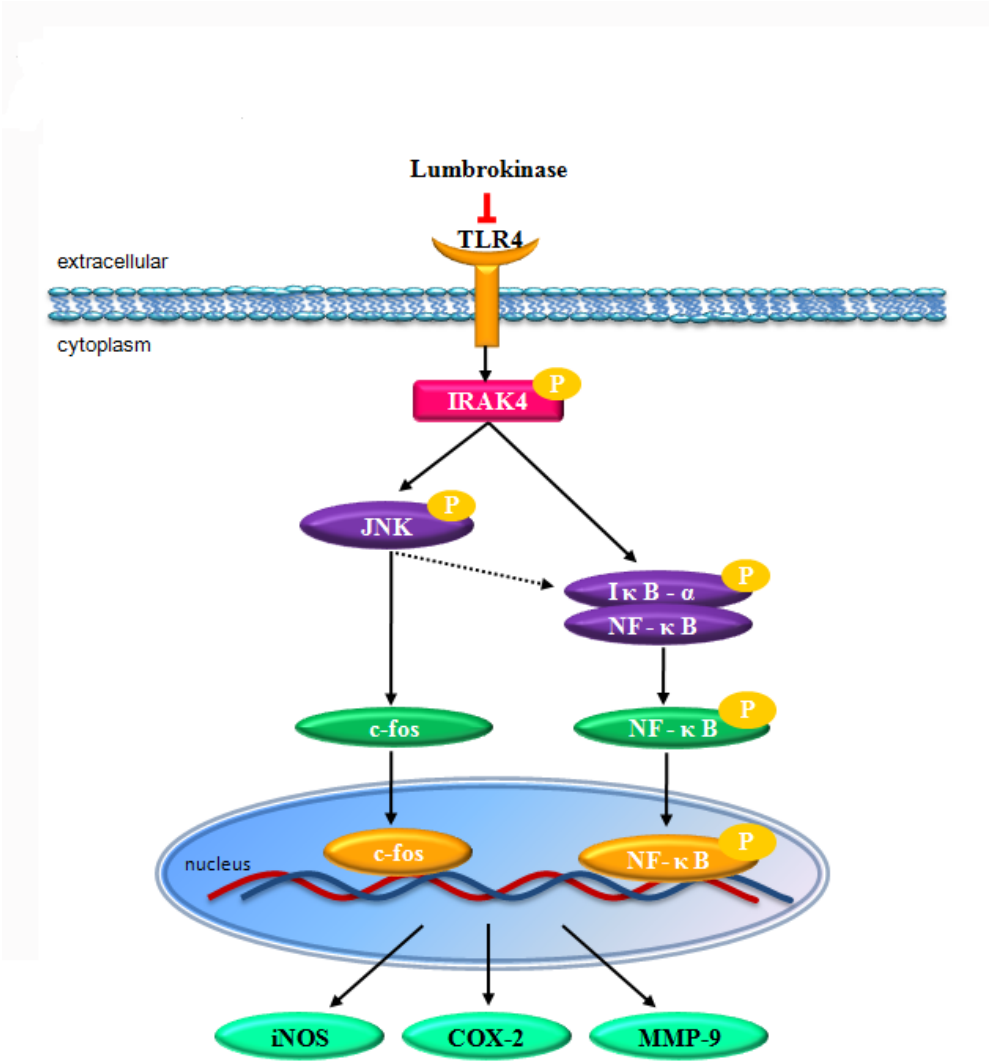
Table 2. Weight and Size of Area at Risk (n=6)

	Vehicle	LK-10
LV weight (g)	0.94±0.05	0.87±0.06
Area at risk (g)	0.46±0.03	0.43±0.03
Area at risk/LV (%)	49.2±0.6	49.4±1.4
Infarct size (g)	0.21±0.04	0.08±0.01*
Infarct size/LV (%)	23.4±6.2	9.7±0.8*
Risk zone infarcted (%)	47.4±1.8	19.6±1.4*
Lactate dehydrogenase (U/L)	6197±1833	1856±53*

Data are shown as the mean ± SEM.

*Statistical difference at the level of $P<0.05$ as compared with vehicle.

LV, left ventricle.



Graphical abstract

Highlights

- Lumbrokinase is a potent cardioprotective drug in rats subjected to myocardial I-R injury.
- Lumbrokinase protects myocardium against I-R injury via TLR4 signaling pathway.
- Lumbrokinase might be a potential therapeutic compound for treatment of acute myocardial infarction.