Ulinastatin protects cardiomyocytes against ischemia-reperfusion injury by regulating autophagy through mTOR activation

JIAN XIAO[1*], XIAOYAN ZHU[2*], GUANGYU JI[1*], QIAN YANG[1*], BO KANG[1], JIANQUAN ZHAO[1], FENG YAO[3], LIHUI WU[1], XIN NI[2] and ZHINONG WANG[1]

[1]Department of Cardiothoracic Surgery, Changzheng Hospital, The Second Military Medical University, Shanghai 200003; [2]Department of Physiology, The Second Military Medical University, Shanghai 200433; [3]Department of Anesthesiology, Shanghai Chest Hospital Affiliated to Shanghai Jiaotong University, Shanghai 200030, P.R. China

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Abstract. Autophagy is significant in myocardial ischemia-reperfusion (IR) injury. Ulinastatin has been demonstrated to protect cardiomyocytes against IR through inducing anti-inflammatory effects. However, whether ulinastatin has an anti-autophagic effect is yet to be elucidated. The present study aimed to investigate the effect of ulinastatin on the regulation of autophagy during IR injury. Cardiomyocytes of neonatal rats were randomly divided into control, hypoxia-reoxygenation (HR) and ulinastatin groups. In order to investigate whether mammalian target of rapamycin (mTOR) is involved in mediating the protective effect of ulinastatin, cells were treated with the mTOR inhibitor, rapamycin 30 min prior to ulinastatin treatment. To demonstrate the anti-autophagic effect of ulinastatin *in vivo*, a rat IR model was established. Ulinastatin ($1x10^4$ U/kg body weight) was administered 30 min prior to the induction of IR via peritoneal injection. Light chain 3 (LC3), phosphorylated (p)-mTOR, p-protein kinase B (Akt) and p-P70S6 kinase (p-P70S6K) protein expression were assessed using western blot analysis. In addition, cell vitality, myocardial infarct size and lactate dehydrogenase (LDH) levels were measured. LC3-II protein expression was found to be downregulated, while p-Akt, p-mTOR and p-P70S6K protein expression were observed to be upregulated by ulinastatin. In addition, cell vitality was found to increase and LDH was observed to decrease in the ulinastatin group compared with the HR group *in vitro*. Furthermore, rapamycin was found to attenuate the myocardial protective effect that is induced by ulinastatin. *In vivo*, ulinastatin was found to downregulate LC3-II protein expression, and reduce myocardium infarct size and LDH serum levels. These findings indicate that ulinastatin exhibits a myocardial protective effect against IR injury by regulating autophagy through mTOR activation.

Correspondence to: Professor Lihui Wu or Professor Zhinong Wang, Department of Cardiothoracic Surgery, Changzheng Hospital, the Second Military Medical University, 415 Fengyang Road, Shanghai 200003, P.R. China
E-mail: wulh@outlook.com
E-mail: wangzn007@hotmail.com

*Contributed equally

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Introduction

Myocardial ischemia-reperfusion (IR) injury is the predominant injury resulting from revascularization treatments, such as coronary thrombolysis, percutaneous coronary interventions, coronary artery bypass grafts and cardiac transplants (1). During ischemia, mitochondria produce reactive oxygen species (ROS) and during reperfusion an extra burst of ROS generation occurs (2). Furthermore, during reperfusion, multiple molecular inflammatory cascades are activated; including those involving interleukin (IL)-1 and tumor necrosis factor (TNF) α. Chemokine production is induced within hours of reperfusion (3), with pro-inflammatory cytokines sequentially inducing cell autophagy (4).

Autophagy is a type of programmed cell death, which has been indicated to be significant in cell homeostasis, as well as cell defense and adaptation to adverse environments (5-7). Autophagy has an important role in the heart and activation of autophagy has been observed in a variety of heart diseases, including cardiac hypertrophy, heart failure and IR injury. Autophagy performs two opposing functions in the heart (8); it has a protective role during nutrient deprivation and other forms of cellular stress, however, excessive autophagy may be used for self-destruction (9).

Ulinastatin is a multivalent enzyme inhibitor, which is predominantly used for the treatment of pancreatitis, severe infection-induced acute circulatory failure, as well as for the prevention of multiple organ failure (10). Recently, ulinastatin has been found to exhibit myocardial protective effects through inhibiting the expression of TNF, reducing levels of oxygen free radicals and increasing levels of endogenous nitric oxide (11,12). During myocardial IR injury, ulinastatin has been reported to show protective effects via the induction of an anti-inflammatory response (1). However, whether the protective effect of ulinastatin in cardiomyocytes is associated

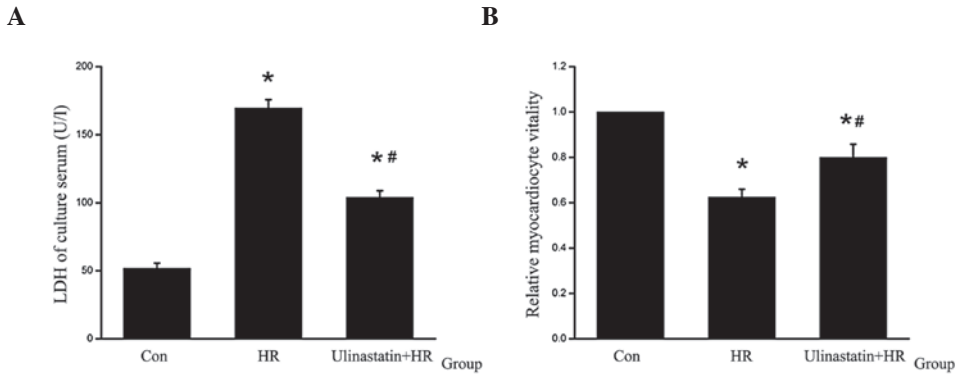

Figure 1. LDH levels in the cell culture serum and cardiomyocyte vitality measured using MTT assay (n=6). LDH levels were found to increase and cell vitality was observed to decrease following HR treatment. HR-induced injury was attenuated by ulinastatin compared with the HR group (n=5). (A) LDH levels in the cardiomyocyte culture serum. (*P<0.001 vs. Con and #P<0.001 vs. HR). (B) Cardiomyocyte vitality measured using MTT assay (*P<0.001, *P=0.005 vs. Con; #P=0.011 vs. HR). LDH, lactate dehydrogenase; Con, control; HR, hypoxia-reoxygenation.

with cardiomyocyte autophagy, is yet to be elucidated. The present study aimed to investigate whether ulinastatin reduces cardiomyocyte injury through regulating autophagy and its associated mechanisms.

Material and methods

Animals. All animal experiments were approved by the Animal Research Ethics Committee of the Second Military Medical University (Shanghai, China). The experiments conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Cell culture and experimental protocols. Neonatal cardiomyocytes were prepared from the hearts of Sprague-Dawley rats (age, <3 days) (13). On day four, the cardiomyocytes were randomly divided into the following three groups: Con, a control group where the cells were cultured in Dulbecco's modified Eagle's medium and incubated in an atmosphere of 5% CO_2 for 24 h; HR, a hypoxia-reoxygenation group where the cells were incubated with 1% O_2, 5% CO_2 and 94% N_2 for 24 h, followed by 5% CO_2 and 95% air for 6 h; and an ulinastatin group, where the cells were treated with $1x10^4$ U/l ulinastatin for 30 min prior to HR, then were incubated under the same conditions as the HR group. In order to investigate whether mammalian target of rapamycin (mTOR) was involved in the protective effect of ulinastatin, the cells were treated with the mTOR inhibitor, rapamycin (20 nM) 30 min prior to ulinastatin treatment.

MTT assay. An MTT assay was used to assess cardiomyocyte vitality. The cardiomyocytes were cultured in 96-well plates at a density of $1x10^3$ cells. MTT solution (10 μl; Sigma, St. Louis, MO, USA) was added to the growing cells and incubated for 4 h. The crystals were then solubilized by adding 100 μl solubilization solution. The absorbance of the purple solution was determined at a wavelength of 450 nm using a microtiter plate reader (Bio-Rad, Hercules, CA, USA).

Western blot analysis. The protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China) according the manufacturer's instructions. Equal quantities of protein (50 μg) from the cardiomyocytes were subjected to western blot analysis using anti-LC3-I, -LC3-II (Sigma-Aldrich, St. Louis, MO, USA), anti-p-Akt, -p-mTOR and -p-P70S6K (Cell Signaling Technology, Inc., Beverly, MA, USA) primary antibodies to detect LC3, p-Akt (Ser-473), p-mTOR (Ser-2448) and p-P70S6K (Thr-389) protein expression, respectively. An enhanced chemiluminescence detection kit (Amersham Biosciences, Picastaway, NJ, USA) was used to detect the immunoreactive protein bands. The autophagy results were presented as the LC3-II/LC3-I expression ratio.

In vivo rat model and experimental protocols. Sprague-Dawley rats weighing between 250 and 300 g were anesthetized using intraperitoneal injection of 10% chloral hydrate (300 mg/kg body weight), prior to endotracheal intubation. IR was induced by ligating the left anterior descending artery as previously reported (14). Thirty rats were randomly divided into three equal groups: Control group, where the rats underwent thoracotomy without ligation; IR group, where the rats were treated with ischemia for 30 min and reperfusion for 6 h; and an ulinastatin group, where the rats were treated with ulinastatin ($1x10^4$ U/kg body weight) by intraperitoneal injection, 30 min prior to IR, followed by the same conditions as the IR group.

Measuring infarct size. Myocardial infarct size was measured as previously described (15). The total left ventricular area, infarct area (INF) and area at risk (AAR) were assessed. The percentage of the INF/AAR was calculated.

Lactate dehydrogenase (LDH) assay. Blood and culture serum were collected following reperfusion from the rats and cultured cardiomyocytes, respectively, in order to determine LDH levels.

Statistical analysis. Quantitative data are presented as the mean ± standard error. Statistical significance was determined using one-way analysis of variance and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Ulinastatin attenuates HR-induced cardiomyocyte injury in vitro. Following HR, a decrease in cardiomyocyte vitality

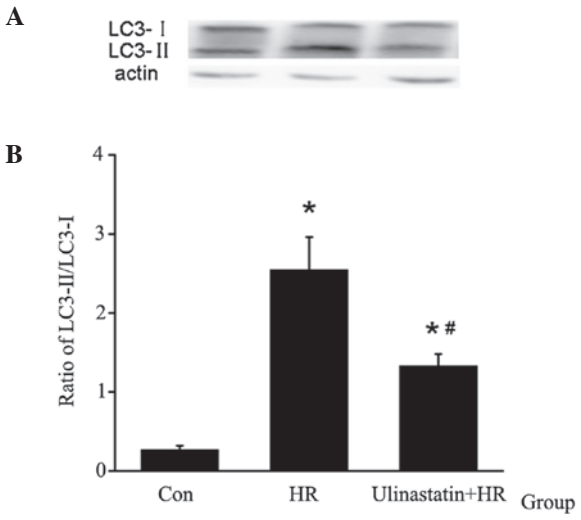

Figure 2. LC3 protein expression detected using western blot analysis (n=5). (A) Western blot showing LC3 protein expression in the different groups. (B) Ratio of LC3-II/LC3-I expression in different groups. LC3-II was upregulated by HR and was attenuated by ulinastatin. *P<0.001, P=0.017 vs. Con and #P=0.008 vs. HR. LC3, light chain 3; Con, control; HR, hypoxia-reoxygenation.

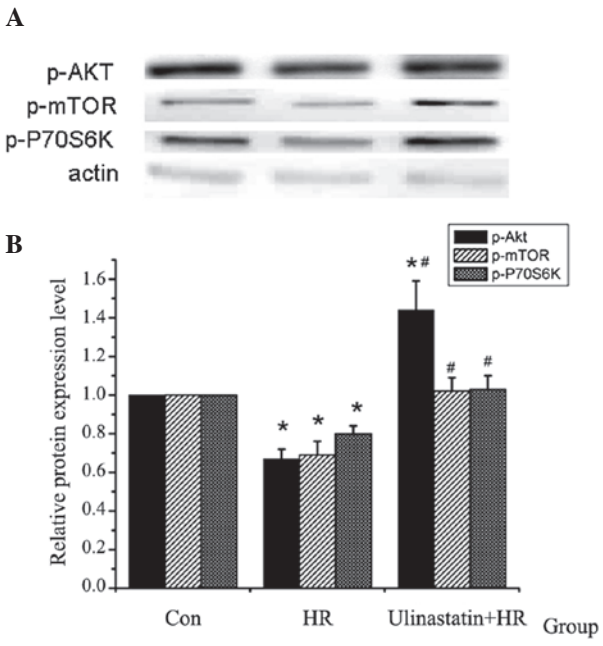

Figure 3. p-Akt, p-mTOR and p-P70S6K protein expression detected using western blot analysis (n=5). (A) Western blot analysis of p-Akt, p-mTOR and p-P70S6K protein expression in the different groups. (B) Relative protein expression of p-Akt, p-mTOR and p-P70S6K in the different groups. p-Akt, p-mTOR and p-P70S6K expression were found to be downregulated by HR, and this HR-induced downregulation was attenuated by ulinastatin. (*P<0.001, P=0.017 vs. Con and #P=0.008 vs. HR). p-, phosphorylated; Akt, protein kinase B; mTOR, mammalian target of rapamycin; P70S6K, P70S6 kinase; Con, control; HR, hypoxia-reoxygenation.

and an increase in LDH levels in the culture serum were observed. To assess the protective effect of ulinastatin, cardiomyocytes were treated with ulinastatin prior to HR. Cell vitality was found to increase and LDH levels in the culture serum were found to decrease in the HR+ulinastatin group compared with the HR group (Fig. 1).

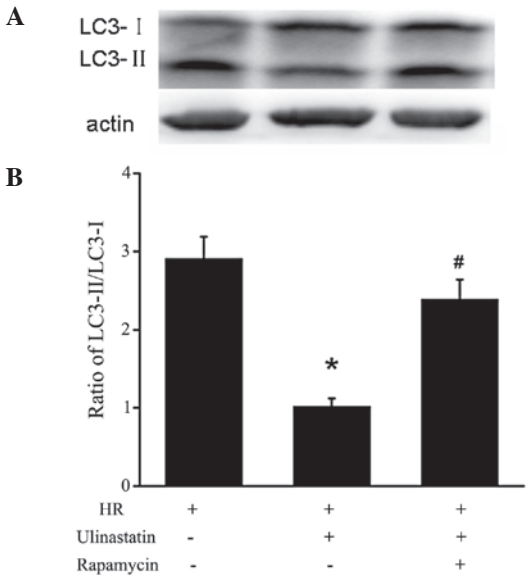

Figure 4. Rapamycin regulates LC3 protein expression (n=4). (A) Western blot of LC3 protein expression from the different groups. (B) Ratio of LC3-II/LC3-I expression in the different groups. Ulinastatin-induced LC3-II downregulation was attenuated by rapamycin during HR *in vitro*. *P<0.001 vs. HR and #P=0.002 vs. ulinastatin. LC3, light chain 3; HR, hypoxia-reoxygenation.

Ulinastatin attenuates HR-induced cardiomyocyte autophagy in vitro. In order to analyze cardiomyocyte autophagy, LC3 protein expression was assessed using western blot analysis. The LC3-II/LC3-I expression ratio was used to measure the relative autophagy levels in cardiomyocytes. HR was found to induce cardiomyocyte autophagy, which was attenuated by ulinastatin treatment (Fig. 2).

Ulinastatin inhibits cardiomyocyte autophagy through mTOR in vitro. In order to investigate the mechanism underlying the anti-autophagic effect of ulinastatin in cardiomyocytes, the protein expression of p-Akt (Ser-473), p-mTOR (Ser-2448) and p-P70S6K (Thr-389) was analyzed. Treatment with ulinastatin prior to HR significantly increased the protein expression of p-Akt, p-mTOR and p-P70S6K compared with the HR group (Fig. 3). Furthermore, the LC3-II/I expression ratio was significantly increased in the cells treated with rapamycin+ulinastatin compared with those treated with ulinastatin, indicating that rapamycin attenutates the ulinastatin-induced inhibition of autophagy (Fig. 4).

Ulinastatin attenuates myocardial IR injury by inhibiting autophagy in vivo. To demonstrate the protective myocardial and anti-autophagic effects of ulinastatin, the rats were treated with ulinastatin ($1x10^4$ U/kg body weight) 30 min prior to IR. Ulinastatin inhibited IR-induced cardiomyocyte autophagy and reduced the relative area of the infarct, serum LDH levels and inflammation (Fig. 5).

Discussion

Ulinastatin has been proposed as a myocardial protective agent. Ulinastatin has been reported to improve myocardial contractility, reduce myocardial infarct size and decrease

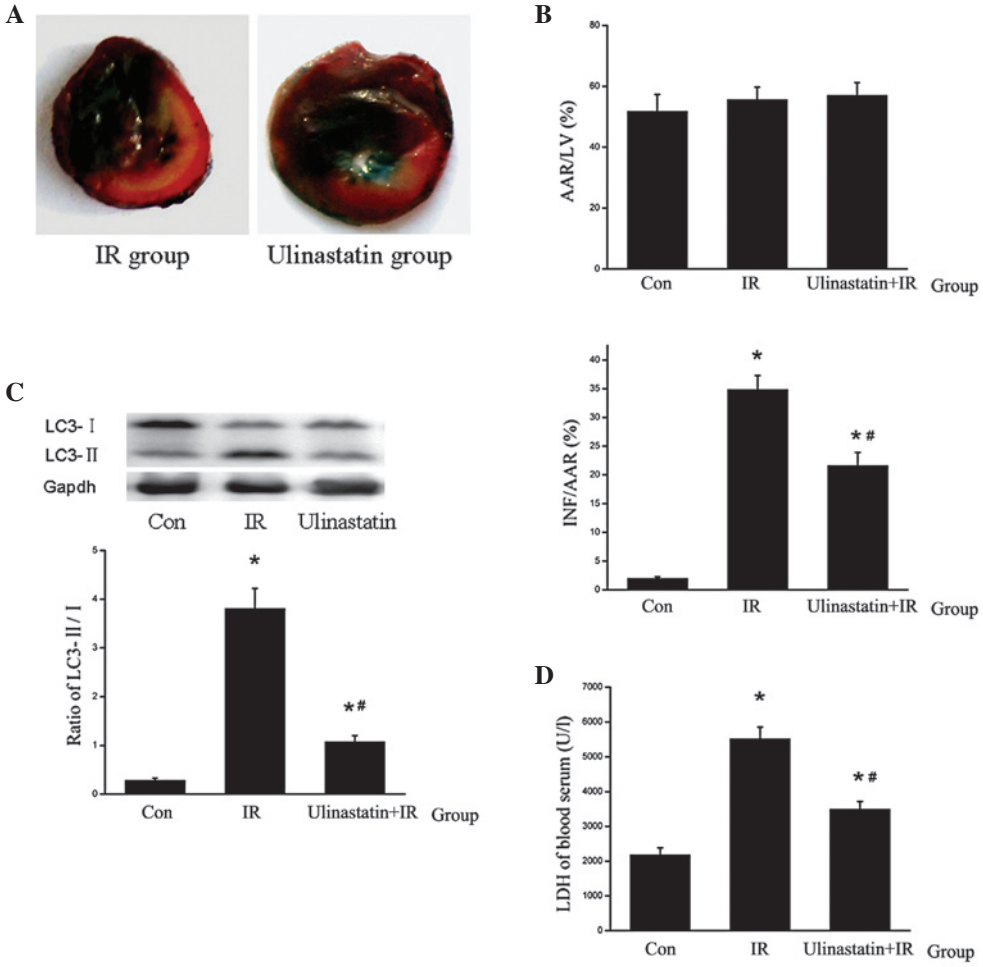

Figure 5. Ulinastatin inhibits cardiomyocyte autophagy and attenuates IR-induced myocardial injury (n=6). (A) Mid-myocardial cross sections of triphenyltetrazolium chloride-stained hearts. Dark blue area, nonischemic zone; white area, INF; red area, AAR (B) AAR/LV and INF/AAR ratios. No significant difference was observed in AAR/LV among the Con, IR and ulinastatin groups. However ulinastatin was found to significantly attenuate the INF/AAR ratio in the ulinastatin+IR group compared with the IR group. *P<0.001 vs. Con and #P<0.001 vs. IR. (C) LC3-II/LC3-I ratio in the different groups. LC3-II was upregulated by IR and downregulated by ulinastatin *in vivo*. *P<0.001, *P=0.047 vs. Con and #P<0.001 vs. IR. (D) LDH levels in the blood serum. Ulinastatin reduced LDH levels in the blood serum compared with the IR group. *P<0.001, *P=0.002 vs. Con and #P<0.001 vs. IR. IR, ischemia reperfusion; AAR, area at risk; LV, left ventricle; Con, control; LC3, light chain 3; INF, infracted tissue.

serum levels of creatine kinase and cardiac troponin I following myocardial IR injury *in vivo* (1). In the present study, cultured primary cardiomyocytes were treated with ulinastatin prior to HR *in vitro*. Ulinastatin was found to improve cell vitality and reduce LDH levels, thus ulinastatin may have a protective effect against HR injury in cardiomyocytes *in vitro*. Furthermore, the myocardial protective effect of ulinastatin was was found to be associated with various mechanisms in an *in vivo* rat heart model. It has previously been reported that ulinastatin may contribute to the recovery of cardiac function following reperfusion by reducing mitochondrial dysfunction and maintaining energy production (16). Moreover, the protective effect of ulinastatin may be associated with anti-inflammatory effects. For example, ulinastatin has been reported to reduce the expression of TNF and IL-6 and upregulate that of IL-10 and -13 (12,17-21).

It has been reported that TNF stimulates autophagy, while IL-13 suppresses autophagy through stimulating the phosphoinositide 3-kinase/mTOR signal transduction pathway (4,9,22). Therefore, ulinastatin may inhibit autophagy via the downregulation of TNF and the upregulation of IL-13. Autophagy is regulated by autophagy-related genes (Atgs), among which beclin 1 is required for the vesicle nucleation step of autophagy. Autophagosome formation involves two complexes, Atg12-Atg5-Atg16 and Atg4-Atg7-Atg3. These complexes are involved in the conversion of the soluble form of LC3 (LC3-I) to the autophagic vesicle-associated form (LC3-II), which is used as a marker of autophagy (23). The LC3-II/LC3-I ratio has been used for analyzing autophagy in numerous studies. In a previous study on an IR model, autophagy was found to be markedly enhanced and inhibition of autophagy, via the downregulation of beclin 1, was observed to have a protective effect *in vivo* (24).

In the present study, HR-induced LC3-II protein expression was found to be downregulated by ulinastatin *in vitro*. Furthermore, ulinastatin was observed to upregulate p-Akt, p-mTOR and p-P70S6K protein expression. To assess whether ulinastatin inhibits HR-induced cardiomyocyte autophagy through activating mTOR, cells were treated with the mTOR-specific inhibitor, rapamycin prior to HR and

ulinastatin treatment. Rapamycin treatment was found to upregulate LC3-II, indicating that rapamycin attenuated the anti-autophagic effect of ulinastatin. Therefore, ulinastatin may protect cardiomyocytes against HR injury by inhibiting autophagy through activating Akt/mTOR. This may be associated with ulinastatin-induced anti-inflammatory effects.

In order to investigate the anti-autophagic effect of ulinastatin *in vivo*, a rat IR model was established. Certain rats were treated with ulinastatin ($1x10^4$U/kg body weight) 30 min prior to IR. Inflammation was found to be reduced by ulinastatin. Furthermore, myocardium LC3-II protein expression, myocardial infarct size and serum LDH levels were observed to be reduced in the ulinastatin group compared with the IR group.

In conclusion, the present study demonstrated that ulinastatin has an important protective role against IR injury by regulating autophagy via the mTOR signaling pathway. Furthermore, the protective effect of ulinastatin may be associated with its anti-inflammatory effect.

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