Ulinastatin alleviated Lps-induced injury of cardiac micro vascular endothelial Cell via NF-κB pathway

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Abstract: The aim of this study to investigate the effect of ulinastatin (UTI) on lipopolysaccharide (LPS) -induced injury of cardiac micro vascular endothelial cell, and explore potential mechanisms Primary cardiac micro vascular endothelial cells were harvested from neonatal Sprague Dawley rats. Cardiac micro vascular endothelial cells were prepared for further treatment after subculture. The experiment was designed into 4 groups: Control group, LPS (0.1U/ml) group, UTI (100U/ml) group and (UTI+LPS) group. MTT assay and scratch test were performed to assess cell viability of cardiac micro vascular endothelial cells. Flow cytometry was performed to examine apoptosis. Western blot was performed to examine expression of multiple proteins, including pAkt, Bcl-2, NF-κB, TNF-α and Caspase-3. Compared with control group, LPS treatment indeed increased protein expression of Caspase-3, and resulted in significant apoptosis of cardiac micro vascular endothelial cells. Compared with LPS group, UTI+LPS group had a higher level of cell viability, verified by MTT assay and scratch test. Moreover, expressions of pAkt, NF-κB and Bcl-2 were decreased after UTI treatment, suggesting UTI significantly alleviated LPS induced-apoptosis of cardiac micro vascular endothelial cell via Akt/NF-κB pathway. Ulinastatin could protect cardiovascular system by alleviating LPS-induced injury of cardiac micro vascular endothelial cell. The potential mechanism is Akt/NF-κB pathway.

Keywords: Ulinastatin, LPS, cardiac microvascular endothelial cell, Akt/NF-κB.

INTRODUCTION

Micro vascular endothelium is a novel research hotspot, which is recently considered as not only barrier of vessels but also organs with multiple physiological functions (Gutterman *et al*., 2016. In addition, studies have showed that dysfunction of cardiac micro vascular endothelial cells resulted in a series of cardiovascular diseases, including myocardial ischemia, angina and arrhythmia (Ter Maaten *et al*., 2016). Moreover, lipopolysaccharide (LPS)-induced injury mainly contributed to dysfunction of cardiac micro vascular endothelial cells (Huang *et al*., 2015), however, detailed mechanisms are unclear on how LPS induced injury of cardiac micro vascular endothelial cells, and novel therapies were required to improve clinical treatment for micro vascular endothelium.

As a Kunitz protease inhibitor, ulinastatin (UTI) is a glycoprotein widely distributed in urine and blood (Lv *et al*., 2016). Previous studies have proved that UTI was an important natural anti-inflammatory with effect to inhibit release of inflammatory mediators Wang *et al*., 2016. Clinical trials demonstrated that UTI alleviated immune response of patients with septicopyemia so as to shorten length of stay and improve prognosis (Wang *et al*., 2015). What's more, UTI has been used as a therapeutic for acute pancreatitis with promising efficacy (Wang *et al*., 2016). There are reports that UTI could maintain intact micro vascular endothelium of multiple organs, but no evidence was about effect of UTI on cardiac micro vascular endothelial cells (Wang *et al*., 2016). In the aspect of molecular mechanism, both apoptosis and autophagy pathways were reported to be influenced by UTI, and expressions of NF-κB were decreased after UTI treatment, verified by animal experiments (Zhang *et al*., 2016; Ma *et al*., 2016; Ouyang *et al*., 2016). All these indicated that UTI could be a potential therapeutic for injury of cardiac micro vascular endothelial cell.

In summary, we hypothesized that UTI could alleviated LPS-induced injury of cardiac micro vascular endothelial cell via apoptosis or autophagy pathways and NF-κB might be a pivotal factor to understand cardioprotective effect of UTI.

MATERIALS AND METHODS

Isolation of cardiac micro vascular endothelial cells

Neonatal Sprague Dawley rats were purchased from Guangdong medical experimental animal center. Isolation of cardiac micro vascular endothelial cells was performed as described previously (Zhu *et al*., 2016). Briefly, the neonatal heart tissue was dissected and homogenized in a sterile environment with cold Phosphate Buffered Saline (PBS) treatment. The tissue homogenate was filtered through a 41 μm nylon mesh (Spectrum), and the nylon mesh was washed three times with PBS. Micro vessels retained on the mesh were then washed off and pelleted by centrifugation at 4000g for 10min at 4°C. The pellets

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were resuspended in 15% dextran T-500 and then added onto 20% dextran T-500, followed by centrifugation at 25,000 g for 10 min at 4°C. The pellets were collected to harvest cardiac micro vascular endothelial cells.

Grouping for cardiac micro vascular endothelial cells

Cardiac micro vascular endothelial cells were cultured for 48 hours in routine medium. Cardiac micro vascular endothelial cells were randomly assigned into four groups. Control group was normally cultured for further experiment. Add LPS solution (0.1U/ml) to induce injury in LPS group. UTI+LPS group was treated with LPS solution (0.1U/ml) and UTI solution (100U/ml), respectively. UTI group was treated with UTI solution (100U/ml) as positive control. Continue culture cells in four groups in the same condition (37^o, 5%CO_2).

MTT assays

MTT (5mg/ml, 30μl) reaction solution was added into four different Cardiac micro vascular endothelial cell groups, cultured at 37□ for 3h. Rinse cells for 3 times, then add DMSO into cardiac micro vascular endothelial cells to terminate reaction for 10min. In the end, the optical density was detected for squamous carcinoma cells Colon16 at 492nm in micro plate reader.

Scratch test

Digest cells into single cell suspension. All cells were inoculated into a 6-well plate with a concentration of 10^6 cells per well overnight, and scratched vertically with a 100μl micro pipette tip on the next day. Thereafter, cells were rinsed twice with PBS and placed in serum-free culture medium. After 24 h and 48 h, cells were counted under an invert phase-contrast microscope in five random fields. Every migration test was performed in triplicate.

Flow cytometry

Flow cytometer was performed according to the introduction of kit, detailed protocols were as following: Adjust the treated cell concentration at 10^5/ml. Cell suspension was mixed with reaction solution and FITC-Annexin V reagents by ratio of 250: 50: 1, reacting for 15min at room temperature. Then expression of phosphatidylserine was detected in different group. Emission and absorb wavelengths were 488nm and 625nm, respectively.

Activity assay was performed according to the introduction of kit, and specific steps were shown as following: Adjust the treated cell concentration at 10^5/ml, then chromophoric substrate were added into the lysis, culturing 30min at 37^o. Finally, absorbance value was assessed at 490nm, which indicated the relative activity of caspase-3.

Western blot

Culture primary cardiac micro vascular endothelial cells were further cultured for 48 hours. Western blot was performed to examine expression of micro vascular endothelial cells. The protocol was as follows: Lyse cells with RIPA Lysis buffer. Transfer protein with PVDF membrane after SDS-PAGE gel electrophoresis. Block transferred PVDF membrane with defatted milk (5%). Incubate with CXCR7 antibody. GAPDH was used as internal control. Examine specific protein bands with chemiluminescence. Gel imaging system was used to analyze relative expression of specific protein bands.

STATISTICAL ANALYSIS

All results were analyzed with SPSS16.0. Single factor variance was applied in the inter group analysis. $P<0.05$ was considered to be statistically significant.

RESULTS

LPS induced injury of cardiac micro vascular endothelial cell

Compared with control group, LPS group had a higher ratio of apoptosis, verified by flow cytometry (fig. 1). In addition, Western blot showed that LPS treatment significantly increased expression of Caspase-3 (fig. 2). All these indicated that LPS treatment indeed caused remarkable injury of cardiac micro vascular endothelial cell, suggesting LPS-induced injury model was successfully established.

UTI improved migration of cardiac micro vascular endothelial cell

Cell wound scratch test demonstrated that UTI treatment improved migration of cardiac micro vascular endothelial cell, and alleviated negative impact of LPS. At 24-hour after culture, migration distances in four groups were 4.23±1.13μm (LPS), 24±6.85μm (UTI), 7.61±2.65μm (LPS+ UTI) and 10.32±3.13μm (control), respectively. At 36-hour after culture, migration distances in four groups were 7.23±2.46μm (LPS), 38±7.14μm (UTI), 14.62±1.97μm (LPS+ UTI) and 18.32±3.41μm (control), respectively (fig. 3). Such findings elucidated UTI treatment significantly promoted migration of cells ($P<0.05$), while LPS-induced injury significantly attenuated migration of cells ($P<0.05$), suggesting UTI could improve migration. What's more, effect of UTI on migration was time-dependent, verified by cell wound scratch test at different time points.

UTI treatment decreased apoptosis of cardiac micro vascular endothelial cell

Compared with LPS group, LPS+ UTI group had a lower ratio of apoptosis and better cell viability, verified by flow cytometry (fig. 1) and MTT assay (fig. 4), respectively. Western blot also showed that UTI+LPS group had a lower level of Caspase-3 than LPS group (fig. 2), suggesting UTI treatment abrogated apoptosis-promoting effect of LPS on cardiac micro vascular endothelial cells.

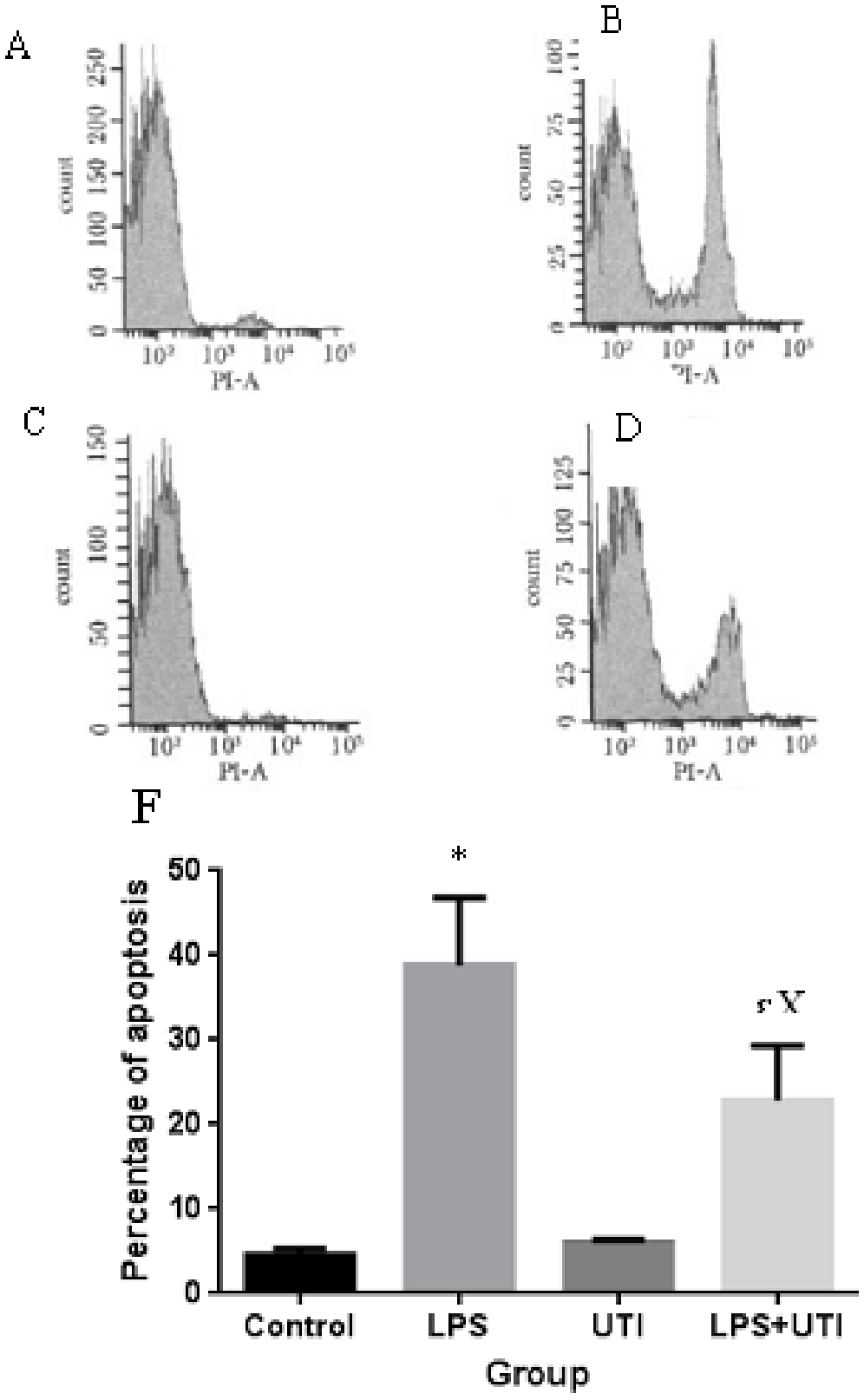


Fig. 1: (A) Cell distribution in control group. (B) Cell distribution in LPS group. (C) Cell distribution in UTI group. (D) Cell distribution in LPS+UTI group. (F) Analysis of apoptosis in four groups.*Compared with control, P<0.05; **Compared with LPS, P<0.05.

UTI decreased expressions of Akt/NF-κB pathway related proteins

Western blot showed that, compared with control group, LPS increased expressions of pAkt, NF-κB, Bcl-2 and TNF-α (fig. 2). Increased expressions of pAkt, NF-κB and Bcl-2were significantly abrogated after UTI treatment, while no difference was observed in expression of TNF-α, suggesting UTI decreased expressions of Akt/NF-κB pathway related proteins (fig. 2). Moreover, there was no difference between control group and UTI group, suggesting UTI did not influence baseline state of cardiac micro vascular endothelial cells.

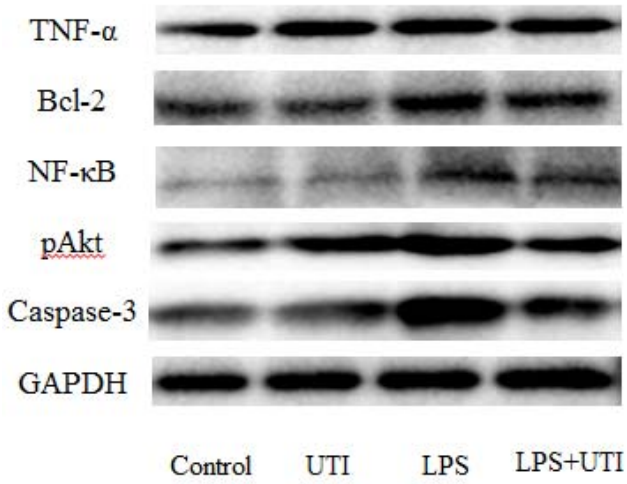


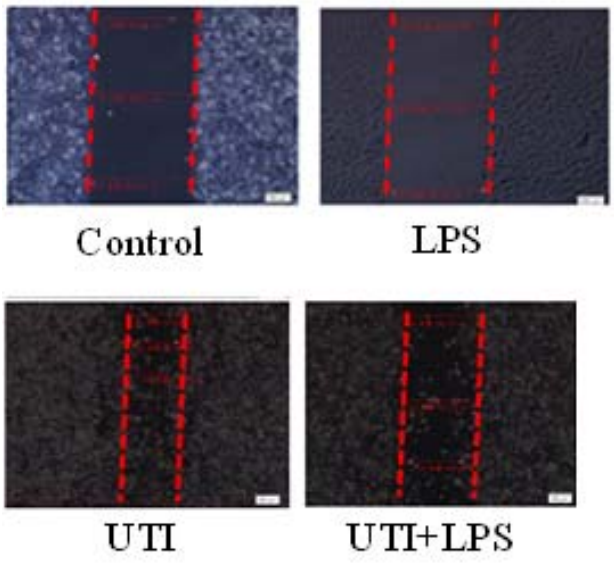
Fig. 2: Western blot for multiple proteins in four groups.

DISCUSSION

Our study confirmed that LPS indeed contributed to injury of cardiac micro vascular endothelium. What's more, we proved that ulinastatin *in vivo* alleviated LPS-induced injury of cardiac micro vascular endothelium via Akt/NF-κB pathway, suggesting ulinastatin could be a potential therapeutic for cardiovascular disease.

Ulinastatin has been widely used for treatment of pancreatitis in clinical scenario due to its promising apoptosis inhibiting effect (Liu *et al*., 2014; Abraham *et al*., 2013). Considering such effect, researcher further explored the role of ulinastatin in endothelium, and found some hints that ulinastatin protect intact structure of endothelium (Zhang *et al*., 2016; Zhu *et al*., 2016; Atal *et al*., 2016). However, it is still unclear whether ulinastatin protects cardiac micro vascular endothelium. Previous study indicated that Akt/NF-κB pathway was under regulation of ulinastatin. In another hand, Akt/NF-κB pathway indeed influenced cardiac micro vascular endothelium (Jiang-Ying *et al*., 2016). Our study demonstrated that ulinastatin protected cardiac micro vascular endothelium via Akt/NF-κB pathway, verified by flow cytometry, scratch test and Western blot. Intriguing, it has been proved that ulinastatin improved healing of pulmonary micro vascular endothelial cells (Li *et al*., 2015), and we broadened roles of ulinastatin in cardiac micro vascular endothelium.

A



B

	Control	UTI	LPS	UTI+LPS
24 hour	10.32±3.13μm	24±6.85μm	4.23±1.13μm*	7.61±2.65μm**
36 hour	18.32±3.41μm	38±7.14μm	7.23±2.46μm*	14.62±1.97μm**

Fig. 4: (A) Image of cell migration. (B) Analysis of migration distances in four groups.*Compared with control, P < 0.05; **Compared with LPS, P<0.05

As a novel field in cardiovascular diseases, cardiac micro vascular endothelium has been proved with pivotal role in protection of heart. Although multiple factors could cause injury of cardiac micro vascular endothelium, lipopolysaccharide (LPS) was a leading factor which contributed to the most injury cases (Nickols *et al*., 2015; Fan *et al*., 2016). LPS is mainly distributed in cell wall of gram negative bacteria, and always has biological toxicity to host (Nickols *et al*., 2015). Further degradation of LPS produces endotoxin, and such substances jeopardize our cardiovascular system (Appleby *et al*., 2016; Gao *et al*., 2016). Our study proved that ulinastatin could alleviate LPS-induced injury, consistent with previous report. Moreover, we found that ulinastatin inhibited ill effect of LPS via regulating Akt/NF-κB pathway. Our study also showed that ulinastatin did not influence expression of TNF-α, but decreased pAkt, Bcl-2 and NF-κB. These finding suggested that ulinastatin might influence autophagy to maintain protective effect, specifically, ulinastatin regulated Akt/NF-κB pathway.

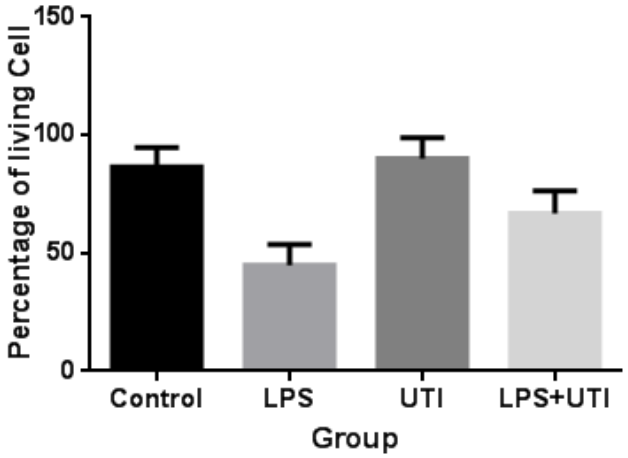


Fig. 4: MTT assays for percentage of living cells in four groups. *Compared with control, P<0.05; **Compared with LPS, P<0.05

In summary, ulinastatin is beneficial for cardiac micro vascular endothelium, and can alleviate LPS-induce injury via Akt/NF-κB pathway. Further animal experiment should be performed to explore detailed effect of ulinastatin for a better understanding.

CONCLUSION

Ulinastatin alleviated LPS-induced injury of cardiac micro vascular endothelial cell via Akt/NF-κB pathway. Ulinastatin can protect cardiovascular system promisingly, and is a potential therapeutic for cardiovascular diseases.

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