Ulinastatin protects against lipopolysaccharide-induced cardiac microvascular endothelial cell dysfunction via downregulation of lncRNA MALAT1 and EZH2 in sepsis

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Abstract. The present study aimed to evaluate the effects of ulinastatin on the permeability and apoptosis of lipopolysaccharide (LPS)-induced cardiac microvascular endothelial cells (CMVECs), and investigate its molecular mechanisms in sepsis. The sepsis rat model was induced by cecal ligation and puncture (CLP), and rat CMVECs were isolated and treated with LPS or/and ulinastatin. Then, cell permeability was evaluated by transendothelial electrical resistance, reactive oxygen species (ROS) levels were assessed by 2,7-dichlorofluorescein diacetate, cell apoptosis was detected using Annexin V-FITC apoptosis detection kit, and the expression levels of Bcl-2, Bax, caspase-3, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and EZH2 were detected by RT-qPCR and/or western blotting. In addition, the relationship of MALAT1 and EZH2 was evaluated by RNA immunoprecipitation (RIP) assay and chromatin immunoprecipitation (ChIP) assay. Compared with LPS-induced CMVECs, treatment with ulinastatin significantly reduced CMVEC permeability and the percentage of apoptotic cells, as well as an increased level of Bcl-2 and inhibited the levels of ROS, caspase-3 and Bax (all p<0.05). In addition, long non-coding RNA (lncRNA) MALAT1 was confirmed to interact with EZH2 in CMVECs. Overexpression of lncRNA MALAT1 and EZH2 was found in the hearts of the sepsis rat and LPS-induced CMVECs, while ulinastatin inhibited the expression of lncRNA MALAT1 and EZH2 in the LPS-induced CMVECs (all p<0.05). Ulinastatin protected against LPS-induced CMVEC cell hyperpermeability and apoptosis via downregulation of lncRNA MALAT1 and EZH2 in sepsis.

Introduction

Sepsis is a severe inflammatory disease in response to bacterial infection (1). It is characterized by systemic inflammatory response syndrome (SIRS) and is frequently caused by hemorrhage, trauma and abdominal surgery (1). In the intensive care unit, severe sepsis is considered as a leading cause of death due to the occurrence of SIRS and multiple organ dysfunctions (2,3). Thus, it is imperative to understand the underlying pathogenesis and search for effective intervention and therapy for sepsis.

Previous study has demonstrated that cardiac dysfunction is induced in ~50% of critically ill patients with sepsis, and the recovery of cardiac function is considered as a key predictor for survival (4). It is reported that cardiac microvascular dysfunction is associated with cardiac dysfunction in patients with sepsis (5). Importantly, cardiac microvascular endothelial cells (CMVECs), accounting for 1/3 of all heart cells, exert an important influence on the normal condition of coronary microvessels and adjacent cardiomyocytes (6). In addition, CMVEC dysfunction, such as hyperpermeability and apoptosis caused by inflammatory responses, is found in myocardial ischemia/reperfusion injury (7,8). Although cardiac microvascular dysfunction is confirmed in sepsis, few studies have investigated the function of CMVECs in sepsis.

Ulinastatin, a protease inhibitor, exhibits anti-inflammatory activity by inhibiting the expression of pro-inflammatory factors and blocking the signaling pathways related to inflammation (9). Several animal experiments have demonstrated a protective role of ulinastatin against sepsis by suppressing the inflammatory response (10-12). Randomized controlled clinical studies have further revealed that ulinastatin can significantly improve organ failure and reduce mortality in patients with sepsis (13,14). However, it is still unclear whether the role of ulinastatin in sepsis is associated with the function of CMVECs.

Currently, increasing evidence has demonstrated that long non-coding RNAs (lncRNAs) are involved in various diseases (15), including neurodegenerative diseases (16), cardiovascular diseases (17) and cancers (18) by regulating target gene transcription. Understanding the underlying mechanisms of lncRNAs in sepsis may contribute to an effective
treatment for sepsis. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was originally known as an lncRNA in patients with non-small cell lung cancer (NSCLC) who have a high risk of metastasis. A recent study reported that MALAT1 is associated with cell proliferation and migration in cancer cells (19). Nevertheless, its role in CMVEC dysfunction of sepsis is still poorly investigated. In the present study, a sepsis model in the rat was induced by cecal ligation and puncture (CLP), and rat CMVECs were isolated and treated by lipopolysaccharide (LPS) or/and ulinastatin. We aimed to evaluate the effects of ulinastatin on the permeability and apoptosis of LPS-induced CMVECs and investigate its molecular mechanisms.

**Materials and methods**

**Ethics statement.** Healthy male Sprague-Dawley (SD) rats (~100 g of body weight) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Approval from the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Xinjiang Medical University was obtained prior to all animal experiments.

**Animal model of sepsis.** A total of 50 SD rats were randomly assigned into two groups: a sham group (n=10) and a sepsis group (n=40). All rats were acclimatized 3-5 days before the experiments. The rat sepsis model was established using CLP according to a previously described (20). CLP induced intra-abdominal infection and the release of LPS, resulting in SIRS and sepsis. Briefly, the rats were anesthetized by an intraperitoneal injection of 10% chloral hydrate (3 ml/kg; Sigma, St. Louis, MO, USA) and fixed in a supine position. A 1.5-cm longitudinal incision was made along the abdominal midline, then the caecum was exposed and separated. For rats in the sepsis group, the caecum below the ileocaecal valve was ligated using 3.0 suture. Then, a 18-gauge needle was used to puncture through the caecum 3 times, and the feces was extruded by gently squeezing the caecum. Lastly, the separated caecum was returned to the abdominal cavity and the incision was sutured layer by layer. Rats in the sham group were treated with similar procedures but without ligation or puncture. The entire operation was carried out under aseptic conditions. The rats were sacrificed 24 h post-operatively, and the heart tissues were collected for the following experiments.

**Isolation of CMVECs.** CMVECs were isolated from SD rats following a previously reported method (21). In brief, two SD rats were anesthetized and then intraperitoneally injected with heparin (300 IU/g; Sigma) for 20 min. Then, the rats were sacrificed by cervical dislocation. After sterilization with 75% alcohol, the thoracic cavity was opened. Then, the heart was removed and rinsed with sterile D-Hanks’ containing heparin. Next, the connective tissue, atria, right ventricle and interventricular septum were removed, and epicardial and endocardial cells from the left ventricle were devitalized by immersing in 75% ethanol for 20-30 sec. The remaining tissue was minced and rinsed with D-Hanks’, and then incubated in 0.02% collagenase type II (dissolved in D-Hanks’) for 30 min and 0.02% trypsin (both from Sigma) for another 10 min at 37°C. The dissociated cells were centrifuged at 1,000 rpm for 10 min, and then resuspended in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Cambridge, MA, USA) containing 15% fetal bovine serum (FBS). Lastly, the cells were cultured in laminin-coated dishes (Corning, Inc., Corning, NY, USA).

**Cell treatment.** LPS was used to induce the sepsis cell model. To determine the optimum inducement time and concentration, the primary CMVECs were treated with various concentrations (0, 10, 50, 100 ng/ml, 1 and 2 µg/ml) for 0, 3, 6, 9 and 12 h, respectively. To investigate the effect of ulinastatin on the LPS-induced CMVECs, the CMVECs were treated with LPS and LPS + ulinastatin (1000, 3000 or 5000 U/l), respectively. In addition, the treated cells were also transfected with the lncRNA MALAT1 overexpression vector (named as pc-MALAT1; RiboBio, Guangzhou, China) using Lipofectamine 2000 (Invitrogen).

**Assessment of cell permeability.** To evaluate cell permeability, transendothelial electrical resistance (TER) was measured using STX2 electrode and EVOM2 meter (World Precision Instruments, Sarasota, FL, USA). CMVECs cells were seeded on fibronectin-coated Transwell membrane inserts (0.4-µm pore size; Corning, Inc.). Then, confluent CMVECs were treated with LPS, LPS + ulinastatin or LPS + ulinastatin + lncRNA MALAT1 overexpression vector, respectively. The value of TER was calculated as the resistance value multiplied by the membrane area.

**Cell apoptosis assay.** The Annexin V-FITC apoptosis detection kit (Invitrogen) was used to quantify cell apoptosis. Briefly, the treated cells were collected by digestion with Trypsin and centrifugation at 1,500 rpm for 6 min. After being washed with phosphate-buffered saline (PBS), the cells were mixed with FITC-Annexin V and propidium iodide (PI) for 15 min at 25°C in the dark. Cells were then added to 400 µl 1X binding buffer and detected using a flow cytometer. Annexin V-positive and PI-negative cells were considered as apoptotic cells.

**Reactive oxygen species (ROS) assay.** The ROS level was assessed by 2,7-dichlorofluorescein diacetate (DCFH-DA; Sigma). After being washed with PBS, the treated cells were incubated with serum-free DMEM containing 10 µM DCFH-DA for 20 min at 37°C in the dark. Next, the cells were collected and analyzed using a Spectra Max M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The fluorescent intensities were measured at a 535 nm absorbance.

**Real-time-quantitative polymerase chain reaction (RT-qPCR) analysis.** Total RNA extraction was carried out using TRIzol reagent (Invitrogen), and complementary DNA was obtained by the reverse transcription of total RNA using the MultiScribe RT kit (Applied Biosystems, Foster City, CA, USA). Then, the levels of target genes, including lncRNA MALAT1, B-cell lymphoma-2 (Bcl-2), Bax, caspase-3, EZH2, DAB2IP and Brachyury were determined using SYBR® Premix Ex Taq™ (Takara Biotechnology Co., Ltd., Dalian, China). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal reference. Primers for these genes are listed in

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Table I. Primer sequences for specific genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
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<tbody>
<tr>
<td>RT-qPCR</td>
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<tr>
<td>MALAT1</td>
<td>F: 5'-AAAGCAAGGTCTCCCACAAG-3'</td>
</tr>
<tr>
<td></td>
<td>R: 5'-GTCAGTGGACTAGTACAAGC-3'</td>
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<tr>
<td>Bcl-2</td>
<td>F: 5'-GATGCGTACCAACAAAG-3'</td>
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<td></td>
<td>R: 5'-AGTGAATGTGGCCGTACC-3'</td>
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<tr>
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<td>F: 5'-GGATGCCCATGACTGACC-3'</td>
</tr>
<tr>
<td></td>
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<td>Caspase-3</td>
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<td></td>
<td>R: 5'-TTGGTGCGGAACGCGTAAAATC-3'</td>
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<tr>
<td>DAB2IP</td>
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<tr>
<td></td>
<td>R: 5'-GCAATAGGTCCTCTTCTTG-3'</td>
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<tr>
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<td>F: 5'-AGGTTGGAGATTTCTTCTC-3'</td>
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<td></td>
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<td></td>
<td>R: 5'-CGTTCATCCCACGACAAACA-3'</td>
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<tr>
<td>ChIP-qPCR</td>
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<td>DAB2IP</td>
<td>F: 5'-CTGTGCTTCTGTTCTCTTCTTG-3'</td>
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<td>R: 5'-GGATGCCCTGACCCGTAATGCC-3'</td>
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<tr>
<td>Brachury</td>
<td>F: 5'-AGGTTGGAGATTTCTTCTC-3'</td>
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<tr>
<td></td>
<td>R: 5'-AGGTTGGAGATTTCTTCTC-3'</td>
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| RNA immunoprecipitation (RIP) assay. Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore) was used to perform RIP assay following the manufacturer's instructions. In brief, CMVECs were treated with LPS for 6 h. Then, the cells were collected and treated with RIPA buffer. After centrifugation, the supernatant was incubated with protein-A/G-Sepharose beads, anti-EZH2 monoclonal antibody and IgG overnight at 4°C. Subsequently, co-precipitated RNA was extracted and then detected by RT-qPCR.

Chromatin immunoprecipitation (ChIP) assay. ChIP assay was carried out using QuickChIP kit (Imgenex, San Diego, CA, USA). In brief, CMVECs were transfected with two siRNAs targeting MALAT1 (si-MALAT1-1 and si-MALAT1-2; Dharmaco, Mickleton, NJ, USA) using Lipofectamine 2000 for 48 h. Then, protein-chromatin cross-linking of cells was induced using 1% formaldehyde for 10 min at 37°C and quenched by glycine. Next, the cells were lysed in lysis buffer and chromatin DNA from cell lysates was sonicated into fragments. Next, chromatin DNA fragments were subjected to immunoprecipitation with EZH2 or methyltransferase catalyzing histone H3 lysine 27 trimethylation (H3K27me3) antibody. After reverse cross-linking, DNA fragments were purified and then detected by RT-qPCR.

Western blotting. RIPA buffer (Sangon Biotech, Shanghai, China) was used to extract protein from the heart tissues and cells. The measurement of protein concentration was conducted using BCA Protein Quantitative assay (Pierce, Appleton, WI, USA). Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then blotted onto nitrocellulose membranes (Millipore, Bedford, MA, USA). After being blocked with 5% nonfat milk in Tris-buffered saline Tween-20 (TBST), the membranes were incubated with rat anti-Bcl-2, Bax, caspase-3, EZH2, DAB2IP, Brachury and GAPDH monoclonal antibodies (1:500-1,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C, respectively. After being washed with TBST, the membranes were incubated with goat anti-rat secondary antibody (1:5,000; Santa Cruz Biotechnology, Inc.) for 2 h at room temperature. Ultimately, the proteins were detected with Enhanced chemiluminescence (Millipore, Bedford, MA, USA).

RNA immunoprecipitation (RIP) assay. Magna RIP RNA-Binding Protein Immunoprecipitation kit (Millipore) was used to perform RIP assay following the manufacturer's instructions.
Ulinastatin inhibits the expression of IncRNA MALAT1 and EZH2 in LPS-induced CMVECs. To investigate the mechanism of ulinastatin in LPS-induced CMVECs, the expression levels of IncRNA MALAT1 and EZH2 were detected. The results revealed that the levels of IncRNA MALAT1 and EZH2 were significantly upregulated in the hearts of rat from the sepsis group in comparison with these levels in the sham group (p<0.01) (Fig. 3A). Similarly, in vitro experiments showed that LPS markedly elevated the expression levels of IncRNA MALAT1 and EZH2 compared with the levels in the untreated cells (p<0.01) (Fig. 3B), while ulinastatin inhibited the upregulation of IncRNA MALAT1 and EZH2 (p<0.05) (Fig. 3B).

IncRNA MALAT1 interacts with EZH2 in CMVECs. RTP assay was performed to evaluate the relationship between IncRNA MALAT1 and EZH2. The results revealed that IncRNA MALAT1 could bind to EZH2 (Fig. 4A), indicating the interaction of IncRNA MALAT1 and EZH2. Then, the IncRNA MALAT1 level was successfully inhibited by the transfection of si-MALAT1-1 and si-MALAT1-2 (p<0.05) (Fig. 4B). Silencing of IncRNA MALAT1 markedly inhibited the expression of...
Figure 3. Ulinastatin inhibits the levels of long non-coding RNA (lncRNA) metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) and EZH2 in lipopolysaccharide (LPS)-induced cardiac microvascular endothelial cells (CMVECs). (A) The levels of lncRNA MALAT1 and EZH2 in the hearts of sepsis and sham rats. (B) The levels of lncRNA MALAT1 and EZH2 in the LPS-induced CMVECs with or without ulinastatin (UTI) treatment. *p<0.05 and **p<0.01 compared with the control group; #p<0.05 compared with the LPS group.

Figure 4. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) interacts with EZH2 in cardiac microvascular endothelial cells (CMVECs). (A) RNA immunoprecipitation assay confirmed that long non-coding RNA (lncRNA) MALAT1 could bind to EZH2. (B) The levels of MALAT1 as well as EZH2 target genes DAB2IP and Brachyury in CMVECs transfected with si-MALAT1-1 and si-MALAT1-2. (C) The levels of DAB2IP and Brachyury in CMVECs when MALAT1-knockdown cells were precipitated with EZH2 by chromatin immunoprecipitation (ChIP) assay. (D) The levels of DAB2IP and Brachyury in CMVECs when MALAT1-knockdown cells were precipitated with histone H3 lysine 27 trimethylation (H3K27me3) by ChIP assay. *p<0.05 compared with the control group.
EZH2 target genes, DAB2IP and Brachyury (p<0.05) (Fig. 4B) compared with that in the untreated cells. Furthermore, the effect of IncRNA MALAT1 on EZH2 was evaluated by ChIP assay. The results revealed that when IncRNA MALAT1-knockdown cells were precipitated with EZH2 or H3K27me3 (the composition of EZH2) antibody, the expression levels of DAB2IP and Brachyury were both inhibited compared with levels in the untreated cells (p<0.05) (Fig. 4C and D).

**Ulinastatin protects against LPS-induced CMVEC dysfunction via downregulation of IncRNA MALAT1 and EZH2.** The above results showed that ulinastatin induced the downregulation of IncRNA MALAT1 in the LPS-induced cells. Thus, we investigated the effect of overexpression of IncRNA MALAT1 on CMVEC permeability and apoptosis following treatment with ulinastatin and LPS. Compared with the untreated cells, the IncRNA MALAT1 level was significantly increased (p<0.01) (Fig. 5A) in cells transfected with pc-MALAT1. Then, the results revealed that upon treatment with ulinastatin, upregulation of IncRNA MALAT1 markedly increased CMEC permeability (Fig. 5B) and apoptosis (Fig. 5C) in the LPS-induced cells (all p<0.05).
Cardiac microvascular dysfunction in sepsis has been demonstrated (5) and the protective role of ulinastatin against sepsis has also been reported (22). Nevertheless, the effects of ulinastatin on CMVEC dysfunction in sepsis are still unclear. The present study revealed that compared with LPS-induced CMVECs, the treatment of ulinastatin significantly reduced CMVEC permeability and apoptosis. In addition, we found upregulated IncRNA MALAT1 and EZH2 in the hearts of sepsis rats and LPS-induced CMVECs, while ulinastatin had an inhibitory effect on the upregulation of IncRNA MALAT1 and EZH2.

Several studies have confirmed the LPS-induced sepsis cell or animal model (23,24). CMVEC dysfunction has been reported to be associated with the occurrence of sepsis (5,25). This study found that LPS treatment promoted CMVEC dysfunction, such as hyperpermeability and apoptosis. Consistently, Janicke et al (26) demonstrated the hyperpermeability and apoptosis in endothelial cells induced by LPS. In addition, we found that ulinastatin reduced LPS-induced CMVEC hyperpermeability and apoptosis. Previous studies have shown that ulinastatin had inhibitory effects on apoptosis, oxidative stress and inflammation (9,27). The protective effect of ulinastatin against vascular endothelial injury was also reported in patients who underwent heart operation (28). Moreover, Li et al (29) reported that ulinastatin inhibited cell hyperpermeability and apoptosis in oxidant-induced human umbilical endothelial cells. Lin et al (30) also revealed that ulinastatin attenuated the hyperpermeability of rat lung microvascular endothelial cells when treated by shock serum. All these results collaborate our results. The present study further revealed that ulinastatin inhibited the levels of ROS, caspase-3 and Bax as well as increased the level of Bcl-2 in LPS-induced CMVECs. It was well known that ROS induce mitochondrial oxidative stress and then promote the release of cytochrome c (31). Oxidative stress plays a vital regulatory role in mitochondria dependent apoptotic signaling (32). Mitochondria-mediated apoptosis could be regulated by anti-apoptotic factor Bcl-2, pro-apoptotic factor Bax (26), as well as caspase-3 activation, which increase membrane permeability (32,33). These results suggest that ulinastatin inhibits LPS-induced cell hyperpermeability and apoptosis through a mitochondrial-dependent pathway.

The present study further investigated the role of IncRNA MALAT1 in CMVEC dysfunction in sepsis. The results revealed the upregulation of IncRNA MALAT1 and EZH2 in vitro and in vivo sepsis model, while ulinastatin inhibited the expression of IncRNA MALAT1 and EZH2. Previous studies have shown overexpression of MALAT1 in cancers, including non-small cell lung (34), bladder (35), colorectal (36) and prostate cancer (37). Recently, MALAT1 has been reported to regulate the production of inflammatory cytokines in cancers (38). Polycomb protein EZH2 was also reported to be overexpressed in cancers (39). EZH2 was considered as an oncogenic gene that interacts with EED and SUZ12 by forming the polycomb repressive complex-2 (PRC2) (40). H3K27me3 is a key functional component of EZH2 (40). This study revealed that IncRNA MALAT1 could bind to EZH2, and MALAT1 silencing inhibited the expression of EZH2 target genes DAB2IP and Brachyury in cells precipitated with EZH2 or H3K27me3. These results indicated the interaction of MALAT1 and EZH2 in CMVECs, which was consistent with the study of Wang et al (41). Increasing evidence has shown that both MALAT1 and EZH2 are associated with cell proliferation, apoptosis and metastasis in cancer cells (42,43). All these results indicate that ulinastatin may inhibit LPS-induced CMVEC dysfunction by inhibiting IncRNA MALAT1 and EZH2. The diagram illustrating the mechanism of ulinastatin in LPS-induced CMVECs is shown in Fig. 6.

Ulinastatin protects against LPS-induced CMVEC hyperpermeability and apoptosis via downregulation of IncRNA MALAT1 and EZH2 in sepsis.

Acknowledgements

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References


