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Inhibition of MRP4 alleviates sepsis-induced acute lung injury in rats

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ABSTRACT

This study was undertaken to examine the regulatory role of multidrug resistance-associated protein 4 (MRP4) in an experimental model of sepsis-induced acute lung injury in rats. Sepsis was induced by cecal ligation and puncture in anesthetized rats. Animals were then randomly assigned to receive intravenous injection of vehicle or MRP4 inhibitor (MK571, 20 mg/kg). The pathological changes were observed by hematoxylin and eosin staining. Lung water content, lung vascular permeability and inflammatory cell count in bronchoalveolar lavage fluid (BALF) were quantified. Serum tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels were measured. In addition, lung tissue cyclic adenosine monophosphate (cAMP) levels were examined by enzyme-linked immunosorbent assay. Furthermore, the effects of MRP4 knockdown on lipopolysaccharide (LPS)-induced endothelial permeability and the cytoskeleton of rat pulmonary microvascular endothelial cells (PMVECs) were detected. The protein expression levels of MRP4, Rac1, VE-cadherin, β-catenin and ZO-1 were measured by Western blot analysis. MK571 significantly reduced lung tissue damage, lung water content and lung vascular permeability. Lung tissue cAMP levels were attenuated in MK571-treated animals compared with vehicle controls. MK571 also decreased sepsis-induced inflammatory cell accumulation in BALF. In addition, the MK571 group had significantly lower serum TNF-α and IL-6 levels compared with vehicle controls. Consistently, knockdown of MRP4 protected against LPS-induced increase in the endothelial permeability and the destruction of cytoskeleton in vitro. Furthermore, silencing MRP4 gene significantly reduced MRP4 protein expression and restored the protein expression of Rac1, VE-cadherin, β-catenin and ZO-1 in rat PMVECs in response to LPS stimulation. These data suggest that inhibition of MRP4 significantly alleviates sepsis-induced acute lung injury in rats.

1. Introduction

Sepsis is a syndrome that is characterized by the systemic inflammation, accelerated activity of the clotting cascade, and endothelial hyperpermeability. Sepsis is associated with significant morbidity and mortality [1]. Although significant progress has been made in the development of therapeutic interventions that can reduce mortality in the past few years, sepsis remains the major cause of death among patients in intensive care units of hospitals. Acute lung injury is one manifestation of sepsis-induced multiple organ dysfunction syndrome, which often indicates the severity of illness [2]. Progressive pulmonary edema typically develops in patients with sepsis, indicating increase in vascular endothelial permeability [3]. Impaired vascular endothelial barrier function is a critical mechanism of edema formation [4]. During sepsis, many cytokines and inflammatory mediators disassemble the intercellular junctions, change the cellular cytoskeletal structure or damage the cell monolayer, and ultimately induce endothelial gaps. This formation of endothelial gap can lead to microvascular leak and pulmonary edema, which are characteristic of sepsis [5]. Thus, therapeutic approaches targeting barrier-stabilizing mechanisms are new strategies for sepsis-induced multiple organ dysfunction syndrome.

It has been established that the RAS superfamily of small guanosine triphosphatases (GTPases) are known to regulate intercellular junctions [6]. Generally, Rac1, Cdc42, and Rap1 primarily act to stabilize endothelial barrier function, whereas RhoA oppositely impairs barrier properties. Recent studies revealed that the second messenger cyclic adenosine monophosphate (cAMP) is involved in stabilizing the endothelial barrier properties [7]. cAMP-elevating agents enhance vascular endothelial barrier functions via activation of Rac1 and Cdc42, as

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well as inhibition of RhoA [8–10]. ATP-binding cassette transporters (ABC transporters) are transmembrane proteins that transport various substrates across cellular membranes by utilizing the energy of ATP binding and hydrolysis [11]. Subfamily ABCC contains thirteen members and nine are referred to as the Multidrug Resistance Proteins (MRPs). MRPs can extrude cAMP from various cells [12]. Pharmacological and genetic inactivation of MRPs has been demonstrated to enhance intracellular cAMP signaling [13,14]. In addition, MRPs are involved in acute inflammatory response by regulating cytokines and inflammatory mediators [15–17]. Multidrug resistance–associated protein 4 (MRP4), a member of ABCC, acts as an energy-dependent transporter for cyclic nucleotides. Recently, MRP4 has been shown as an endogenous regulator of intracellular cAMP level [13]. Inhibition of MRP4 prevents and reverses pulmonary hypertension in mice. In a murine model of zymosan-induced peritonitis, Leite, et al. [18] found that inhibition of MRP4 significantly decreased vascular permeability, edema and inflammation. These observations indicate that MRP4 may exert profound regulatory effects on vascular endothelial barrier dysfunction during sepsis. However, to the best of our knowledge, the effect of inhibition of MRP4 on sepsis-induced vascular endothelial barrier dysfunction and acute lung injury has not yet been studied.

Here, we first report that inhibition of MRP4 by significantly reduced sepsis-induced lung tissue damage, lung water content and lung vascular permeability. Inhibition of MRP4 also decreased sepsis-induced inflammatory cell accumulation in BALF and serum inflammatory factors levels. Similarly, knockdown of MRP4 protected against LPS-induced increase in the endothelial permeability and the destruction of cytoskeleton in vitro. Our findings suggest that inhibition of MRP4 is a novel therapeutic strategy for the treatment of sepsis-induced acute lung injury.

2. Materials and methods

2.1. Animals and sepsis model

Sixty male Sprague-Dawley rats (180–200 g), were purchased from the Laboratory Animal Center of Renmin Hospital of Wuhan University (Wuhan, China). All experiments were approved by the Animal Care and Use Committee of Renmin Hospital at Wuhan University and confirmed the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (the 8th Edition, NRC 2011).

Rats were randomly divided into the Sham group, Sepsis group and Sepsis + MK571 group. Sepsis was induced by cecal ligation and puncture (CLP) as previously described [19]. Briefly, rats were anesthetized with i.m. ketamine (60 mg/kg) and xylazine (10 mg/kg). Then, the cecum was ligated and punctured twice with an 18-gauge needle. Sham control animals underwent the same procedure without CLP. Following CLP, animals in the Sepsis + MK571 group received intraperitoneal injection of 20 mg/kg MK571 (Cayman Chemical Company, USA), and animals in the Sepsis group received the same volume of normal saline. All animals were subcutaneously resuscitated with normal saline (3 ml/100 g body weight) immediately after surgery. At 24 h after CLP, the experiment was terminated.

2.2. Arterial blood gas analysis

Approximately 0.5 ml of arterial blood was collected at the end of the experiment through right carotid puncture for blood gas analysis, and the PaO2/FiO2 ratio was calculated.

2.3. Pathological examination

Lung tissues were fixed with 4% paraformaldehyde for hematoxylin and eosin staining. Lung morphologic changes were observed by light microscopy. The degree of pathological injury was scored based on edema, neutrophil infiltration, hemorrhage, and disorganization of lung parenchyma, as a previously scoring system described [20]. The degree was graded numerically from 0 to 4. Higher scores suggest more severe lung damage.

2.4. Lung vascular permeability assay

Lung vascular permeability was evaluated by measuring Evan's blue dye leakage. Briefly, 1% Evans blue dye (2 ml/kg) was injected intravenously into rats and allowed to circulate for 0.5 h. Then, the pulmonary artery was cannulated and perfused with saline. The left atrium was opened with an incision to allow for drainage effluent. The lungs (100 mg) were then removed and homogenized. The homogenate was incubated for 24 h at 60 °C and then centrifuged for 10 min at 4000*g*. The supernatants were collected and the absorbance was determined by spectrophotometric analysis at a wavelength of 630 nm.

2.5. Wet to dry weight ratio of lung tissues

The lower lobe of left lung was excised, weighed, and then dried for 48 h at 72 °C. Lung wet-to-dry (W/D) weight ratio was measured by dividing wet weight by the dry weight.

2.6. Measurement of cAMP levels in lung tissues

Lung samples were treated with isobutyryl methylxanthine to inhibit phosphodiesterases. Then, the samples were homogenized and the cAMP levels were detected with enzyme linked immunosorbent assay (ELISA) using a commercial cAMP ELISA kit according to the manufacturer's instructions.

2.7. Determination of inflammatory cell influx into the airways

The right main bronchus was clamped, and then the left lung was lavaged three times by instillation of 0.5 ml Hank's balanced salt solution. Bronchoalveolar lavage fluid (BALF) was immediately harvested and centrifuged at 2000 rpm and 4 °C for 10 min to collect the cell pellet. BALF cell counting was performed using a standard hemocytometer.

2.8. Biochemical measurement

Rats were anesthetized and abdominal aorta blood was collected using heparinized syringes. The blood was centrifuged at 2000 rpm and 4 °C for 10 min and the supernatants were separated for tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) determination using Enzyme immunoassay kits according to the operation manual of the kits.

2.9. Cultured pulmonary microvascular endothelial cells and in vitro endothelial permeability assays

Rat pulmonary microvascular endothelial cells (PMVECs) were pursued from Bena Culture Collection (Beijing, China). PMVECs were cultured in Dulbecco's modifed Eagle's medium (DMEM), with 10% fetal bovine serum (Gibco, Invitrogen, Carlsbad, USA), humidified incubator under 37 °C, 5% CO2 when the cells reached 90% confluency by passaging with trypsin-ethylenediaminetetraacetic acid (EDTA; Gibco). To knockdown MRP4 expression, we infected PMVECs with AdshMRP4, or AdshRNA (Hanbio Biotechnology Co., Ltd., Shanghai, China) at a multiplicity of infection of 50. PMVECs were exposed to 1×10^{10} pfu/ml for 24 h, followed by LPS (10 μg/ml) stimulation. Endothelial permeability was determined by horseradish peroxidase (HRP) permeability assay, as previously described [21]. After 2 h, 6 h, 12 h and 24 h, HRP activity was measured with a microplate spectrophotometer at OD450. The morphological characteristic and distribution of F-actin was measured by laser confocal fluorescence microscope.

2.10. Western blotting

The PMVECs were stimulated with LPS for 12 h, and then washed with PBS (4 °C) and lysed using RIPA buffer. After the total cellular protein concentration was determined by BCA Protein Assay kit. The protein samples were loaded on a 10% SDS-polyacrylamide gel and underwent electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After the membranes were blocked by TBST containing 5% non-fat milk for 2 h at room temperature, the membranes were subsequently incubated with primary antibodies: rabbit anti-VE-cadherin, rabbit anti-β-catenin , rabbit anti-ZO-1 and rabbit anti-RAC1 (Bioss, Beijing, China), rabbit anti-MRP4 (Cell Signaling Technology, USA) antibodies overnight at 4 °C with constant agitation. The membranes were then washed and incubated with secondary antibodies: goat anti-rabbit (Invitrogen). All immunoblots were scanned and quantified using the Odyssey Infrared Imaging System, and all values were normalized to β-Actin.

2.11. Statistical analysis

Data are expressed as mean ± SD. Statistical analysis was performed using SPSS 11.0 statistic software. Differences among the different groups were analyzed for statistical significance by one-way ANOVA, followed by LSD-*t*-test. A P value of < 0.05 was considered statistically significant.

3. Results

3.1. Arterial blood gas analysis

As shown in Fig. 1, values of PaO2 and PaO2/FiO2 in the Sepsis group were significantly smaller than those in the Sham group, suggesting that lungs of rats were damaged by sepsis. These values in the Sepsis + MK571 group were significantly improved when compared with the Sepsis group ($P < 0.05$).

3.2. Lung histological alteration

Fig. 2 exhibited the results of lung tissue pathological examination. As demonstrated in Fig. 2A, no pathological changes were observed in the Sham group, however, there were serious injuries in the Sepsis group. After administration of MK571, lung injury mitigated to different degrees. Fig. 2B showed that the histological scores were significantly higher in the Sepsis group than that in the Sham group ($P < 0.01$). Administration of MK571 alleviated the lung injury as suggested by decreasing histological scores.

3.3. Lung vascular permeability and lung tissue W/D ratio

As shown in Fig. 3A, sepsis led to a significant increase in lung vascular leakage as indicated by a prominent increase in vascular Evans blue leakage. Sepsis-induced vascular leakage was significantly less after treatment with MK571. Fig. 3B showed that the W/D ratio was significantly higher in the Sepsis group than that in the Sham group ($P < 0.01$). Compared with the Sepsis group, the W/D ratio in the Sepsis + MK571 group was decreased obviously ($P < 0.01$).

3.4. The cAMP concentration of lung tissue

As shown in Fig. 4, the cAMP levels in lung tissues were significantly lower in the Sepsis group than that in the Sham group and it was up-regulated in the Sepsis + MK571 group.

3.5. Serum TNF-α and IL-6 Levels and BALF inflammatory cell counting

For the Sham group, TNF-α and IL-6 were maintained at a low level in the serum. After CLP, TNF-α and IL-6 levels was sharply elevated, suggesting the great release of inflammatory factors. When MK571 was administered, production of TNF-α and IL-6 was notably decreased (Fig. 5A/B). There was excessive inflammatory cell infiltration into the lungs of the Sepsis group rats compared with the Sham group, which were dramatically reduced in rats treated with MK571 (Fig. 5C).

3.6. Endothelial permeability and cytoskeleton reorganization in rat PMVECs

Compared with the control cells, treatment of the PMVECs with LPS in vitro resulted in a significant increase in endothelial monolayer permeability. However, AdshMRP4 infection remarkably attenuated LPS-induced PMVECs hyperpermeability (Table 1). The majority of F-actin was localized to the cell periphery in the control cells. Consistently with the protective effects of MRP4 inhibition against LPS-induced hyperpermeability, LPS-induced F-actin rearrangement with stress fiber formation was dramatically attenuated by AdshMRP4 infection (Fig. 6).

3.7. Expression of MRP4, Rac1, VE-cadherin, β-catenin and ZO-1 in rat PMVECs

As shown in Fig. 7, the protein level of MRP4 was significantly up-regulated in the LPS group compared with the control group. Conversely, the protein levels of Rac1, VE-cadherin, β-catenin and ZO-1 were remarkably down-regulated. Silencing MRP4 gene significantly reduced MRP4 protein expression and restored the protein expression of Rac1, VE-cadherin, β-catenin and ZO-1 in the Ad-shMRP4 group compared with the LPS group.

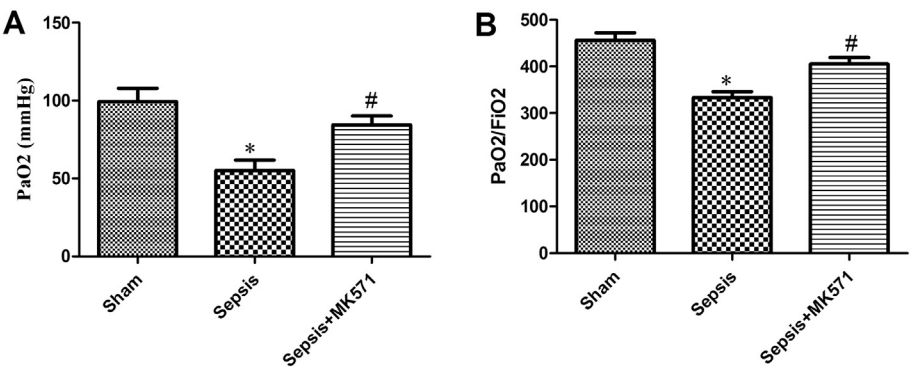

Fig. 1. Changes of PaO2 and PaO2/FiO2 values in the three groups. The values are expressed as mean ± SD. *$P < 0.05$ compared with the Sham group mice; #$P < 0.05$ compared with the Sepsis group.

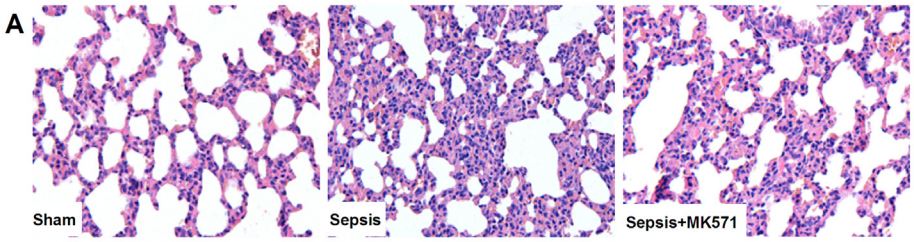

Fig. 2. Pathological changes of lung tissues in the three groups. (Left) The lung tissues were histologically analyzed by hematoxylin and eosin in different groups (×200). A: Sham group, B: Sepsis group, C: Sepsis + MK571 group. (Right) Lung injury was assessed by histological scores for each group. The values are expressed as mean ± SD. $*P < 0.05$ compared with the Sham group mice; $\#P < 0.05$ compared with the Sepsis group.

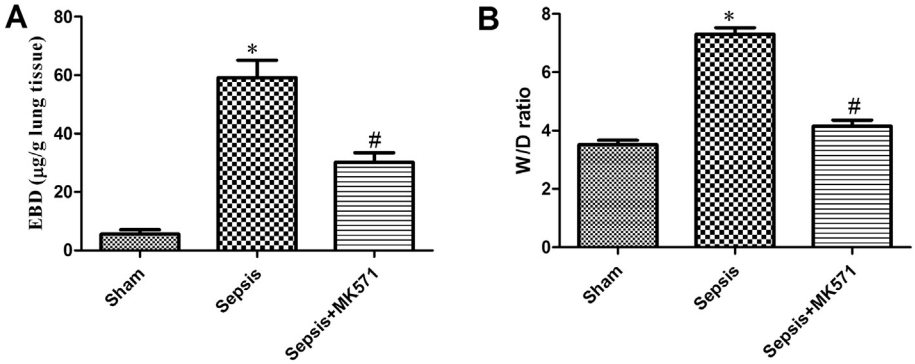

Fig. 3. Lung vascular Evans blue leakage (A) and W/D ratio (B) in the three groups. The values are expressed as mean ± SD. $*P < 0.05$ compared with the Sham group mice; $\#P < 0.05$ compared with the Sepsis group.

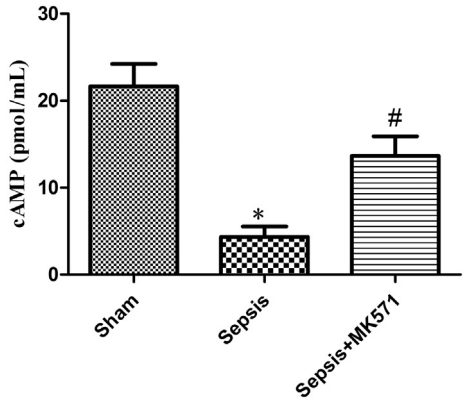

Fig. 4. Lung tissue cAMP concentration in the three groups. The values are expressed as mean ± SD. $*P < 0.05$ compared with the Sham group mice; $\#P < 0.05$ compared with the Sepsis group.

4. Discussion

In the present study, we find that inhibition of MRP4 alleviated sepsis-induced acute lung injury. These novel findings clearly indicate that MRP4 might play a critical role in sepsis-induced acute lung injury. Thus, inhibition of MRP4 may serve as a new therapeutic strategy for the treatment of sepsis-induced acute lung injury.

First, we establish acute lung injury model induced by sepsis and study the role of MRP4. Pathological examination of lung tissue in the Sepsis group shows typical acute lung injury. Inhibition of MRP4 could mitigate acute lung injury as evidenced by histological examination. Pulmonary edema is assessed by vascular leakage and W/D ratio analyses. We find that sepsis-induced acute lung injury led to a significant increased vascular leakage and W/D ratio, indicating the presence of pulmonary edema. Inhibition of MRP4 is demonstrated to mitigate the vascular leakage and W/D ratio and alleviate the pulmonary edema. Furthermore, knockdown of MRP4 dramatically protects against LPS-induced PMVECs hyperpermeability and cytoskeleton reorganization in vitro. Similarly, previous study reports that MRPs inhibitors probenecid and MK571 prevents the increase in vascular permeability induced by zymosan [18]. More importantly, PaO2 and SpO2 levels decrease in acute lung injury rats, with significant restoration after inhibition of MRP4. The pulmonary microvascular endothelial barrier function is impaired after the occurrence of acute lung injury, which will damage the gas exchange [22]. When the vascular leakage and pulmonary edema are reduced by inhibiting MRP4, we find that the PaO2 and PaO2/FiO2 values are improved obviously, which suggests that pulmonary ventilation function is improved effectively.

Mechanistically, the protective effect of MRP4 inhibition on sepsis-induced acute lung injury might be exerted by reducing lung vascular hyperpermeability, and by decreasing inflammatory response. Previous

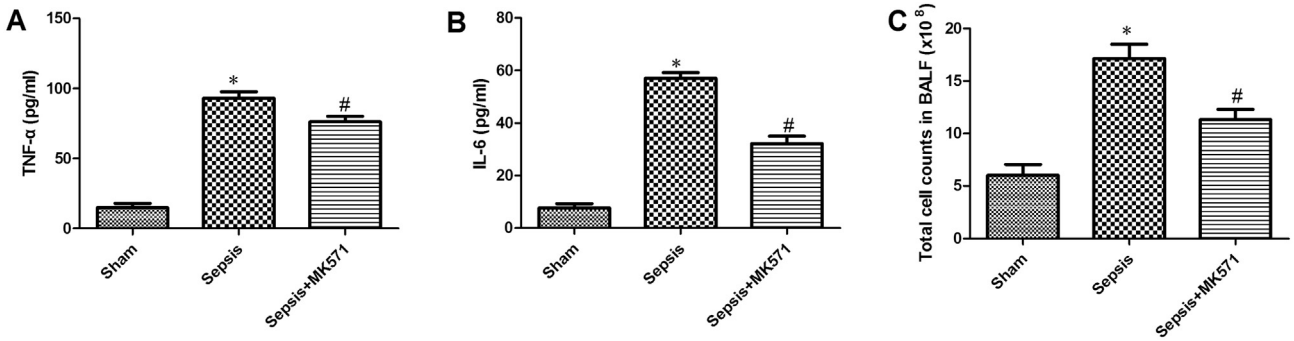

Fig. 5. Serum TNF-α (A) and IL-6 levels (B) and BALF cell counting (C) in the three groups. The values are expressed as mean ± SD. *$P < 0.05$ compared with the Sham group mice; #$P < 0.05$ compared with the Sepsis group.

Table 1
Endothelial permeability in rat PMVECs.

Group	2 h	6 h	12 h	24 h
Control	0.281 ± 0.02	0.298 ± 0.01	0.327 ± 0.04	0.322 ± 0.03
LPS	0.406 ± 0.04*	0.558 ± 0.04*	1.121 ± 0.17*	0.793 ± 0.02*
Ad-shRNA	0.405 ± 0.03*	0.515 ± 0.03*	1.103 ± 0.18*	0.796 ± 0.04*
Ad-shMRP4	0.320 ± 0.02*,#	0.386 ± 0.03*,#	0.702 ± 0.07*,#	0.573 ± 0.05*,#

Endothelial permeability was determined by horseradish peroxidase (HRP) permeability assay and presented by the OD value at 450 nm.
* $P < 0.05$ for control group.
# $P < 0.05$ for Ad-shRNA/LPS group.

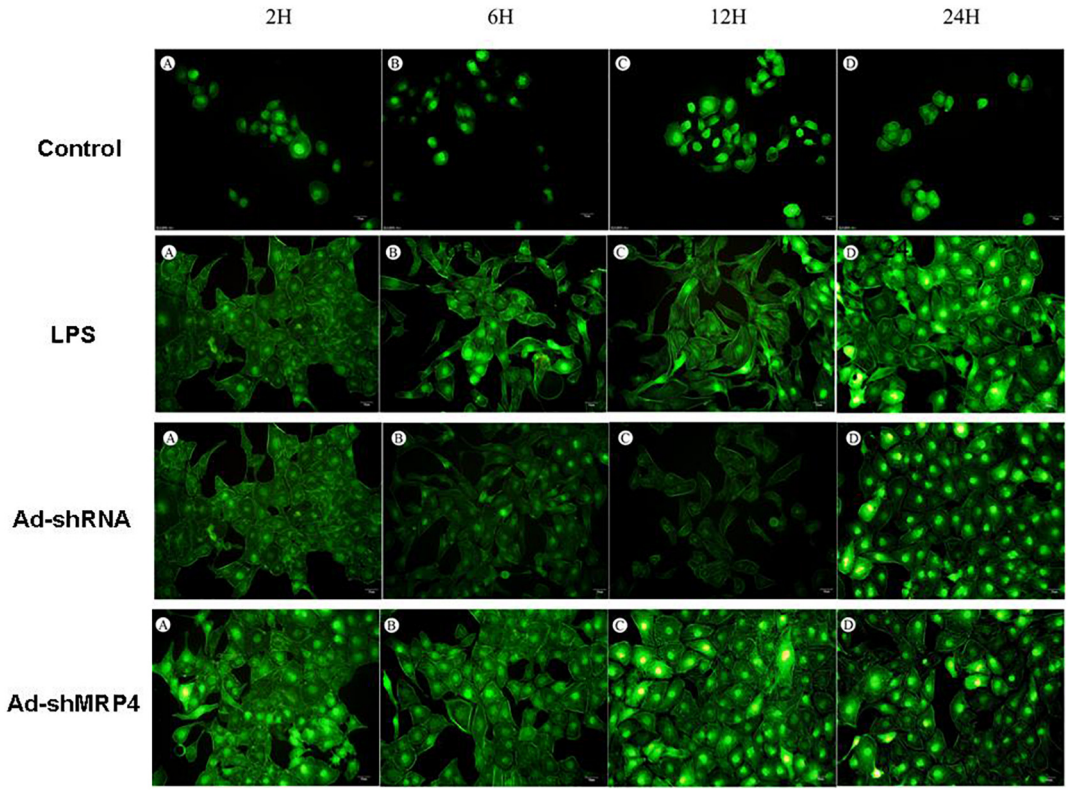

Fig. 6. Cytoskeleton reorganization in rat PMVECs. Immunofluorescence staining was used to detect F-actin and remodeling of actin cytoskeleton. Control group: PMVECs without LPS stimulation; LPS group: PMVECs with LPS stimulation; Ad-shRNA group: PMVECs with Ad-shRNA infection, followed by LPS stimulation; Ad-shMRP4 group: PMVECs with Ad-MRP4 infection, followed by LPS stimulation.

studies have shown that elevated endothelial cAMP levels reduced vascular permeability [8,9]. Activation of the cAMP has recently been shown to be critical for the maintenance of endothelial barrier function. Vice versa, inactivation of the cAMP played an important role in barrier disruption. For example, previous studies find that prostaglandins and atrial natriuretic peptide stabilize endothelial barrier via increasing intracellular cAMP levels followed by activation of Rac1 and Cdc42, and therefore mitigate ventilator-induced lung injury in vivo [23,24]. Co-treatment of forskolin and rolipram prevent the increase of permeability in microvascular endothelial cells by increasing cAMP levels

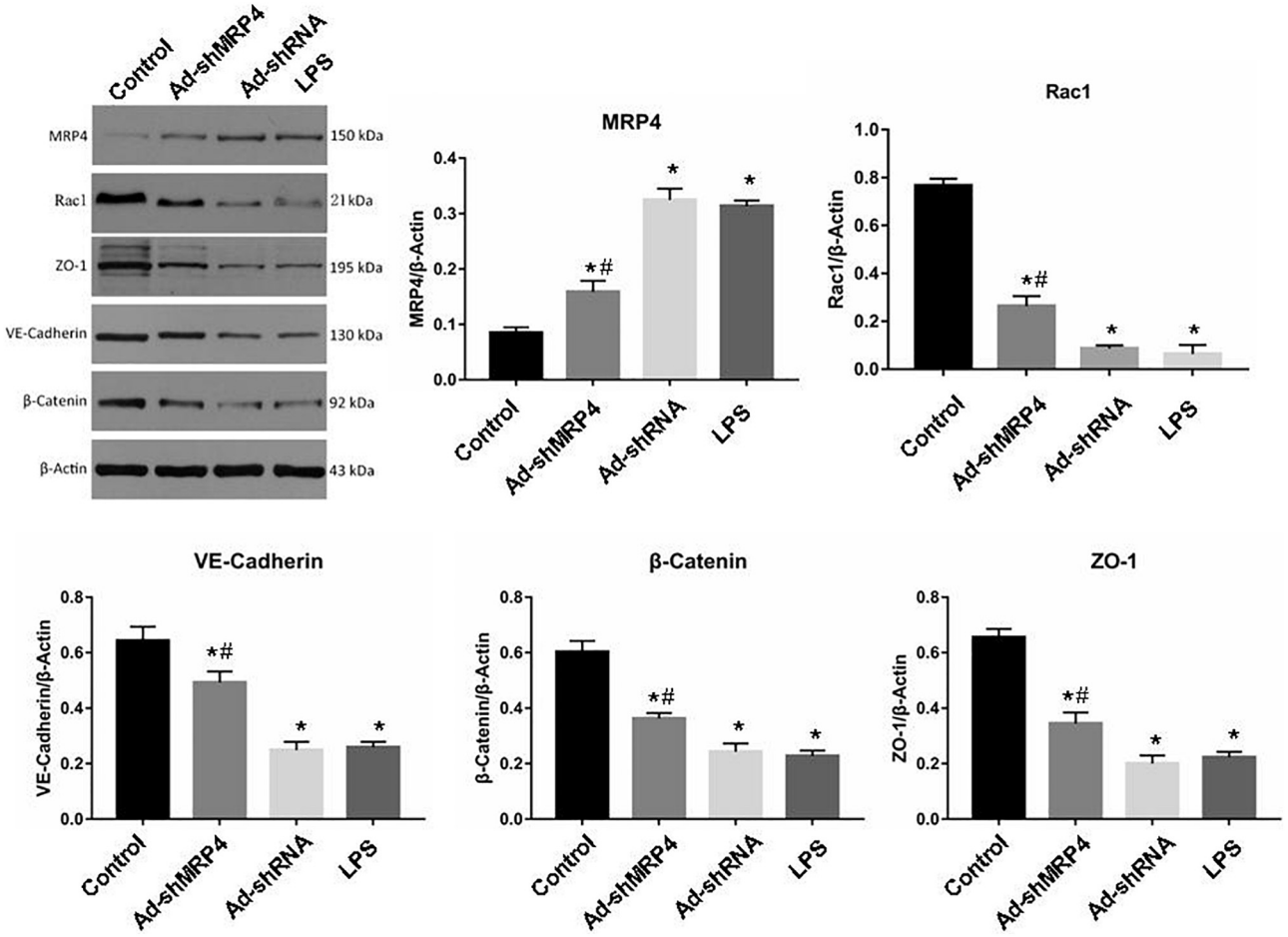

Fig. 7. Expression of MRP4, Rac1, VE-cadherin, β-catenin and ZO-1 rat PMVECs. A. Representative blots of MRP4, Rac1, VE-cadherin, β-catenin and ZO-1 after treatment with LPS in rat PMVECs infected with Ad-shRNA or Ad-shMRP4. B-E. Quantitative results of MRP4, Rac1, VE-cadherin, β-catenin and ZO-1. $*P < 0.05$ for control group; $\#P < 0.05$ for Ad-shRNA/LPS group.

[25]. MRPs have been shown to act as a physiological cAMP regulator in various cells. Inhibition of MRP4 is shown to protect mice from pulmonary hypertension by increasing intracellular cAMP levels [13]. In our study, we find that MK571 elevates cAMP levels in lung tissue of septic rats and silencing MRP4 gene increases the cAMP levels and protein level of Rac1 in rat PMVECs after LPS treatment, suggesting that the protective effect of MRP4 inhibition on sepsis-induced acute lung injury is associated with the elevation of cAMP levels.

Leukocyte recruitment to the inflammatory site involves cytokines. It has been demonstrated that MRPs transport leukotriene C4 and prostaglandin E2, which involve in the influx of polymorphonuclear cells in the peritonitis model induced by zymosan [18]. Schultz et al. [26] find that MRP1 knockout mice inoculated with *S. pneumoniae* exhibited a significant reduction in neutrophil influx and TNF-α level. MK571 suppresses cytokines, such as TNF-α and interleukins secretion from cells during inflammation [17,27]. Furthermore, we observed that MK571 significantly decreased serum TNF-α and IL-6 levels, and reduced inflammatory cell accumulation in BALF. Therefore, the reduction on inflammatory cell influx into the airways observed in our study may be associated with the decreased levels of inflammatory cytokines.

The maintenance of endothelial barrier function is vital for its permeability. Adherens and tight junctions are intercellular junctions crucial for endothelial barrier function. VE-cadherin and β-catenin are critical parts of adherens junctions and participate in the control of the endothelial integrity and permeability. LPS-induced endothelial hyperpermeability is accompanied by decreased expression of the adherens junction proteins, endothelial VE-cadherin and beta-catenin [28]. Claudins and occludins are principal tight junctional constituents, locate on the intracellular side of plasma membrane associated with ZO-1, peripheral membrane protein, anchoring the strands to the actin. Tornavaca et al. found that ZO-1 depletion resulted in tight junction disruption, redistribution of active myosin II from junctions to stress fibers [29]. Our study found that MRP4 inhibition significantly increased the expression levels of VE-cadherin, β-catenin and ZO-1, and therefore attenuated the impairment of endothelial barrier function in rat PMVECs stimulated by LPS.

Nevertheless, there are some limitations in the present study needed to be addressed. First, only the acute effect of MRP4 inhibition on sepsis-induced acute lung injury are examined; the chronic effect remain to be elucidated in the future. Second, we do not measure the dose-response curve of MK571 on sepsis-induced acute lung injury, although the dose used in the present study has been found to exhibit protective effects previously. Third, MK571 is not a unique inhibitor for MRP4. It has also been reported to be an inhibitor of MRP1, MRP4 and MRP5 [30,31]. Third, several cell types participate in the pathogenesis of sepsis-induced acute lung injury, such as endothelial cells, epithelial cells and inflammatory cells, etc. However, the present study focused on endothelial cells, which play an important role. Given that MRP4 is present in various cell types and regulates a variety of biological processes, the regulatory effects of MRP4 on other cell types needs to be further determined. Finally, protective effect of MRP4 inhibition on acute lung injury may be associated with the elevation of lung cAMP levels and alleviation of inflammatory response. However, the exact mechanism needs to be further demonstrated.

5. Conclusions

In summary, the present study shows that inhibition of MRP4 significantly alleviates sepsis-induced acute lung injury in rats. The protective effect of MRP4 inhibition on sepsis-induced acute lung injury is mainly mediated via maintenance of endothelial barrier function. Our observations provide new insights into the mechanisms of sepsis-induced acute lung injury, and have potential implication for developing novel treatment strategies for sepsis-induced acute lung injury.

Disclosure

None.

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