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Original Research

Sustenance of endothelial cell stability in septic mice through appropriate activation of transient receptor potential vanilloid-4

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Abstract: Therapeutic target transient receptor potential vanilloid-4 (TRPV-4) is frequently applied in endotoxemia research. It has been reported that HC067047, an inhibitor of TRPV-4, mitigated LPS-induced injury. However, the inhibition of TRPV-4 with HC06047 did not attenuate LPS-induced symptoms and exaggerated pathology. This study was carried with a view to unravelling the reason**(s)** behind these conflicting results. Different doses of the inhibitor were used in the same degree of sepsis, and their effects were determined through assays for sepsis-related physiological indicators such as endothelial injury markers, coagulation index, organ damage indicators, inflammatory factor levels, and cell apoptosis. The results showed that high or low inhibitor levels had no significant effect on sepsis-related physiological indicators. These findings suggest that proper activation of TRPV-4 in sepsis is important for maintaining normal physiological function. Thus, the degree of TRPV-4 activation should match the severity of sepsis.

Key words: Transient receptor potential vanilloid 4; Sepsis; HC06047; Lipopolysaccharide; Endothelial injury.

Introduction

Sepsis is a fatal clinical condition that results from a systemic host response to invading pathogens. It damages organs and tissues of the host, and it may progress to multiple organ failure and hypotensive shock (1). The TRPV-4 is described in the vasculature (2). Its influence on the septic process makes it a potential therapeutic target (3). Interestingly, there are inconsistent reports on the effect of TRPV-4 inhibition (3-5). Some researchers have reported that the inhibition of TRPV-4 with HC067047 reduced mortality in a lipopolysaccharide (LPS)-induced sepsis model (5). In contrast, other workers found that the inhibition of TRPV-4 with HC067047 was unable to attenuate LPS-induced symptoms and exaggerated pathology (4). Moreover, it has been reported that TRPV-4 blockage brought about impairment of immune response at the initial stages of sepsis, while the same TRPV-4 inhibition was beneficial to anti-inflammatory effects and barrier stabilization in sepsis-induced acute respiratory distress syndrome (3). Following careful analyses of these findings, we speculate that the conflicting results may be related to a mismatch between the activation level of TRPV-4 and the severity of sepsis. In the studies analyzed (3-5), the same dose of TRPV-4 inhibitor (HC067047) was used, but there were differences in the severity of sepsis. Thus, we tried to match the dose of the inhibitor used with the severity of sepsis, and then assayed some key sepsis-related physiological indicators such as endothelial injury markers, coagulation index, organ damage indicators, inflammatory factor levels, and cell apoptosis state, to assess influence of inhibitor concentration. The results showed that excessive or insufficient inhibitors did not significantly improve sepsis-related physiological indicators. Thus, proper TRPV-4 activation in sepsis is important for maintaining normal physiological function.

Materials and Methods

Mice grouping and treatments

Male C57BL/6J mice were kept in a room at 23 °C and relative humidity of 50 ± 8 %, and were permitted unrestrained access to drinking water and feed. The mice were aged 9 to 10 weeks, and weighed 23 - 25 g. The animal treatments were as described by Sand *et al.* (4). We produced the sepsis model through intravenous (*i.v.*) injection of 12.5 mg/kg LPS. Normal saline and dimethyl sulphoxide (DMSO) were used as vehicles for LPS and GSK2193874, respectively. Ethical approval for the animal handling protocol was given by Hangzhou Normal University Animal Ethics Committee, and the execution of study was consistent with the directives of the National Institute of Health on animal experimentations.

Six groups of mice were used in this study**:** 12.5 mg/kg LPS group (LPS) (4), 12.5 mg/kg LPS + 0.5 mg/kg HC067047 (intraperitoneal injection, *i.p.*) group (LPS + 0.5HC), 2.5 mg/kg HC067047 (*i.p.*) + 12.5 mg/kg LPS group (LPS+2.5HC), 12.5 mg/kg LPS + 5 mg/kg HC067047 (*i.p.*) group (LPS+5HC), 12.5 mg/kg LPS + 10 mg/kg HC067047 (*ip*) group (LPS+10HC), and control placed on normal saline (4 – 5). At 16 hours after endotoxemia induction, the injuries induced in the various groups were assessed.

Determination of endothelial injury

Blood specimens were collected from the hearts of anaesthetized mice 16 hours after endotoxemia induction, and centrifuged to obtain plasma samples used to measure biomarkers of endothelial injury, such as soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble intercellular adhesion molecule-1 (sICAM-1), E-selectin (sEs), and soluble von Willebrand factor (vWF), as indexes of endothelial lesions in various diseases (6-7). The vWF was determined using an immunoturbidimetric assay (Siemens Healthcare, Erlangen, Germany). Other markers were assessed by ELISA kits (eBioscience, USA) and Infinite® 200 Pro NanoQuant microplate reader (Tecan, Männedorf, CH) in line with the stipulated operating instructions.

Assay of markers of inflammation and hemostasis

The plasma concentrations of TNF)-α and IL-6 were determined using ELISA kits (eBiosciences, Thermo, Waltham, MA) and Infinite® 200 Pro NanoQuant microplate reader (Tecan, Männedorf, CH) according to the manufacturer's protocols. Soluble endothelial protein C receptor (sEPCR) and soluble thrombomodulin (sTM) were also assayed with ELISA kits (Novatein Biosciences, Cambridge, USA). The levels of these markers in the plasma were calculated from standard curves.

Plasma levels of prothrombin time (PT), D-dimer and activated partial thromboplastin time (APTT) were determined using coagulation analyzer (Sysmex CA-1500, Japan), in line with the manufacture's protocols. The reagents for PT, APTT and D-dimer were obtained from Siemens Healthcare (Erlangen, Germany).

Analysis of blood biochemistry

Blood biochemistry was assessed immediately after collection using HITACHI 7600 automated biochemistry analyzer (Tokyo Japan), according to the manufacture's protocols. Liver and kidney function markers such as aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), blood urea nitrogen (BUN) and plasma creatinine (Cr) were assessed. The reagents used for ALT, AST, LDH, Cr, and BUN assays were products of Medical System (Ningbo, China).

Assay of caspase-3 activity

Caspase-3 Activity Assay Kit (Beyotime, Nanjing) was used to assay for the activity of caspase-3 in the kidney according to manufacturer's protocol. Kidney tissues were ground in cell lysis buffer, and aliquots of the supernatant collected after centrifugation at 16,000 × g for 10 min were added to Ac-DEVD-*p*NA substrate and reaction buffer, and incubated in the dark for 90 min at 37 ºC. Caspase-3 activity was based on the absorbance at 405 nm in microplate reader (Tecan, Männedorf, CH). Data were normalized against the control.

Statistical analysis

Each assay was carried out in triplicate. Data are presented as mean ± standard error of the mean (SEM), and were analyzed for statistically significant using ANOVA and *t*-test. All analyses were carried out with SPSS version13.0. Statistical significance was fixed at $p < 0.05$.

Results

Treatment with 2.5 mg/kg HC067047 reduced the levels of endothelial markers in mice given 12.5 mg/kg LP

Endotoxemia induced an increase in plasma endothelial injury markers, relative to control (Figure 1), suggesting that vascular endothelial cell were severely damaged, which induced significant disruption of endothelial barrier. There were significant reductions in sVCAM-1, vWF, sEs, sVCAM-1, and sICAM-1 in LPS+2.5HC group, relative to LPS, LPS+0.5HC, or LPS+10HC groups (vWF: p = 0.011, 0.019, or 0.016; sVCAM-1: p = 0.022, 0.034, or 0.032; sICAM-1: p = 0.012, 0.028, or 0.030; sEs: p = 0.014, 0.026, or 0.030; respectively; Figure. 1). The LPS+2.5HC and LPS+5HC groups were comparable ($p > 0.05$; Figure. 1). The difference between LPS and LPS+5HC (vWF: p = 0.025; sVCAM-1: p = 0.037; sICAM-1: p = 0.024; sEs: p = 0.018; respectively; Figure. 1) was smaller than that between LPS and LPS+2.5HC (vWF: p = 0.011; sVCAM-1: p = 0.022; sICAM-1: p = 0.012; sEs: p = 0.014; respectively; Figure. 1). The changes among LPS, LPS+0.5HC, or LPS+10HC were not significant ($p > 0.05$; Figure. 1). These results demonstrate that 2.5 mg/kg HC067047 mitigated vascular endothelial injury and maintained barrier stabilization in 12.5 mg/kg LPS-treated mice.

Treatment with 2.5 mg/kg HC067047 maintained anti-coagulation in mice given 12.5 mg/kg LPS.

The plasma levels of sTM and sEPCR in LPS + 2.5HC

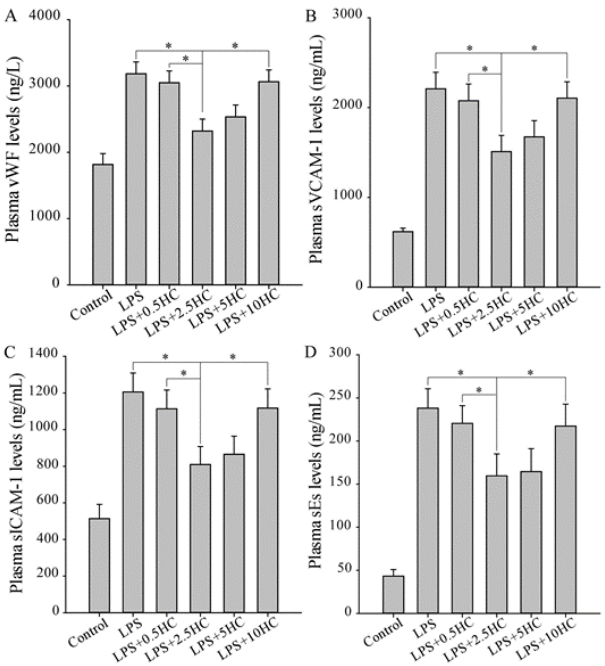

Figure 1. Concentrations of vascular endothelial injury parameters. The vascular endothelial injury was assessed by the levels of vWF (A), sVCAM-1 (B), sICAM-1 (C), and sEs (D) in endotoxemia mice (n=8). The levels of these markers were increased in 12.5 mg/kg LPS-treated mice, when compared with those in the controls. There were decreases in sICAM-1, vWF, sEs and sVCAM-1 in LPS+2.5HC group when compared to LPS, LPS+0.5HC, or LPS+10HC group (vWF: p = 0.011, 0.019, or 0.016; sVCAM-1: p = 0.022, 0.034, or 0.032; sICAM-1: p = 0.012, 0.028, or 0.030; sEs: p = 0.014, 0.026, or 0.030; respectively). *: p<0.05.

Table 1. Comparison of blood biochemical markers in the various groups.

	Control	LPS	LPS+0.5HC	LPS+2.5HC	LPS+5HC	LPS+10HC
ALT (U/L)	23.64 ± 4.11	322.36 ± 30.17	300.4 ± 29.53	82.42 ± 9.61**	100.35 ± 10.16	304.34 ± 30.01
AST (U/L)	22.79 ±4.06	441.29 ± 40.62	419.26 ± 37.43	177.59 ± 25.10**	190.21 ± 19.69	421.17 ± 36.22
LDH (U/L)	69.57 ± 7.43	452.05 ± 40.34	440.24 ± 40.38	170.54 ± 25.19**	191.57 ± 22.26	447.09 ± 41.61
Cr (μmol/L)	28.79± 4.54	197.21± 21.4	183.29 ± 18.76	111.38 ± 15.11*	120.25 ± 12.49	187.25 ± 22.8
BUN (mmol/L)	2.97 ± 0.45	20.37 ± 2.81	19.35 ± 2.05	10.71 ± 1.1*	12.24 ± 1.14	19.36 ± 2.41

Results are expressed as mean ± SEM. *p < 0.01. ** p < 0.001, relative to the LPS group.

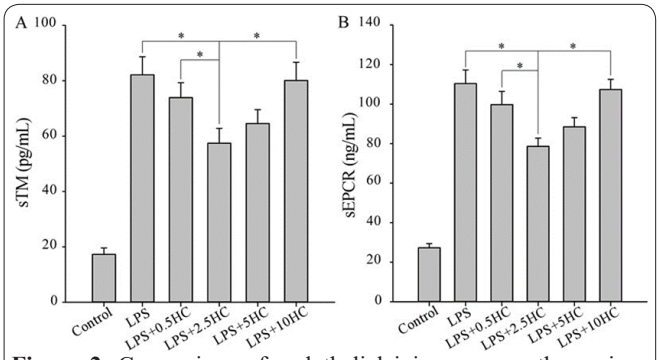

Figure 2. Comparison of endothelial injury among the various groups. (A) Plasma levels of sTM were assessed at 16 hours after induction of endotoxemia (n=8). There was significant decrease in the level of sTM in LPS+2.5HC, relative to LPS, LPS+0.5HC, and LPS+10HC groups (p = 0.017, 0.036, and 0.029, respectively). (B) In the LPS+2.5HC group, sEPCR was significantly decreased, when compared to corresponding levels in LPS, LPS+0.5HC and LPS+10HC groups (p = 0.014, 0.031, and 0.020, respectively). *p < 0.05.

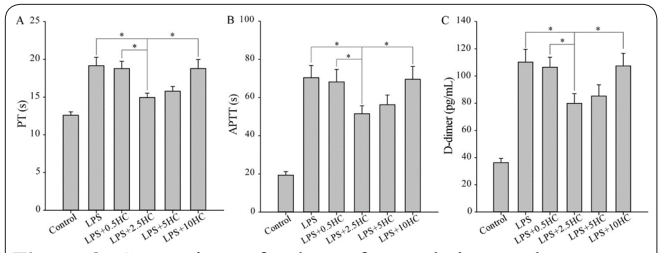

Figure 3. Comparison of values of coagulation markers among the various groups Coagulation markers were assessed at 16 h after induction of endotoxemia (n=8). (A) The values of PT in LPS+2.5HC were shorter than those in LPS, LPS+0.5HC, and LPS+10HC groups (p = 0.028, 0.035 and 0.03, respectively). (B) The changes in APTT in LPS+2.5HC group were similar to those in LPS, LPS+0.5HC, and LPS+10HC groups (p = 0.018, 0.025, and 0.021, respectively). (C) Plasma levels of D-dimer in LPS+2.5HC group were lower than those in LPS, LPS+0.5HC, or LPS+10HC group (p = 0.017, 0.026, or 0.025, respectively).*p < 0.05.

group were lower than those in LPS, LPS+0.5HC, and LPS+10HC groups (sTM: p = 0.017, 0.036, and 0.029, respectively; sEPCR: p = 0.014, 0.031, and 0.020, respectively; Figures 2A and 2B). The plasma levels of sTM and sEPCR in LPS + 5HC group were also lower than those in LPS group (sTM: p = 0.036; sEPCR: p = 0.028; Figures 2A and 2B), but the p values between LPS and LPS+2.5HC groups were smaller than p values between LPS and LPS+5HC groups. This demonstrates that endothelial injury in LPS+2.5HC group was mild, when compared with endothelial injuries in LPS, LPS+0.5HC, LPS+5HC and LPS+10HC groups.

2.5 mg/kg HC067047 treatment improved clotting time in mice treated with 12.5 mg/kg LPS.

Results showed that PT in LPS+2.5HC group was shorter, when compared with PT in LPS, LPS+0.5HC, or LPS+10HC group (p = 0.028, 0.035, or 0.033, respectively; Figure 3A). A similar pattern of changes was seen with APTT in LPS+2.5HC group, when compared with LPS, LPS+0.5HC, or LPS+10HC group (p = 0.025; Figure 3B). The plasma levels of D-dimer in LPS+2.5HC group decreased significantly, relative to the other groups (p < 0.026; Figure 3C). In addition, the levels of PT, APTT and D-dimer in LPS+5HC group were lower than those in LPS group (PT: p = 0.044; APTT: p = 0.038; D-dimer: p = 0.035; respectively; Figures 3A, 3B and 3C), but the LPS+2.5HC and LPS+5HC groups were comparable (PT: p = 0.83; APTT: p = 0.79; D-dimer: p = 0.85; Figures 3A, 3B and 3C). These results demonstrate that the degree of injury to coagulation function in LPS+2.5HC group was mildest among the 12.5 mg/kg LPS-treated groups.

2.5 mg/kg HC067047 treatment reduced levels of blood biochemistry markers in 12.5 mg/kg LPS-treated mice.

Markers of liver and kidney injury were assessed at 16 hours after induction of endotoxemia. The levels of ALT, AST, LDH, Cr, and BUN in LPS+2.5HC group decreased significantly, relative to corresponding values in LPS, LPS+0.5HC, and LPS+10HC groups, but group LPS+2.5HC and group LPS+5HC were comparable (Table 1). Thus, the liver and kidney injuries in LPS+2.5HC group were milder than those in LPS, LPS+0.5HC or LPS+10HC groups.

HC067047 treatment decreased TNF-α and IL-6 concentrations in 12.5 mg/kg LPS-exposed mice.

Plasma levels of IL-6 and TNF-α in 12.5 mg/kg LPS-treated mice were significantly increased, relative to mice in the control group (Figures 4A and 4B), while Il-6 and TNF-α concentrations in plasma of the LPS+2.5HC group were significantly lower than those in LPS group (p = 0.029 and 0.011, respectively); LPS+5HC group (p = 0.028), and LPS+10HC group (p = 0.023). On the other hand, values of IL-6 and TNF-α were comparable among LPS+2.5HC, LPS+5HC, and LPS+10HC groups (p = 0.86). Therefore, in septic mice, the administration of HC067047 suppressed the increase in plasma concentrations of IL-6 and TNF-α 16 hours after induction of endotoxemia.

2.5 mg/kg HC067047 treatment reduced caspase-3 activity in 12.5 mg/kg LPS-treated mice.

Treatment with 12.5 mg/kg LPS markedly increased the levels of cleaved caspase-3 in the left kidney tissue, when compared with those in the controls at 16 hours after endotoxemia induction (Figure 5). Howe-

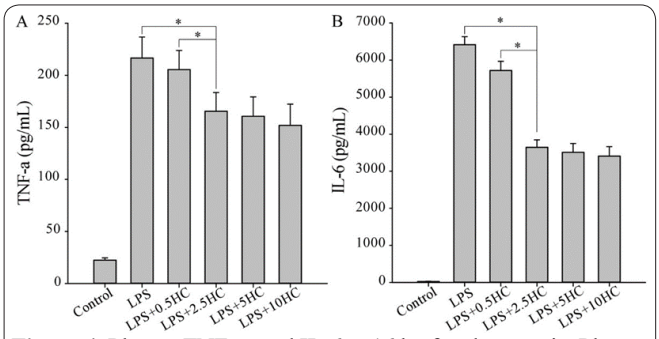

Figure 4. Plasma TNF-α and IL-6 at 16 h of endotoxemia Plasma IL-6 and TNF-α were assessed at 16 hours after endotoxemia induction (n=8). LPS (12.5 mg/kg) induced the secretions of IL-6 and TNF-α (A) Plasma TNF-α in LPS group were higher than those in LPS+2.5HC, LPS+5HC, or LPS+10HC group (p = 0.029, 0.028, or 0.023, respectively). (B) The levels of IL-6 in LPS group were also higher than those in LPS+2.5HC, LPS+5HC, and LPS+10HC groups (p=0.011, 0.01, and 0.009, respectively). *p < 0.05.

ver, the levels of caspase-3 in LPS+2.5HC mice were significantly decreased, relative to corresponding values in LPS, LPS+0.5HC, or LPS+10HC mice (p = 0.021, 0.033, or 0.032, respectively). Caspase-3 levels in LPS+5HC group were also significantly reduced, relative to the LPS group (p = 0.041), but to a lesser extent in LPS+2.5HC group, when compared to the LPS group. These findings suggest that 2.5 mg/kg HC067047 reduced cell apoptosis, but excessive or insufficient HC067047 administration did not attenuate cell apoptosis.

Discussion

Endothelial cells produce E-selectin, ICCAM-1 and VCAM-1, and induce the downstream vWF release (7). Inflammation induces endothelial injury and increases in the levels of vWF, sVCAM-1, sICAM-1, and sEs (7). Therefore, these parameters serve as indicators of endothelial injury (7). In our study, 12.5 mg/kg LPS markedly induced increases in these indicators . The TRPV-4 is very important in endothelial function (2). For example, the activation of TRPV-4 in endothelial cells induces vascular permeability (8). It is also implicated in irisin-induced endothelium-dependent vasodilation (2). Studies have shown that inappropriate activation of TRPV-4 leads to endothelial injury and acute circulatory collapse (8). It has been reported that excessive activation of TRPV-4 in sepsis contributes to lethal endothelial failure and vascular permeability, while TRPV-4 inhibitors reduce these responses, increase cell survival, and improve vascular permeability during sepsis (5, 8). Our results showed that 2.5 mg/kg HC067047 treatment significantly reduced these markers levels in 12.5 mg/kg LPS-treated mice. However, the blockage of TRPV-4 activity with HC067047 sometimes produces contradictory results (9). This is consistent with the inability of 10 mg/kg HC067047 to reduce the levels of endothelial injury markers in 12.5 mg/kg LPS-treated mice. Thus, excessive inhibition of TRPV-4 may be undesirable in the treatment of sepsis. This implies that appropriate activation of TRPV-4 may be necessary and indeed beneficial for resisting endotoxemia and maintaining normal physiological function. In a related report, it has been demonstrated that TRPV-4 deficiency aggravated kidney injury after acute renal ischemia reperfusion (9). On the other hand, blockage of TRPV-4 alleviated myocardial ischemia/reperfusion injury in mice (10).

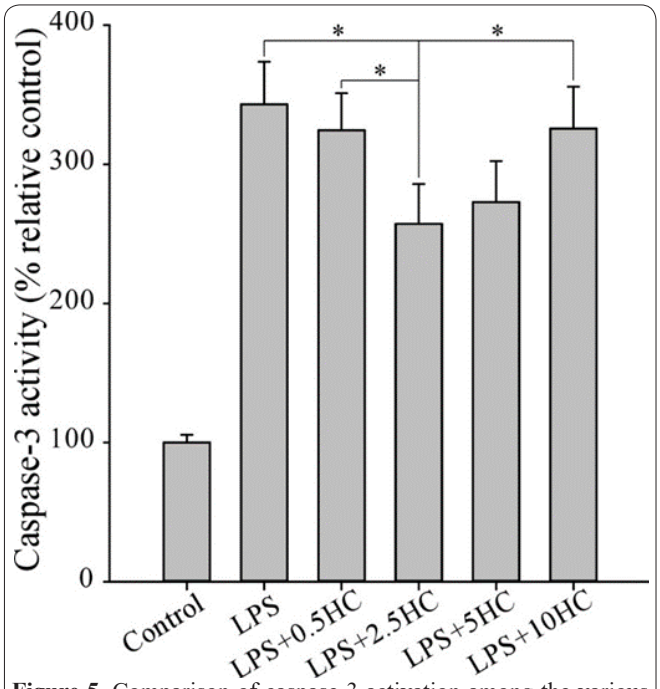

Figure 5. Comparison of caspase-3 activation among the various groups Endotoxemia-induced caspase-3 activity at 16 hours after endotoxemia induction. Caspase-3 activity in LPS+2.5HC mice was lower than those in LPS, LPS+0.5HC, or LPS+10HC group (p = 0.021, 0.033, or 0.032; respectively). *p < 0.05.

The expressions of TM and EPCR are almost exclusive to vascular endothelial cells, and increased levels of sTM and sEPCR serve as markers of endothelial injury in sepsis (11). In our study, the concentrations of sTM and sEPCR in LPS+2.5HC mice group were the lowest among the 12.5 mg/kg LPS-treated mice groups. This suggests that vascular endothelial cell injury in LPS+2.5HC was milder than the degree of injury in any of the other LPS-treated mice. Furthermore, in normal situations, the endothelial cell layer provides an anticoagulant surface by expressing TM and EPCR, and supports thrombin to generate activated protein C (APC) that limits the progression of the coagulation cascade (11, 12). The endothelial surface is cleaved by elastase to liberate EPCR and TM during sepsis; the resultant sTM and sEPCR can also bind APC, but they inhibit its anticoagulant activity (11). Endotoxemia induces dysfunctional coagulation (11). The accepted markers of coagulation function are APTT, PT, and D-dimer (13). In LPS+2.5HC group, 2.5 mg/kg HC067047 treatment markedly reduced the values of PT, APTT, and D-dimer, when compared with LPS, LPS+0.5HC, and LPS+10HC groups, thereby attenuating coagulation dysfunction. These findings were consistent with the plasma levels of sTM and sEPCR. Dysfunctional coagulation aggravates hypoxic-ischemic injury in organs and tissues, and results in multiple organ failure during sepsis (14, 15). The lowest values of ALT, AST, LDH, Cr, and BUN (markers of liver and kidney injury) were seen in LPS+2.5HC group. Thus, 2.5 mg/kg HC067047 treatment effectively maintained endothelial cell stability, improved coagulation function, and alleviated organ injury in LPS+2.5HC group.

Endotoxemia induces expressions of IL-6 and TNF-α

(3, 5). These factors are involved in organ injuries, including endothelium injury (16). In sepsis, the release of IL-6 and TNF-α is inhibited by HC067047 administration (5). Our results also showed that HC067047 reduced the 12.5 mg/kg LPS-induced increases in levels of IL-6 and TNF-α in mice. However, these inflammatory markers were significantly lower in the LPS+10HC group than in LPS+2.5HC group, but the injuries induced by endotoxemia in LPS+10HC were more serious than those in LPS+2.5HC group.

Exocytosis and endocytosis depend on perfect tuning of intracellular calcium signaling with the actin cytoskeleton (17). Calcium levels are regulated intracellularly through the membrane pumps and Ca^{2+} **c**hannels [18]. Indeed, TRPV-4 is a calcium channel (5). Excess levels TRPV-4 inhibitor (HC067047) blocked TRPV-4 activation, reduced Ca^{2+} influx, interfered with actin dynamics, and suppressed secretion of more inflammatory cytokines in LPS+10HC mice. The dynamic balance in actin cytoskeleton between polymerization and de-polymerization is very important for the survival of LPS-treated cells (19). It is known that TRPV-4 induces actin de-polymerization through the Ca^{2+}/calcineurin/LIM-kinase1/cofilin pathway (5, –20-21). The results obtained in this study suggest that the 10 mg/kg HC067047 treatment in LPS+10HC group might have resulted in underactivity of TRPV-4, which in turn reduced actin de-polymerization and increased the amount of stress fibers in endothelial cells (5). An increase in the number of actin stress fibers has been shown to induce cell apoptosis (22). Thus, 10 mg/kg HC067047 aggravated 12.5 mg/kg LPS-induced apoptosis. Therefore, HC067047 did not attenuate the 12.5 mg/kg LPS-induced injury.

In summary, these conflicting results suggest that when TRPV-4 is used as therapeutic target, it should be determined whether the resultant activation levels can cause disease remission. Further studies should look for methods which can be used to assess the relationship between disease severity and TRPV-4 activation levels. This is important because the methods may be able to guide a doctor to either activate or inhibit TRPV-4 when treating sepsis.

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Conflict of Interest
There are no conflicts of interest in this study.

Author's contribution
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Zhen-Ming Pei; Yi-Hua Zhu1, Zhen-Ming Pei collected and analysed the data; Yi-Hua Zhu wrote the text and all authors have read and approved the text prior to publication.

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