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PCSK9 Promotes Endothelial Dysfunction in Sepsis via TLR4/MyD88/NF-κB and NLRP3 Pathways

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Research Article

Keywords: PCSK9, sepsis, endothelial dysfunction, inflammation

Posted Date: February 11th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1325215/v1

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Abstract

Endothelial dysfunction often accompanies sepsis. We aimed to explore the role of PCSK9 in septic endothelial dysfunction. Sepsis was induced by Lipopolysaccharide (LPS) treatment on human umbilical vein endothelial cells (HUVECs) *in vitro* and Cecal ligation and puncture (CLP) surgery mice *in vivo*. Evolocumab (EVC) and Pep 2-8, PCSK9 inhibitors, was subsequently used to determine the role of PCSK9 in septic endothelial dysfunction in vitro and in vivo, respectively. And TLR4 agonist, Kdo2-Lipid A ammonium (KLA), was used to determine the related mechanism. Expression of eNOS, VE-Cadherin, PCSK9, TLR4, MYD88, p-p65, p65, NLRP3, ASC and Caspase1 p20 in the aortas and HUVECs were measured by Western blot and mRNA expression of TNFα, IL-1 and IL-6 were determined by PCR. Nitric oxide (NO) content in the medium and cell viability were examined. Vasodilation function of the aorta was detected by vascular reactivity experiments. The 48-h survival rate after CLP was observed. The results demonstrated that the expression of eNOS and VE-Cadherin decreased and PCSK9 expression increased in septic HUVECs or mice. Nevertheless, inhibition of PCSK9 could improve the eNOS and VE-Cadherin expression. The activation of TLR4/MyD88/NF-κB and NLRP3 pathways should be responsible for the PCSK9-induced endothelial dysfunction in sepsis. Vascular reactivity test and survival study shows that inhibiting PCSK9 could prevent the decline of endothelium-dependent vasodilation function and improve the survival rates of septic mice. In summary, our results suggest that increased PCSK9 in sepsis activates the TLR4/MyD88/NF-κB and NLRP3 pathways to induce inflammation, which results in vascular endothelial dysfunction and decrease of survival rates. However, inhibition of PCSK9 could improve the vascular endothelial function in sepsis, which may be a potential way for clinical treatment.

Introduction

Sepsis is a horrible disease, which usually leads to poor prognoses and even death for the patients[1]. Once sepsis enters the advanced stage, the inflammatory storm leads to multiple organ dysfunction and the disease becomes irreversible[2]. It is worth noting that hemodynamic disorder is one of the most crucial causes of disease progression[3]. In sepsis, the inflammatory factors damage blood vessels, which may present as disturbance of vascular tone regulation and a loss of effective blood volume due to excess fluid spilling out of the vessels, and lead to blood pressure instability and shock[4].

Vascular endothelium is one of the most important components of blood vessels[5]. Although it is only distributed on the inner surface of blood vessels, it plays a very important role in regulating vascular function, such as the vasodilation function, vascular barrier function and anti-inflammatory effect[6]. Vascular endothelial dysfunction in sepsis could further aggravate the disease, and ultimately lead to individual death[7]. Recent studies have shown that protecting the endothelium is effective in treating the sepsis[8]. However, the mechanism of endothelial dysfunction in sepsis is not fully understood, and a further study is needed.

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a member of the proprotein convertase and can regulate the LDL and cholesterol metabolism[9]. PCSK9 is associated with the formation of

atherosclerotic plaques and inhibition of it has been showed to be beneficial to vascular function[10]. In addition, the negative role of PCSK9 in sepsis has also been gradually revealed by some studies[11, 12]. Nevertheless, the relationship between PCSK9 and vascular endothelial function in sepsis remains unknown. This study explored the role of PCSK9 in septic endothelial dysfunction and further explored the possible mechanisms, providing new insights for the treatment of sepsis.

Material And Method

Reagents

Primary antibodies against p65 (10745-1-AP) and β-actin (20536-1-AP), secondary antibodies (goat against rabbit, SA00001-2) and secondary antibodies (goat against mouse, SA00001-1) were purchased from Proteintech (Rosemont, IL, USA). Primary antibodies against Caspase-1 p20 (WL02996a) ,TLR4 (WL00196) and MyD88 (WL02494) was purchased from Wanleibio (Shenyang, Liaoning, China). Primary antibody against ASC (sc-514414) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies against p-p65 (3033T) and eNOS (32027) were purchased from Cell Signaling Technology (Danvers, MA, USA). Primary antibodies against NLRP3 (ab214185) and VE-Cadherin (ab33168) were purchased from Abcam (Cambridge, UK). Lipopolysaccharide (LPS) (L2880-100MG) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Evolocumab (EVC) (1256937-27-5) was obtained from Shanghai TheraMabs Bio-technology co., LTD (www.theraprotein.com.cn) (Shanghai, China). Kdo2-Lipid A ammonium (KLA) (HY-N8277) was obtained from MedChemExpress (Monmouth Junction, NJ, USA). Pep 2-8 (1541011-97-5) was obtained from Good Laboratory Practice Bioscience (Montclair, CA, USA). Cell Counting Kit-8 (CCK8) was purchased from Bimake (Shanghai, China). NO Detection kit was obtained from Beyotime (Shanghai, China).

Animals

Healthy male C57BL/6 mice (age 8 weeks) were purchased from the Animal Center of Chongqing Medical University. All animals were housed in a controlled rodent facility (20~22°C; 12-h light/dark cycle) and free to acquire standard rodent chow and water. Animal experiments were performed in accordance with the National Animal Protection and Use Guidelines and approved by the local ethical committee of the First Affiliated Hospital of Chongqing Medical University (No: 2021-706).

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were cultured in Medium 199 (fetal bovine serum 20%, glutamine 0.1%, heparin 0.01%, and endothelial growth factor 0.01%). Six to nine generations of HUVECs were used in experiments. Newborn umbilical cord was obtained from the First Affiliated Hospital of Chongqing Medical University, and the experiments were approved by the local ethical committee of the First Affiliated Hospital of Chongqing Medical University (No: 2021-689).

Cell viability assay

Cell viability was measured using the CCK-8) (Bimake, Shanghai, China) as described by the manufacturer. Briefly, cells were seeded in 96-well plates at a density of 1 × 104 cells/well and cultured for 1day. Next, different treatments were added in the medium of the HUVECs for 24h. The medium was removed and 100 μl of DMEM together with 10 μl of the CCK8 solution were added into each well. The plated were incubated at 37◦C for 1–4 h. Then, OD450 was measured using a microplate spectrophotometer (Thermo Scientific, USA).

Measurement of NO

The NO content in the medium was determined using a NO Detection kit (Beyotime, Shanghai, China) as described by the manufacturer. In brief, 50 μL/well standard or sample were added to a 96-well plate followed by Griess Reagent I and Griess Reagent II. The absorbance was measured at 540 nm with a a microplate spectrophotometer (Thermo Scientific, USA).

Cecal Ligation and Puncture (CLP)

CLP was performed to induce sepsis in mice and the procedure is described previously. Briefly, the mice were anesthetized with 2–3% isoflurane in 100% oxygen, and an incision (1.0 cm) in the midline abdomen was performed. The cecum was exteriorized and 4-0 silk thread was used to ligate it at one half. 21-gauge needle was used to puncture the cecum and squeezed the cecum to assure patency of the puncture hole. The incision was sutured in layers after returning the cecum to the abdomen. For resuscitation, 5 mL/100 g prewarmed saline solution was injected to all the mice subcutaneously. Sham-operated mice received the same procedures except punctures. Animals were sacrificed by cervical dislocation, and the aortas were isolated to be used for subsequent experiments. Pep 2-8 (10 μg/kg) and KLA (1 μg/g) were administrated 2 h before surgery by intraperitoneal injection, with the dosage based on previous studies[13, 14].

Western Blot Analysis

Proteins were extracted from the aorta and HUVECs with RIPA lysis buffer. Proteins were separated on 10~12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Skim milk in TBST buffer was used to block the membranes 1h at room temperature, and then the membranes were incubated with appropriate primary antibodies overnight at 4°C. The blots were then incubated with secondary antibodies conjugated with horseradish peroxidase and ultimately visualized using chemiluminescence.

Vascular Reactivity

DMT620 Multiwire Myograph System (DMT, Hinnerup, Denmark) was used to record the isometric tension. Rings (2–3mm in length) were placed under a resting tension of 4 mN in a chamber filled with 37ºC, aerated (95% O_2, 5% CO_2) physiological saline solution (NaCl 119 mM, KCl 4.7 mM, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 5.5 mM glucose). Using 10^{-5} M acetylcholine (ACh)-induced relaxation in vessels contracted with 10^{-7} M norepinephrine to determine the integrity of the vascular endothelium. Concentration-response curves for ACh and sodium nitroprusside

(SNP) were then conducted (10^{-9} to 10^{-5} M). The EC_{50} and Emax were determined by nonlinear regression analyses using the version 8.0 of GraphPad software (GraphPad, San Diego, CA, USA). The sensitivity was expressed as pD_2 = log-EC_{50}.

Real-time PCR

To assess the inflammation in HUVECs and aortas, the mRNA expression levels of TNFα, IL1 and IL18 were determined by quantitative real-time PCR. Total RNA was extracted from the aorta or HUVECs with TRIzol reagent as described previously. Then, 1 μg of the extracted RNA was reverse transcribed. Real-time PCR amplification of the reverse transcribed cDNA was performed with SYBR green. The primers used in this study are shown as followed: TNFα (human), forward GAGGCCAAGCCCTGGTATG, reverse CGGGCCGATTGATCTCAGC; TNFα (mice), forward GAGAAAGTCAACCTCCTCTCTG, reverse GAAGACTCCTCCCAGGTATATG; IL-1 (human), forward CTTCTGGGAAACTCACGGCA, reverse AGCACACCCAGTAGTCTTGC ; IL-1 (mice), forward TTCAGGCAGGCAGTATCACTC, reverse GAAGGTCCACGGGAAAGACAC; IL-18 (Human) forward TGGCTGCTGAACCAGTAGAA, reverse ATAGAGGCCGATTTCCTTGG; IL-18 (mice), forward TGGTTCCATGCTTTCTGGACTCCT, reverse TTCCTGGGCCAAGAGGAAGTGATT; β-actin (human), forward CCTTCCTGGGCATGGAGTC, reverse TGATCTTCATTGTGCTGGGTG; β-actin (mice), forward GGCAAATTCAACGGCACA, reverse GTTAGTGGGGTCTCGCTCTG.

Survival Study

After surgery, the animals in each group (n = 10) were observed for 48 h and No other experimental procedures were performed. Pep 2-8 (10 μg/kg) and KLA (1 μg/g) were injected intraperitoneally 2 h before surgery and re-administrated 24 h after CLP.

Statistical Analysis

All data are expressed as the mean ± SD. All statistical analyses were performed using GraphPad Prism 8.0 software. Statistical evaluation was performed by using one-way analysis of variance followed by the Bonferroni post hoc test. Survival curves for all groups were performed using Kaplan-Meier survival curves and analyzed using log-rank tests. Differences were considered statistically significant at $P < 0.05$.

Results

LPS decreased eNOS and VE-Cadherin expression and increased PCSK9 expression in HUVECs in a dose-dependent manner.

The expression of eNOS and VE-Cadherin reflects endothelial function. We treated HUVECs with different concentrations of LPS for 24 h, and the expression of eNOS, VE-Cadherin and PCSK9 was detected by Western blot. As shown in Fig. 1A-D, LPS decrease the eNOS and VE-Cadherin expression and increased PCSK9 expression in HUVECs in a dose-dependent manner ($P<0.05$, n=4). NO, a vasodilators, is produced under eNOS catalysis, and the content of it in the culture medium of HUVECs was determined. As shown

in Fig. 1E, NO content decreased with the increase of LPS concentration (P<0.05, n=4). CCK8 experiment demonstrated that the cell viability of HUVEC was decreased by LPS in a dose-dependent manner (Fig. 1F, P<0.05, n=4). Since 10 μg/ml LPS treatment leads to the most obvious changes, this concentration was selected and used in subsequent experiments.

Inhibition of PCSK9 reversed LPS-induced declines in eNOS and VE-Cadherin expression, NO content and cell viability.

To investigate the role of PCSK9 in septic endothelial dysfunction, we used EVC to specifically inhibit PCSK9 in HUVECs. The results showed that LPS induced significant declines in eNOS (Fig. 2A and B) and VE-Cadherin (Fig. 2A and C) expression, NO content (Fig. 2E) and cell viability (Fig. 2F), and up-regulation of PCSK9 expression (P<0.05, n=4). However, these effects could be reversed by EVC treatment in a dose-dependent manner (P<0.05, n=4). Since the 200μM EVC treatment leads to the most obvious changes, this concentration was selected and used in subsequent experiments.

Inhibition of PCSK9 reversed LPS-induced activation of TLR4/MyD88/NF-κB and NLRP3 pathways and increased production of inflammatory cytokines.

Our previous study has shown that TLR4/MyD88/NF-κB and NLRP3 pathways play a critical role in septic endothelial dysfunction and another studies manifested that PCSK9 could activate TLR4 to induce myocardial injury in sepsis. We therefore measure the expression of TLR4/MyD88/NF-κB and NLRP3 pathways and the mRNA expression of related inflammatory factors. The result displayed that LPS induced increases in expression of TLR4 (Fig. 3A and B), MyD88 (Fig. 3A and C), p-p65 (Fig. 3A and D), NLRP3 (Fig. 3A and E), ASC (Fig. 3A and F), and Caspase1 p20 (Fig. 3A and G) and mRNA expression of TNF-α (Fig. 3H), IL-1(Fig. 3I) and IL-18(Fig. 3J) (P<0.05, n=4). However, these effects could be reversed by EVC treatment (P<0.05, n=4).

The therapeutic effects of inhibiting PCSK9 in septic endothelial dysfunction were counteracted by agonists of TLR4.

To determine the role of TLR4/MyD88/NF-κB and NLRP3 pathways in PCSK9-induced septic endothelial dysfunction, we used KLA to specifically activate TLR4. The Western blot results showed that inhibition of PCSK9 with EVC, compared with LPS group, could increase the expression of eNOS (Fig. 4A and B) and VE-Cadherin (Fig. 4A and C) and decrease the expression of TLR4 (Fig. 4A and E), MyD88 (Fig. 4A and F), p-p65 (Fig. 4A and G), NLRP3 (Fig. 4A and H), ASC (Fig. 4A and I) and Caspase1 p20 (Fig. 4A and J) (P<0.05, n=4). However, these effects was diminished by KLA treatment (P<0.05, n=4). In addition, compared with LPS group, inhibition of PCSK9 with EVC decreased mRNA expression of TNF-α (Fig. 3H), IL-1(Fig. 3I) and IL-18(Fig. 3J) and increased of NO content and cell viability(P<0.05, n=4), while, these effects was reversed by KLA treatment (P<0.05, n=4). Although EVC could induce a decrease of PCSK9 (Fig. 4A and D) expression in HUVECs compared with LPS group (P<0.05, n=4), KLA treatment have little effects on PCSK9 expression compared with LPS + EVC group (P>0.05, n=4).

PCSK9 impaired endothelial function by activating the TLR4/MyD88/NF-κB and NLRP3 pathways to increase the production of inflammatory cytokines in vivo.

To determine the relationship between PCSK9 and septic endothelial dysfunction in vivo, CLP was performed on mice to induce sepsis and PCSK9 inhibitor Pep 2-8 were used. The results showed that CLP decreased the expression of eNOS and VE-Cadherin and increased the expression of TLR4 (Fig. 4A and E), MyD88 (Fig. 4A and F), p-p65 (Fig. 4A and G), NLRP3 (Fig. 4A and H), ASC (Fig. 4A and I) and Caspase1 p20 (Fig. 4A and J) and the mRNA expression of TNF-α (Fig. 3H), IL-1(Fig. 3I) and IL-18(Fig. 3J) in aortas of mice (P<0.05, n=4). However, these effects could be reversed by Pep 2-8 treatment (P<0.05, n=4). The KLA treatment could abolish the effects of Pep 2-8 (P<0.05, n=4). Pep 2-8 could reversed the increased PCSK9 expression in CLP group(P<0.05, n=4) and KLA treatment have little effects on PCSK9 expression compared with the CLP + Pep 2-8 group(P>0.05, n=4).

PCSK9 impaired endothelium-dependent vasodilation function and induced death in septic mice.

Vascular endothelium-dependent vasodilation function and survival rate in sepsis mice were measured. As shown in Fig. 6A, inhibition of PCSK9 by Pep 2-8 significantly reversed CLP-induced vascular endothelium-dependent vasodilation dysfunction (pD2: Sham 7.307, CLP 6.525, CLP + Pep 2-8 7.157) (P<0.05, n=4). On the other hand, activating the TLR4 with KLA abolish the therapeutic effects of Pep 2-8 (pD2: CLP + Pep 2-8 7.157, CLP + Pep 2-8 + KLA 6.727) (P<0.05, n=4). The results of Survival Study displayed that the onset of death was 12 h after CLP, 60% of CLP mice died within 24 h, and the septic mice all died at 48 h after surgery. Inhibiting PCSK9 with Pep 2-8 could significant improve the survival rate of septic mice. However, the protective effects of Pep 2-8 can be abolished by the KLA (P<0.05, n=4).

Discussion

This study mainly explored the role of PCSK9 in septic endothelial dysfunction. It was found that, in sepsis, PCSK9 damaged vascular endothelial cells and decreased endothelium-dependent vasodilation function and barrier function, in which the TLR4/MyD88/NF-κB and NLRP3 pathways were involved. Our study suggests that the role of PCSK9 and the impairment of vascular endothelial function should be considered in the treatment of patients with sepsis.

Sepsis is a terrible systemic disease and causes damage to almost all organs of the body[15]. Pathogens and inflammatory cytokines spread throughout the body along the bloodstream, and severely damage the function of various organs[16]. In its advanced stages, multiple organ dysfunction usually occurs and individual death ensues[17]. Modern medicine has made great progress in the treatment of sepsis. Nevertheless, it still kills a large number of patients each year[18]. Exploring its mechanism does favor to further understand this disease and find more effective treatment.

In sepsis, vascular dysfunction leads to hemodynamic instability and decreases circulating blood volume, and severe hypotension and even shock occur[19]. The blood stream is unable to bring the oxygen, nutrients and therapeutic drugs to the organs, and the patient's chances of survival are slim[20]. In

addition, the blood vessels are ubiquitous in the body, and they are not only the channels for transporting blood, but also important components of each organ[21]. Thus, protecting vascular function in sepsis is of great significance.

Endothelium is attached to the inner surface of blood vessels and plays an important role in regulation of vascular function[22]. It regulates tension of blood vessel, prevents too much fluid from leaking from blood into tissues, and reduces inflammation[23]. Endothelial nitric oxide synthase (eNOS) and Vascular endothelial cadherin (VE-Cadherin) directly affect endothelial cell function. eNOS is an important enzyme catalyzing nitric oxide synthesis and plays an important role in vasodilation regulation[24]. VE-Cadherin promotes the endothelial cell-to–cell adhesion and maintains endothelial integrity[25]. In our study, LPS and CLP decreased the expression of eNOS and VE-Cadherin, suggesting that endothelial function impairment occurred in sepsis.

PCSK9 is involved in lipid metabolism[26]. Clinical studies have proved that the level of PCSK9 in serum of patients with sepsis increased, and PCSK9 is regarded as a biomarker of sepsis[27]. Another studies have shown that the expression level of PCSK9 is positively correlated with the pathological damage of liver and kidney in septic mice[12, 28]. In the current study, we found for the first time that PCSK9 increased in LPS-treated HUVECs and the aortas of CLP-induced septic mice. Subsequently, we found that inhibition of PCSK9 reversed the sepsis-induced decline in eNOS and VE-Cadherin expression in vitro and in vivo. This confirms our hypothesis that the elevation of PCSK9 in sepsis induces endothelial dysfunction.

Toll-like receptor 4 (TLR4) is a vital component of innate immunity system[29]. A number of exogenous and endogenous ligands bind the TLR4 and activate TLR4/MyD88/NF-κB signaling pathway[30]. p65 is an important subunit of NF-κB. Phosphorylation of p65 promotes NF-κB translocation to the nucleus and increases transcription of inflammatory cytokines to induce inflammation[31]. Our previous study has manifested that this pathway involves in septic endothelial dysfunction[32]. Recent studies showed that PCSK9 could activate TLR4 to induce inflammatory responses in atherosclerosis[33]. NLRP3 inflammasomes is a complex composed of NLRP3,ASC and Caspase1. which play a critical role in vascular endothelial function impairment in sepsis[34]. Activation of NF-KB increases the transcription of each component of NLRP3 inflammasomes[35]. Next, stimulated by some substances, NLRP3 was activated and caspase1 is recruited and cleaved into caspase1 p20 which increase the transcription of IL-1and IL-18[36]. Therefore, we hypothesized that the TLR4/MyD88/NF-κB and NLRP3 pathways is associated with the PCSK9-induced vascular endothelial cells in sepsis. In this study, we found that activation of the TLR4/MyD88/NF-κB and NLRP3 pathways and up-regulated mRNA expression levels of inflammatory cytokines can be reversed by PCSK9 inhibition. However, specific activation of TLR4 by KLA could abolish the protective effect of PCSK9 inhibition, although PCKS9 expression is still inhibited. Our data suggest that PCSK9 activated the TLR4/MyD88/NF-κB and NLRP3 pathways to induces an inflammatory response that leads to vascular endothelial injury.

Vascular reactivity test and survival study of septic mice can more directly reflect the vascular function and status of the organism. Results from these confirmed our conclusion that inhibiting PCSK9 to prevent the activation of TLR4 in sepsis could improve the vascular function and survival rates of septic mice.

In summary, our study suggests that PCSK9 activates TLR4/MyD88/NF-κB and NLRP3 pathways to trigger inflammatory response and damage vascular endothelial function in sepsis. Inhibition of PCSK9 may have a great potential in clinical treatment.

Declarations

AUTHOR DECLARATIONS

Ethics Approval and Consent to Participate Animal experiments were performed in accordance with the National Animal Protection and Use Guidelines and approved by the local ethical committee of the First Affiliated Hospital of Chongqing Medical University (No: 2021-706). Newborn umbilical cord was obtained from the First Affiliated Hospital of Chongqing Medical University, and the experiments were approved by the local ethical committee of the First Affiliated Hospital of Chongqing Medical University (No: 2021-689).

Consent for Publication The manuscript is approved by all authors for publication.

Availability of Data and Materials The data and materials used in this study are available from the corresponding author on reasonable request.

Competing Interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding This work was supported by the National Natural Science Foundation of China (82070238).

Authors' Contributions SL and YX conceived this idea; LH and ZC performed the experiments in vitro. LH and ZL performed the experiments in vivo. YL participated in the collection, analysis and interpretation of data. LH wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgements Not applicable

Authors' Information Not applicable

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Figures

Figure 1

LPS decreased eNOS and VE-Cadherin expression and increased PCSK9 expression in HUVECs in a dose-dependent manner. Western blot was used to measure eNOS, VE-Cadherin, PCSK9, and β-actin expression in HUVECs treated with 0, 0.5, 1, 2, 5, 10 μg/ml LPS for 24 hours (A). Quantifification of eNOS, VE-Cadherin and PCSK9 of Western blots (B-D). NO release into the culture medium of HUVECs treated with 0, 0.5, 1, 2, 5, 10 μg/ml LPS for 24 hours was measured (E). Cell viability of HUVECs treated with 0, 0.5, 1, 2, 5, 10 μg/ml LPS for 24 hours was measured (F). n=4, $^{**}P<0.01$.

Figure 2

Inhibition of PCSK9 reversed LPS-induced declines in eNOS and VE-Cadherin expression, NO content and cell viability. Western blot was used to measure eNOS, VE-Cadherin, PCSK9, and β-actin expression in HUVECs treated with 0 μg/ml LPS and 10 μg/ml LPS+EVC (0, 50, 100, 200 μM) for 24 hours (A). Quantification of eNOS, VE-Cadherin and PCSK9 of Western blots (B-D). NO release into the culture medium of HUVECs treated with 0 μg/ml LPS and 10 μg/ml LPS+EVC (0, 50, 100, 200 μM) for 24 hours was measured (E). Cell viability of HUVECs HUVECs treated with 0 μg/ml LPS and 10 μg/ml LPS+EVC (0, 50, 100, 200 μM) for 24 hours was measured (F). n=4, *P<0.05, **P<0.01.

Figure 3

Inhibition of PCSK9 reversed LPS-induced activation of TLR4/MyD88/NF-κB and NLRP3 pathways and increased production of inflammatory cytokines. Western blot was used to measure TLR4, MyD88, p-p65, p65, NLRP3, ASC, Caspase1 p20, and β-actin expression in HUVECs treated 0 μg/ml LPS + 0 μM EVC, 10 μg/ml LPS + 0 μM EVC and 10 μg/ml LPS + 200 μM EVC for 24 hours (A). Quantification of TLR4, MyD88, p-p65/p65, NLRP3, ASC and Caspase1 p20 of Western blots (B-G). Real-time PCR was used to measure TNFα (H), IL-1 (I) and IL-18 (J) mRNA expression in HUVECs treated 0 μg/ml LPS + 0 μg/ml EVC, 10 μg/ml LPS + 0 μM EVC and 10 μg/ml LPS + 200 μM EVC for 24 hours. n=4, *P<0.05, **P<0.01.

Figure 4

The therapeutic effects of inhibiting PCSK9 in septic endothelial dysfunction were counteracted by agonists of TLR4. Western blot was used to measure eNOS, VE-Cadherin, PCSK9, TLR4, MyD88, p-p65, p65, NLRP3, ASC, Caspase1 p20, and β-actin expression in HUVECs treated 10 μg/ml LPS + 0 μM EVC + 0 μg/ml KLA, 10 μg/ml LPS + 200 μM EVC +0 μg/ml KLA and 10 μg/ml LPS + 200 μM EVC + 10 μg/ml KLA (A). Quantification of eNOS, VE-Cadherin, PCSK9, TLR4, MyD88, p-p65/p65, NLRP3, ASC and Caspase1 p20 of Western blots (B-J). Real-time PCR was used to measure TNFα (K), IL-1 (L) and IL-18 (M) mRNA expression in HUVECs treated 0 μg/ml LPS + 0 μg/ml EVC, 10 μg/ml LPS + 0 μM EVC and 10 μg/ml LPS + 200 μM EVC for 24 hours. NO release into the culture medium of HUVECs treated 0 μg/ml LPS + 0 μg/ml EVC, 10 μg/ml LPS + 0 μM EVC and 10 μg/ml LPS + 200 μM EVC for 24 hours was measured (N). Cell viability of HUVECs treated 0 μg/ml LPS + 0 μg/ml EVC, 10 μg/ml LPS + 0 μM EVC and 10 μg/ml LPS + 200 μM EVC for 24 hours was measured (O). n=4, **P<0.01.

Figure 5

PCSK9 impaired endothelial function by activating the TLR4/MyD88/NF-κB and NLRP3 pathways to increase the production of inflammatory cytokines in vivo. Western blot was used to measure eNOS, VE-Cadherin, PCSK9, TLR4, MyD88, p-p65, p65, NLRP3, ASC, Caspase1 p20, and β-actin expression in aortas of mice from Sham group, CLP group, CLP + 10 μg/kg Pep 2-8 group, and CLP + 10 μg/kg Pep 2-8 + 1 μg/g KLA at 24 h after CLP induction (A). Quantification of eNOS, VE-Cadherin, PCSK9, TLR4, MyD88, p-p65/p65, NLRP3, ASC and Caspase1 p20 of Western blots (B-J). Real-time PCR was used to measure TNFα (K), IL-1 (L) and IL-18 (M) mRNA expression in aortas of mice from Sham group, CLP group, CLP + 10 μg/kg Pep 2-8 group, and CLP + 10 μg/kg Pep 2-8 + 1 μg/g KLA at 24 h after CLP induction. n=4, $^{**}P<0.01$.

Figure 6

PCSK9 impaired endothelium-dependent vasodilation function and induced death in septic mice in septic mice. Acetylcholine-induced relaxation (A) and sodium nitroprusside-induced relaxation (B) of the aorta of mice from Sham group, CLP group, CLP + 10 μg/kg Pep 2-8 group, and CLP + 10 μg/kg Pep 2-8 + 1 μg/g KLA were measured at 24 h after CLP induction (n = 4). Survival within 48 h after CLP (C) was observed (n = 10). $^{*}P < 0.05$, $^{**}P<0.01$ compared with the Sham group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with the CLP group; $^{\&}P < 0.05$, $^{\&\&}P < 0.01$ compared with the CLP + Pep 2-8 group.