Abstract. Sepsis is characterized by injury to the microvasculature and the microvascular endothelial cells, leading to barrier dysfunction. However, the specific role of injury in septic endothelial barrier dysfunction remains to be elucidated. In the present study, it was hypothesized that endothelial cell inflammatory injury is likely required for barrier dysfunction under septic conditions in vitro. 2,3,5,4'-Tetrahydroxystilbene-2-0-β-D-glucoside (TSG), a compound extracted from Chinese herbs, is able to inhibit the inflammatory injury of septic-serum in endothelial cells. In the present study, cell viability was assayed by CCK-8 method; mRNA and protein expression was identified by RT-qPCR, western blot or Elisa, respectively and the production of reactive oxygen species was observed by a fluorescence microscope. The present study indicated that septic serum significantly decreased the cell viability of pulmonary aortic endothelial cells (PAECs) following co-cultivation for 6 h, which occurred in a time-dependent manner. TSG notably increased the viability of PAECs in a time- and concentration-dependent manner. Further investigations revealed that septic serum increased the secretion of interleukin (IL)-1β, IL-6 and C-reactive protein in PAECs, whereas pretreatment with TSG significantly decreased the secretion of these inflammatory factors. These data indicated that septic serum increased inflammatory injury to the PAECs, and TSG decreased this injury via the reactive oxygen species-mitogen-activated protein kinase-nuclear factor-κB signaling pathway.

Introduction

The organ dysfunction induced by sepsis is largely due to systemic inflammation, which results in dysfunction of the microvasculature, particularly microvascular endothelial cells (1,2). Impaired barrier function in the vascular wall, neutrophil influx into organs, impaired distribution of blood flow in microvascular beds and microvascular thromboses are the most common forms of dysfunction of microvasculature (3). The dysfunction of microvasculature is important clinically, as it has been documented early in the course of sepsis in humans and is associated with increased mortality rates, particularly when it persists over time (4,5).

Endothelial dysfunction is an early stage in several vascular diseases and inflammation is involved in the pathological process of organ dysfunction, primarily by motivating endothelial dysfunction. One of the most common results of inflammation is the activation of endothelial cell apoptosis (6-9).

The lungs are important organs and the systemic inflammatory response caused by sepsis is an important risk factor for pulmonary microvascular dysfunction (10-12). Multiple mechanisms promote septic pulmonary microvascular dysfunction, including activation by mechanical interaction with activated leukocytes, inflammatory cytokines, and exposure to harmful leukocyte-derived molecules, including oxidants (13). These factors result in pulmonary microvascular endothelial cell abnormalities, including disruption of inter-pulmonary microvascular endothelial cell junctions and cytoskeleton-driven retraction. In addition, the locally produced inflammatory factors in target organs may have a direct and important effect in the pathological process.
2,3,5,4′-Tetrahydroxystilbene-2-O-β-D-glucoside (TSG; Fig. 1) is one of the major bioactive constituents extracted from *Polygonum multiflorum* Thunb, and exhibits various pharmacologic activities, including anti-inflammatory, antioxidant, and anti-atherosclerotic effects, improvement of memory and learning ability, neuroprotection, anti-aging, attenuation of human platelet aggregation and promotion of hair growth (14-19). However, the protective effect of TSG on sepsis-induced inflammatory injury remains to be fully elucidated.

Sepsis can induce the expression of several inflammatory factors (20,21) and the anti-inflammatory effect of TSG is well-documented (22-25). However, the pro-inflammatory mechanisms in sepsis and the mechanism underlying the anti-inflammatory effect of TSG induced by septic serum remain to be elucidated. The present study provided evidence that inflammatory factors were produced by activated endothelial cells via the reactive oxygen species (ROS)-mitogen-activated protein kinase (MAPK)-nuclear factor (NF)-κB signaling pathway. TSG exerted a protective effect via interfering with this signaling pathway.

Materials and methods

Reagents. TSG was obtained from the National Food and Drug Testing Institute (Beijing, China). Tissue culture medium 199 (M199) was purchased from GE Healthcare Life Sciences (South Logan, UT, USA) and fetal bovine serum (FBS) were acquired from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Endothelial cell growth factor (ECGF) was purchased from Roche Diagnostics (Basel, Switzerland). TRIzol and a One-Step RT-PCR kit were from Invitrogen; Thermo Fisher Scientific, Inc. Extracellular signal-regulated kinase (ERK) 1/2 inhibitor PD98059, p38 inhibitor SB203580, c-Jun N-terminal kinase (JNK) inhibitor SP600125, antioxidant N-acetylcysteine (NAC), and NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) were from Sigma-Aldrich; Merck Millipore (Darmstadt, Germany). Rabbit NF-κB antibody (cat. no. ab207297) and rabbit CD31 antibody (cat. no. ab228364) were provided by Abcam (Cambridge, MA, USA). Rabbit monoclonal β-actin antibody (cat. no. NC011) was obtained from Zhuzhu Biotech (Xi’an, China). ERK1/2 (cat. no. AF1051) and phosphorylated (phospho)-ERK1/2 (cat. no. AM071) antibodies, and 2,7′-dichlorodihydrofluororescein diacetate (H₂DCF-DA) were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Phospho-p38 (cat. no. 8632S) and p38 (cat. no. 8690T) antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Goat anti-rabbit IgG, HRP conjugated (cat. no. CW0103) or Goat anti-mouse IgG, HRP conjugated (cat. no. CW0110S) secondary antibodies were provided by CW Biotech Co., Ltd. (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for detecting IL-1β, IL-6 and C-reactive protein (CRP) were from West Tang Biotechnology (Shanghai, China). All other materials, except where indicated were from Sigma-Aldrich; Merck KGaA, and were of analytical grade.

Preparation of septic serum. The male SD rats (n=16, weight: 160-180 g) were randomly assigned into two groups. Septic model rats were established according to the experiment procedure described by Niwa et al (27) as follows: A laparotomy was performed through a midline incision of each animal under ether anesthesia. The cecum was filled with feces by milking stools back from the ascending colon, following which the distal one third of the cecum was tied off. The ligated region of the cecum was punctured twice with an 18-gauge needle, following which the bowel was replaced in the peritoneal cavity and the abdomen closed. In the control group, the rats were treated in the same manner as the septic rat models, but without ligation of the cecum and puncturing. At 7 h post-surgery, serum samples were collected via the abdominal aorta.

Cell viability assay. The CCK-8 method was used to detect the cell viability of PAECs. Approximately 1-1.5x10⁶ cells were incubated with septic serum for 12 h, incubated with different concentrations of TSG for 12 h or subjected to septic serum treatment in the absence/presence of TSG in 96-well plates at 37°C. The experimental protocol was as follows: 10 µl CCK-8 solution was added to each well, followed by incubation for 4 h at 37°C. The optical density was measured at 450 nm using a microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA). The mean optical density of six wells was used to calculate the cell viability percentage.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. The PAECs were cultured in 6-well plates immunofluorescence staining with CD31 antibody (Fig. 2). Before the experiment, all rats had free access to food and water, and were maintained in a constant environment with a conventional 12/12 h light/dark cycle. The cells were cultured in M199 media supplemented with 20% FBS, 100 U/ml streptomycin, 100 µl penicillin, 95 µg/ml heparin and 20 µg/ml ECGF. The cells were maintained in a 5% humidified air CO₂ atmosphere at 37°C. Cells at passages 3-8 at 80-90% confluence were used in the present study. All experimental procedures were performed in strict accordance with the international, national and institutional rules, and approved by the Institutional Animal Care Committee of Sun Yat-Sen University (Guangzhou, China).

Primary culture of pulmonary aortic endothelial cells (PAECs). The rat PAECs were isolated from the pulmonary aortae of male Sprague-Dawley (SD) rats (6 weeks old) based on previously described methods (26) and identified using molecular structure of 2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside.
Run in triplicate, and analyzed using the 2-ΔΔCq method as previously described (28). ELISA analysis. The PAECs (1-1.5x10⁶) were cultured in a 96-well plate and stimulated with septic serum for 12 h or pretreated with TSG for 2 h. The supernatant was then collected, and the levels of IL-1β, IL-6 and CRP in the supernatant were assayed using ELISA kits specific for rat IL-1β, IL-6 and CRP.

Immunofluorescence staining. The PAECs were incubated with the primary rabbit anti-cd31 antibody (1:300 dilution) overnight at 4°C and were then incubated with the secondary Alexa fluor 488-conjugated antibody (1:500 dilution) for 3 h at room temperature. Subsequently, the cells were stained in 2-(4-aminophenyl)-6-indolecarbamidine dihydrochloride solution. Each of these steps was followed by washing with ice-cold PBS three times. Finally, the cells on coverslips were preserved in anti-fade mounting medium and the expression of CD31 was observed under a fluorescent microscope.

Western blot analysis. Following the indicated treatments, the cells were washed twice with ice-cold PBS (pH 7.4) and lysed in lysis buffer supplemented with protease inhibitor cocktail and phosphatase inhibitors (Roche Diagnostics; 100 µl per well of a 6-well plate). The concentration of protein was measured using a BCA protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal quantities of the protein (30 µg) were loaded and separated by 10% SDS-PAGE, and blotted onto a PVDF membrane (0.45 µm; GE Healthcare Life Sciences). The membranes were incubated with anti-β-actin (1:2,000), anti-p38 (1:1,000) or anti-phospho-p38 (1:500), anti-ERK1/2 (1:1,000) or anti-phospho-ERK1/2 (1:800) antibodies at 4°C overnight. Following washing with PBS three times, the membranes were incubated with the goat anti-rabbit IgG HRP conjugated or goat anti-mouse IgG HRP conjugated secondary antibodies (1:2,500) for 3 h. The immune complexes were enhanced using chemiluminescence and band intensities were measured and analyzed by Image-Pro Plus software (Version 10; Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. The results are represented as the mean ± standard error of the mean. Statistical analysis was performed using the Steer-Dwass or Mann-Whitney multiple comparison test. Analysis was performed using GraphPad prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

TSG increases the cell viability of septic serum-induced PAECs. As shown in Fig. 3A, the septic serum significantly decreased the cell viability of the PAECs following incubation for 12, 24 or 48 h. Treatment with TSG alone did not affect the viability of the PAECs (Fig. 3B). However, pretreatment of the cells with TSG at a concentration of 80 µmol/l for 2 h, followed by co-incubation with septic serum for another 12 h increased the viability of the PAECs, in a concentration-dependent manner, compared with the viability in the septic serum group (Fig. 3C).

TSG decreases septic serum-induced inflammatory cytokine expression in PAECs. The mRNA levels of IL-1β, IL-6 and CRP were determined using RT-qPCR analysis. As
shown in Fig. 4A-C, the mRNA levels of IL-1β, IL-6 and CRP in the PAECs were significantly increased following exposure to septic serum for 12 h (P<0.01 vs. control). By contrast, pretreatment with TSG at a concentration of 40 µmol/l for 2 h, followed by co-incubation with septic serum significantly decreased the expression of mRNA in a concentration-dependent manner.

The results from the ELISA analysis indicated that the protein expression levels of IL-1β and IL-6 in the supernatant in a concentration-dependent manner (Fig. 4D and E). Pretreatment with TSG at a concentration of 80 µmol/l for 2 h, followed by co-incubation with septic serum notably decreased the level of CRP in the supernatant in a concentration-dependent manner (Fig. 4F).

**TSG decreases septic serum-induced ROS generation in PAECs.** As shown in Fig. 5 septic serum increased the
generation of intracellular ROS in PAECs, whereas pretreatment with TSG for 2 h significantly eliminated the septic serum-induced intracellular expression of ROS (Fig. 5).

**TSG decreases septic serum-induced protein expression levels of phosphorylated ERK1/2, p38 and JNK.** As it is reported that MAPK is important in the expression of several inflammatory cytokines, the upregulated expression of inflammatory cytokines by septic serum in PAECs may be associated with MAPK signaling. The results of the present study showed that the protein levels of phospho-ERK1/2, phospho-P38 and phospho-JNK in the PAECs were increased following stimulation with septic serum (Fig. 6A-C). Pretreatment with TSG for 2 h significantly reduced the septic serum-induced protein expression of phospho-ERK1/2, phospho-P38 and phospho-JNK in the PAECs. Subsequent experiments indicated that pretreatment with the antioxidant NAC for 1.5 h, alone or in combination with TSG decreased the protein expression levels of phospho-ERK1/2, phospho-P38 and phospho-JNK. These results indicated that the MAPK pathway may be involved in septic serum-induced inflammatory cytokine expression, whereas TSG eliminated the septic serum-induced expression of inflammatory cytokines in the PAECs. Taken together, these results showed that TSG inhibited septic serum-induced inflammatory injury via interfering with the ROS-MAPK signaling pathway in PAECs.

**TSG decreases septic serum-induced levels of NF-κB in PAECs.** MAPK and NF-κB are involved in the expression of several inflammatory cytokines, and septic serum-induced inflammation in PAECs may be associated with ROS-MAPK-NF-κB signaling. The results of the present study showed that the nuclear expression of NF-κB in PAECs was increased following stimulation with septic serum for 12 h. Pretreatment of the cells with NAC (antioxidant), PD98059 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor) or PDTC (NF-κB inhibitor) for 1.5 h significantly reduced the septic serum-induced nuclear expression of NF-κB (Fig. 7). These results also indicated that ROS was involved in the septic serum-induced nuclear expression of NF-κB in PAECs.

**TSG decreases septic serum induced inflammation via the ROS-MAPK-NF-κB signaling pathway in PAECs.** As shown in Fig. 4, the protein levels of IL-1β, IL-6 and CRP in the cells were significantly decreased following pretreatment with TSG for 1 h (P<0.05 or P<0.01 vs. control). As a positive control, the cells pretreated with PD98059 (ERK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor) or antioxidant NAC for 1 h showed similar effects (Fig. 8). These results indicated that the ROS-MAPK-NF-κB signaling pathway was involved in septic serum-induced inflammation in the PAECs.
Discussion

In the present report, in vitro conditions of septicemia were established to examine the protective effect of TSG on septic serum-induced inflammatory injury in PAECs. The results confirmed that the stimulation of PAECs with septic serum led to upregulation in the protein expression levels of IL-1β, IL-6 and CRP, whereas pretreatment with TSG significantly reduced the expression of these inflammatory cytokines in PAECs, in a concentration-dependent manner. Subsequent experiments indicated that the protective effect of TSG was predominantly through interfering with the ROS-MAPK-NF-κB signaling pathway.

In sepsis, multiple organ dysfunction is mainly due to systemic inflammatory injury of the microvasculature, particularly microvascular endothelial cells (29-32). Numerous clinical and laboratory investigations have indicated that, for microvascular and microvascular endothelial cells, dysfunction or injury are the initial stages of organ dysfunction (33). In addition, increased numbers of circulating endothelial cells and soluble markers of endothelial cell damage correlate with increased severity of sepsis and higher mortality rates (34,35). In addition, this septic microvascular dysfunction is clinically relevant, as the presence of microvascular dysfunction in human sepsis is associated with more severe sepsis, organ dysfunction, and increased mortality rates (36,37). The clinical outcomes, including survival rates, have been reported to be particularly poor if septic microvascular dysfunction persists over time despite usual clinical management (13,27,38-40). In the present study, a rat model of sepsis was established, and rat septic serum was used to investigate the pro-inflammatory effect of septic serum and the protective effect of TSG. The results showed that septic serum significantly induced the expression levels of IL-1β, IL-6 and CRP, whereas TSG eliminated the pro-inflammatory effects of septic serum in the endothelial cells.

The activation of an inflammatory effect occurs via multiple pathways, and data from clinical and experimental investigations have shown that oxidative damage to endothelial cells is severe in sepsis (41-43). ROS are important secondary messengers and are directly involved in oxidative stress. The present study showed that ROS were important in the septic serum-induced expression of inflammatory factors. Pre-treatment with antioxidant NAC 10⁻² M significantly inhibited the protein expression levels of IL-1β, IL-6 and CRP in PAECs, whereas cells co-cultivated with TSG reduced septic serum-induced superoxide anion generation in PAECs.
MAPK and NF-κB signaling are pivotal in inflammation (44-49). The activation of NF-κB is responsible for the expression of several inflammatory cytokines. The results of the present study showed that p38 MAPK and NF-κB were involved in the protein expression of IL-1β, IL-6 and CRP induced by septic serum; the selective p38 MAPK and NF-κB inhibitor, PDTC, significantly inhibited the protein expression of IL-1β, IL-6 and CRP in PAECs, and TSG had a similar effect to the specific inhibitor.

In conclusion, the present study demonstrated that septic serum induced the protein expression of IL-1β, IL-6 and CRP in PAECs, and that TSG inhibited the septic serum-induced inflammatory injury via interfering with the ROS-p38MAPK-NF-κB signaling pathway. These results provide novel evidence supporting the potential inflammatory effect of septic serum and the anti-inflammatory effect of TSG.

References


