Doxycycline Hyclate Protects Lipopolysaccharide-Induced Endothelial Barrier Dysfunction by Inhibiting the Activation of p38 Mitogen-Activated Protein Kinase

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Doxycycline hyclate (DOX-h) attenuates inflammatory conditions independent of its antimicrobial effect. This study aimed to observe the effects of DOX-h on lipopolysaccharide (LPS)-induced endothelial barrier dysfunction. The endothelial monolayer permeability of human umbilical vein endothelial cells (HUVECs) was monitored by transendothelial electrical resistance (TEER). The phosphorylation of mitogen-activated protein kinases (MAPKs) and the arrangement of F-actin were detected. The results showed that both pretreatment and simultaneous treatment with DOX-h markedly attenuated the LPS-induced reduction in TEER and the disorganization of F-actin on HUVECs in a dose- and time-dependent manner. LPS mediated the phosphorylation of all three MAPKs (p38, extracellular signal-regulated kinase (ERK)1/2, and c-Jun N-terminal kinase (JNK)), but DOX-h was only able to inhibit the LPS-induced phosphorylation of p38 and JNK. The data further suggested that DOX-h alleviated LPS-evoked TEER reduction and F-actin redistribution by inhibiting the phosphorylation of p38 and its downstream target, heat shock protein (HSP)27. Thus, DOX-h attenuates LPS-induced endothelial barrier dysfunction via inhibition of the p38 MAPK-HSP27-F-actin pathway.

Key words: doxycycline hyclate; lipopolysaccharide; endothelial barrier dysfunction; mitogen-activated protein kinase; F-actin; human umbilical vein endothelial cell

Sepsis is an important, resource-consuming public health concern and one of the leading causes of morbidity and mortality in the world.1) The increased vascular permeability and subsequent loss of plasma and fluid are significant pathological processes during the development of sepsis and septic shock.2,3) As an important contributory pathogenic factor in sepsis, lipopolysaccharide (LPS), also named as endotoxin, is capable to damage the vascular endothelial barrier integrity by activating tremendous inflammatory signal transduction, causing a state of circulatory disorder and dysfunction during the development of sepsis.4,5) The most affected organs and tissue(s) would be lung and blood-brain barrier.5,6) The disruption of endothelial barrier in those tissues will result in acute lung injury (ALI) and acute respiratory distressed syndrome (ARDS), as well as the dysfunction of central nerve system.6,7) The blockade of hyperpermeability response could improve the survival of LPS-challenged mice8) and allogeneic human lung lobe.9)

Despite progress in diagnosis and supportive care, sepsis is still challenging to the field of medicine, and its occurrence appears to be increasing.1,10) There is therefore an urgent need to develop new therapies to ameliorate sepsis-associated morbidities. Tetracyclines are well-known antibiotics that are extensively used against a wide variety of common infections.11) Doxycycline, commonly used in the form of doxycycline hyclate (DOX-h), has been shown to inhibit some matrix metalloproteinases (MMP) independently of its antimicrobial effect.12) There is a report demonstrating that doxycycline attenuates burn-induced microvascular hyperpermeability in rats through MMP-9 inhibition.13) It is possible that DOX-h has the potential to protect vascular endothelial barrier function from LPS damage. The first aim of this study was to verify that DOX-h could work as an inhibitor of LPS-induced endothelial hyperpermeability.

The mitogen-activated protein kinase (MAPK) family, with p38 MAPK, the c-Jun N-terminal kinase (JNK/SAPK), and the extracellular signal-regulated kinase (ERK)1/2 as three major members, plays a key role in modulating the actin–myosin cytoskeleton and regulating vascular permeability.14) LPS is a well-known activator of MAPKs, and LPS-MAPK pathway interactions are critical in the development of sepsis.15) It has been reported that DOX-h can attenuate the phosphorylation of JNK1/2, ERK1/2, and p38 MAPKs in the corneal epithelium, while DOX-h suppresses p38 MAPK in murine LA4 lung epithelial cells stimulated by cytokines.16,17) It is therefore reasonable to speculate that DOX-h might protect the endothelial barrier from LPS insult by inhibiting the activation of MAPKs.

In this study, we aimed to investigate the protective effects of DOX-h on the permeability of the human umbilical vein endothelial cell (HUVEC) monolayer and cytoskeletal remodeling in response to LPS, and to explore whether these processes depend on the inhibition of MAPK activity.

MATERIALS AND METHODS

Reagents Mouse polyclonal antibodies against human β-actin, JNK and p-JNK (1 : 1000, Santa Cruz, CA, U.S.A.), rabbit polyclonal antibodies against human p38, p-p38, ERK, p-ERK, p-heat shock protein (HSP)27 (1 : 1000, Cell Signaling, MA, U.S.A.) were used. The inhibitors of p38 (SB203580), JNK (SP600125) and LPS from Escherichia coli 055:B5 were...
obtained from Sigma (St. Louis, MO, U.S.A.). Rhodamine–phalloidin was obtained from Molecular Probes (Carlsbad, CA, U.S.A.). HSP27 small interfering RNA (siRNA) and scramble siRNA were purchased from GenePharma (Shanghai, China). Trizol kit and MMLV-RT reverse transcriptase were obtained from Invitrogen (Carlsbad, CA, U.S.A.). Unless specified, biochemical reagents were obtained from Sigma.

**Cells and Cell Culture** HUVECs were purchased from ScienCell (Carlsbad, CA, U.S.A.). HUVECs were discarded following passage 10. Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium was from Hyclone (Logan, UT, U.S.A.). Other culture additives were from Gibco BRL (Grand Island, NY, U.S.A.). HUVECs were maintained at 37°C in a humidified atmosphere with 5% CO₂. When the cultures reached saturation, cells were subcultured by fourfold dilution.

**The Evaluation of Endothelial Barrier Function** Firstly, the effects of DOX-h on endothelial barrier dysfunction induced by LPS were evaluated by measurements of trans-endothelial electrical resistance (TEER) across a HUVEC monolayer grown in a Transwell chamber. DOX was determined using an STX2 electrode and EVOM2 meter according to the manufacturer’s instruction manual (World Precision Instruments, Sarasota, FL, U.S.A.). HUVECs were seeded fibronectin-coated 6.5-mm Transwell filters (0.4-µm pore size). Resistance values for an experimental group on multiple Transwell inserts were measured sequentially, and the mean was expressed in the common unit (Ω cm²) after subtraction of the value for a blank (a cell-free filter). The result was presented as normalized data (i.e., ratio of resistance at a given time to resistance at “zero” time).

The endothelial barrier function was further evaluated by measuring the leakage of fluorescent probe fluorescein isothiocyanate (FITC)-labeled bovine serum albumin (BSA). HUVECs were seeded on fibronectin-coated transwell-clear polyester membranes (Corning Costar, Acton, MA, U.S.A.). Cells were exposed to different reagents as indicated. Fluorescein isothiocyanate-labeled bovine serum albumin (3.5 mg/mL) was then added to the upper chambers for 45 min. Samples were collected from both the top (luminal) and bottom (abluminal) chambers for fluorometry analysis. Albumin concentrations were detected using a HTS 7000 microplate reader (Perkin-Elmer, Yokohama, Japan) with a standard curve. The permeability coefficient for albumin (Pa) was calculated as follows: 

\[ Pa = \frac{A}{V} \times \frac{1}{\Delta A} \times \frac{1}{V} \times \frac{1}{L} \]

\[ \Delta A = \left( \frac{V}{L} \right) \times \frac{1}{V} \times \frac{1}{L} \]

where \( A \) is the abluminal albumin concentration, \( t \) is time (in s), \( A \) is the area of the membrane (in cm²), \( V \) is the volume of the abluminal chamber, and \( [L] \) is the luminal albumin concentration.

**Western Immunoblotting** Total cell extracts were prepared by lysis and sonication of the cells in lysis buffer with protease and phosphatase inhibitors. Samples were subjected to SDS-PAGE, and proteins were transferred to polyvinylidene difluoride membranes. Blots were blocked for 1 h with 5% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.5% Tween 20 (TBS-T), and then incubated overnight on a rocker at 4°C with primary antibodies against the indicated protein. The blots were washed and incubated with the respective species-specific horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After three 5-min washes in TBS-T, protein bands were visualized by chemiluminescence. Densitometric analysis was done using a Kodak IS2000R Imaging Station. The density ratio of phosphorylated protein and total protein was calculated at first. The normalized data was the ratio of density ratio in a given condition to the density ratio in control.

**Fluorescence Staining** After treatment, endothelial monolayers plated on glass cover slips were washed with phosphate-buffered saline (PBS), fixed with 3.7% formaldehyde solution, permeabilized with 0.1% Triton X-100 in PBS–Tween, and blocked with 5% BSA. Cells were then stained with rhodamine-conjugated phalloidin for 1 h at room temperature to detect F-actin. Cells were visualized using an LSM510 Meta Confocal System (Zeiss) equipped with an Axio Observer Z1 microscope (Zeiss).

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**Figure 1. Time-Dependent Effect of DOX-h on LPS-Induced Hyperpermeability Response in HUVEC Monolayer**

DOX-h (3.0 µmol/L) was applied 12 h before or at the time of treatment with LPS (200 ng/mL) to a confluent, filter-grown HUVEC monolayer. TEER was measured 1, 2, 4, and 8 h after LPS treatment. All data are presented as means±S.D. from four independent experiments. *p<0.05 vs. control, †p<0.05 vs. LPS.

**Figure 2. Dose-Dependent Effect of DOX-h on LPS-Induced Hyperpermeability Response in HUVEC Monolayer**

Various doses of DOX-h were applied 12 h before (A) or at the same time as (B) treatment with LPS, and TEER was measured 8 h after LPS application. All data are presented as means±S.D. from four independent experiments. *p<0.05 vs. control, †p<0.05 vs. LPS.
Stimulation In all experiments, HUVECs were cultured to 90% confluence and then starved of serum for 24h before the experiments. Two hundred nanogram per milliliter LPS was applied to stimulate HUVECs. To clarify the preventive and remedial effects of DOX-h on LPS-induced responses, different doses (0.3, 3, and 30µmol/L) of DOX-h were added to HUVECs, either 12h before LPS treatment (defined as the pre-treated group), or at the same time as LPS treatment (defined as simultaneously treated group). The concentrations of DOX-h used in this study were decided from Franco et al.’s report and the dose range was to ensure the efficiency of DOX-h. The HUVEC monolayer TEER was measured after 1, 2, 4, and 8h. To evaluate the effects of DOX-h on LPS-induced MAPK phosphorylation, cells were treated with DOX-h at 3.0µmol/L, and MAPK activation was monitored 8h after LPS treatment. To confirm the role of p38 or JNK on LPS-evoked vascular hyperpermeability, HUVECs were treated with inhibitors specific for p38 (SB203580) or JNK (SP600125) at 20µmol/L 1h before incubation with LPS for 8h.

Transfection of HSP27 siRNA in HUVECs Transfection was performed according to the protocols provided by the manufacturer. Briefly, HUVECs were transfected using 10nmol/L siRNA targets human HSP27 site (5'-GUCUCAUCCGAAUUUGCAGC-3'), or scramble siRNA, or under mock conditions using siRNA transfection reagent alone. Forty-eight hours after transfection, 200ng/mL LPS was added to HUVECs and TEER was detected in different groups.

Quantitative Real-Time Polymerase Chain Reaction (PCR) Total RNAs were isolated from cultured cells using a Trizol kit according to the manufacturer’s recommended manual. Then cDNAs were synthesized by using total RNAs and MMLV-RT reverse transcriptase. The reaction mixture for real-time PCR was prepared according to the manufacturer’s instructions. The PCR primers used were: 5’-ATGACCAGCGCCCGTGCCCTTCTCG-3’ (HSP27, sense), 5’-CTTAATGGCTCAAGAATGTTCCGAAC-3’ (HSP27, antisense), 5’-TTCATTTGCCCTCAAATCTAGTACG-3’ (glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense), and 5’-GTGAGTAGATGCTGAGAGAC-3’ (GAPDH, antisense).

Statistical Analysis Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) 13.0 software. Data were normalized to the control and expressed as percentages of the basal value as means±S.D. from at least three independent experiments. The significance among groups was tested by ANOVA, followed by post hoc comparisons. A p<0.05 was considered statistically significant.

RESULTS Effects of DOX-h on LPS-Induced Hyperpermeability in HUVECs The results showed that LPS significantly decreased TEER in a HUVEC monolayer, indicating compromise of the endothelial barrier, with the maximal response attained 2h after initiation of LPS stimulation, and sustained at nearly the same level for at least 8h. Both pre-treatment and simultaneous treatments with 3.0µM DOX-h protected against
LPS-induced HUVEC hyperpermeability, as demonstrated by the partial restoration of TEER after 1 h for the pre-treated group, and after 2 h for the simultaneously treated group (Fig. 1). The effects of DOX-h on LPS-induced hyperpermeability also revealed a dose-dependent pattern, as demonstrated by the rates of TEER restoration by pre- (Fig. 2A) or simultaneous (Fig. 2B) treatments with 0.3, 3.0, and 30 µM DOX-h. Incubation of HUVECs with DOX-h alone had no effect on endothelial monolayer permeability.

**Effect of DOX-h on LPS-Induced Activation of MAPK**

HUVECs were stimulated with DOX-h (3.0 µmol/L) 12 h before or at the time of LPS treatment (A). The effect of DOX-h alone on the phosphorylation of MAPKs was detected 8 h and 12 h after DOX-h application (B). Phosphorylation of MAPKs was assessed by Western blotting with specific antibodies as described in Materials and Methods. The immunointensity ratios of phosphorylated p-p38, p-JNK, and p-ERK to total p38, JNK, and ERK were calculated, respectively. The results are expressed as means ± S.D. from three independent experiments. *p<0.05 vs. control. †p<0.05 vs. LPS.
The phosphorylation of p38, JNK, and ERK1/2 was monitored to identify the effects of DOX-h on LPS-induced MAPK activation. While all three MAPK members were phosphorylated by LPS (Fig. 3), only p38 and JNK phosphorylation was attenuated by DOX-h treatment. LPS-induced phosphorylation of ERK1/2 was not affected by DOX-h (Fig. 4A). At the time points of 8h and 12h, treatment with DOX-h (3.0 µmol/L) alone had no significant effect on the phosphorylation of all three MAPK members (Fig. 4B).

DOX-h Attenuates LPS-Induced HUVEC Barrier Dysfunction via Inhibition of p38 MAPK Activation  

The roles of MAP kinases were first confirmed in LPS-induced increase in endothelial monolayer permeability. Pharmacological inhibition of p38 MAPK by SB203580 attenuated the LPS-induced increase in HUVEC endothelial monolayer permeability. In contrast, inhibition of JNK with SP600125 had no effect on the LPS-induced endothelial hyperpermeability response (Fig. 5A). These results demonstrate that the p38 MAPK signal transduction pathway is involved in increased endothelial monolayer permeability induced by LPS. Because DOX-h suppressed the phosphorylation of both p38 and JNK, it is speculated that DOX-h attenuates LPS-induced HUVEC hyperpermeability by inhibition of the p38 MAPK pathway.

F-Actin plays an important role in maintaining intercellular junctions and vascular permeability.22) We investigated the effect of DOX-h on F-actin cytoskeletal rearrangement induced by LPS. Control group, F-actin was mainly distributed in the cortex area of HUVECs, forming a complete and continuous F-actin belt. Eight hours after LPS treatment, transcellular F-actin stress fibers were formed, and cellular tension was increased. In pre- and simultaneous DOX-h treatment groups, F-actin cytoskeletal rearrangement was significantly reduced, and the F-actin filaments basically remained at the cellular cortex border (Fig. 5B), leading, respectively, to the preservation or restoration of the HUVEC monolayer integrity. Consistent with the endothelial monolayer permeability results, inhibition of p38 MAPK with SB203580 attenuated F-actin stress fiber formation in response to LPS, and F-actin distribution was not obviously disrupted compared with the control group. However, LPS-induced disturbance of F-actin organization was not improved significantly by JNK inhibition (Fig. 5B).

To further confirm the attenuating effects of DOX-h on LPS-induced HUVEC barrier dysfunction via inhibition of p38 MAPK activation, we conducted an experiment by measuring the leakage of fluorescent probe FITC-labeled bovine serum albumin through endothelial monolayer. Similar with TEER data, the results revealed that both pre- and concomitant-treatment of DOX-h significantly attenuated LPS-induced leak out of albumin through endothelial monolayer. The inhibition of p38 phosphorylation with SB203580 mimicked this attenuation, while inhibition of JNK with SP600125 showed no such effect on albumin leakage (Fig. 6A), although both inhibitors could attenuated LPS-induced phosphorylation of p38 and JNK (Fig. 6B). Combining the results of inhibition of p38 phosphorylation by DOX-h, we conclude that DOX-h protects endothelial barrier function by suppressing p38 activation.

The Activation of p38 Downstream Signal HSP27 is Suppressed by DOX-h Treatment  

Regarding p38-induced increase in cell contractility, HSP27 is p38’s major downstream target that directly links to contractile F-actin in the cytoskeleton.23,24) Because LPS-induced p38 activation is down-regulated by DOX-h, its downstream functions should also be inactivated. The results in present study first showed that LPS induced the phosphorylation of HSP27, while both pre- and concomitant-treatment of DOX-h abolished this effect (Fig. 7A). The inhibition of p38 also attenuated LPS-induced HSP27 phosphorylation (Fig. 7B). The results further demonstrated that the transfection of siRNA HSP27 protected TEER in LPS-treated HUVECs, while control siRNA had no such effects (Fig. 7C), indicating the involvement of HSP27 in LPS-induced endothelial barrier dysfunction. These data further confirm that down-regulation of p38 pathway plays a role in DOX-h-mediated attenuation of LPS-induced HUVEC barrier dysfunction.

DISCUSSION  

Our study is the first to confirm that DOX-h exhibits potent protective effects on the barrier functions of HUVECs exposed to the inflammatory mediator, LPS, and that p38-HSP27-F-actin interactions are involved in the process.

Increased vascular permeability plays an important role in the occurrence, development, and outcome of bacterial and viral sepsis.25) Perturbation of endothelial cells caused by...
physicochemical stimuli induces cell activation and has functional consequences, resulting in cell egress to extravascular sites and increased vascular permeability. F-Actin acts as a basic cytoskeleton in maintaining the shape and polarity of cells, as well as an important part of the intercellular junction complex. Incorrect distribution of F-actin and the formation of stress fibers are essential for the stimulus-induced alteration of endothelial cell shape, resulting in the disturbance of intercellular junctions and disruption of endothelial integrity.

DOX-h has been used safely and efficaciously in the clinical setting for decades. Previous studies have shown that DOX-h can inhibit RgpB-induced periodontitis and hyperpermeability in the vascular system of the retina due to VEGF-induced changes. In present study, the data show that DOX-h exhibits a strong protective effect against LPS-induced hyperpermeability of the endothelial monolayer in a dose-dependent manner. This effect is both protective and remedial in that DOX-h demonstrated its preservation of endothelial barrier function even when it was applied at the same time as LPS.

Consistent with the permeability measurements, the present results also reveal that morphologically, DOX-h attenuated LPS-induced stress fiber formation by F-actin, which further confirms that DOX-h exhibits a protective effect on increased vascular permeability induced by LPS.

Activation of MAPK proteins is critical in cellular responses associated with inflammatory stimuli such as LPS. The present results are consistent with reports that LPS enhanced significant phosphorylation of all three MAPK members. It is interesting that DOX-h only suppressed the activation of p38 and JNK but not of ERK. This may due to the particular signature motif of each MAPK member. Different MAPKs may transmit different or even opposing signals in the same cells, depending on the selection of binding motifs and scaffold proteins that guarantee the activation of a specific response to a particular signal. In addition, they are phosphorylated by different cascade reactions triggered by their respective upstream signals. We may therefore safely conclude that DOX-h can selectively inhibit a specific MAPK activation.
induced by LPS.

The present results further demonstrate that the p38 MAPK is responsible for LPS-induced F-actin rearrangement and increased endothelial monolayer permeability, whereas JNK is involved neither of these. This is consistent with our previous finding that JNK was not involved in the development of endothelial barrier dysfunction induced by burn injury.33) The explanation may be that p38 and JNK could independently modulate endothelial cell function via distinct substrate phosphorylations. JNK seems to be responsible more for alterations of endothelial adhesive function. The present findings also suggest that attenuation by DOX-h of vascular hyperpermeability induced by LPS might depend on p38 MAPK.

HSP27 has been reported to be the p38 downstream signal that interacts directly with the actin contractile cytoskeleton. Phosphorylation of HSP27 disassociates it from actin, allowing it to polymerize into stress fibers.22) Other studies, including our own, have shown that p38 inhibitor, SB203580, or p38 siRNA attenuate HMGB1 and burn-induced HSP27 phosphorylation.34,35) Because DOX-h attenuates the activation of LPS-induced p38 phosphorylation, it is reasonable to speculate that it also abolishes HSP27 phosphorylation. The result in present study showed that LPS did enhance HSP27 phosphorylation. Both inhibition of p38 and DOX-h treatment attenuated this

Fig. 7. The Involvement of HSP27 in LPS-Induced Endothelial Dysfunction

LPS (200 ng/mL) was applied to confluent, filter-grown HUVEC monolayers for 8 h. In the DOX-h treated groups, HUVECs were stimulated with LPS 12 h after or at the time of treatment with DOX-h (3.0 µmol/L) (A). SB203580 was applied 30 min before LPS treatment. Phosphorylation of HSP27 was assessed by Western blotting with specific antibody (B). HUVECs transfected with control or HSP27 siRNA were treated with 200 ng/mL LPS for 12 h and TEER was measured (C, left panel). Silencing HSP27 led to a 78% reduction of the relative mRNA expression compared with the control condition by quantitative real-time PCR (C, right panel). The results are expressed as means ± S.D. from three independent experiments. *p<0.05 vs. control. #p<0.05 vs. LPS.
enhancement (Figs. 7A, B). Since there is no specific chemical inhibitor for HSP27, HUVECs were transfected with HSP27 siRNA to selectively inhibit the expression of HSP27. The results of TEER detection demonstrated that HSP27 siRNA transfection attenuated LPS-induced decrease of TEER, while control siRNA had no such effects. HSP27 and control siRNA alone had no influence on TEER (Fig. 7C). This data clarified that HSP27 phosphorylation was involved in LPS-induced endothelial dysfunction. DOX-h induced inactivation of p38 as well as its downstream signal HSP27.

In conclusion, our novel findings provide evidence that DOX-h selectively inhibits LPS-induced MAPK pathways in HUVECs. DOX-h attenuated LPS-induced endothelial stress fiber formation and endothelial barrier dysfunction via inhibition of the p38MAPK-HSP27-F-actin pathway. These observations indicate that DOX-h may be an effective drug for treating sepsis in the clinic. Finally, our data suggest that p38 MAPK-HSP27-F-actin may be a novel target in the treatment of infectious diseases.

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