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Original Article

Dexmedetomidine ameliorates lipopolysaccharide-induced endothelial barrier disruption and inflammation by inhibiting NF-κB activity and activating α2-adrenergic receptor

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Abstract: Endothelium is a function barrier that protects the vascular from potentially cytotoxic substances. Lipopolysaccharide (LPS) is known to induce inflammation and endothelial barrier disruption in sepsis. Dexmedetomidine has been suggested to ameliorate endotoxin-induced lung injury due to its anti-inflammatory capacity. However, the effects of dexmedetomidine on LPS-induced endothelial barrier disruption and inflammation remain unknown. In the present study, human umbilical vein endothelial cells (HUVECs) were treated with LPS in the presence of various concentrations of dexmedetomidine. Inflammatory parameters including stress fibers formation, endothelial permeability, monocytes migration and adhesion proteins expressions were examined. In addition, the activity and translocation of NF-κB-p65 were evaluated. These results showed that LPS treatment significantly increased stress fiber formation and endothelial permeability, in parallel with the lowered expression of VE-cadherin and claudin-5, which are essential to maintain adherens- and tight-junctions in HUVECs. Moreover, LPS dramatically increased ICAM-1 and VCAM-1 expressions and secretions, as well as NF-κB activity and translocation. All these alterations induced by LPS were remarkably inhibited by dexmedetomidine in a concentration-dependent manner. Furthermore, the inhibitory effects of dexmedetomidine on adhesion molecules expressions and NF-κB activity were reversed by the α2-adrenergic receptor antagonist yohimbine. In conclusion, our findings reveal that dexmedetomidine ameliorates LPS-induced endothelial barrier disruption and inflammation by inhibiting NF-κB activity and activating α2-adrenergic receptor. This study suggests that dexmedetomidine may be able to preserve vascular barrier integrity of endothelial cells in endotoxin-stimulated diseases such as sepsis.

Keywords: Dexmedetomidine, endothelial permeability, inflammation, NF-κB, α2-adrenergic receptor

Introduction

The endothelium functions as a barrier that protects against neurotoxic substances and facilitates the exchange of waste products and nutrients between the vascular and blood, thus playing major roles in homeostasis including immune response, fibrinolysis and coagulation [1, 2]. Endothelial barrier dysfunction has been suggested to be a common feature of many diseases, which can be induced by lipopolysaccharides (LPS) [1]. LPS is a major component of the outer membrane of Gram-negative bacteria, and it has been found in high level in patients with infection or sepsis [3, 4]. LPS-induced sepsis reflects an uncontrolled systemic inflammatory response via NF-κB activation [5, 6]. Moreover, LPS upregulates adhesion molecules expressions and dissociates the tight- and adherens-junctional proteins to facilitate endothelial permeability, eventually leading to monocytes recruitment and vascular barrier dysfunction [7, 8].

Dexmedetomidine, a sedative and analgesic agent that exerts its effects by selectively agonizing α2-adrenergic receptor, is widely used for sedation in intensive care units [9]. It has been reported that dexmedetomidine protects against ischemia-reperfusion-induced injury in heart, kidney, brain and intestine [10-13]. In addition, accumulating evidences suggest that dex-

medetomidine processes anti-inflammatory capacity. Dexmedetomidine ameliorates sepsis-induced lung injury in endotoxemia rats [14]. In glial cells, dexmedetomidine significantly inhibits LPS-induced the increase of pro-inflammatory cytokines, such as tumor necrosis factor-α, prostaglandin E2, IL-1β, and IL-6 [15, 16]. Moreover, dexmedetomidine also suppresses LPS-induced the secretion and translocation of high mobility group box 1 and subsequently inhibits inflammation in macrophages [17]. However, whether dexmedetomidine influences endothelial permeability and inflammation is largely unknown. Therefore, the primary of this study was to investigate the effects of dexmedetomidine on LPS-induced endothelial permeability and the expression of adhesion molecules, and to further explore the potential mechanisms behind these effects.

Materials and methods

Materials and reagents

M199 medium, RPMI 1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, L-glutamine, human endothelial growth factor β (β-ECGF), FITC-phalloidin and lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA, USA). Dexmedetomidine, lipopolysaccharide (LPS), Triton X-100, bovine serum albumin (BSA), FITC-dextran, calcein-AM and yohimbine were purchase from Sigma Chemical Co. (St. Louis, MO, USA). CCK-8 reagent, RIPA lysis buffer and BCA Protein Assay Kit were obtained from Beyotime (Nanjing, Jiangsu, China).

Cell culture

Human umbilical vein endothelial cells (HUVECs) and THP-1 monocytes were obtained from ATCC (Rockville, MD, USA). HUVECs were cultured in M199 medium supplemented with 20% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 25 U/ml heparin and 5 ng/ml β-ECGF. THP-1 cells were cultured in RPMI 1640 medium with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin. All cultures were maintained in an incubator with 5% CO_2 and 95% O_2 at 37°C.

Cell viability

Viability of HUVECs was analyzed using CCK-8 assay. The cells (2×10^3) were seeded in 96-well culture plates and rendered quiescent by culturing in serum-free M199 medium overnight at 37°C. Following treatment with different concentrations of dexmedetomidine (0.01, 0.1, 1, 10 or 100 μM) for 48 h, CCK-8 was added to cells at final concentration of 500 μg/ml for 30 min. The absorbance was read with a microplate reader (SpectraMax MAX190 spectrophotometry, Sunnyvale, CA, USA) at 540 nm.

Actin filaments visualization

Phalloidin is compound that binds specifically to actin filaments. HUVECs were treated with various concentrations of dexmedetomidine (0.01, 0.1, 1, 10 or 100 μM) for 48 h in the presence of LPS treatment. The cells were fixed with 4% formaldehyde in phosphate buffer saline (PBS) for 10 min, permeated with 0.2% Triton X-100 for 5 min, blocked with 1% BSA for 1 h, and stained with FITC-phalloidin for 1 h. Actin filaments were visualized using a laser-scanning confocal microscopy (LSM 710, Carl Zeiss, München, Germany).

Western blotting

Western blotting analysis was performed as described previously [8]. HUVECs were washed with phosphate-buffered saline (PBS) and lysed in lysis buffer containing 1 mM protease inhibitor (Merck, Darmstadt, Germany). To investigate the nuclear translocation of nuclear factor kappa-B (NFκB) p65, nuclear and cytosol proteins were isolated with a Nuclear/Cytosol Fractionation Kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. The proteins concentrations were assessed using BCA Protein Assay Kit. 50 μg proteins were fractionated in 10%-12% SDS-polyacrylamide gel and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp, Billerica, MA, USA). The membranes were blocked with 5% skim milk in PBS containing 0.1% Tween 20 and incubated with rabbit polyclonal anti-VE-cadherin, rabbit polyclonal anti-claudin-5 (1:500; Santa Cruz Technology, Santa Cruz, CA, USA), mouse monoclonal anti-ICAM-1, rabbit polyclonal anti-VCAM-1, rabbit polyclonal anti-p65 and mouse monoclonal anti-β-actin (1:1000; Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Immunoblotting was carried out by incubation with HRP-conjugated secondary antibodies (1:4000;

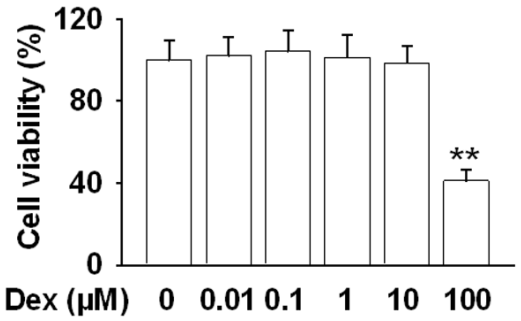

Figure 1. Dexmedetomidine is non-cytotoxic at 100 μM and below in HUVECs. The cells were seeded in 96-well culture plates overnight and then treated with different concentrations of dexmedetomidine (0.01, 0.1, 1, 10 or 100 μM) for 48 h. Cell viability was determined by CCK-8 assay. All data were expressed as mean ± SEM. **P<0.01 vs. 0 μM, n=6.

Cell Signaling Technology) for 1 h at room temperature and detected by an enhanced chemiluminescence reagent (Thermo Scientific, Pittsburgh, PA, USA).

Cell monolayer permeability assay

HUVECs (2×10^4) were grown on collagen-coated FluoroBlok-tinted tissue culture inserts (3 μm polycarbonate membrane, Franklin Lakes, NJ, USA). Cells on the inserts were co-incubated with dexmedetomidine and LPS for 48 h, and treated with 1 mg/mL FITC-dextran for the last 60 min. Sample were collected in the lower chamber and the amount of FITC-dextran diffused through the endothelial monolayer was measured by a microplate reader (SpectraMax MAX190 spectrophotometry) at an excitation of 488 nm and an emission of 520 nm.

Monocytes migration

HUVECs (2×10^4) were seeded on collagen-coated FluoroBlok-tinted tissue culture inserts and co-incubated with dexmedetomidine and LPS for 48 h. THP-1 monocytes were labeled with 5 μM calcein-AM for 30 min and added to the upper chamber for 2 h. The fluorescence of calcein-AM-labelled THP-1 cells migrated into the lower chamber was measured with by a microplate reader (SpectraMax MAX190 spectrophotometry) with emission and excitation wavelength of 485 nm and 535 nm.

Enzyme linked immunosorbent assay (ELISA)

The concentration of ICAM-1 and VCAM-1 were measured in the supernatants of HUVECs using an ELISA kit (Abcam, Cambridge, MA, USA). All measurements were performed as recommend by the manufacturer.

Transient transfection and luciferase reporter gene assay

HUVECs (2×10^5) in 6-well plates were transfected with NFκB promoter-luciferase (Clontech, CA, USA) and β-galactosidase plasmid for 48 h using lipofectamine 2000 according to the manufacturer's instructions. After co-incubation with dexmedetomidine and LPS for 48 h, cell lysates were assayed for luciferase activity and β-galactosidase activity using a Secrete-PairTMDual Luminescence Assay kit (Gene Copoeia, MD) as measured with a microplate reader (SpectraMax MAX190 spectrophotometry).

Statistical analysis

All data were presented as mean ± SEM. n represents the number of independent experiments on different batches of cells. The statistical significance between samples was evaluated by the unpaired two-tailed Student's or the one-way analysis of variance (ANOVA). The level of $P<0.05$ was considered statistically significant.

Results

Effect of dexmedetomidine on viability of HUVECs

The viability of HUVECs after incubation with different concentrations of dexmedetomidine (0.01, 0.1, 1, 10 or 100 μM) was measured using CCK-8 assay. The results revealed that increasing concentrations of dexmedetomidine (0.01, 0.1, 1 and 10 μM) had no significant effect on cell viability (**Figure 1**), indicating dexmedetomidine is non-cytotoxic at 10 μM and below, and is cytotoxic at 100 μM and above.

Dexmedetomidine inhibits LPS-induced endothelial permeability

Stress fibers play an important role in contraction and increase in intracellular gaps [18]. To investigate the effect of Dexmedetomidine on LPS-induced stress fibers formation, actin filaments were visualized by FITC-phalloidin staining. Under normal conditions, actin filaments were distributed throughout the cells and locat-

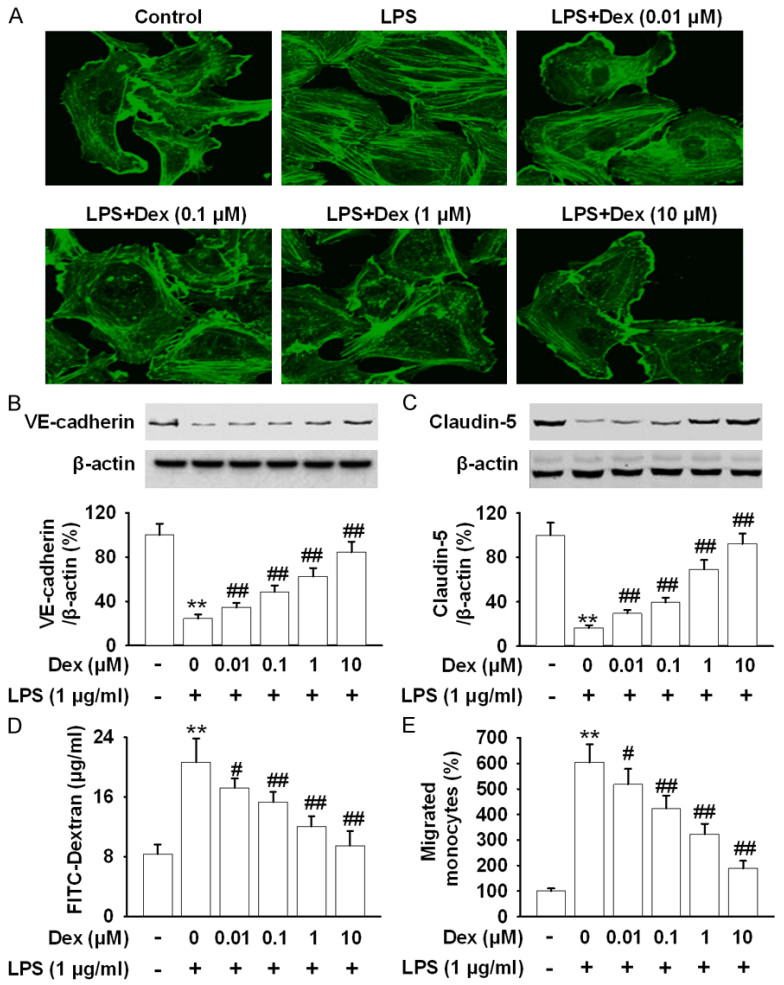

Figure 2. Dexmedetomidine inhibits LPS-induced endothelial permeability in HUVECs. (A) The cells were co-incubated with LPS (1 μg/ml) and different concentrations of dexmedetomidine (0.01, 0.1, 1, or 10 μM) for 48 h. Actin filaments were stained with FITC-phalloidin and observed by confocal microscopy. (B and C) HUVECs were treated as mentioned in (A), the protein expression of VE-cadherin (B) and claudin-5 (C) were determined using western blotting. (D) Following treatment mentioned in (A), FITC-dextran was added to the cells for the last 60 min. The amount of FITC-dextran in the lower chamber was measured by a microplate spectrofluorometer. (E) THP-1 monocytes were labeled with 5 μM calcein-AM for 30 min. Then the labeled monocytes were added to the HUVECs treated as mentioned in (A) and then migrate for 2 h. The fluorescence of the migrated monocytes was measured with a microplate spectrofluorometer. All data were expressed as mean ± SEM. **P<0.01 vs. control, #P<0.05, ##P<0.01 vs. LPS without dexmedetomidine treatment, n=6.

ed on the cellular periphery. LPS activated the formation of stress fibers extending over the cytoplasm. However, dexmedetomidine treatment was shown to have less amount of stress fibers with localization on the cellular periphery (**Figure 2A**). Additionally, LPS significantly stimulated the disassembly of adherens-junction and tight-junction, as demonstrated by decreased VE-cadherin and claudin-5 expressions. After dexmedetomidine treatment, this disassembly was inhibited (**Figure 2B** and **2C**). To further examine whether the restoration of adherens-junction and tight-junction proteins by dexmedetomidine contributes to inhibition of endothelial permeability, endothelial monolayer permeability was measured using FITC-dextran flux in HUVECs. As shown in **Figure 2D**, LPS increased endothelial permeability, and this was inhibited by dexmedetomidine in a concentration-dependent manner. Moreover, we examined the capability of dexmedetomidine in inhibiting monocytes migration to activated endothelial cells. Our results showed that monocytes migration was dramatically increased following LPS stimulation, whereas dexmedetomidine effectively inhibited the migration of THP-1 cells across the HUVECs monolayer (**Figure 2E**). Collectively, these results suggest that dexmedetomidine protects against LPS-induced endothelial leakage.

Dexmedetomidine suppresses LPS-induced ICAM-1 and VCAM-1 expressions in HUVECs

To investigate whether LPS-induced endothelial permeability is due to increased inflammatory response in HUVECs, we determined the expressions of ICAM-1 and VCAM-1, which play critical role in mediating the adhesion of monocytes towards endothelial cells. Western blotting results showed that LPS significantly increased ICAM-1 and VCAM-1 protein expression. However, dexmedetomidine treatment was found to be effectively in inhibiting the above proteins expressions in a concentration-dependent ma-

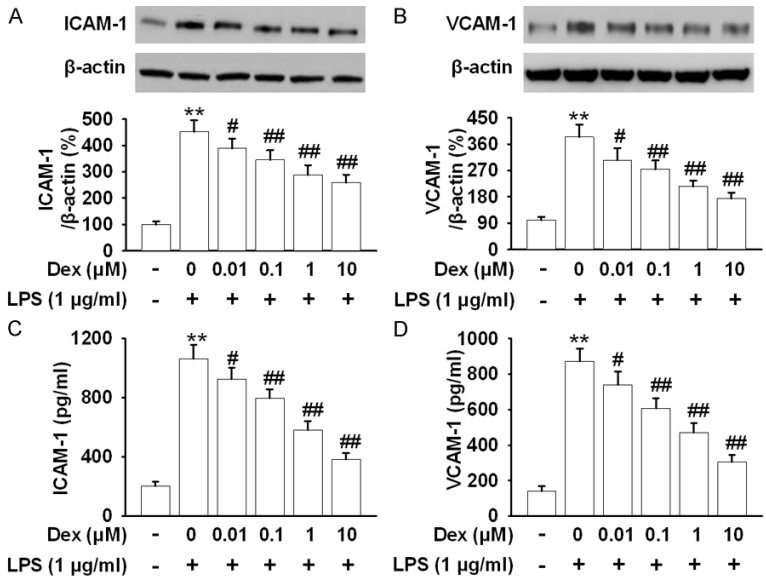

Figure 3. Dexmedetomidine suppresses LPS-induced ICAM-1 and VCAM-1 expression and secretion. (A and B) HUVECs were treated with different concentrations of dexmedetomidine (0.01, 0.1, 1, or 10 μM) in the presence of LPS (1 μg/ml) for 48 h. The expression of ICAM-1 (A) and VCAM-1 (B) were detected by western blotting. (C and D) The cells were treated as described above. The secretion of ICAM-1 (C) and VCAM-1 (D) were measured with ELASA assay. All data were expressed as mean ± SEM. **P<0.01 vs. control, #P<0.05, ##P<0.01 vs. LPS without dexmedetomidine treatment, n=4.

nner (**Figure 3A** and **3B**). Consistent with these results, ELISA assay showed that dexmedetomidine treatment also concentration-dependently suppressed the secretions of ICAM-1 and VCAM-1 in HUVECs (**Figure 3C** and **3D**). These data indicate a protective role of dexmedetomidine in LPS-induced inflammation in HUVECs.

Dexmedetomidine attenuates LPS-induced NF-κB activation and translocation

Since NFκB have been suggested to play an important role in the regulation of inflammation [19], we next investigated whether dexmedetomidine suppressed LPS-induced inflammation via inhibition of NFκB. Luciferase reporter gene assay showed that LPS significantly increased NF-κB promoter luciferase activity, which was remarkably after dexmedetomidine treatment (**Figure 4A**). Moreover, NF-κB nuclear translocation was analyzed by western blotting using anti-p65 antibody. Western blotting showed that the translocation of NF-κB subunit p65 from cytoplasm to nucleus was increased after LPS treatment, indicating the activation of NF-κB. However, following dexmedetomidine treatment, the ability of LPS to induce p65 nuclear translocation was abolished (**Figure 4B** and **4C**).

Inhibition of α2-adrenergic receptor blocks the inhibitory effect of dexmedetomidine on LPS-induced inflammatory response

To further explore the possibility whether dexmedetomidine inhibited LPS-induced inflammation via α2-adrenergic receptor, HUVECs were pretreated with α2-adrenergic receptor antagonist yohimbine for 5 min before co-incubation with LPS and dexmedetomidine for another 48 h. ELISA assay showed that the inhibitory effects of dexmedetomidine on ICAM-1 and VCAM-1 secretion in HUVECs were dramatically reversed by yohimbine (**Figure 5A** and **5B**). Consequently, NF-κB activity was significantly increased after pretreatment with yohimbine, as compared with LPS plus dexmedetomidine treatment (**Figure 5C**). These results suggest that dexmedetomidine inhibits NF-κB-mediated inflammatory response through activating α2-adrenergic receptor.

Discussion

Our study is the first to show the inhibitory effects of dexmedetomidine on endothelial permeability and inflammation in HUVECs. The salient findings of this study were summarized as follows: (1) Dexmedetomidine ameliorated LPS-induced stress fibers formation, adherens- and tight-junction disassembly and monocytes migration across endothelium. (2) We observed that dexmedetomidine also inhibited the secretion and expression of ICAM-1 and VCAM-1 from LPS-activated endothelial cells. (3) LPS-induced NF-κB activation and translocation were blocked by dexmedetomidine treatment. (4) α2-adrenergic receptor was involved in the effects of dexmedetomidine on endothelial permeability and inflammation, and consequently in regulating NF-κB activation.

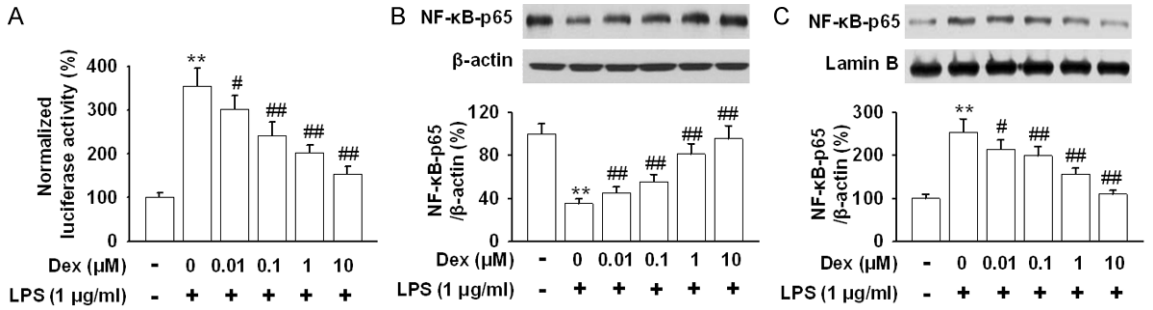

Figure 4. Dexmedetomidine attenuates LPS-induced NF-κB activation and translocation. (A) HUVECs were co-incubated with LPS (1 μg/ml) and different concentrations of dexmedetomidine (0.01, 0.1, 1, or 10 μM) for 48 h. NF-κB luciferase activity was measured using the β-galactosidase activity as an internal control. (B and C) Cytosol (B) and nuclear (C) proteins were isolated and detected by western blotting using p65 antibody. The cells were treated as described in (A). Data were represented as mean ± SEM. **P<0.01 vs. control, #P<0.05, ##P<0.01 vs. LPS without dexmedetomidine treatment, n=4.

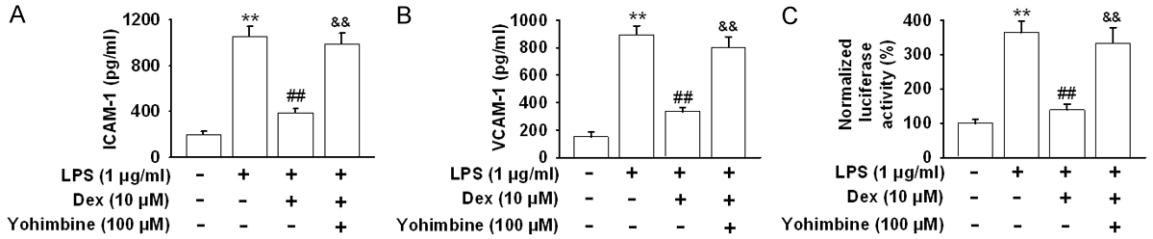

Figure 5. Inhibition of α2-adrenergic receptor blocks the inhibitory effect of dexmedetomidine on NF-κB-mediated inflammation induced by LPS. (A) HUVECs were pretreated with yohimbine (100 μM) for 5 min in prior to co-incubation with LPS (1 μg/ml) and dexmedetomidine (10 μM) for another 48 h. NF-κB luciferase activity was measured using the β-galactosidase activity as an internal control. (B and C) The cells were treated as mentioned in (A), the secretion of ICAM-1 and VCAM-1 were measured with ELISA assay. All data were represented as mean ± SEM. **P<0.01 vs. control, #P<0.05, ##P<0.01 vs. LPS without dexmedetomidine treatment, &&P<0.01 vs. LPS with dexmedetomidine treatment, n=4.

The endothelium is a functional barrier, which plays a dominant role in the regulation of paracellular flux and permeability [1, 2]. When endothelial cells are activated, the endothelial barrier is breakdown, leading to loss of selective permeability and vascular leakage that may eventually result in shock [20]. Previous study has indicated that LPS can induce barrier disruption in endothelial cells [21]. Its effects on endothelium often lead to shock due to increase endothelial permeability [1]. Thus, attenuation of endothelial leakage may be potential strategy to prevent LPS-induced systemic inflammation such as sepsis and endotoxemia. Increasing evidences reported that dexmedetomidine, a potent and highly selective α2-adrenergic receptor agonist, was found to have inhibitory effects on inflammation in endotoxemia rats [14]. Further, dexmedetomidine inhibited LPS-induced up-regulation of inflammatory molecules in macrophages and glial cells [15-17]. Consistent with these studies, in the present study, dexmedetomidine was shown to reduce adherens- and tight-junction proteins expressions, as companied by decreased permeability of FITC-dextran and transendothelial migration of THP-1 monocytes through LPS-stimulated HUVECs. Our results further confirm that dexmedetomidine inhibited monocytes adhesion molecules such as ICAM-1 and VCAM-1 via inactivation of NF-κB, which can be reversed by the α2-adrenergic receptor antagonist yohimbine.

However, there are some limitations in our study. The in vitro data may be not fully representative in the body. Therefore, our results should be further investigated in vivo. In addition, the specificity of α2-adrenergic receptor subtypes in inhibiting NF-κB-mediated inflammation would be further explored in the future, because α2-adrenergic receptor has been

shown to consist of three subtypes: α2A, α2B and α2C.

In summary, our findings provide new insight for us to better understand the anti-inflammatory effects of dexmedetomidine in endothelial cells, and help to find a novel treatment of endothelial barrier dysfunction in inflammatory diseases.

Disclosure of conflict of interest

None.

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