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-
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medetomidine processes anti-inflammatory capacity. Dexmedetomidine ameliorates sep-
sis-induced lung injury in endotoxemia rats [14]. In glial cells, dexmedetomidine significant-
ly inhibits LPS-induced the increase of pro-
inflammatory cytokines, such as tumor necro-
sis factor-α, prostaglandin E2, IL-1β, and IL-6 [15, 16]. Moreover, dexmedetomidine also sup-
presses LPS-induced the secretion and translo-
cation of high mobility group box 1 and subsequenty inhibits inflammation in macrophages [17]. However, whether dexmedetomidine influences endothelial permeability and inflamma-
tion 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, lipopolysaccha-
ride (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 buf-
cer and BCA Protein Assay Kit were obtained
from Beyotime (Nanjing, Jiangsu, China).

Cell culture

Human umbilical vein endothelial cells (HUVE-
Cs) and THP-1 monocytes were obtained from
ATCC (Rockville, MD, USA). HUVECs were cul-
tured in M199 medium supplemented with
20% FBS, 100 U/ml penicillin, 100 U/ml strep-
tomycin, 2 mM L-glutamine, 25 U/ml heparin
and 5 mg/ml β-ECGF. THP-1 cells were cultured in
RPMI 1640 with 10% FBS, 100 U/ml penicillin
and 100 U/ml streptomycin. All cul-
tures were maintained in an incubator with 5% CO₂
and 95% O₂ at 37°C.

Cell viability

Viability of HUVECs was analyzed using CCK-8
assay. The cells (2×10⁵) were seeded in 96-well
culture plates and rendered quiescent by cul-
turing in serum-free M199 medium overnight at
37°C. Following treatment with different con-
centrations 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 micro-
plate reader (SpectraMax MAX190 spectropho-
tometry, 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 inhibi-
tor (Merck, Darmstadt, Germany). To investi-
gate the nuclear translocation of nuclear factor
kappa-B (NFκB) p65, nuclear and cytosol pro-
teins 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-polya-
crylamide gel and then transferred to polyvinyl-
diene 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 polyl-
conal anti-VE-cadherin, rabbit polyclonal anti-
claudin-5 (1:500; Santa Cruz Technology, San-
ta 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. Immuno-
blotting was carried out by incubation with HRP-conjugated secondary antibodies (1:4000;
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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 (2×10⁴) 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 (2×10⁴) 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 (2×10⁵) 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 Secret-e-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-
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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-
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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 ELISA 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.

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.
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...
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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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