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Original Article

α_2 adrenoceptor protects human pulmonary microvascular endothelial cells from TNF-α induced hyper-permeability

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Abstract: It has been controversial about the role of α_2 adrenoceptor (α_2AR) in endothelial function. This study aimed to examine the effects of α_2AR on the integrity of human pulmonary microvascular endothelial cells (HPMECs) upon TNF-α-induced endothelial dysfunction, including apoptosis, hyperpermeability, and increased leukocyte adhesion. HPMECs were treated with TNF-α in the presence or absence of α_2AR agonist dexmedetomidine and antagonists yohimbine, BRL44408, or JP1302. The expression of α_2AR subtypes and VE-cadherin was analyzed by reverse transcription polymerase chain reaction (RT-PCR) and western blot. HPMEC apoptosis, monolayer permeability, cytoskeleton reorganization, and leukocyte adhesion were assessed by annexin V/PI staining, transwell HRP assay, phalloidin-FITC staining, and *in vitro* adhesion assay, respectively. Dexmedetomidine ameliorated TNF-α induced endothelial permeability, cytoskeleton reorganization, inhibition of VE-caherin expression, and increases of ICAM-1 level and leukocyte adhesion to HPMECs. Yohimbine and the subtype-specific antagonists, BRL44408 and JP1302, exhibited opposite effects to those of dexmedetomidine. These data indicated that α_2AR protected HPMECs from TNF-α induced endothelial dysfunction.

Keywords: α_2 adrenoceptor, HPMEC, endothelial barrier dysfunction, cytoskeleton reorganization, ve-cadherin, leukocyte adhesion

Introduction

Sepsis, dysregulated host responses to infection caused severe organ dysfunction [1], causes millions of deaths annually in the world [2]. Among multifaceted host responses, inflammatory mediator surge induced injuries in lung, kidney, brain, or other organs are the signature symptom [3]. It is common for severe sepsis to develop into acute lung injury (ALI) or acute respiratory distress syndrome (ARDS), which has a mortality rate ranging from 34.9% to 46.1% [4]. Pulmonary endothelial damage plays a critical role in inflammation-induced lung diseases [5].

The role of α_2 adrenergic receptor (α_2AR) in inflammatory responses to endotoxemia is still controversial based on the data generated from its agonists and antagonists. α_2AR agonist dexmedetomidine was shown to reduce mortality rate of sepsis and inhibited inflammation during endotoxemia [6, 7]. The anti-inflammatory effect of dexmedetomidine might exert through cholinergic pathway [8]. Furthermore, dexmedetomidine protected against sepsis-induced lung injuries in septic animal models [8-10]. On the other hand, α_2AR antagonist yohimbine was also shown to protect against endotoxin-induced acute lung injury [11] and cardiac dysfunction [12].

Pulmonary microvascular endothelial cells (PMECs) dysfunction is one of the most important components of sepsis-induced ALI [13, 14]. PMECs form an endothelial barrier to control paracellular and transcellular extravasation of proteins, solute, fluids and gases, and the dysfunction of PMECs leads to pulmonary edema and impaired gas exchange [15]. The

effects of α_2AR on endotoxin-induced pulmonary endothelial dysfunction are still poorly understood. This study aims to elucidate the role of α_2AR in TNF-α induced endothelial damage of HPMECs using α_2AR agonist and antagonists.

Materials and methods

Chemicals

Recombinant human TNF-α was purchased from PeproTech (Rocky Hill, NJ, USA). Yohimbine was procured from Dalian Meilun Biotechnology Co. Ltd. (Dalian, China). Dexmedetomidine was obtained from Jiangsu Hengrui Medicine Co. Ltd. (Jiangsu, China). BRL44408, the antagonist for α_{2A} adrenoceptor, was purchased from Sigma-Aldrich (St. Louis, MO, USA), and JP-1302, the antagonist for α_{2c} adrenoceptor, was from Tocris Bioscience (Bristol, UK).

The polyclonal antibodies for ELISA assay of ICAM-1 on cell surface was obtained from Multisciences biotech (Hangzhou, China). For western blot analysis, the polyclonal antibodies against α_{2A} and α_{2B} adrenocepter subtypes were purchased from Proteintech Group Inc (Chicago, IL, USA). The antibody against α_{2C} was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The primary antibody against vascular endothelial (VE)-cadherin was from Abcam (Cambridge, MA, USA).

Cell culture and drug treatment

HPMECs (ScienCell, San Diego, CA) were cultured in endothelial cell medium (ScienCell) according to manufacturer's instruction and used between passage 3 and 7. Cells were grown until 90% of confluence and subject to further treatment. In the six-group experiments, they were exposed to 10 ng/ml TNF-α (TNF-α group), 50 μM of dexmedetomidine for 1 h and then addition of 10 ng/ml TNF-α (dex group), 50 μM of yohimbine for 5 min and then addition of 10 ng/ml TNF-α (yohimbine group), 100 μM of BRL44408 for 5 min and then addition of 10 ng/ml TNF-α (BRL44408 group), 1 μM of JP-1302 for 5 min and then addition of 10 ng/ml TNF-α (JP-1302 group), as well as no treatment as a control group, respectively.

Reverse transcription-PCR (RT-PCR)

RNA was extracted from HPMEC with Trizol reagent (Invitrogen, Shanghai, China) and reverse transcribed into cDNA using Fast Quant RT Super Mix (Tiangen Biotech, Beijing, China). Primers for human α_{2A}, α_{2B}, α_{2C} adrenoceptor mRNAs and internal reference mRNA (β-actin) were from GenScript (Nanjing, China). The primer sequences were as follows: α_{2A}, forward, 5'-ATCATCGCCGTGTTCACGAGC-3', and reverse, 5'-AAGAAGGAGCCGATGCACGAC-3' (455 bp); α_{2B}, forward, 5'-AGGGTGTTTGTGGGGCATCT-3', and reverse, 5'-CAAGCTGAGGCCGGAGACACT -3' (112 bp); α_{2C}, forward, 5'-CCAACGAGCTC-ATGGC CTAC-3', and reverse, 5'-GAGATGACG-GCCGAGATGAG-3' (220 bp); β-actin, forward, 5'-AGCGAGCATCCCCCAAAGTT-3', and reverse, 5'-GGGCACGAAGGCTCATCATT-3' (285 bp).

RT-PCR was carried out using the following conditions: 94°C for 4 min; 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 25 seconds; then 72°C for 4 minutes, and finally 4°C for 4 minutes.

The PCR products were visualized by agarose gel electrophoresis.

Western blot

HPMECs were subject to cell lysis and centrifugation (10,000 rpm, 10 min, 4°C). The supernatants were transferred into new tubes, heated at 100°C for 10 min, added 4×SDS loading buffer, and then centrifuged again (12000 g, 1 min, 4°C). Aliquots of supernatants were subjected to SDS-PAGE (8%) and then transferred onto PVDF membranes (Millipore). Membranes were blocked for 1 h in the blocking buffer (1×PBS, 0.1% Tween-20, 5% w/v non-fat milk) and then incubated with primary antibodies at 4°C overnight. β-actin (Sigma-Aldrich, St. Louis, MO, USA) was used as loading control in detection of the α_2AR subtypes. GAPDH (Santa Cruz, CA, USA) was used as loading control in detection of VE-cadherin (ab166715, Abcam, Cambridge, MA, USA). NaK-ATPase (ab185065, Abcam) was used as loading for ICAM-1 (ab20, Abcam) assessment. The secondary antibodies were horseradish peroxidase-conjugated and ECL reagent was used for detection.

Permeability assay

Permeability was assessed essentially according to method described previously [16]. Briefly, HPMVECs at a concentration of 10^5/ml were seeded in transwell inserts (0.4 μm pore size; Costar, Corning incorporated, USA) and grown

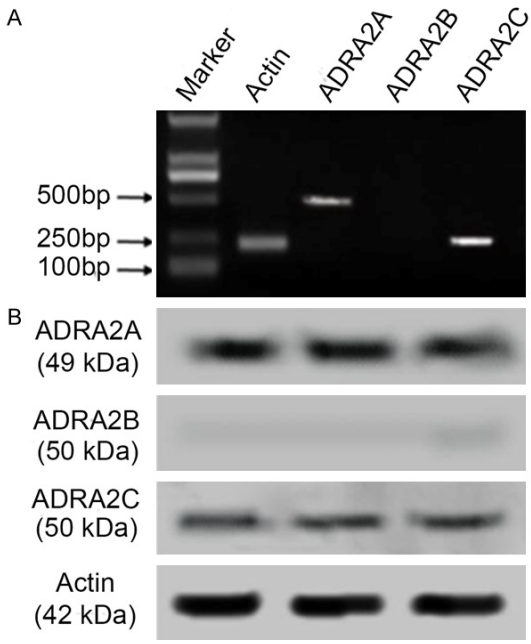

Figure 1. HPMECs express α_2ARs. A. PCR amplicons from α_2AR subtypes in resting HPMECs run in agarose gel electrophoresis. B. Western blot of protein extracts from resting HPMECs, b-actin was used as a loading control. ADRA2A = α_2A adrenergic receptor; ADRA2B = α_2B adrenergic receptor; ADRA2C = α_2C adrenergic receptor.

in endothelial cell medium, which was kept at the same level both at upper and lower chambers. Meanwhile, transendothelial electric resistance was monitored. The resistance value higher than 100 $\Omega \cdot cm^2$ was taken as a signal that the endothelial cells had grown into confluence. Prior to the experiment, the culture medium in the two compartments was replaced with fresh medium, and 50 μg/ml horse radish peroxidase (HRP; Biosharp, Hefei, China) was added into the medium in upper chamber. 50 μl medium from lower chamber was collected after 4 h, 8 h, 12 h, and 24 h and incubated with tetramethylbenzidine (TMB; Biosharp, Hefei, China) at room temperature for 5 min. The reactions were terminated by adding 1 M H_2SO_2. HRP activity was calculated from the increase in absorbance at 450 nm, and the percentages of HRP infiltrated to the lower chamber were recorded as final data.

Immunofluorescence

HPMECs at a concentration of 5×10^5 cells/ml were grown on a 48-well culture plate for 24 h, with each well containing 300 μl ECM. Then they were serum-starved for 12 h and allotted to different groups. After another 12 h the treatments were terminated, and the cells were fixed with a solution of 4% paraformaldehyde for 30 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After blocking with 5% BSA, cells were exposed to phalloidin-FITC for 1 h for visualization of F-actin. Fluorescence was observed with a Leica fluorescence microscope (Leica IX71, Germany) and analyzed using ImageJ (NIH, Bethesda, MD).

Annexin V/PI assays for apoptosis

HPMECs of the six groups were collected 12 h later, and were washed with cold phosphate-buffered saline (PBS), stained with Annexin V-FITC and PI and then evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (Bestbio, Shanghai, China). Both early apoptotic (Annexin V-positive, PI negative) and late apoptotic (Annexin V-positive and PI-positive) cells were counted.

ELISA assay

HPMECs were seeded into 96-well plates and grown to 80-90% confluence, then subject to different treatments. 12 h later, cell monolayers were washed, fixed for 15 min in 2% paraformaldehyde and blocked with 2% normal goat serum. Primary antibody for ICAM-1 (1:10000, rabbit anti-human) was added to each well and incubated for 1 h at 37°C. The cells were then washed and then incubated with horseradish peroxide (HRP)-conjugate goat anti-rabbit second antibody (Santa Cruz). The surface ICAM-1 expression was determined by addition of 100 μl of TMB substrate solution. The reaction was stopped with 100 μl of 0.18 M H_2SO_4. The samples were read in a plate reader at OD 450 nm.

Leukocyte adhesion assay

MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich, St Louis, MO, USA) labeled HL60 cells were used to assess the adhesion of leukocytes to endothelial monolayer according to previously described method [17]. HPMECs at a concentration of 1×10^5 cells/ml were seeded in 96-well culture plate, with each well containing 100 μl of ECM. Cells were grown to confluent monolayers and subject to different treatments. Meanwhile,

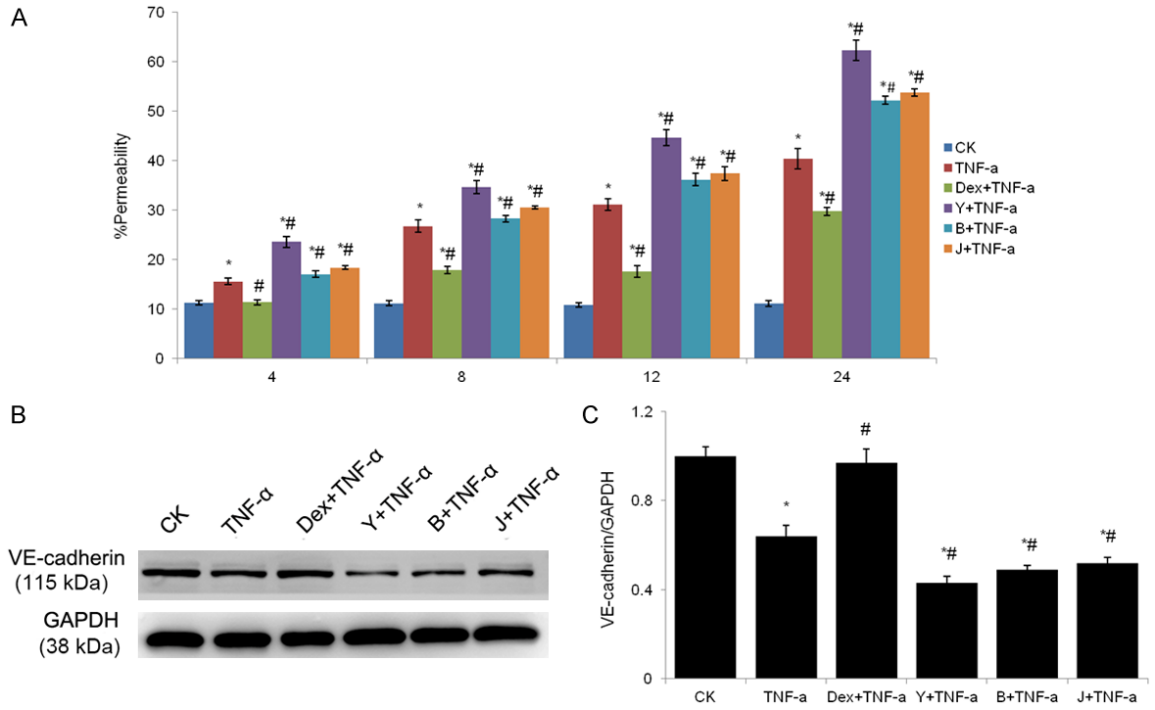

Figure 2. Activation of α_2AR inhibited TNF-α induced permeability of HPMEC monolayer. A. Permeability of HRP at different time points. B. Western blot analysis of VE-cadherin expression. C. Quantitative analysis of VE-cadherin protein levels in HPMECs after different treatment. CK = control group; Dex = dexmedetomidine; Y = yohimbine; B = BRL44408; J = JP-1302. *, P<0.05 vs. control group; #, P<0.05 vs. TNF-α group.

HL-60 cells at a concentration of 1×10^6 were prepared by adding 1/4 volume of 5 mg/ml MTT and incubated for 30 min at 37°C in 5% CO_2:95% air. Then the labeled cells were centrifuged, washed twice with the RPMI 1640 medium, and cultured in RPMI 1640 medium containing 10% fetal bovine serum.

After HPMECs being treated with TNF-α in the absence or presence of dexmedetomidine, yohimbine, BRL44408, or JP1302 for 12 h, medium was removed and cells were washed twice with PBS. MTT-labeled HL60 cells (100 μl, at a concentration of 1×10^6/ml) were added in each well and incubated for 30 min at 37°C in 5% CO_2:95% air. Then the supernatant was removed and wells were washed with PBS. 50 μl of Dimethylsulfoxide (DMSO, Invitrogen, Carlsbad, CA, USA) was added to each well. Absorbance at 540 nm was measured with a microplate reader (MK3, Thermo scientific, USA) after 15 min incubation at room temperature in the dark.

Statistical analysis

Experiments were repeated three times in triplicates. Data were shown as mean ± standard deviations (SD). Graphpad prism 6.0 was used to perform statistical analyses. The between group differences were evaluated using student's t test. A *p* value less than 0.05 was considered statistically significant.

Results

α_{2A} and α_{2C} adrenoceptor were expressed by HPMECs

To assess the potential roles of α_2AR subtypes in the inflammatory responses of HPMECs, we first detected the expression of the three subtypes of α_2AR with RT-PCR and western blot analyses. RT-PCR revealed that α_{2A} and α_{2C} but not α_{2B} adreoceptors were expressed in HPMECs (**Figure 1A**). The western blot analysis confirmed this result (**Figure 1B**).

The permeability of HPMEC monolayer was reduced by α_2AR activation

The percentage of HRP infiltrated from top chamber into the lower chamber of transwell culture system was employed as a proxy for endothelial monolayer permeability. The permeability of HPMEC monlayer was significantly

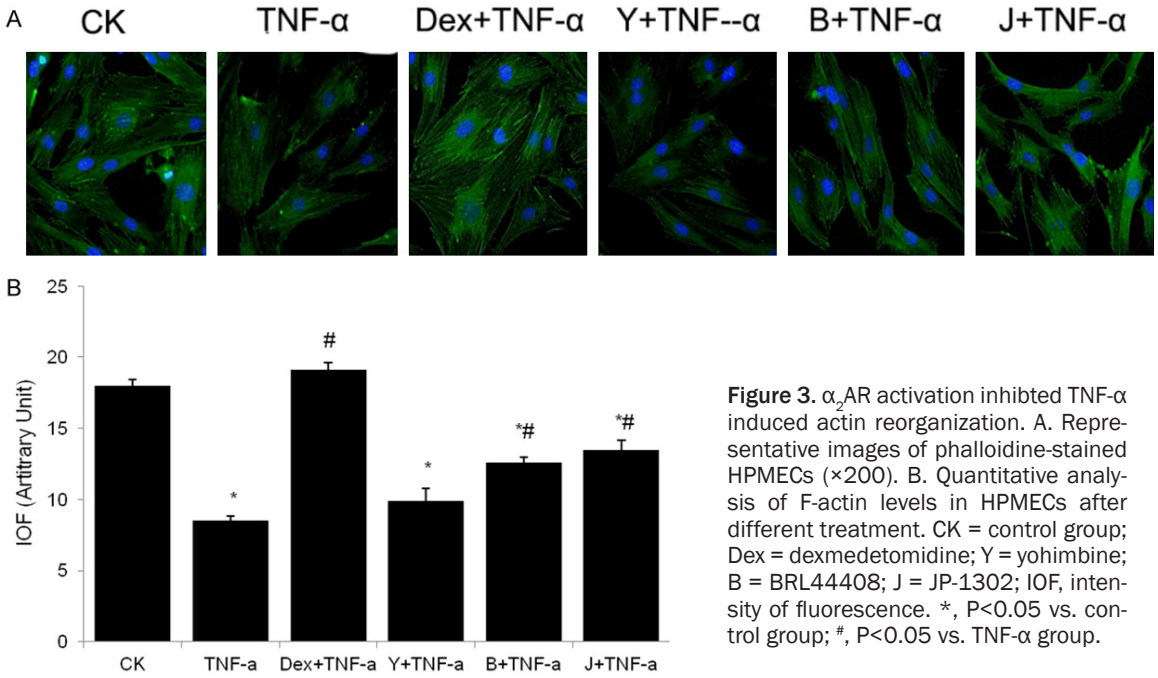

Figure 3. α_2AR activation inhibted TNF-α induced actin reorganization. A. Representative images of phalloidine-stained HPMECs (×200). B. Quantitative analysis of F-actin levels in HPMECs after different treatment. CK = control group; Dex = dexmedetomidine; Y = yohimbine; B = BRL44408; J = JP-1302; IOF, intensity of fluorescence. *, P<0.05 vs. control group; #, P<0.05 vs. TNF-α group.

increased after 4 h TNF-α treatment and further increased at least after 24 h TNF-α treatment, which was aggravated by pretreatment with α_2AR antagonists yohimbine, BRL44408, and JP-1302 whereas alleviated by α_2AR agonist dexmedetomidine (**Figure 2A**). As VE-cadherin is required for maintaining a functional endothelial barrier, we then looked into the changes of VE-cadherin levels of HPMEC upon TNF-α with or without α_2AR agonist and antagonists treatments. The expression of VE-cadherin in HPMECs was significantly down-regulated by TNF-α (**Figure 2B**, **2C**). Dexmedetomidine suppressed the inhibition of VE-cadherin expression whereas yohimbine, BRL-44408, and JP1302 caused further down-regulation of the VE-cadherin expression by TNF-α (**Figure 2B**, **2C**).

α_2AR activation inhibited TNF-α induced actin reorganization

To better understand the mechanisms involved in endothelial permeability changes, we next investigated cytoskeleton reorganization of HPMECs upon TNF-α treatment by staining the polymerized F-actin with phalloidine. In control group, the cells were in normal shape, presented prominent peripheral actin-staining cortical band, and maintained tight intercellular contacts. TNF-α caused actin rearrangement by distorting the normal cell shape, disconnecting the adjacent cells, reducing F-actin content, and increasing stress fiber formation. Dexmedetomidine alleviated the changes caused by TNF-α whereas yohimbine, BRL44408, and JP-1302 all exacerbated the effects of TNF-α (**Figure 3**).

Activation of α_2AR did not protect endothelial cell from apoptosis

TNF-α induced apoptosis of HPMECs (**Figure 4**). Pretreatment with dexmedetomidine and BRL44408 had no obvious effect on the TNF-α induced apoptosis, while the TNF-α induced apoptosis was exacerbated by yohimbine and JP-1302 (**Figure 4**).

α_2AR inhibited leukocyte adhesion to endothelial monolayers

TNF-α treatment resulted in significant increase of cell surface expression of ICAM-1 in HPMECs (**Figure 5A**, **5B**) and leukocyte adhesion (**Figure 5C**). Dexmedetomidine inhibited TNF-α induced ICAM-1 upregulation and leukocyte adhension while yohimbine, BRL44408, and JP1302 promoted TNF-α induced increase of ICAM-1 expression (**Figure 5A**, **5B**) and leukocyte adhesion (**Figure 5C**).

Discussion

α_2AR agonist dexmedetomidine protected endothelial monolayer integrity from TNF-α

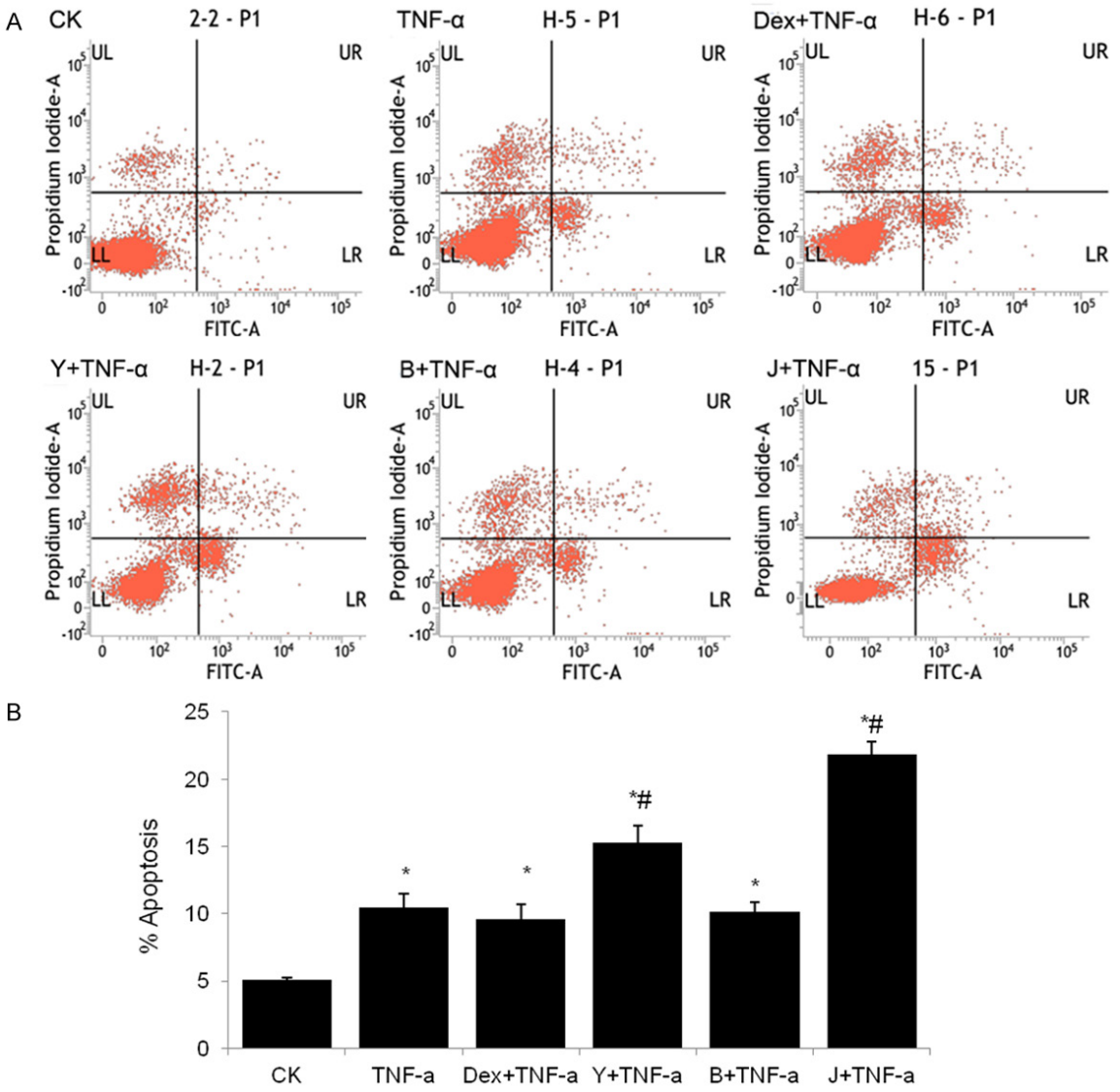

Figure 4. Apoptosis assessed by Annexin V/PI staining. CK = control group; Dex = dexmedetomidine; Y = yohimbine; B = BRL44408; J = JP-1302. *, P<0.05 vs. control group; #, P<0.05 vs. TNF-α group.

induced endothelial dysfunctions whereas the antagonists yohimbine, BRL44408, and JP1302 aggravated the TNF-α induced endothelial damages, which seemed not to be subtye-specific as α_2A antagonist BRL44408 and α_2C antagonist JP-1302 showed similar effects. Moreover, the effects of yohimbine were the additive effects of both BRL44408 and JP-1302, indicating that both α_2A and α_2C adenoceptors were involved in protecting HPMEC against inflammatory insults. Activating α_2AR with agonist dexmedetomidine ameliorated TNF-α induced endothelial monolayer permeability, actin reorganization, VE-cadherin destabilization, and leukocyte recruitment whereas antagonists had the opposite effects. However, dexmedetomidine did not protect HPMECs from TNF-α induced apoptosis.

Microvascular endothelium is essential for vascular homeostasis. The microvascular endothelial permeability is critically regulated by cytoskeletal structures (F-actin, microtubules, and intermediate filaments) and cell contact protein complexes (focal adhesions, adherens junction and tight junctions) [18]. TNF-α induces cellular retraction of cultured endothelial monolayers, which can be attributed to actin reorganization [19, 20]. Meanwhile, the intercellular VE-cadherin is phosphorylated and

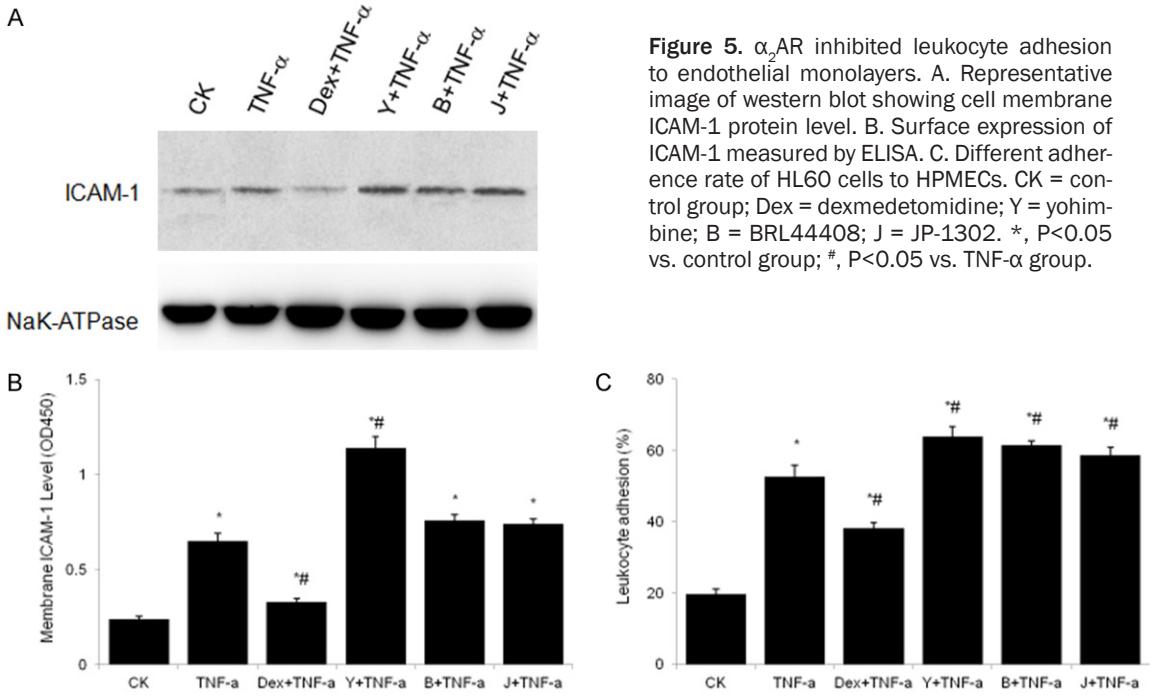

Figure 5. α_2AR inhibited leukocyte adhesion to endothelial monolayers. A. Representative image of western blot showing cell membrane ICAM-1 protein level. B. Surface expression of ICAM-1 measured by ELISA. C. Different adherence rate of HL60 cells to HPMECs. CK = control group; Dex = dexmedetomidine; Y = yohimbine; B = BRL44408; J = JP-1302. *, P<0.05 vs. control group; #, P<0.05 vs. TNF-α group.

down-regulated, which destabilizes adherens junction [21-23]. These two pathways are considered as the prerequisites of TNF-α induced endothelial barrier dysfunction [24]. Our data agree with the notion that dexmedetomidine might protect the endothelial barrier through maintaining adhesive force of VE-cadherin and suppressing TNF-α induced actin reorganization.

Moreover, dexmedetomidine also inhibited TNF-α caused increase of ICAM-1 expression and leukocyte recruitment by HPMECs. ICAM-1 is an adhesion molecule constitutively expressed on HPMECs, and remarkably increased along with many other adhesion molecules (PECAM-1, VCAM-1, and ELAM-1) by TNF-α treatment, which in turn promotes the adhesion of leukocytes to endothelial cells [25-27]. Furthermore, the engagement of ICAM-1 by leukocytes also results in phosphorylation of VE-cadherin [28, 29], which contributes to the endothelial barrier dysfunction.

It was controversial on the role of endothelial apoptosis in endothelial barrier dysfunction. While antiapoptotic treatment could prevent endothelial barrier dysfunction [30, 31], others studies showed that TNF-α-induced permeability did not dependent on endothelial cell apoptosis [32, 33]. Our result was in agreement with the notion that apoptosis was not the primary cause of TNF-α induced endothelial dysfunction as dexmedetomidine protected endothelial barrier but did not reduce TNF-α induced HPMEC apoptosis. Moreover, the impairment of endothelial barrier function by the α_2AR antagonists did not show obvious subtype-specific characteristics, which was different from the trends of apoptosis.

Yohimbine inhibited lipopolysaccharide (LPS) induced inflammatory cytokines production and acute lung injury in rats [11]. In mice, yohimbine relieved the inhibition of gastrointestinal motility and the overexpression of iNOS caused by LPS [34]. However, the current study did not find any protective effect of yohimbine on TNF-α induced endothelial barrier dysfunctions. On the contrary, yohimbine and other α_2AR antagonists exacerbate TNF-α induced loss of endothelial integrity.

Different downstream signaling pathways could mediate the effects of α_2AR [24, 35, 36]. Focal adhesion kinase pathway was shown involved in dexmedetomidine attenuation of lung injury and pulmonary microvascular hyper-permeability caused by renal ischemia/reperfusion [24]. The effects of α_2AR could also be mediated by inhibition of adenylyl cyclase [34] or through anti-oxidative stress mechanisms [36]. The cur-

rent data demonstrated that intercellular adhesion complex component VE-cadherin and cytoskeleton were partially responsible for mediating the effects of α_2AR on pulmonary microvascular endothelial permeability.

Taken together, to our best knowledge, this was the first study that investigated the conflicting conclusions about the effects of α_2AR on inflammation and organ injuries based on treatment with α_2AR agonists and antagonists. It was clear that activating α_2AR on activation of HPMECs induced by TNF-α, and provides an explanation to the with agonist dexmedetomidine attenuated HPMEC hyper-permeability caused by TNF-α through upregulating VE-cadherin, inhibiting actin reorganization and expression of adhesion molecules. However, α_2AR antagonists aggravated TNF-α induced HPMEC permeability in a subtype insensitive fashion. These results suggested that α_2ARs may be effective targets in reducing pulmonary endothelial permeability and controlling inflammation-caused lung injury.

Disclosure of conflict of interest

None.

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