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M_3 receptor is involved in the effect of penehyclidine hydrochloride reduced endothelial injury in LPS-stimulated human pulmonary microvascular endothelial cell

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ABSTRACT

LPS has been recently shown to induce muscarinic acetylcholine 3 receptor (M_3 receptor) expression and penehyclidine hydrochloride (PHC) is an anticholinergic drug which could block the expression of M_3 receptor. PHC has been demonstrated to perform protective effect on cell injury. This study is to investigate whether the effect of PHC on microvascular endothelial injury is related to its inhibition of M_3 receptor or not.

HPMVECs were treated with specific M_3 receptor shRNA or PBS, and randomly divided into LPS group (A group), LPS + PHC group (B group), LPS + M_3 shRNA group (C group) and LPS + PHC + M_3 shRNA group (D group). Cells were collected at 60 min after LPS treatment to measure levels of LDH, endothelial permeability, TNF-α and IL-6 levels, NF-κB p65 activation, I-κB protein expression, p38MAPK, and ERK1/2 activations as well as M_3 mRNA expression.

PHC could decrease LDH levels, cell permeability, TNF-α and IL-6 levels, p38 MAPK, ERK1/2, NF-κB p65 activations and M_3 mRNA expressions compared with LPS group. When M_3 receptor was silence, the changes of these indices were much more obvious.

These findings suggest that M_3 receptor plays an important role in LPS-induced pulmonary microvascular endothelial injury, which is regulated through NF-κB p65 and MAPK activation. And knockout of M_3 receptor could attenuate LPS-induced pulmonary microvascular endothelial injury. Regulative effects of PHC on pulmonary microvascular permeability and NF-κB p65 as well as MAPK activations are including but not limited to inhibition of M_3 receptor.

1. Introduction

Microvascular endothelial barrier failure is a characteristic hallmark of acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS), so the mechanisms of endothelial injury are of great therapeutic interest [1]. LPS is a major component of Gram-negative bacteria outer membranes which is believed to be the main initiator for the microvascular abnormalities in ALI [2]. Pulmonary microvascular endothelial cells (PMVECs), as a barrier between the blood and microvessel wall, are described as a target for LPS during Gram-negative sepsis [3]. LPS has been recently shown to induce muscarinic receptors (mAChR) expressions and the blockage of mAChR exerts anti-inflammatory properties in the LPS-induced lung inflammation. Moreover, mAChR antagonist could inhibit ACh-induced airway microvascular leakage in which M_1 and M_3 receptors play a major role [4–6].

Penehyclidine hydrochloride (PHC) is a new anticholinergic drug which had both antimuscarinic and antinicotinic activities and retained potent central and peripheral anticholinergic activities. Owing to selectively blocking M_1, M_3 receptors and N receptor, PHC has few M_2 receptor-associated cardiovascular side effects [7]. Our previous studies have found that M_3 receptor expressed most highest among the four kinds of subtype (M_1-M_4) in human pulmonary microvascular endothelial cells (HPMVECs) and M_3 receptor was activated after LPS challenge [8]. It has been shown that PHC pretreatment attenuated the LPS-activated cell injury [9]. However, it is unknown whether the effect of PHC on pulmonary microvascular endothelial injury is related to its inhibition of M_3 receptor or not. Then in this study HPMVECs were

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transfected with a shRNA-containing plasmid that specifically targets M_3 receptor. LDH level, microvascular permeability, TNF-α and IL-6 concentrations, MAPK and NF-κB activation, I-κB protein expression, as well as M_3 receptor mRNA expression, were examined in the incubation of LPS and PHC in HPMVEC.

2. Materials and methods

2.1. Materials

PHC was provided by Lisite Corporation (Chengdu, China). LPS (*Escherichia coli* 0111: B4) and RPMI 1640 were purchased from Sigma (USA). LDH kits were purchased from Jiancheng Biologic Project Company (Nanjing, China). Antibody against NF-κB p65 was purchased from Abcam Inc (Abcam Inc, Cambridge, United Kingdom), and anti-I-κB antibody was provided by Cell Signaling Technology (CST Inc, Danvers, Massachusetts, USA). Antibody against p38 MAPK was purchased from Epitomics Inc (Epitomics Inc, Burlingame, CA, USA), anti-ERK1/2 antibody was purchased from Bioworld company (Bio-world, Dallas, Texas, USA), and anti-β-actin antibody was provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA). ELISA kit was available from R&D Systems (R&D Systems Inc. Minneapolis, MN, USA).

2.2. Cell lines and cell culture

HPMVEC were purchased from the ScienCell Research Laboratories (ScienCell, CA, USA). Cells were cultured in RPMI1640, 10% standard newborn calf serum, 50 μg ml^{-1} streptomycin, and 50 IU·ml^{-1} penicillin in a 5% humidified CO_2 atmosphere at 37 °C (E191TC, SIM CO_2 INCUBATOR, USA). The medium was changed every other day, and cells were characterized by a typical cobblestone appearance under phase contrast microscope. When the cells were cultured to 80% confluency, the medium was changed and washed twice with PBS. The cells were digested with 0.25% trypsin for 2–3 min and mixed into a suspension. Take the 4-6 generation cells for the later experiment.

2.3. Depletion of M_3 receptor in HPMVEC

The plasmid constructs expressing shRNA directed against human M_3 mRNA were manufactured by Wuhan Guge biological co. The shRNA sequences were as follows: M_3 shRNA: 5′-AGCTC AAAAAA CCACTCTACCTCTGTCCTT cgtcttgaa AAGGACAGAGGTAGAGTGG-3′. The recombinant plasmid was identified with restriction endonuclease analysis. The plasmid expressing M_3 shRNA contained with green fluorescent protein (GFP) gene. The transfection efficiency of M_3 shRNA was observed by fluorescence microscope. The effects of M_3 knockdown were determined using RT-PCR and we were able to achieve more than 60% transduction efficiency using the plasmid transfection system (data not shown).

2.4. Cell groups

It has been shown that LPS could increase LDH level, p38 MAPK and NF-κB activations in our previous study [10,11], so this study is mainly to determine the mechanism of PHC on these indices. In this study, HPMVEC were treated with specific M_3 receptor shRNA or PBS. Cells were seeded in 6-well plates (2 ml per well) or in culture flasks (4 ml per flask) with the density of 1×10^5 per ml, and randomly divided into LPS group (A group), LPS + PHC group (B group), LPS + M_3 shRNA group (C group) and LPS + PHC + M_3 shRNA group (D group). Cells were treated with 0.1 μg ml^{-1}LPS in A group and C group for 60 min, and cells were treated with 2 μg ml^{-1} PHC for 60 min and then stimulated with a 0.1 μg ml^{-1} concentration of LPS in B group and D group for 60 min.

2.5. Determination of LDH levels

Culture supernatants were collected for determination of LDH as an indicator of cell survival. The levels of LDH were measured by 2, 4-dinitrophenylhydrazine chromogenic assay according to the manufacturer's instructions (LDH kit, Nanjing Jiancheng Bioengineering Institute, Nan Jing, China).

2.6. Permeability assay

Cells were seeded onto polycarbonate membrane Transwell inserts (6.5 mm diameter, 0.4 μm pore size; Corning Incorporated, Corning, NY, USA) precoated with collagen. Culture medium volumes in the upper and lower compartments of the Transwell were 100 and 600 μl, respectively. After reaching confluence, cells were treated according to experimental protocols and fluorescein isothiocyanate (FITC) -labeled bovine serum albumin (BSA, 1 mg ml^{-1}) (Sigma, USA) were added to the upper compartment. The cells were then incubated in a humidified atmosphere with 5% CO_2 at 37 °C. After 60 min, the medium was removed from the lower compartment, and the fluorescence intensity was measured with a fluorescence spectrophotometer (excitation: 490 nm; emission: 525 nm). To allow quantitative comparison among all experiment groups, data are expressed as the relative concentration of FITC-BSA when compared with the group A.

2.7. Measurement of TNF-α and IL-6

Using commercially available kits (R&D Systems, USA), TNF-α and IL-6 levels were measured by a two-step sandwich ELISA method, according to the manufacturer's instructions. Background absorbency of blank wells was subtracted from the standards and unknowns prior to determination of sample concentrations.

2.8. Western blot analysis

Equal amounts of proteins (40 μg) were loaded onto SDS-polyacrylamide gel, transferred to nylon membranes, and incubated with primary antibody overnight at 4 °C. Excess antibody was then removed by washing the membranes in PBS-0.05% Tween-20, and the membranes were incubated in secondary antibodies for 30 min. After being washed in PBS-0.05% Tween 20, the bands were detected by enhanced chemiluminescence (ECL) and the density of the individual bands was quantified by densitometry using AlphaEase FC software (Alpha Innotech, USA).

2.9. Real-time quantitative PCR analysis

Cells were harvested and total RNA was extracted using Trizol Reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. 2 μg of total RNA was subjected to reverse transcription using TOYOBO First Strand cDNA Synthesis Kit. Reverse transcription was performed at 70 °C for 5 min, 0 °C for 3 min, 42 °C for 30 min, and 80 °C for 5 min. The mRNA levels of M_3 receptor were measured by quantitative PCR respectively. qPCR amplifications were performed in triplicate using the SYBR Green I assay. The reactions were carried out in 25 μl reactions containing 2.0 μl cDNA, 2.0 μl mixed gene-specific forward and reverse primers (10 mM each), 12.5 μl of 2 × qPCR Mix and 8.5 μl double-distilled H_2O. The amplification reaction was carried out in an initial 1 min predenaturation at 95 °C, 40 cycles at 95 °C for 15 s, 58 °C for 20 s, 72 °C for 20 s followed by the protocol for the melting curve with an increase of 1 °C between each 20s from 72 °C to 95 °C. β-actin gene was used as an internal control for normalization of RNA quantity and quality differences in all samples. For each analyzed sample, qPCR provides a CT value where the fluorescence signal is detectable. All of CT was dependent on the starting amount of cDNA. β-actin CT value was used to confirm that the starting amount of cDNA in