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NPS 2143, a selective calcium-sensing receptor antagonist inhibits lipopolysaccharide-induced pulmonary inflammation

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ABSTRACT

NPS 2143, a novel and selective antagonist of calcium-sensing receptor (CaSR) has been reported to possess anti-inflammatory activity. In the present study, we examined the protective effect of NPS 2143 on lipopolysaccharide (LPS)-induced acute lung injury (ALI). NPS 2143 pretreatment significantly inhibited the influx of inflammatory cells and the expression of monocyte chemoattractant protein-1 (MCP-1) in the lung of mice with LPS-induced ALI. NPS 2143 decreased the levels of neutrophil elastase (NE) and protein concentration in the bronchoalveolar lavage fluid (BALF). NPS 2143 also reduced the production of inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in the BALF and serum. In addition, NPS 2143 attenuated the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), and increased the activation of AMP-activated protein kinase (AMPK) in the lung. NPS 2143 also downregulated the activation of nuclear factor-kappa B (NF-κB) in the lung. In LPS-stimulated H292 airway epithelial cells, NPS 2143 attenuated the releases of IL-6 and MCP-1. Furthermore, NPS 2143 upregulated the activation of AMPK and downregulated the activation of NF-κB. These results suggest that NPS 2143 could be potential agent for the treatment of inflammatory diseases including ALI.

1. Introduction

Acute lung injury (ALI) is a serious and progressive clinical disorder that may be caused by a variety of factors including bacterial endotoxins (Baudiss et al., 2016). The high mortality rate of ALI has not been changed over time (Phua et al., 2009). Acute airway inflammation is the major characteristic in ALI. Bacterial infection is the major cause of airway inflammation, and is found in ALI patients (Fagon and Chastre, 2003). Neutrophils and macrophages are responsible for the airway inflammatory response, which is characterized by the overproduction of inflammatory molecules in the bronchoalveolar lavage fluid (BALF) (Grommes and Soehnlein, 2011). Neutrophils contribute to the progression of ALI by means of migration into the lung and secretion of granule proteins (Li et al., 2016). The increased level of neutrophil elastase (NE) leads to a protease-antiprotease imbalance, which causes lung damage and injury (Tsai et al., 2015). Macrophages are the predominant inflammatory cells and key participants in the pathogenic process of pulmonary inflammatory response by increasing inflammatory molecules (Liou et al., 2016). The enhanced levels of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) lead to a hyper-inflammation that plays an essential pathogenic role in pulmonary inflammation (Li et al., 2016). Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are important inflammatory mediators, and increased levels of these molecules have been shown in ALI models (Speyer et al., 2003; Tsai et al., 2015). Monocyte chemoattractant protein-1 (MCP-1) is one of the key chemokines that

Abbreviations: ALI, acute lung injury; LPS, lipopolysaccharide; BALF, bronchoalveolar lavage fluid; NE, neutrophil elastase; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; MCP-1, monocyte chemoattractant protein-1; AMPK, 5′ adenosine monophosphate-activated protein kinase; IκB, inhibitor of NF-κB; NF-κB, nuclear factor-κB; NPS 2143, 2-Chloro-6-[(2R)-3-[[1,1-dimethyl-2-(2-naphthalenyl)ethyl]amino]-2-hydroxypropoxy]-benzonitrile hydrochloride

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regulates the recruitment of inflammatory cells such as neutrophils and macrophages (Deshmane et al., 2009). Inhibition of MCP-1 effectively attenuated the pulmonary inflammation by decreasing the influx of inflammatory cells in the ALI animal models (Kimura et al., 2015). The activation of AMP-activated protein kinase (AMPK) suppresses inflammatory response in a murine endotoxemia model through the inhibition of inflammatory cytokines and NF-κB activation (Liu et al., 2016). Nuclear factor kappa-B (NF-κB) signaling pathway regulate inflammatory molecules, including iNOS, TNF-α, IL-6 and MCP-1 in LPS-induced inflammatory response (Lee et al., 2016c; Messina et al., 2011). Researchers have reported that the modulation of NF-κB has potential therapeutic advantages for airway inflammatory diseases including ALI (Fagon and Chastre, 2003; Li et al., 2016; Yeh et al., 2014).

The calcium-sensing receptor (CaSR) is a member of the G-protein coupled receptor (GPCR) superfamily that is expressed in multiple tissues, including human lung tissue (Milara et al., 2010; Wang et al., 2013) and is an important regulator of Ca^{2+} homeostasis (Kos et al., 2003). Altered CaSR is associated with several pathological condition including inflammation (Lee et al., 2012; Paccou et al., 2014; Riccardi and Kemp, 2012; Rossol et al., 2012). Especially, it is well known that knockdown of CaSR inhibits inflammasome activation (Lee et al., 2012). NPS 2143 is a selective potent CaSR antagonist, which has been reported to possess various biological properties such as anticancer (Joeckel et al., 2014) and anti-inflammatory activities (Mine and Zhang, 2015). Protective effect of NPS 2143 on the airway inflammation is well established in allergic asthma (Yarova et al., 2015). In our recent study, we confirmed that NPS 2143 has an anti-inflammatory activity in cigarette smoke extract (CSE)-stimulated H292 human airway epithelial cells (Lee et al., 2016b). Thus, interference with CaSR activation may be more effective approach to inflammatory diseases including ALI. However, the role of NPS 2143 remains unexplored in LPS-induced ALI. Therefore, we investigated the protective effect of NPS 2143 on the pulmonary inflammation using mouse models with LPS-induced ALI.

2. Materials and methods

2.1. Chemical reagents and cell culture

NPS 2143, a CaSR allosteric antagonist (calcilytic) and dexamethasone (DEX) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and were solubilized with dimethyl sulfoxide (DMSO). Airway epithelial NCI-H292 cells were obtained from the American Type Culture Collection (CRL-1848; ATCC, Manassas, VA, USA) and were grown in RPMI 1640 medium (Gibco, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, GE Healthcare, United Kingdom) in the presence of penicillin (100 U/ml), streptomycin (100 μg/ml), and HEPES (25 mM) and incubated at 37 °C in a 5% CO_2 incubator. The cells were pretreated with NPS 2143 (0.5 and 1 μM) 1 h prior to incubation with LPS (10 μg/ml) for 24 h.

2.2. Animal models of LPS-induced ALI

Six-week-old C57BL/6 male mice were purchased form the Koatech Co. (Pyeongtaek, Korea). All of the animal care and experimental procedures were performed under specific pathogen-free conditions in compliance with the National Institutes of Health Guidelines and approved from the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology. ALI mouse model was established by LPS intranasal administration to explore the effect and its underlying mechanism of NPS 2143 as previously described (Lee et al., 2016c; Yuk et al., 2017). Briefly, the mice (n = 7, each group) were randomly divided into 4 groups as follows: NC; normal control, LPS; lipopolysaccharide, DEX; LPS + dexamethasone (1 mg/kg, p.o.), NPS 2143; LPS + NPS 2143 (2.5 or 5 mg/kg, p.o.). NPS 2143 and DEX were dissolved with 0.5% DMSO in PBS, and were administered orally from day 0 to day 1. To induce ALI, the mice were exposed to LPS (10 μg dissolved in 50 μl/per mouse) intranasally 1 h after the final NPS2143 and DEX administration. DEX was used as a positive control.

2.3. Bronchoalveolar lavage fluid (BALF) and inflammatory cells counts

The BALF collection was previously described by Lee et al. (Lee et al., 2016a). In brief, the mice were given an intraperitoneal (i.p.) injection with pentobarbital (100 mg/kg; Hanlim Pharm, Co., Seoul, Korea) 24 h after the LPS administration, and a tracheostomy was performed. To obtain the BALF, 0.7 ml of ice-cold PBS was infused into the lung via tracheal cannulation and extraction was acquired by two times (total volume, 1.4 ml). To determine the number of different types of cells, 100 μl of the BALF was centrifuged onto a glass slides using a Cytospin (Hanil Science Industrial, Seoul, Korea) for 5 min at 1000 rpm. The slides were dried at RT for 1 h, and then the cells were fixed and stained using Diff-Quik® staining kit (B4132-1A; IMEB Inc., Deerfield, IL, USA), according to the manufacturer's instruction.

2.4. Neutrophil elastase (NE) activity and total protein concentration in the BALF

The activity of neutrophil elastase (NE) was determined using N-succinyl-(Ala)3-*p*-nitroanilide (Sima-Aldrich) in 37 °C for 90 min, according to the protocol described by Sakuma et al. (Sakuma et al., 1998). Determination of protein levels in the BALF was quantified with a protein assay kit according to the manufacturer's instruction (Bio-Rad) (Lee et al., 2015). The absorbance was determined using the microplate reader at 595 nm.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The levels of inflammatory cytokines (TNF-α and IL-6) and chemokine (MCP-1) in the BALF were measured according to the manufacturer's protocol (BD Bioscience, San Jose, CA, USA and R & D Systems, Minneapolis, MN, USA). The absorbance was measured at 450 nm using a Spark™ 10 M multimode microplate reader (Tecan system inc., CA, USA).

2.6. Histology

After the BALF were collected, the lung tissues samples were obtained from the mice and fixed in 10% (v/v) neutral buffered formalin. For histological analysis, the lung tissues were embedded in paraffin and were sectioned at 4 μm thickness using a rotary microtome. The lung sections were stained with hematoxylin and eosin (H & E) solution (Sigma-Aldrich Inc, St. Louis, MO, USA) to estimate inflammatory cells influx into the lung.

2.7. Western blot analysis

The expression levels of proteins were determined using western blot analysis. Briefly, lung tissues were obtained 6 or 24 after the last challenge with LPS and were harvested, lysed, and homogenized. The levels of AMPK and NF-κB activation were evaluated using lung tissues that were obtained 6 h after the administration with LPS. The expression of iNOS, COX-2 and MCP-1 were determined with lung tissues that were obtained 24 h after the administration with LPS. Equal amount of protein was denatured and resolved on 8–12% SDS polyacrylamide gels, and transferred to Hyond PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were incubated with blocking buffer (5% skim milk in TBST) for 1 h at room temperature. Specific antibodies against iNOS, COX-2, MCP-1, phosphorylated (p)-AMPK (rabbit polyclonal antibody, Santa Cruz, 1;1000), *p*-p65, p65, *p*-IκB and β-actin (rabbit polyclonal antibody, Cell signaling