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 release 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 (Liu 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...
regulates the recruitment of inflammatory cells such as neutrophils and macrophages (Deshmans 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 (Mila et al., 2010; Wang et al., 2013) and is an important regulator of Ca²⁺ 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 (Joecel 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) to estimate inflammatory cells counts. 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) 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.

2.2. 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% 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, p-IkB and β-actin (rabbit polyclonal antibody, Cell signaling
Technology; 1:2500) were incubated overnight at 4 °C in 5% skim milk. The membranes were washed in TBST buffer, and then developed by enhanced chemiluminescent (ECL) reagent (Amersham Bioscience, Cambridge, U.K).

2.8. Statistical analysis

All values shown in the data are presented as the mean ± standard error of the mean (S.E.M). Statistical significance was carried out by Student’s t test and one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test using SPSS software 12 K (SPSS, Chicago, IL, USA). Data with values of \( p < 0.05 \) were considered statistically significant. Single (*) and double (**) marks represent statistical significance of \( p < 0.05 \) and \( p < 0.01 \), respectively.

3. Results

3.1. Effect of NPS 2143 on the recruitment of neutrophils and macrophages in the BALF of mice with LPS-induced ALI

Upon LPS treatment, the numbers of inflammatory cells were markedly increased in the BALF, whereas these events were significantly decreased with NPS 2143 pretreatment (Fig. 1A and B). The inhibition rates of neutrophils influx are 52.1% (DEX), 37.0% (NPS 2.5) and 49.3% (NPS 5). The inhibition rates of macrophages influx are 48.8% (DEX), 35.5% (NPS 2.5) and 46.3% (NPS 5). There was no significant difference in the numbers of neutrophils and macrophages in the BALF of mice treated with NPS 2143 (5 mg/kg) or DEX (1 mg/kg) alone compared to normal control (NC) group (Fig. 1B).

3.2. Effect of NPS 2143 on the levels of NE activity and protein concentration in the BALF

The levels of NE activity and protein concentration were significantly increased in LPS group compared with NC group (\( p < 0.01 \)) (Fig. 2A and B). However, NPS 2143 pretreatment effectively decreased these levels (compared with LPS group, \( p < 0.05 \)). The inhibition rates of NE activity were 59.01% (DEX), 43.28% (NPS 2.5) and 56.13% (NPS 5) (Fig. 2A). The inhibition rates of BALF protein concentration were 37.56% (DEX), 37.70% (NPS 2.5) and 41.41% (NPS 5) (Fig. 2B). To compare the effect of pre- or post-treatment of NPS 2143 on NE activity, NPS 2143 was treated 1 h before LPS administration or 1 h after LPS administration. 24 h after LPS administration, the level of NE activity was evaluated (Fig. 2C). The inhibition rates of NPS 2143 pretreatment on NE activity were 37.2% (DEX), 34.1% (NPS 2.5) and 40.7% (NPS 5). The inhibition rates of NPS 2143 post-treatment were 39.0% (DEX), 33.9% (NPS 2.5) and 34.6% (NPS 5) (compared with LPS group, \( p < 0.05 \)). The effect of 2.5 or 5 mg/kg of NPS 2143 were similar to those of 1 mg/kg of DEX, which is used for positive control. There was no significant difference of the level of NE activity in the BALF of mice with treated with NPS 2143 (5 mg/kg) or DEX (1 mg/kg) alone compared to NC group (Fig. 2C).

![Fig. 1. Effect of NPS 2143 on the influx of neutrophils and macrophages in the BALF of ALI mice. (A) The image of inflammatory cells (magnification, × 400). (B) The counts of neutrophils and macrophages. Data are expressed as mean ± standard error of the mean (S.E.M). *p < 0.01 indicates statistically significantly different from normal control (NC) group. *p < 0.05 and **p < 0.01 indicate statistically significant difference compared to lipopolysaccharide (LPS) group.](image-url)


3.3. Effect of NPS 2143 on the production of inflammatory cytokines in the BALF and serum

Increased levels of TNF-α and IL-6 were detected in the BALF and serum of LPS group compared with NC group (p < 0.01) (Fig. 3A and B). These levels were effectively down-regulated with NPS 2143 pre-treatment (compared with LPS group, p < 0.05). There was no significant difference of the levels of those cytokines in the BALF or serum of mice treated with NPS 2143 (5 mg/kg) or DEX (1 mg/kg) alone compared to NC group.

3.4. Effect of NPS 2143 on the expression of iNOS and COX-2 in the lung

In ALI cases, large amounts of inflammatory molecules such as iNOS and COX-2 were detected in the lung tissue (Liou et al., 2016). Therefore, we examined whether NPS 2143 affects the increased levels of these molecules. As shown in Fig. 4A and B, LPS administration markedly upregulated the expression of iNOS and COX-2 in the lung (compared with NC group, p < 0.05). However, NPS 2143 pretreatment significantly decreased the expression of these molecules in a concentration-dependent manner (compared with LPS group,

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**Fig. 2.** Effect of NPS 2143 on the activity of NE and protein concentration in the BALF. The levels of (A and C) NE activity and (B) BALF protein concentration. Data are expressed as mean ± standard error of the mean (S.E.M). *p < 0.01 indicates statistically significantly different from NC group. *p < 0.05 and **p < 0.01 indicate statistically significant difference compared to LPS group.

**Fig. 3.** Effect of NPS 2143 on the production of inflammatory cytokines in the BALF and serum. (A-B) The levels of TNF-α and IL-6 in the BALF and serum. Data are expressed as mean ± standard error of the mean (S.E.M). *p < 0.01 indicates statistically significantly different from NC group. *p < 0.05 and **p < 0.01 indicate statistically significant difference compared to LPS group.
NPS 2143 upregulates AMPK activation using western blot analysis. As shown in Fig. 6A, the phosphorylation of AMPK was significantly increased with NPS 2143 compared with NC or DEX group (p < 0.05).

Upon endotoxin stimulation, IκB is phosphorylation, ubiquitination and degradation, resulting in NF-κB phosphorylation and nucleus translocation (Xia et al., 2004). Based on this report, we measured the levels of NF-κB and IκB phosphorylation using western blot analysis. The increase of NF-κB and IκB phosphorylation was seen in LPS group compared with NC group (p < 0.01), whereas this increase were significantly decreased in NPS 2143 pretreatment group (compared with LPS group, p < 0.01).

3.7. Effect of NPS 2143 on LPS-stimulated H292 airway epithelial cells

We examined the anti-inflammatory effect of NPS 2143 in LPS-stimulated H292 airway epithelial cells. The marked increase of IL-6 and MCP-1 were detected by LPS administration (Fig. 7A and B). However, NPS 2143 pretreatment significantly decreased the levels of these molecules. NPS 2143 (1 μM) and DEX (1 μM) were equally effective in reducing levels of IL-6 and MCP-1 production (IL-6: 69.43 vs 69.63, MCP-1: 80.21 vs 77.11, p < 0.05). To evaluate the post-treatment of NPS 2143 on LPS-induced IL-6, NPS 2143 was treated 1 h after LPS administration. The inhibition rates of IL-6 were 41.37% (DEX), 15.34% (NPS 0.5), 33.80% (NPS 1) and 51.18% (NPS 2) (Fig. 7C). The activation of AMPK were upregulated with NPS 2143 administration (Fig. 7D). In addition, the activation of NF-κB was attenuated by NPS 2143 pretreatment (Fig. 7E and F). No obvious toxicity was observed upon treatment with NPS 2143 or DEX in the concentration range (data not shown).

4. Discussion

Increasing evidences have shown that CaSR are related in inflammatory diseases (Lee et al., 2012; Rossol et al., 2012). Interestingly, recent reports have suggested that CaSR may be a promoter of airway inflammation (Diaz-Soto et al., 2016; Yarova et al., 2015). Our present study demonstrates that CaSR inhibitor NPS 2143 exert anti-inflammatory activities in ALI animal models and LPS-stimulated airway epithelial cells. These results imply that the blockade of CaSR may provide a novel therapeutic strategy for the treatment of pulmonary inflammatory diseases.

Macrophage-derived TNF-α and IL-6 play a critical role in combat with the bacterial endotoxin. However, uncontrolled production of these molecules leads to the tissue and organ damage (Yang et al., 2017). Thus, the regulation of these overproduction is important point. It is well known that CaSR is expressed in the T lymphocyte and its activation promote the inflammatory molecules, and the administration of NPS 2143 effectively decrease the levels of TNF-α and the influx of inflammatory cells in inflammatory disease models (Wu et al., 2015; Yarova et al., 2015). NPS 2143 also inhibits the levels of TNF-α, IL-6 and MMP-9 in CSE-stimulated human airway cells (Lee et al., 2016b). These results means that blocking the activation of CaSR is meaningful in reducing inflammatory molecules. We therefore evaluated the effect of NPS2143 on TNF-α and IL-6 induced by LPS. We found that the levels of these cytokines were increased in the BALF or in the serum of ALI mice, whereas NPS 2143 pretreatment reduced these secretion (Fig. 3A and B). Simultaneously, we found that the numbers of macrophages were much lower compared with LPS group (p < 0.01) (Fig. 1A and B). These results imply that NPS2143 affect not only macrophages influx but also pro-inflammatory cytokines production against bacterial infection.

In the acute phase of ALI, macrophages produce inflammatory chemokines, such as MCP-1, which induces the recruitment of inflammatory cells including neutrophils (Duan et al., 2014; Liu et al., 2015). These pathophysiological characteristics were reduced with anti-inflammatory agent, which act as a MCP-1 inhibitor (Yang et al., 2016).
Therefore, inhibition of MCP-1 is important strategic plan in the treatment of ALI. As shown in Fig. 5C, LPS administration markedly increased the release of MCP-1 in the BALF. (Compared with NC group, \( p < 0.01 \)). However, this feature was reduced by NPS 2143 pretreatment (Fig. 5C). This result reflects that inhibition of MCP-1 may affect neutrophils recruitment (Fig. 1A and B). Next measured the expression of MCP-1 in the lung. As we expected, the expression of MCP-1 were significantly decreased with NPS 2143 pretreatment compared with LPS group (\( p < 0.01 \)) (Fig. 5B). This effect of NPS 2143 on MCP-1 expression may contribute to the attenuation of inflammatory cells influx into the lung (Fig. 5A). These results suggest that NPS2413 may act as a MCP-1 inhibitor in inflammatory disease.

The high levels of neutrophils are detected in patients with ALI and in animal models with LPS-induced ALI (Lee et al., 2016c). Neutrophil-derived elastase initiates the macrophages recruitment and is responsible for the breakdown of lung tissue (Masood et al., 2015; Tsai et al., 2015). It is also well known that specific inhibition of NE reduced the symptoms of ALI, and therefore inhibition of NE is therapeutically...
useful in the treatment of ALI (Kawabata et al., 2002). Thus, an approach to inhibiting neutrophils influx and NE activity may be crucial to attenuate symptoms of ALI. We found that NPS 2143 pretreatment attenuates the recruitment of neutrophils in LPS-induced ALI (Fig. 1A and B). Increased levels of NE activity were also inhibited by NPS 2143 pre- or post-treatment (Fig. 2A and C). These results imply that NPS 2143 may be a controller of neutrophils influx and a potent inhibitor of NE.

Now, we know that NPS2143 could attenuate the influx of inflammatory cells and the production of inflammatory chemokine/ cytokines. We also confirmed that NPS 2143 pretreatment dose-dependently led to a significant reduction of iNOS and COX-2 expression (Fig. 4A and B). Therefore, it is necessary to explore the underlying mechanism of NPS 2143, which will effectively utilize the NPS 2143 for clinical development. Recently, AMPK has been recognized as an important regulator of endotoxin-induced inflammation by reducing the recruitment of neutrophils and macrophages (Kim et al., 2016; Li et al., 2015). Therefore, we focused on AMPK activation. Obviously, exposure to LPS decreased the AMPK activation compared with those in normal control group. Pretreatment with NPS 2143 (2.5 and 5 mg/kg) significantly increased AMPK activation compared with those in the DEX or LPS group (Fig. 6A).

Recently, inhibitory effect of NPS 2143 on NF-κB activation has been revealed in T lymphocyte in cecal ligation and puncture (CLP) rat (Wu et al., 2015). NPS 2143 inhibits nuclear translocation of NF-κB in CSE-stimulated airway epithelial cells (Lee et al., 2016b). These results suggest that NPS 2143 may have potential in regulating NF-activation. In our study, the activation of NF-κB was observed to be greater in LPS group compared to NC group (Fig. 6B and C). However, NPS 2143 group mice showed NF-κB inactivation compared to LPS group mice. The effects of NPS 2143 (5 mg/kg) were comparable to the effect of DEX. Therefore, we expect that NPS 2143 may increase resistance to pulmonary inflammation through the NF-κB pathway in treatment of ALI.

It is well known that IL-6 amply the pulmonary inflammation and lead to the pathogenesis of endotoxin-induced ALI (Niu et al., 2017; Shao et al., 2017; Zhu et al., 2017). NPS 2143 inhibited the over-production of IL-6 induced by CSE in H292 cells (Lee et al., 2016b). In our study, NPS 2143 pretreatment effectively reduced the levels of IL-6 and MCP-1 in LPS-stimulated H292 cells (Fig. 7A and B). NPS 2143 post-treatment also significantly decreased LPS-induced IL-6 (Fig. 7C). Interestingly, the levels of IL-6 were reduced by NPS 2143 pretreatment and post-treatment in LPS-stimulated H292 cells (Fig. 7A and C). These results suggest that NPS 2143 may be useful as an inhibitor of IL-6 in inflammatory diseases. Moreover, NPS 2143 led to AMPK activation and NF-κB inactivation (Fig. 7D–F). These results indicate that NPS 2143 has anti-inflammatory properties in LPS-stimulated H292 airway epithelial cells.

In conclusion, exposure to LPS has been shown to induce a marked inflammatory cells and toxic molecule in mice, and to produce inflammatory cytokine/chemokine in H292 airway epithelial cells. Pretreatment of NPS 2143 effectively reduced those events by NF-κB inactivation and AMPK activation in vivo and in vitro. Considering the importance of regulation of neutrophils influx and these cells-derived toxic molecule, our results suggest that NPS 2143 may be a useful therapeutic adjuvant in ALI. Further studies are required to clarify the role and efficacy of NPS2143 in ALI.

Conflict of interest

The authors declare that there are no conflict of interest.

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