Anti-inhibitory potential of an ethanolic extract of *Distromium decumbens* on pro-inflammatory cytokine production in *Pseudomonas aeruginosa* lipopolysaccharide-stimulated nasal polyp-derived fibroblasts

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Abstract. Marine algae are rich sources of biologically active compounds that may present useful leads in the development of pharmaceuticals, nutraceuticals, and functional foods. The main aim of this study was to identify the possible anti-inflammatory effects of *Distromium decumbens* in nasal polyp-derived fibroblasts (NPDFs) and its associated mechanism of action. NPDFs were stimulated by *Pseudomonas aeruginosa* lipopolysaccharide (*PA*-LPS) and treated with an ethanolic extract of *Distromium decumbens* (DDE). The production of interleukin-6 (IL-6) and IL-8 in the supernatant, the phosphorylation of mitogen-activated protein kinase (MAPK) molecules [extracellular signal-related kinase 1/2 (ERK1/2), c-Jun N-terminal kinase and p38 MAPK] and Akt, and the activation of nuclear factor-κB (NF-κB) were assayed in the *PA*-LPS-stimulated NPDFs untreated or treated with DDE. The expression levels of IL-6 and IL-8 in *PA*-LPS-exposed NPDFs were detected using enzyme-linked imm\unosorbent assays. The mechanisms by which DDE regulates cellular signaling cascades were investigated using electrophoretic mobility shift assays and western blot analysis. Functional validation was performed by measuring the inhibitory effects of DDE on neutrophil migration *in vitro*. DDE reduced the expression of IL-6 and IL-8 stimulated by *PA*-LPS in NPDFs. The activation of ERK1/2, Akt and NF-κB by *PA*-LPS was inhibited by DDE. Inhibitors of ERK1/2, Akt and NF-κB inhibited the expression of IL-6 and IL-8. In addition, DDE significantly attenuated *PA*-LPS-induced migration of differentiated HL-60 cells. The present findings suggest that DDE potently inhibits inflammation through the ERK1/2, Akt and NF-κB signaling pathways in NPDFs.

Introduction

Nasal polyps (NPs) cause considerable morbidity, including nasal congestion, rhinorrhea, anosmia, decreased taste, sinusitis, olfactory dysfunction and headaches, which significantly deteriorate the quality of life of patients (1). NPs are swellings of the lining of the nasal passages and sinuses. They are characterized by tissue remodeling consisting of basement membrane thickening, goblet cell hyperplasia, epithelial proliferation, pseudocyst formation, focal fibrosis, inflammatory cell infiltration and edema (1,2). NPs are a common chronic inflammatory disease of the mucous membranes with a high recurrence rate in the nose and paranasal sinuses, and are generally related with chronic rhinosinusitis (CRS) (3). While many studies on NPs have been carried out, the pathogenesis of NPs has not been elucidated thus far.

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Many types of cells, such as epithelial cells, T cells, mast cells and fibroblasts, are related with the pathogenesis of NPs (3). Among these, fibroblasts are the critical component of the polyp organization. Fibroblasts are relevant regulators of local inflammation due to a source of biological mediators in initiating and amplifying inflammation (4). Stimulated fibroblasts contribute towards the inflammatory response by releasing inflammatory mediators. *Pseudomonas aeruginosa (P. aeruginosa)* can be frequently found in nasal smears of patients with persistent sinus symptoms after sinus surgery (5). Previous studies have shown that lipopolysaccharide (LPS) derived from *P. aeruginosa* induces interleukin-1 (IL-1), IL-6, monocyte chemoattractant protein-1 (MCP-1), and tumor necrosis factor-α (TNF-α) in airway inflammation and goblet cell hyperplasia (6). Among these inflammatory mediators, IL-6 and IL-8 were found to be produced in LPS-stimulated NP-derived fibroblasts (NPDFs) (4). NPDF activation by LPS appears to play a crucial role in the initiation and progression of NPs. Therefore, regulation of NPDF activation and inhibition of these inflammatory mediators may be therapeutic targets to reduce the development of NPs.

Marine algae have been used as dietary and medicinal component in Asia and are rich in dietary fibers, minerals, vitamins, polysaccharides, proteins and various functional polyphenols (7). Previous studies have supported the pharmaceutical and the medicinal importance of seaweed in treating NFs (7,8). The authors of the present study have found that the ethanolic extract of *Distromium decumbens* (DDE) has the highest quantity of total polyphenol content (21.27±0.09%). In our previous study, DDE was found to have more than 89.57±0.43% 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity (9). In addition, DDE inhibited nitric oxide, reactive oxygen species, and prostaglandin E_2 production in RAW cells when LPS was used as an inducer (unpublished data). These inflammatory mediators serve important roles in inflammation-related disorders. Taken together, these results address the hypothesis that DDE may exert anti-inflammatory effects in NPs.

Therefore, as a part of our ongoing study to screen and evaluate the anti-inflammatory efficacy of natural bioactive materials, we focused on the anti-inflammatory effects of DDE and the mechanism related in NPDFs, which can be stimulated using LPS to mimic the conditions of NPs *in vitro*.

Materials and methods

Reagents. LPS from *P. aeruginosa* (*PA*-LPS) was purchased from Merck Millipore (Darmstadt, Germany). An antibody against nuclear factor-κB (NF-κB) (cat. no. 14-6731) was purchased from eBioscience (San Diego, CA, USA). Antibodies against phospho (p)-extracellular signal-related kinase (ERK) (cat. no. 9106), c-Jun N-terminal kinase (JNK) (cat. no. 9252), p-JNK (cat. no. 9251), p-p38 mitogen-activated protein kinase (MAPK) (cat. no. 9211), Akt (cat. no. 9272), and p-Akt (cat. no. 4058) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against ERK (cat. no. sc-94), and p38 MAPK (cat. no. sc-535) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Inhibitors of ERK kinase (U0126) and Akt (LY294002) were purchased from Calbiochem (Billerica, MA, USA) and were dissolved in dimethyl sulfoxide (DMSO). BAY 11-7082 and parthenolide were purchased from Santa Cruz Biotechnology, Inc..

Preparation of ethanol extract from D. decumbens. Marine brown alga, *D. decumbens*, was collected from the Jeju Island, Korea. After collection, *D. decumbens* was washed with tap water to remove slats, epiphytes, and sand attached to the surface of the samples, and they were then maintained at -20˚C. The samples were lyophilized and homogenized using a grinder. The dried powder was extracted with 70% EtOH (1:10 w/v) for 1 h (5 times) by sonication, and then the extract was evaporated *in vacuo*. The extract was dissolved in DMSO prior to use in the experiment.

NP-derived fibroblast culture. Fifteen subjects with NPs and 15 subjects with deviated nasal septum were recruited from the Department of Otorhinolaryngology, Inje University Busan Paik Hospital, Busan, Republic of Korea. Based on the minimum criteria for chronic sinusitis with NPs, individuals were diagnosed as having NPs (10). All patients who participated in this study provided informed consent and the procedure was approved by our local ethics committee. A NP was defined as the presence of an endoscopically visible bilateral polyp growing from the middle nasal meatus into the nasal cavity, and affecting the ethmoid and maxillary sinuses as observed using computed tomography of the paranasal sinus. NPs were obtained from the region of the middle meatus at the beginning of the surgical procedure. The subjects had no history of nasal allergy, aspirin sensitivity or asthma. No patient had prescribed steroids (topical or systemic), nonsteroidal anti-inflammatory drugs, antihistamines, or macrolide antibiotics during the 4 weeks before the biopsy. NPDFs were separated from tissues by enzymatic treatment with collagenase (500 U/ml), hyaluronidase (30 U/ml), and DNase (10 U/ml) (all from Sigma-Aldrich, St. Louis, MO, USA). Dulbecco's modified Eagle's medium was applied to culture the cells containing 10% (v/v) heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1,000 μg/ml streptomycin (Invitrogen, Carlsbad, CA, USA), and 1,000 U/ml penicillin. The purity of the NPDFs was confirmed by flow cytometry and characteristic spindle-shaped cell morphology. Experimental cells were used in the fourth cell passage.

Cell line culture. RPMI-1640 medium supplemented with fetal bovine serum (10%) was used to maintain HL-60 cells. To achieve the expression of the neutrophilic phenotype, the HL-60 cells were induced to differentiate (dHL-60) with 1.75% (vol/vol) DMSO for 3-4 days.

Determination of cell viability. The Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) method was applied to determine the cellular viability. Briefly, wells ($1x10^5$ cells/ml) were incubated with DDE for 24 h and then the cells were washed with PBS twice. CCK-8 was added to each well and incubated at 37˚C for 1 h, followed by analysis at 450 nm using a plate reader (model EL800; Bio-Tek Instruments, Inc., Winooski, VT, USA).

Enzyme-linked immunosorbent assay (ELISA). The cytokine levels in the culture media were detected using ELISA. ELISA

kits, which were used to measure IL-6 and IL-8 expression levels, were purchased from BioLegend (San Diego, CA, USA). The absorbance at 450 nm was measured using a plate reader (model EL800; Bio-Tek Instruments, Inc.).

Cell migration assay. The dHL-60 cell migration was measured using a 24-well plate system at densities of $1x10^6$ cells/ml. The dHL-60 cells were added to the upper chambers of Transwell cluster plates (24-well companion plate; BD Biosciences, Franklin Lakes, NJ, USA) with 3-μm pore filters. *PA*-LPS-induced NPDFs were added to the lower wells of the plates. The cells were allowed to migrate for 24 h at 37˚C in 5% CO_2. The transferred cells from the lower chamber were collected and then centrifuged at 400 x g for 10 min. The number of cells that had migrated to the lower well was counted with a hemocytometer. Each experiment was performed in triplicate and repeated at least 3 times.

Western blot analysis. The cells were lysed with lysis buffer (Mammalian Cell-PE LB; G-Biosciences, St. Louis, MO, USA). Equal amounts of protein were separated on 10% SDS-polyacrylamide mini-gels and transferred onto nitrocellulose membranes (GE Healthcare Life Sciences, Chalfont, UK). Following incubation with the appropriate primary antibodies (ERK, p-ERK, p38, p-p38, JNK, p-JNK, Akt, p-Akt and NF-κB) at a dilution of 1:1,000 overnight at 4˚C, the membranes were incubated with horseradish peroxidase conjugated anti-rabbit (cat. no. 31460; Pierce Biotechnology, Inc., Rockford, IL, USA) or anti-mouse (cat. no. sc-2031; Santa Cruz Biotechnology, Inc.) secondary antibodies. Following three washing with Tris-buffered saline Tween-20 (TBST), immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) detection system (Pierce Biotechnology, Inc.).

Electrophoretic mobility shift assay (EMSA). Nuclear extract was prepared using the NE-PER nuclear extraction reagent (Pierce Biotechnology, Inc.). An oligonucleotide containing the immunoglobulin κ-chain binding site (κB, 5'-GATCTCAGA GGGGACTTTCCGAGAGA-3') was synthesized as a probe for the gel retardation assay. A non-radioactive method, in which the 3' end of the probe was labeled with biotin, was used (Pierce Biotechnology, Inc.). The binding reactions contained 5 μg nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 50 ng poly(dI-dC) and 20 fM biotin-labeled DNA. The reactions were incubated at room temperature for 20 min to a final volume of 20 μl. The competition reactions were performed by adding a 100-fold excess of unlabeled κB to the reaction mixture. The mixture was then separated by electrophoresis on a 5% polyacrylamide gel in 0.5X Tris-borate buffer and transferred to nylon membranes. The biotin-labeled DNA was assessed using a LightShift Chemiluminescent EMSA kit (Pierce Biotechnology, Inc.).

Statistical analysis. Data values represent the means ± standard deviation. To analyze the data produced from the experiments with two independent variables, one-way analysis of variance was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Values of $P<0.05$ and $P<0.01$ were considered significant.

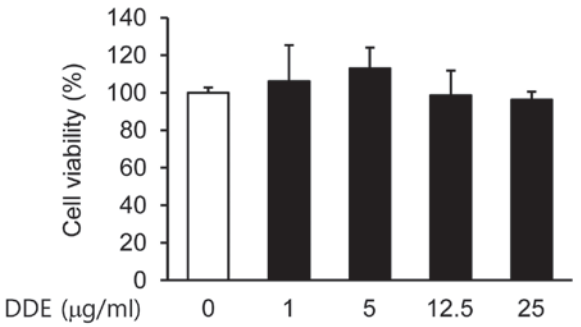

Figure 1. Effect of *Distromium decumbens* (DDE) on the viability of nasal polyp-derived fibroblasts (NPDFs). The cells were pretreated with the indicated concentrations of DDE (1-25 μg/ml) for 24 h. Cell viability was examined using the Cell Counting Kit-8 (CCK-8) assay, and the results are expressed as the percentage of surviving cells compared to control cells (DMSO). Each value indicates the mean ± standard deviation and represents results obtained from three independent experiments.

Results

Effects of DDE on the viability of NPDFs. Initially, we examined the viability of NPDFs treated with DDE by using a CCK-8 assay. No significant cytotoxicity to NPDFs was observed at doses up to 25 μg/ml (Fig. 1). Based on these results, a concentration range of 1 to 25 μg/ml was selected for the subsequent experiments.

Effects of DDE on the expression of IL-6 and IL-8 in PA-LPS-induced NPDFs. The protein expression levels of IL-6 and IL-8 were markedly increased after the stimulation of NPDFs with *PA*-LPS (Fig. 2). To assess the effect of DDE on the protein production of IL-6 and IL-8 in NPDFs, we pretreated the cells with DDE (1, 5, 12.5 and 25 μg/ml) before stimulation with *PA*-LPS (10 μg/ml). The treatment with DDE attenuated the LPS-induced expression of IL-6 and IL-8 proteins.

Effects of DDE on the migration of dHL-60 cells towards PA-LPS-stimulated NPDFs. To examine whether DDE affects the migration of dHL-60 cells, migration assays were performed using Transwell cluster plates (Fig. 3). The number of migrated dHL-60 cells co-cultured with *PA*-LPS-treated NPDFs was 2 times greater than that of the vehicle-treated dHL-60 cells. However, the levels of infiltration of the dHL-60 cells were significantly attenuated by DDE treatment in a concentration-dependent manner when compared with the infiltration of the *PA*-LPS-induced group.

Effects of DDE on the activation of the Akt and MAPK signaling pathways in PA-LPS-induced NPDFs. To clarify the mechanisms underlying the effects of DDE on the expression of inflammatory mediators, we inspected the activation of Akt and MAPKs using western blot analysis. The stimulation of NPDFs with *PA*-LPS resulted in an increase in the phosphorylation of Akt and ERK, but not of JNK and p38. Pretreatment for 1 h with DDE (1, 12.5 and 25 μg/ml) attenuated the phosphorylation of Akt and ERK induced by a 2-h incubation with 10 μg/ml *PA*-LPS (Fig. 4A). To verify whether the Akt and ERK signaling pathways are involved in the expression of IL-6

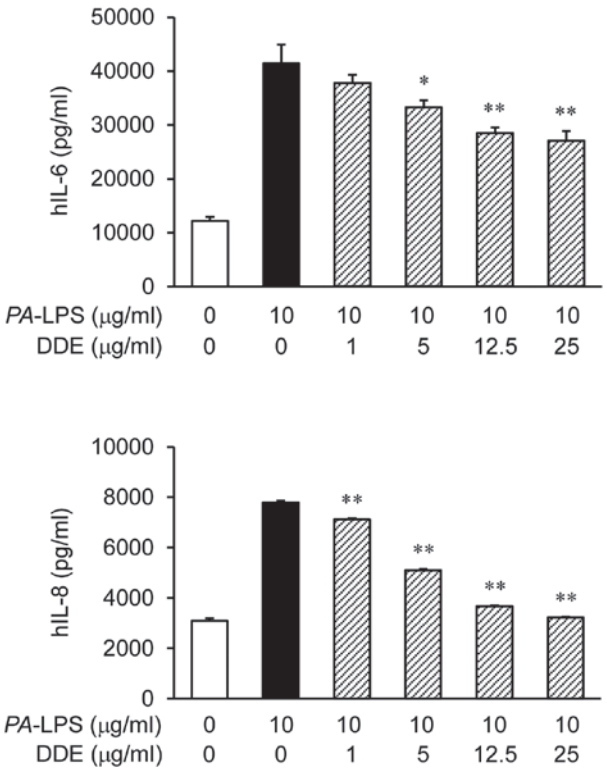

Figure 2. Effect of *Distromium decumbens* (DDE) on the expression of inflammatory cytokines in *Pseudomonas aeruginosa*-lipopolysaccharide (*PA*-LPS)-stimulated nasal polyp-derived fibroblasts (NPDFs). The expression levels of interleukin-6 (IL-6) and IL-8 proteins were determined by enzyme-linked immunosorbent assay (ELISA). The cells were pretreated with DDE (1-25 μg/ml) for 1 h prior to *PA*-LPS stimulation for 24 h. Each bar represents the mean ± standard deviation from three independent experiments. *P<0.05 and **P<0.01 vs. *PA*-LPS-stimulated values.

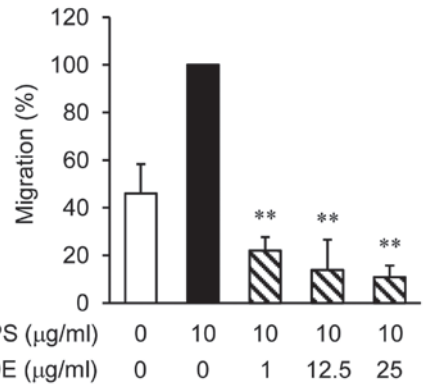

Figure 3. *In vitro* migration of dHL-60 cells. dHL-60 cells were added to the upper wells of Transwell cluster plates with 3 μm pore filters. The cells were pretreated with *Distromium decumbens* (DDE) (1-25 μg/ml) for 1 h prior to *Pseudomonas aeruginosa*-lipopolysaccharide (*PA*-LPS) stimulation for 24 h. *PA*-LPS-stimulated nasal polyp-derived fibroblasts (NPDFs) were added to the lower chambers of the plates. The numbers of cells that had migrated to the lower wells were counted using a hemocytometer. Each bar represents the mean ± standard deviation from triplicates from three independent experiments. **P<0.01 vs. *PA*-LPS-stimulated values.

and IL-8, NPDFs were treated with *PA*-LPS with or without DDE, Akt or ERK inhibitors. The upregulated expression of IL-6 and IL-8 was significantly inhibited by U0126 (an inhibitor of ERK) and LY294002 (an inhibitor of Akt) (Fig. 4B).

Effects of DDE on the activation of NF-κB in PA-LPS-stimulated NPDFs. The production of pro-inflammatory cytokines is regulated by the transcription factor NF-κB in *PA*-LPS stimulation (11). Therefore, to inspect the mechanism by which DDE affects the expression of IL-6 and IL-8, we assessed the effects of DDE on NF-κB activation. We found that DDE inhibited the *PA*-LPS-induced translocation of NF-κB p65 into the nuclear compartment (Fig. 5A). We next examined the effect of DDE on the DNA-binding activity of NF-κB using EMSA (Fig. 5B). *PA*-LPS treatment caused a significant increase in the DNA-binding activity of NF-κB, whereas treatment with DDE markedly reduced the *PA*-LPS-induced DNA-binding activity of NF-κB.

Effects of NF-κB inhibitors on the expression of IL-6 and IL-8 in PA-LPS-induced NPDFs. To confirm whether the NF-κB p65 signaling pathway is involved in the expression of IL-6 and IL-8, NPDFs were treated with *PA*-LPS with or without DDE or NF-κB inhibitors (BAY 11-7082 and parthenolide, respectively). As shown in Fig. 5C, BAY 11-7082 and parthenolide inhibited the expression of IL-6 and IL-8 in the *PA*-LPS-stimulated NPDFs.

Discussion

Seaweed has long been traditionally consumed for medicinal and dietary purposes. Marine algae are known to produce natural bioactive materials, such as dietary fibers, protein, polysaccharides, vitamins, minerals and various functional polyphenols (12). They are rich sources of anti-inflammatory therapeutic agents. A pro-inflammatory response is initiated as a defense reaction following stimulation by environmental stimuli such as pathogens and damage, resulting in the expression of pro-inflammatory mediators. These pro-inflammatory factors are regarded as responsible for the pathological states associated with NPs (4). Therefore, the present study was attempted to examine the pharmacological effects of DDE on the expression of inflammatory cytokines and the migration of neutrophils. In addition, the mechanism of action of *PA*-LPS-induced stimulation of NPDFs was investigated.

Inflammatory factors are expressed in response to gram-negative bacterial infections, mediated by LPS, a cell wall component of gram-negative bacteria. LPS is a pathogen-associated molecular pattern that provokes pathogenicity. Fibroblasts are the major structural components of tissues and play an augmenting effector role in the inflammatory response (13). Nasal fibroblasts are the principal structural components of the nose and are involved in NPs, which are common findings in chronic rhinosinusitis (14). Nasal fibroblasts produce pro-inflammatory cytokines, including IL-6, and chemokines, including IL-8, in nasal fibroblasts upon LPS stimulation (3).

We first investigated *PA*-LPS-induced production of IL-6 and IL-8 in HNDFs. IL-6, a pleiotropic cytokine, has a wide range of biological activity, which is associated with the production of acute phase proteins. IL-6 is associated with the pathogenesis of diverse inflammatory diseases, including multiple myeloma, rheumatoid arthritis, Crohn's disease,

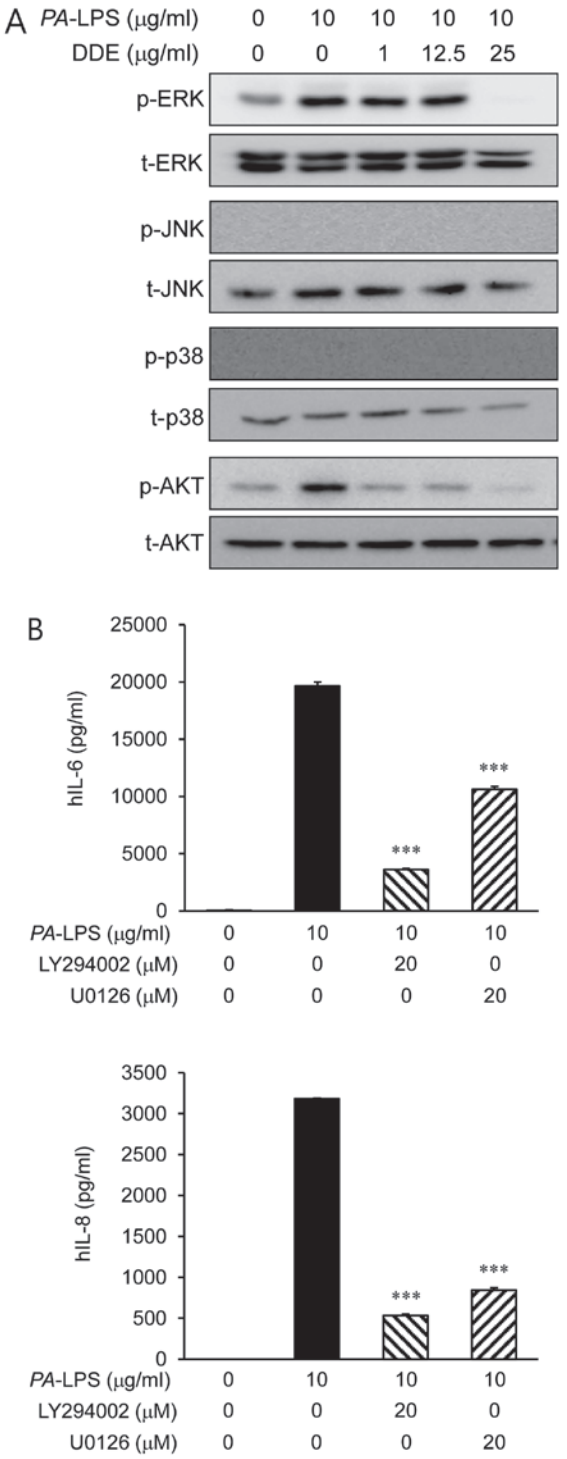

Figure 4. Effect of *Distromium decumbens* (DDE) on *Pseudomonas aeruginosa*-lipopolysaccharide (*PA*-LPS)-induced phosphorylation of MAPKs and Akt in nasal polyp-derived fibroblasts (NPDFs). (A) NPDFs were treated with a vehicle or the indicated concentrations of DDE for 1 h prior to stimulation with *PA*-LPS (10 μg/ml) for 2 h. Cell extracts were then prepared and subjected to western blotting with antibodies specific to the phosphorylated forms of extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK), p38 and Akt. The results presented are representative of three independent experiments. (B) The expression levels of interleukin-6 (IL-6) and IL-8 in the culture medium were determined by enzyme-linked immunosorbent assay (ELISA). NPDFs were treated with LY294002 (an Akt inhibitor) and U0126 (an ERK inhibitor) 30 min before *PA*-LPS stimulation for 24 h.

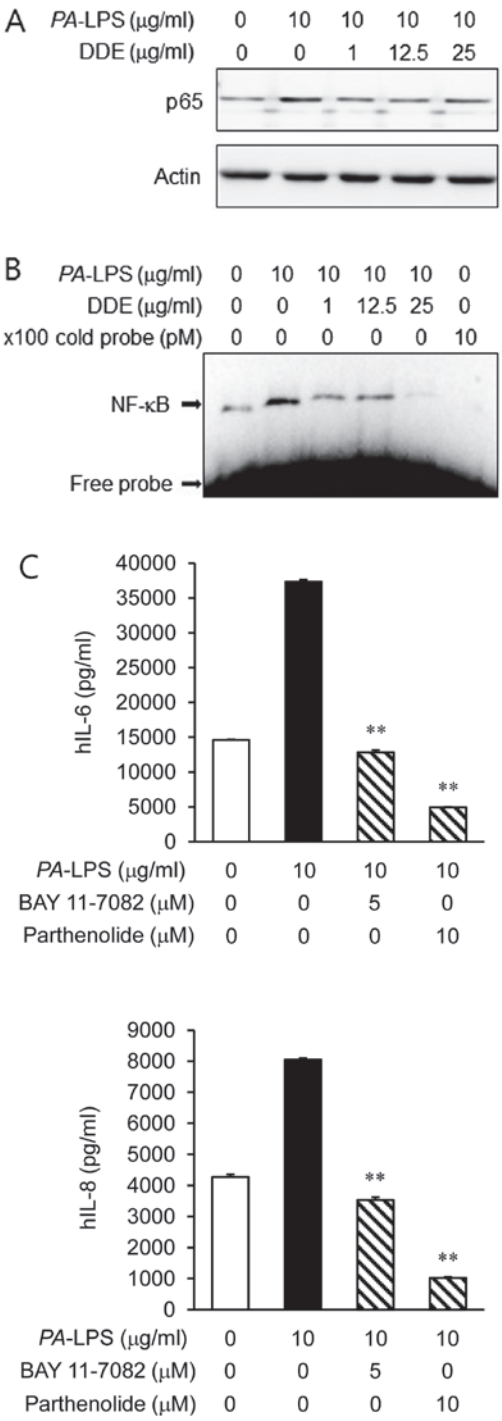

Figure 5. Effect of *Distromium decumbens* (DDE) on the translocation and binding of nuclear factor-κB (NF-κB) into the nuclei of *Pseudomonas aeruginosa*-lipopolysaccharide (*PA*-LPS)-stimulated nasal polyp-derived fibroblasts (NPDFs). The NPDFs were pretreated with DDE (1, 12.5 or 25 μg/ml) for 1 h and then stimulated with *PA*-LPS for 2 h. (A) Nuclear extracts were evaluated for NF-κB via western blot analysis. (B) Nuclear extracts were evaluated for NF-κB via electrophoretic mobility shift assay (EMSA). (C) The production of interleukin-6 (IL-6) and IL-8 was measured using an enzyme-linked immunosorbent assay (ELISA) kits. NPDFs were pretreated with NF-κB inhibitors, BAY 11-7082 or parthenolide, for 30 min, and were then stimulated with *PA*-LPS for 24 h. **P<0.01 vs. *PA*-LPS-stimulated values.

asthma and lupus (15-18). IL-6-induced signaling can be mostly seen in a relatively stingy number of IL-6-reactive cells, whereas in a chronic inflammatory phase it is able to activate nearly all cells of the body (19). A recent study confirmed elevated IL-6 protein expression in polyp tissue compared to its expression in middle turbinate in the same patients with

CRS with NPs (CRSwNP) (20). These results suggest that IL-6 plays a pathogenic role in CRSwNP. Although IL-8 synthesis aims at combating and eliminating the pathogen, persistent production of IL-8 plays a crucial role in the accumulation of neutrophils in sites of inflammation (21). The concentrations of IL-6 and IL-8 are increased in nasal lavage and NPs in CRS, and they activate NP growth (22). As shown in Fig. 2 DDE inhibited the *PA*-LPS-induced production of IL-6 and IL-8 at concentrations of DDE that were not cytotoxic to the NPDFs. Next, we performed functional validation by assessing the attenuation effect of DDE on the migration of neutrophils *in vitro*. Neutrophils release matrix metalloproteinases and induce tissue destruction (23,24). Because fibroblasts can produce a variety of chemokines, they are considered responsible for local recruitment of inflammatory cells (25,26). A human promyelocytic HL-60 cell line that can be induced to differentiate using agents such as DMSO is a useful model system for studying neutrophil migration (27). We therefore used dHL-60 cells to evaluate the anti-migratory effects of DDE. Fig. 3 shows that treatment of dHL-60 cells with DDE (1, 12.5 or 25 μg/ml) decreased migration dose-dependently.

As DDE inhibited the expression of IL-6 and IL-8 in *PA*-LPS-stimulated NPDFs, we further investigated the mechanism underlying the inhibitory actions of DDE. LPS promotes the transcription of a variety of inflammatory genes related to inflammatory responses, including MAPKs (ERK, JNK and p38 MAPK) and the Akt pathway, in various types of cells (28). MAPKs play an important role in the control of cell responses. MAPKs have been implicated as important mediators of the signaling pathway that appears to play a critical role in the inflammatory response (29). In addition, Akt is essential in LPS-stimulated inflammation progression (30), since LPS induces inflammatory responses via Akt signaling in fibroblasts (31). Therefore, modulation of the Akt axis is considered to be an effective approach towards the treatment of many inflammatory disorders. A previous study demonstrated that LPS stimulates IL-6 and IL-8 expression through the MAPK and Akt signaling pathways in NPDFs (4). As expected, when NPDFs were treated with *PA*-LPS, ERK and Akt were phosphorylated, whereas pretreatment with DDE significantly reduced the LPS-induced phosphorylation of ERK and Akt (Fig. 4A). In addition, treatment with these kinase inhibitors effectively inhibited *PA*-LPS-stimulated expression of IL-6 and IL-8 (Fig. 4B). Therefore, the present study demonstrated a significant inhibition of *PA*-LPS-stimulated phosphorylation of ERK and Akt by DDE in NPDFs.

Transcription factor NF-κB is known to be a multifunctional regulator of various genes involved in the expression of many inflammatory mediators involved in inflammatory process (32). Activation of NF-κB causes phosphorylation, ubiquitination and proteasome-mediated degradation of the inhibitory IκB proteins and then nuclear transfer (33). Therefore, we examined the effects of DDE on the *PA*-LPS-stimulated activation of NF-κB by NPDFs. Since activated NF-κB enters the nucleus and induces IL-6 and IL-8 expression, we studied the nuclear translocation of the NF-κB subunit. Western blot analysis showed that stimulation with *PA*-LPS induced translocation of NF-κB to the nuclear compartment. However, nuclear translocation of *PA*-LPS-induced NF-κB was attenuated in the presence of DDE. According to EMSA data, *PA*-LPS stimulation increased the DNA-binding activity of NF-κB. As shoen in Fig. 5B, pretreatment with DDE effectively inhibited the *PA*-LPS-stimulated DNA-binding activity of NF-κB. To confirm the above results, we investigated the effects of the NF-κB inhibitors, BAY 11-7082 and parthenolide, on the *PA*-LPS-induced production of IL-6 and IL-8 by NPDFs (Fig. 5C). These data demonstrated that DDE inhibits NF-κB activity in *PA*-LPS-stimulated NPDFs by suppressing the nuclear translocation of p65, resulting in attenuation of IL-6 and IL-8 production.

In conclusion, the results of the present study demonstrated that *PA*-LPS increased the release of IL-6 and IL-8 from cultured NPDFs. Pretreatment with DDE significantly attenuated the expression of IL-6 and IL-8 in NPDFs under *PA*-LPS stimulated inflammatory conditions by suppressing the activation of the ERK, Akt, and NF-κB signaling pathways. These results suggest that the anti-inflammatory activity of bioactive compounds contained in *D. decumbens* ethanolic bioactive extract have the potential to be developed as therapeutic agents for the management of NPs.

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