



# Prostaglandin E2 Induces IL-6 and IL-8 Production by the EP Receptors/Akt/NF- $\kappa$ B Pathways in Nasal Polyp-Derived Fibroblasts

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**Purpose:** Interleukin 6 (IL-6) and IL-8 participate in the pathogenesis of chronic rhinosinusitis with nasal polyps, and their levels are increased by prostaglandin E2 (PGE2) in different cell types. The purposes of this study were to determine whether PGE2 has any effect on the increase in the levels of IL-6 and IL-8 in nasal polyp-derived fibroblasts (NPDFs) and subsequently investigate the possible mechanism of this effect. **Methods:** Different concentrations of PGE2 were used to stimulate NPDFs at different time intervals. NPDFs were treated with agonists and antagonists of E prostanoid (EP) receptors. To determine the signaling pathway for the expression of PGE2-induced IL-6 and IL-8, PGE2 was treated with Akt and NF- $\kappa$ B inhibitors in NPDFs. Reverse transcription-polymerase chain reaction for IL-6 and IL-8 mRNAs was performed. IL-6 and IL-8 levels were measured by enzyme-linked immunosorbent assay (ELISA). The activation of Akt and NF- $\kappa$ B was evaluated by western blot analysis. **Results:** PGE2 significantly increased the mRNA and protein expression levels of IL-6 and IL-8 in NPDFs. The EP2 and EP4 agonists and antagonists induced and inhibited IL-6 expression. However, the EP4 agonist and antagonist were only observed to induce and inhibit IL-8 expression level. The Akt and NF- $\kappa$ B inhibitors significantly blocked PGE2-induced expression of IL-6 and IL-8. **Conclusions:** PGE2 increases IL-6 expression via EP2 and EP4 receptors, and IL-8 expression via the EP4 receptor in NPDFs. It also activates the Akt and NF- $\kappa$ B signal pathways for the production of IL-6 and IL-8 in NPDFs. These results suggest that signaling pathway for IL-6 and IL-8 expression induced by PGE2 might be a useful therapeutic target for the treatment of nasal polyposis.

**Key Words:** Nasal polyps; prostaglandins E; interleukin-6; interleukin-8; E prostanoid receptor; Akt

## INTRODUCTION

Nasal polyposis (NP) is a common chronic inflammatory disease of the mucous membranes in the nose and paranasal sinuses. NP is characterized by eosinophilic infiltration and tissue remodeling consisting of epithelial proliferation, goblet cell hyperplasia, pseudocyst formation, basement membrane thickening, focal fibrosis and edema.<sup>1</sup> NP often accompanies chronic rhinosinusitis (CRS), and symptoms of NP include nasal congestion, loss of smell, and headache caused by secondary infection.<sup>2</sup> Although many studies for NP have been reported, its pathogenesis remains poorly understood and the treatment of NP treatment is limited.

Prostaglandin E2 (PGE2), a metabolite of arachidonic acid, has multiple physiological effects.<sup>3</sup> These effects are mediated by 4 different E prostanoid (EP) receptors that belong to the G protein-coupled receptor family: EP1 increases Ca<sup>2+</sup> channel gating via Gq, EP2 and EP4 increase the levels of cyclic adenos-

ine monophosphate (cAMP) via Gs, whereas EP3 decreases cAMP via Gi.<sup>4</sup> In previous studies, low production of PGE2 has been reported in NP and in both fibroblasts and epithelial cells.<sup>5</sup> PGE2 suppresses eosinophilia-associated cellular responses induced by staphylococcal enterotoxin, dominantly through an EP2-mediated pathway in NP.<sup>6</sup> These observations suggest that PGE2 plays a role as an inflammatory mediator to induce inflammatory diseases, such as NP, allergic rhinitis, and bronchial asthma. However, the effect of PGE2 on the production of interleukin 6 (IL-6) and IL-8 in NP is unknown.

Many kinds of cells such as epithelial cell, T cell, mast and fi-

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broblast, are involved in the pathogenesis of NP. Among these cells, fibroblasts play a key role in the structural modification of the sinonasal mucosa. Fibroblasts differentiate into myofibroblasts and produce large amounts of extracellular matrix molecules such as collagen and fibronectin.<sup>7</sup> Recent studies have shown that fibroblasts are not just a structural modifier, but also important modulators of local inflammation.<sup>8</sup> Fibroblasts express many receptors for cytokines, growth factors, and hormones.<sup>9</sup> Therefore, they play a role for mediator of immune function and have the capacity to release a variety of pro-inflammatory mediators such as eotaxin, IL-6 and IL-8.<sup>10,11</sup> Previous studies revealed that IL-6 and IL-8 are increased by PGE2 via the EP4 receptor in different cell types.<sup>12,13</sup> However, it is unknown whether PGE2 induces expression of IL-6 and IL-8 in nasal polyp-derived fibroblasts (NPDFs).

The purposes of this study were to determine whether PGE2 has any effect on the increase of IL-6 and IL-8 in NPDFs, and subsequently to investigate the possible mechanism underlying this effect.

## MATERIALS AND METHODS

### Reagents

PGE2, purchased from Sigma (St. Louis, MO, USA) was dissolved in dimethyl sulfoxide (Sigma) and then diluted with complete medium to concentrations suitable for use in this experiment. The receptor agonists and antagonists, obtained from Cayman Chemical (Ann Arbor, MI) were as follows: EP1 receptor and EP3 receptor agonist (Sulprostone, 10 nM), EP2-receptor agonist (Butaprost, 10  $\mu$ M), EP4 receptor agonist (CAY10580, 10  $\mu$ M), EP2 receptor antagonist (AH6809, 10  $\mu$ M) and the EP4 receptor antagonist (AH23848, 10  $\mu$ M). Akt inhibitor (LY294002, 10  $\mu$ M) was purchased from Calbiochem (Billerica, MA, USA). NF- $\kappa$ B inhibitor (BAY-11, 1  $\mu$ M) was bought from Sigma. NPDFs were previously exposed to PGE2 (20  $\mu$ M) after pre-treatment for 1 hour with all agonists, antagonists and inhibitors.

### Isolation and induction of NPDFs

Fibroblasts were cultured from 8 patients (4 women and 4 men;  $32.3 \pm 5.2$  years of age) who underwent endoscopic sinus surgery for CRS with NP at the Department of Otorhinolaryngology Head and Neck Surgery of the Korea University Medical Center. The study protocol was approved by the Institutional Review Board of the Korea University College of Medicine. NPDFs were isolated from surgical tissues and purified according to our previous study.<sup>14</sup> Cells used for the experiments were obtained from the fourth cell passage.

### Reverse transcription-polymerase chain reaction (RT-PCR)

NPDFs were stimulated with PGE2 in time (0-24 hours) and dose (0-20  $\mu$ M, 12 hours) dependent manner. NPDFs were

stimulated with PGE2 (20  $\mu$ M), with or without Sulprostone (10 nM), Butaprost (10  $\mu$ M), CAY 10580 (10  $\mu$ M), AH6809 (10  $\mu$ M), AH23848 (10  $\mu$ M), LY294002 (10  $\mu$ M) and BAY-11 (1  $\mu$ M) for 12 hours. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and 2  $\mu$ g of the RNA were reverse-transcribed using MMLV reverse transcriptase (Invitrogen). PCR was performed using the primer pairs targeting specific genes, as follows: *IL-6* (sense sequence, 5'-GCCTTCGGTCCAGTTGCC-3'; anti-sense sequence, 5'-GCGCAGAATGAGATGAGTTGTCATG-3'; 566 bp), *IL-8* (sense sequence, 5'-ATGACTTCCAAGCTGGCC-3'; anti-sense sequence, 5'-TCTTCAAAAA CTCTCCACAA CCC-3'; 282 bp), *GAPDH* (sense sequence, 5'-GTGGATATTGTTGCCATCAATGACC-3'; anti-sense sequence, 5'-GCCCC AGCCTTCTTCATGGTGGT-3'; 271 bp). Amplification reactions were performed as follows: the initial denaturation step was performed at 94°C for 5 minutes, followed by 30 cycles performed successively at 94°C for 45 seconds, 55-65°C for 45 seconds, and 72°C for 45 seconds. The final extension step was performed at 74°C for 5 minutes. All these reactions were performed in a volume of 20  $\mu$ L and the products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide. Gel images were acquired using the Molecular Imager Chemi-Doc XRS + (Bio-Rad, Hercules, CA, USA).

### Enzyme-linked immunosorbent assay (ELISA) of IL-6 and IL-8

NPDFs were stimulated with PGE2 for 48 hours in dose (0-20  $\mu$ M)-dependent manner. NPDFs were stimulated with PGE2 (20  $\mu$ M), with or without Sulprostone (10 nM), Butaprost (10  $\mu$ M), CAY10580 (10  $\mu$ M), AH6809 (10  $\mu$ M), AH23848 (10  $\mu$ M), LY294002 (10  $\mu$ M) and BAY-11 (1  $\mu$ M) for 48 hours. IL-6 and IL-8 production in the medium derived from NPDFs was determined by ELISA (R&D Systems, Minneapolis, MN, USA). This assay was performed according to the manufacturer's instructions.

### Western blot analysis

NPDFs were stimulated with PGE2 (20  $\mu$ M), with or without LY294002 (10  $\mu$ M) or BAY-11 (1  $\mu$ M) for 1 hour. The fibroblasts were lysed in PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, Korea); proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Millipore Inc., Billerica, MA, USA). These membranes were incubated with anti-rabbit polyclonal phosphorylated Akt, p50, and GAPDH (Santa Cruz, CA, USA). After incubation, the membranes were washed 3 times (5 minutes per wash) and treated with peroxidase-conjugated anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) for 1 hour. After washing, a substrate obtained from an enhanced chemiluminescence reagent kit (Du Pont, Boston, MA, USA) was added to the membranes. The membranes were then exposed to X-ray films.

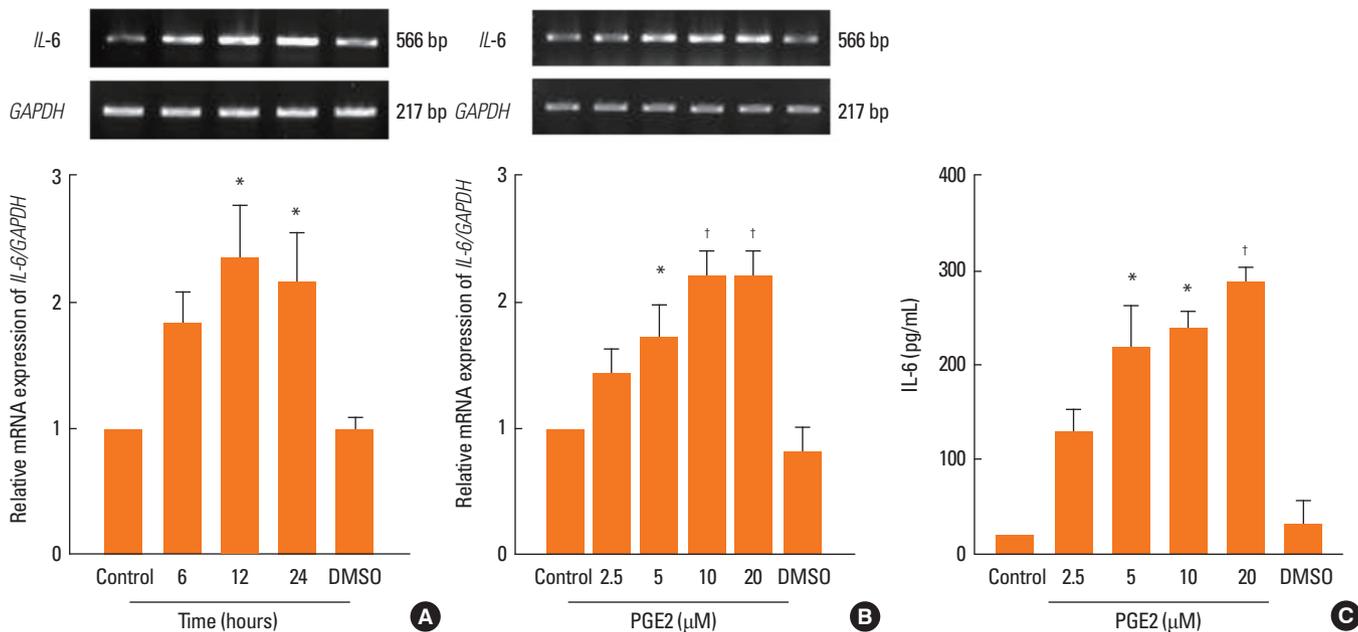
### Statistical analysis

The statistical significance of the difference between the control and experimental data was analyzed using Tukey's test (GraphPad Prism, version 5; GraphPad Software, San Diego, CA, USA). A  $P$  value of  $<0.05$  was considered statistically significant.

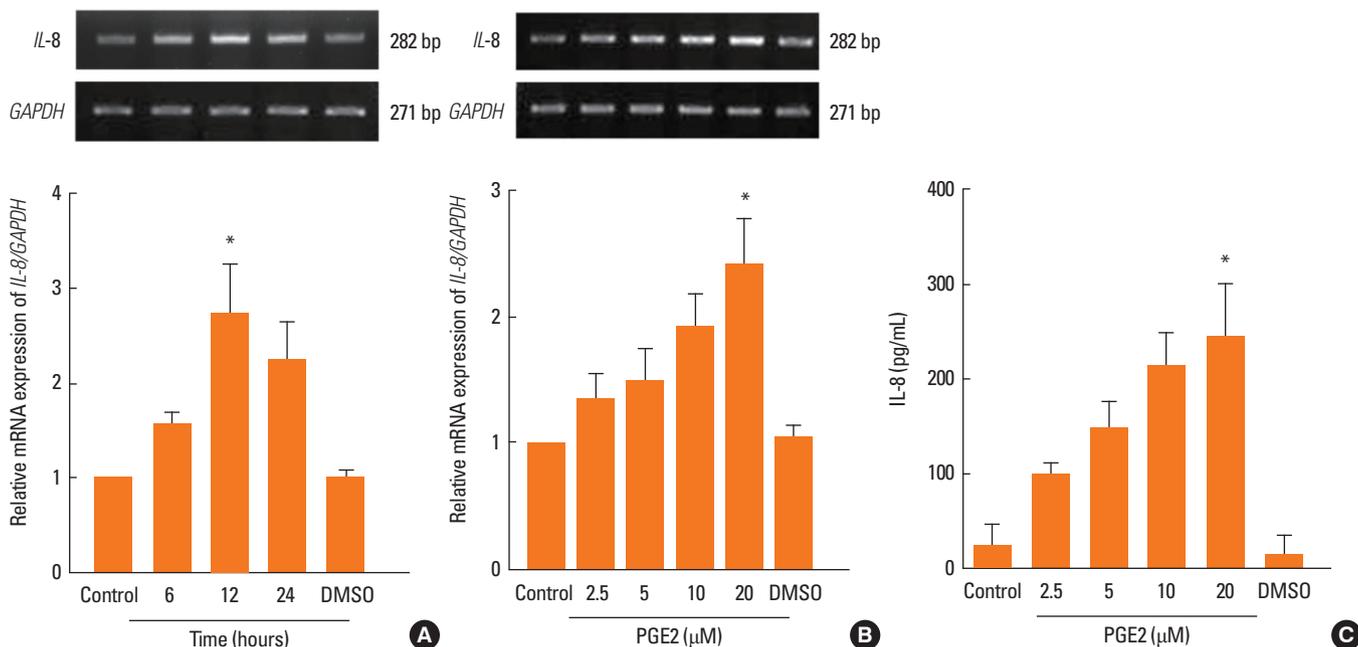
### RESULTS

#### PGE2 induces IL-6 and IL-8 expressions in NPDPs

To determine the effect of PGE2 on IL-6 and IL-8 expressions in NPDPs, NPDPs were stimulated with PGE2 for 12 or 48



**Fig. 1.** Effect of PGE2 on IL-6 expression in NPDPs. (A) NPDPs were stimulated with PGE2 (20 μM) in a time-dependent manner. The expression level of *IL-6* mRNA was examined using RT-PCR and quantified. (B, C) NPDPs were stimulated with PGE2 in a dose-dependent manner. The mRNA and protein expression levels of IL-6 were examined using RT-PCR for 12 hours (B) and ELISA for 48 hours (C). Values are the mean  $\pm$  SEM of three independent samples. \* $P < 0.05$ , † $P < 0.01$  as compared to the mean IL-6 values of control cells.



**Fig. 2.** Effect of PGE2 on IL-8 expression in NPDPs. (A) NPDPs were stimulated with PGE2 (20 μM) in a time-dependent manner. The expression level of *IL-8* mRNA was examined using RT-PCR and quantified. (B, C) NPDPs were stimulated with PGE2 in a dose-dependent manner. The mRNA and protein expression levels of IL-8 were examined using RT-PCR for 12 hours (B) and ELISA for 48 hours (C). \* $P < 0.05$  as compared to the mean IL-8 values of control cells.

hours. PGE2 significantly increased IL-6 and IL-8 mRNA expression levels in time-dependent (Fig. 1A and 2A) and dose-dependent (Fig. 1B and 2B). Also, PGE2 induced production of IL-6 and IL-8 in dose-dependent manner (Fig. 1C and 2C).

**PGE2-induces IL-6 production increases with EP2 and EP4 in NPDFs**

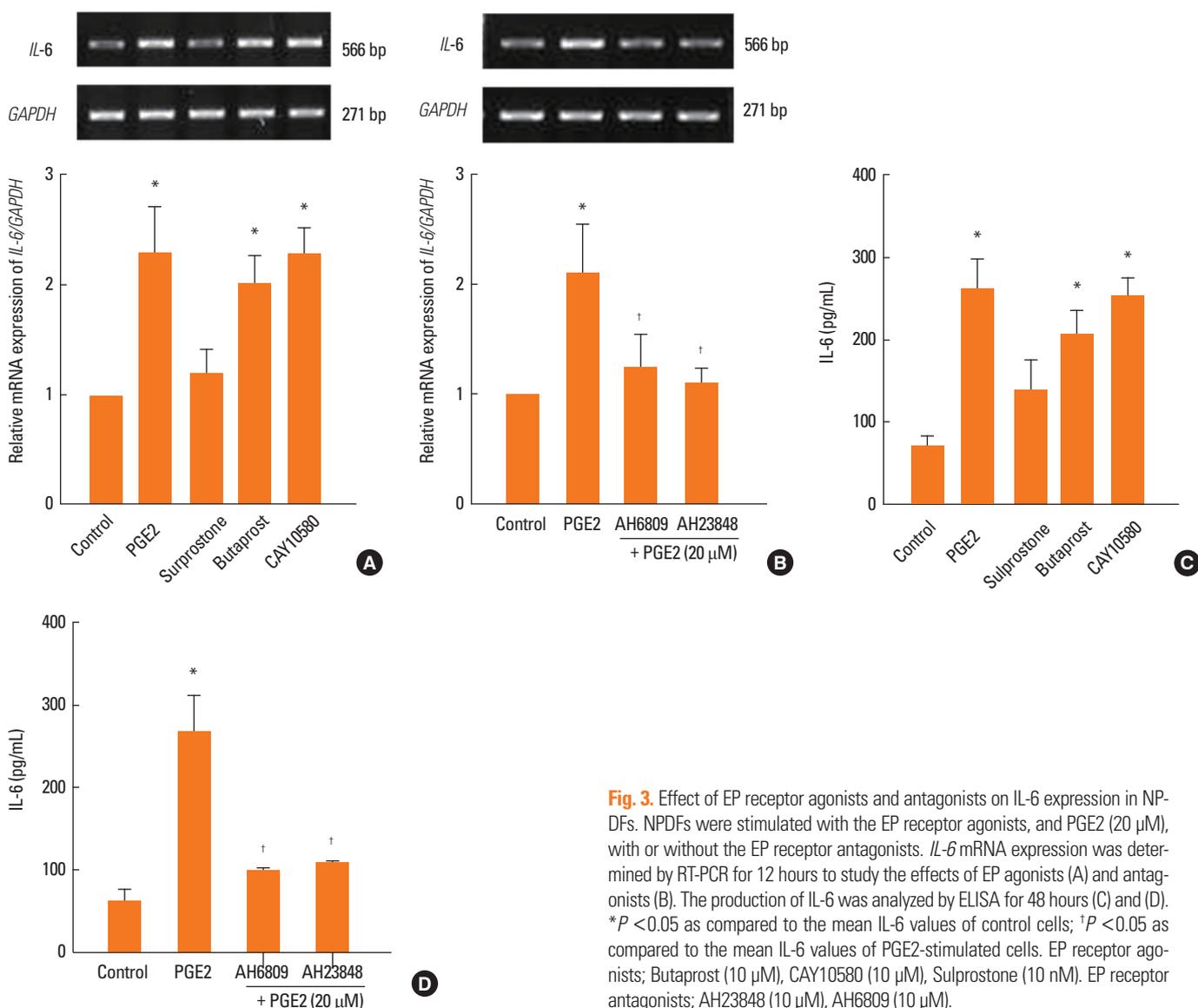
To identify the receptors that mediate the effect of PGE2-induced IL-6 expression, we used agonists and antagonists specific to the corresponding receptors. The specific EP2 agonist (Butaprost, 10 μM) and EP4 agonist (CAY10580, 10 μM) significantly induced IL-6 expression in NPDFs. However, the EP1 and EP3 agonist (Sulprostone, 10 nM) did not induce IL-6 expression in NPDFs (Fig. 3A and C). The increased expression of IL-6 was inhibited by both EP4 antagonist (AH23848, 10 μM) and EP2 antagonist (AH6809, 10 μM) (Fig. 3B and D).

**PGE2-induces IL-8 production increases with only EP4 in NPDFs**

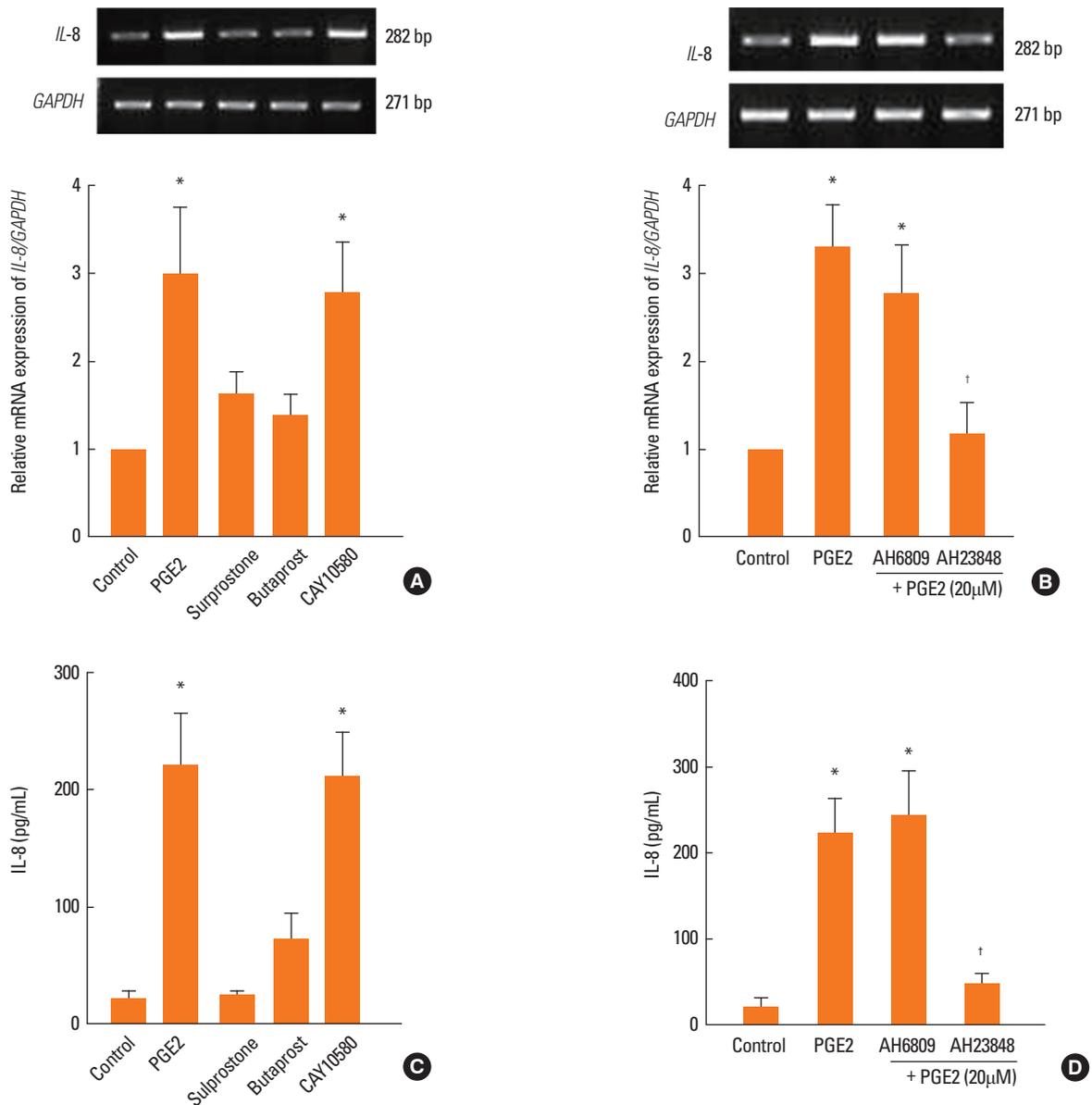
To examine receptors that mediate PGE2-induced IL-8 expression, we treated PGE2 with and without specific agonists and antagonists. The EP4 agonist (CAY10580, 10 μM) significantly induced IL-8 expression in NPDFs. However, EP1 and EP3 agonist (Sulprostone, 10 nM) and EP2 agonist (Butaprost, 10 μM) did not increase IL-8 expression in NPDFs (Fig. 4A and C). IL-8 expression was inhibited by the EP4 antagonist alone (AH23848, 10 μM) (Fig. 4B and D).

**PGE2 increases IL-6 and IL-8 expression via the Akt pathway**

To determine the signal pathway for PGE2-induced IL-6 and IL-8 production in NPDFs, we evaluated the stimulation of Akt as a downstream marker of IL-6 and IL-8 signaling by western blot analysis and RT-PCR. In western blot analysis, PGE2-in-



**Fig. 3.** Effect of EP receptor agonists and antagonists on IL-6 expression in NPDFs. NPDFs were stimulated with the EP receptor agonists, and PGE2 (20 μM), with or without the EP receptor antagonists. IL-6 mRNA expression was determined by RT-PCR for 12 hours to study the effects of EP agonists (A) and antagonists (B). The production of IL-6 was analyzed by ELISA for 48 hours (C) and (D). \*P < 0.05 as compared to the mean IL-6 values of control cells; †P < 0.05 as compared to the mean IL-6 values of PGE2-stimulated cells. EP receptor agonists; Butaprost (10 μM), CAY10580 (10 μM), Sulprostone (10 nM). EP receptor antagonists; AH23848 (10 μM), AH6809 (10 μM).



**Fig. 4.** Effect of EP receptor agonists and antagonists on IL-8 expression in NPDLs. NPDLs were stimulated with the EP receptor agonist, and PGE2 (20 μM), with or without the EP receptor antagonist. *IL-8* mRNA expression was determined by RT-PCR for 12 hours to study the effects of EP agonists (A) and antagonists (B). Production of IL-8 was analyzed by ELISA for 48 hours (C) and (D). \* $P < 0.05$  as compared to the mean IL-8 values of control cells; † $P < 0.05$  as compared to the mean IL-8 values of PGE2-stimulated cells. EP receptor agonists; Butaprost (10 μM), CAY10580 (10 μM), Sulprostone (10 nM). EP receptor antagonists; AH23848 (10 μM), AH6809 (10 μM).

duced stimulation of Akt decreased significantly when an Akt inhibitor (LY294002, 10 μM) was treated (Fig. 5A). Moreover, PGE2-induced expression levels of IL-6 and IL-8 were specifically inhibited by the Akt inhibitor (Fig. 5B-E).

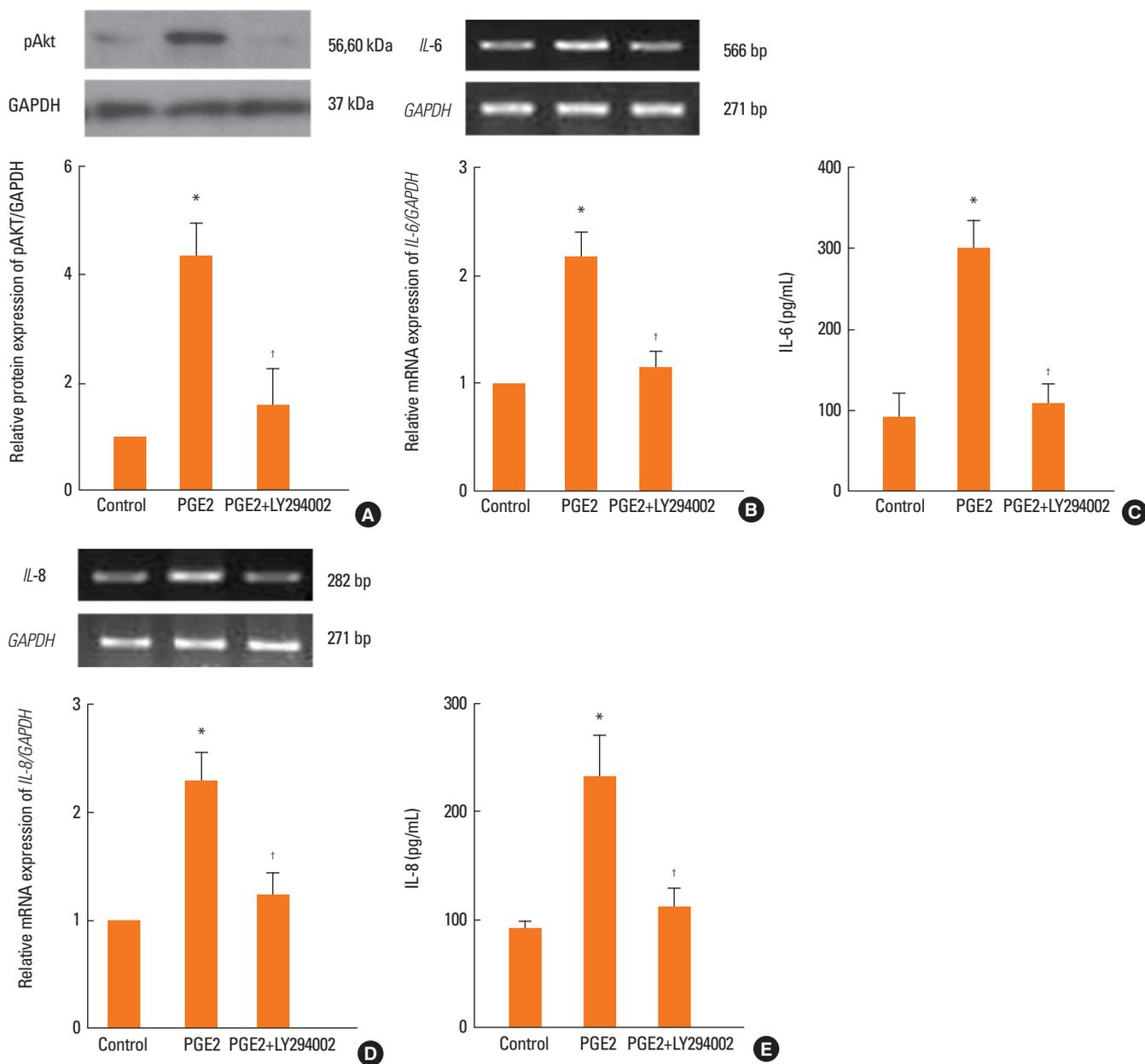
#### PGE2 increases IL-6 and IL-8 expression via the NF-κB transcription factor

To determine whether the NF-κB transcription factor was involved in PGE2-induced IL-6 and IL-8 production in NPDLs, we treated PGE2 under two conditions: in the presence of the

NF-κB inhibitor (BAY-11, 1 μM) and in the absence of the inhibitor. The western blot analysis showed that PGE2 increased the expression of p50 (subunit of NF-κB) and that p50 expression decreased in the inhibitor-treated NPDLs (Fig. 6A). The production of IL-6 and IL-8 was specifically inhibited by treatment with the NF-κB inhibitor (Fig. 6B and C).

#### DISCUSSION

In this study, we found that PGE2 significantly induced the ex-

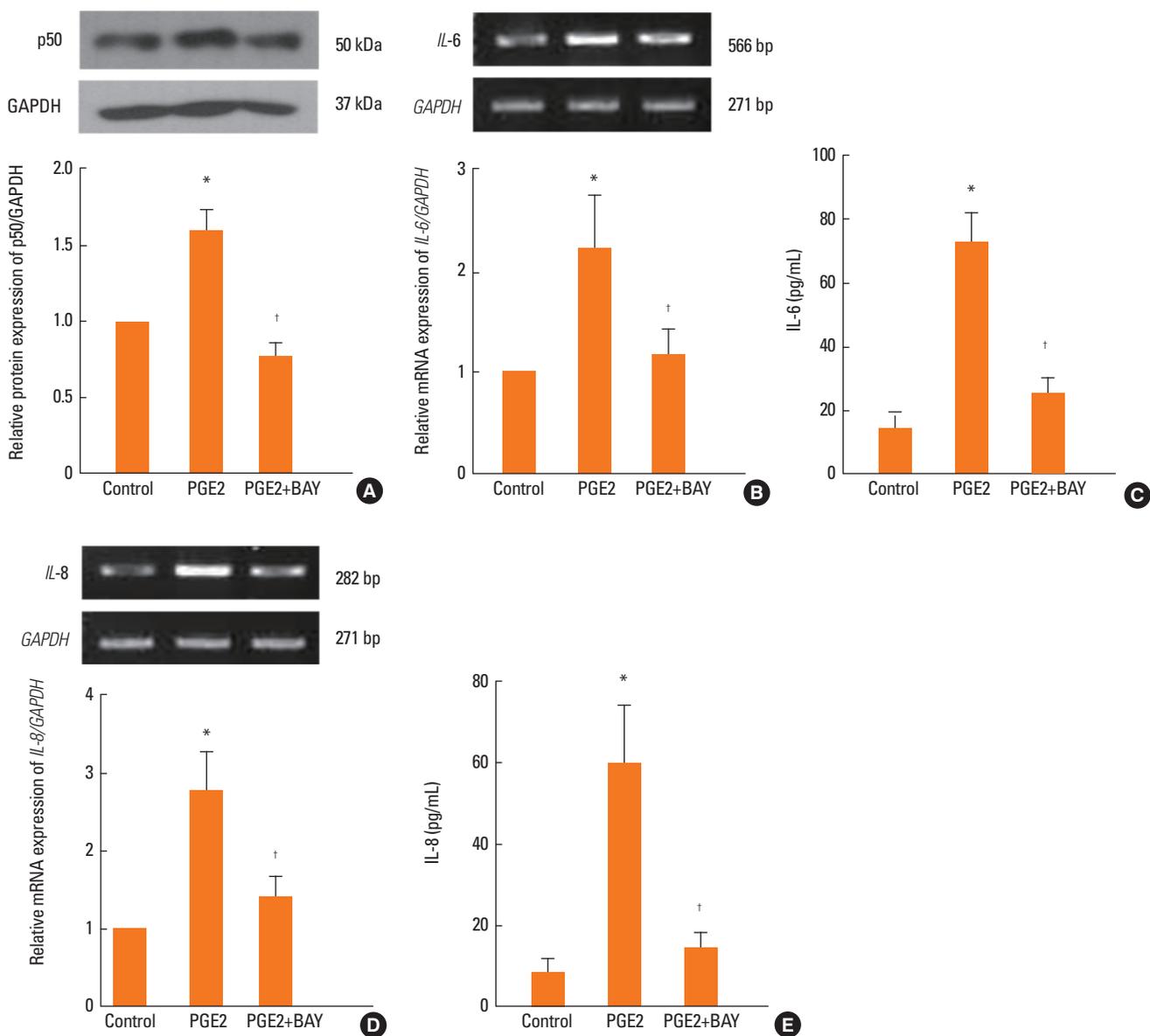


**Fig. 5.** The role of Akt in the expression of IL-6 and IL-8 in NPDFs. NPDFs were stimulated with PGE2 (20 μM), with or without the Akt inhibitor (LY294002, 10 μM). The activation of Akt was determined by western blot analysis (A). Akt inhibition of IL-6 expression was analyzed by RT-PCR for 12 hours and ELISA for 48 hours (B and C). Akt inhibition of IL-8 expression was analyzed by RT-PCR for 12 hours and ELISA for 48 hours (D and E). \**P* < 0.05 as compared to the mean IL-6 or IL-8 values of control cells; †*P* < 0.05 as compared to the mean IL-6 or IL-8 values of PGE2-stimulated cells.

pression of IL-6 and IL-8 in NPDFs. PGE2 increased IL-6 expression via EP2 and EP4, however, increased IL-8 expression via EP4 alone. PGE2 activated the Akt and NF-κB signal pathways for IL-6 and IL-8 expression.

The roles of cytokines in the development of NP are widely investigated. IL-6 has a chemotactic effect on eosinophils and IL-8 affects both neutrophils and eosinophils, causing their migration to the site of inflammation.<sup>15</sup> According to recent studies, increased levels of IL-6 and IL-8 may participate in the primary pathogenesis of CRS and NP as well as in the recurrent

episodes.<sup>16-18</sup> IL-6 is a multifunctional cytokine implicated in various inflammatory conditions including NP pathogenesis. It stimulates fibroblast proliferation, increases collagen deposition, and decreases collagen breakdown. IL-8 releases other inflammatory mediators such as histamine and leukotriene B4.<sup>19,20</sup> IL-8 is a critical cytokine in the pathogenesis of CRS, and high levels of IL-8 have been detected within NP.<sup>21,22</sup> PGE2-induced IL-6 and IL-8 release is mediated by EP4 receptor in colonic epithelial cells, pulmonary endothelial cells, and astrocytes.<sup>12,13,23</sup> In this study, PGE2 induced both IL-6 and IL-8 ex-



**Fig. 6.** Role of NF- $\kappa$ B on the expression of IL-6 and IL-8 in NPDPs. NPDPs were stimulated with PGE2 (20  $\mu$ M), with or without the NF- $\kappa$ B inhibitor (BAY-11, 1  $\mu$ M). Western blot analysis showed increased expression of NF- $\kappa$ B in case of when PGE2-treated NPDPs and decreased expression of NF- $\kappa$ B in case of BAY-11-treated NPDPs (A). Inhibition of IL-6 or IL-8 expression by BAY-11 treatment was analyzed by RT-PCR for 12 hours (B and D) and ELISA for 48 hours (C and E). \* $P < 0.05$  as compared to the mean IL-6 or IL-8 values of control cells; † $P < 0.05$  as compared to the mean IL-6 or IL-8 values of PGE2-stimulated cells.

pression in NPDPs. PGE2-induced IL-6 expression was mediated by EP2 and EP4 receptors. On the other hand, PGE2 induced IL-8 expression only by the EP4 receptor.

PGE2 are mediated via four different G-protein-coupled PGE receptors (e.g. EP1, EP2, EP3, and EP4), which are involved in the activation of phospholipase C (EP1) and activation (EP2, EP4) or inhibition (EP3) of adenylyl cyclase.<sup>24</sup> Recent studies showed EP4 agonist induced potent relaxations in asthma.<sup>25</sup> PGE2 decreases proliferation of bronchial smooth muscle cell via EP2 and EP4 receptors in nonasthmatic eosinophilic bronchitis.<sup>26</sup> In our data, activation of EP2 and EP4 receptor induced

expression of IL-6 and activation of EP4 receptor stimulated expression of IL-8 in NPDPs. However, sulprostone (EP1/3 agonist) did not induce both IL-6 and IL-8 expression in NPDPs. These data suggest that PGE2 induced expression of IL-6 and IL-8 via EP2 and/or EP4 receptor(s) in NPDPs.

Activation of EP receptors can initiate kinase signaling by phosphatidylinositol 3-kinase (PI3K)/Akt pathways and then affects target gene transcription.<sup>27</sup> A previous study has demonstrated that PGE2 induces IL-6 expression in human chondrocytes via the PI3K/Akt-dependent pathway.<sup>28</sup> Therefore, we evaluated Akt as a downstream molecule for PGE2-induced

IL-6 and IL-8 signaling. In western blot analysis, phosphorylation of Akt significantly increased in PGE2-stimulated NPPDFs. Additionally, treatment with the Akt inhibitor (LY294002) specifically inhibited the activation of Akt in PGE2-stimulated NPPDFs. In RT-PCR and ELISA data, the expression levels of IL-6 and IL-8 were also inhibited by treatment with LY294002. These findings show that PGE2-induced IL-6 and IL-8 production was mediated by the Akt pathway in NPPDFs.

Activated Akt can phosphorylate I $\kappa$ B. The phosphorylated I $\kappa$ B frees from NF- $\kappa$ B, allowing translocation to the NF- $\kappa$ B nucleus. The translocated NF- $\kappa$ B subsequently activates target genes.<sup>29,30</sup> Activation of Akt regulates the binding of the NF- $\kappa$ B to the IL-6 promoter and mediates PGE2-induced IL-6 expression.<sup>28</sup> We demonstrated that NF- $\kappa$ B is a transcription factor for PGE2-induced IL-6 and IL-8 signaling. PGE2 increased the expression level of p50, a subunit of NF- $\kappa$ B, and p50 was shown to be inhibited by the NF- $\kappa$ B inhibitor (BAY-11) in western blot analysis. PGE2-induced IL-6 and IL-8 production was blocked when treated with the NF- $\kappa$ B inhibitor, as found by ELISA. These findings show that PGE2-induced IL-6 and IL-8 production is mediated by the Akt pathway in NPPDFs. Our data reveal that PGE2 enhances IL-6 and IL-8 expression via the activation of NF- $\kappa$ B pathway in NPPDFs.

In conclusion, we have shown that PGE2 increases the expression of IL-6 and IL-8 in NPPDFs. PGE2-induced expression of IL-6 and IL-8 is mediated by EP2 and/or EP4 receptor(s) and via Akt and/NF- $\kappa$ B downstream pathways in NPPDFs. Our findings show the effect of PGE2 on the expression of IL-6 and IL-8 and the underlying pathway in NPPDFs. These results suggest that signaling pathway of PGE2 induced-IL-6 and IL-8 expression might provide a therapeutic target for the treatment of NP.

## ACKNOWLEDGMENTS

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