Suppressive Effect of 4-Hydroxy-2-(4-Hydroxyphenethyl) Isoindoline-1,3-Dione on Ovalbumin-Induced Allergic Asthma

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Abstract

4-Hydroxy-2-(4-hydroxyphenethyl)isoindoline-1,3-dione (PD1) is a synthetic phthalimide derivative of a marine compound. PD1 has peroxisome proliferator-activated receptor (PPAR) γ agonistic and anti-inflammatory effects. This study aimed to investigate the effect of PD1 on allergic asthma using rat basophilic leukemia (RBL)-2H3 mast cells and an ovalbumin (OVA)-induced asthma mouse model. In vitro, PD1 suppressed β-hexosaminidase activity in RBL-2H3 cells. In the OVA-induced allergic asthma mouse model, increased inflammatory cells and elevated Th2 and Th1 cytokine levels were observed in bronchoalveolar lavage fluid (BALF) and lung tissue. PD1 administration decreased the numbers of inflammatory cells, especially eosinophils, and reduced the mRNA and protein levels of the Th2 cytokines including interleukin (IL)-4 and IL-13, in BALF and lung tissue. The severity of inflammation and mucin secretion in the lungs of PD1-treated mice was also less. These findings indicate that PD1 could be a potential compound for anti-allergic therapy.

Key Words: PD1, Anti-allergic, Anti-asthma, Ovalbumin, RBL-2H3

INTRODUCTION

Allergic asthma has become a global health problem that affects at least 334 million people worldwide (Bui et al., 2017). Allergic asthma is a complex inflammatory lung disease characterized by chronic airway inflammation and hyper-responsiveness (AHR). The airways of patients with asthma often show infiltration of eosinophils, mucus overproduction, bronchial mucosal thickening, and bronchial wall remodeling (Girodet et al., 2011). In allergic asthma, T helper (Th) effector cells play a crucial role in the release of inflammatory cytokines and chemokines, mucus secretion, and AHR (Foster et al., 2001; Umetsu et al., 2002; Cohn et al., 2004). Currently, the most effective drug treatments for asthma are corticosteroids, which are unfortunately associated with the limitation of serious adverse effects with long-term use, such as weight gain, adrenal insufficiency, hypertension, diabetes, and osteoporosis.

Peroxisome proliferator-activated receptor (PPAR) γ is a ligand-activated transcription factor of the nuclear receptor superfamily, which controls the expression of a very large number of genes. It has been known that PPAR γ plays an important role in regulating adipocyte differentiation, fatty acid storage, and glucose metabolism (Spiegelman, 1998). Recent researches have explored that PPAR γ modulated immune and inflammatory responses (D’Agostino et al., 2007; Ahmadian et al., 2013). Results from clinical trials suggest that natural PPAR γ agonists may be beneficial to human health by acting as anti-inflammatory molecules (Majdalawieh and Ro, 2010; Tyagi et al., 2011). Activation of PPAR γ inhibits the nuclear factor kappa B (NF-κB) pathway, leading to the repression of pro-inflammatory genes, such as iNOS, COX-2, and IL-6 (Ricote et al., 1998; Bo et al., 2016). 4-Hydroxy-12-(4-hydroxyphenethyl)isoindoline-1,3-dione (PD1), is a synthetic phthalimide derivative of a marine natural PPAR γ agonist, paecilocin A (structure in Fig. 1A) (Xiao et al., 2014; Su et al., 2017). PD1 is an analog compound synthesized from paecilocin and was first confirmed as a PPAR γ agonist and subsequently, it was found to have anti-inflammatory effects. PD1 exhibited PPAR γ agonistic activity in Ac2F and HepG2 cells (Xiao et al., 2014; Eom et al., 2016). Recently, Su et al. (2017) reported that PD1 significantly suppressed lipopolysaccharide (LPS)-induced inflammation in RAW264.7 macrophages and showed impressive anti-inflammatory activity in vivo in a carrageenan-induced acute inflammatory rat paw edema model.
MATERIALS AND METHODS

Materials

PD1 was synthesized in the Laboratory of Marine Natural Products (Xiao et al., 2014). Purity of the final compound was about 90%. Other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

Rat basophilic leukemia (RBL)-2H3 mast cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). RBL-2H3 cells were cultured at 37°C in a 5% CO2-humidified incubator, and maintained in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/mL penicillin, and 50 μg/mL streptomycin (Lee et al., 2013).

Animals

Female Balb/c mice (5-week-old) were purchased from Daehan Biolink (DBL, Seoul, Korea) and housed under standard laboratory conditions (22°C ± 2°C under a 12-h light/dark cycle) with free access to food and water in the laboratory animal facility at Pusan National University (PNU). The animal protocol used in this study was reviewed and approved by the PNU Institutional Animal Care Committee (PNU-IACUC) with respect to the ethics of the procedures and animal care.

Assessment of degranulation

Degranulation was detected by measuring β-hexosaminidase release (Lee et al., 2016). Briefly, RBL-2H3 cells (1×10⁶ cells/well in a 24-well plate) were sensitized with 0.2 g/mL monoclonal anti-dinitrophenyl mouse immunoglobulin E (DNP-IgE, D8406, Sigma-Aldrich) overnight at 37°C in a 5% CO2 incubator. Then, the cells were washed twice with piperazine-N,N’-bis-(2-ethanesulfonic acid) (PIPES) buffer (pH 7.2) containing 25 mM PIPES, 110 mM sodium chloride (NaCl), 5 mM potassium (KCl), 5.6 mM glucose, 0.4 mM magnesium chloride (MgCl2), 0.1% bovine serum albumin (BSA), and 1 mM calcium chloride (CaCl2) to remove the DNP-IgE. After incubation with different concentrations of PD1 at 37°C for 30 min, the cells were treated with 1 μg/mL human DNP-albumin (DNP-hAb, A6661, Sigma-Aldrich), and then incubated for an additional 30 min at 37°C to induce degranulation. Then, 25 μL of the supernatant was transferred to a 96-well microplate and incubated for 2 h with 25 μL 5 mM 4-nitrophenyl N-acetyl-b-D-glucosaminide (NS376, Sigma-Aldrich) in 0.1 M citrate buffer (pH 4.5). The reaction was terminated by adding 200 μL stop buffer (0.05 M sodium carbonate [Na2CO3/0.05 M sodium bicarbonate [NaHCO3], pH 10), and the optical density at 405 nm was measured.

Induction of asthma in Balb/c mice and PD1 administration

Six-week-old female Balb/c mice (20 g) were divided into the following three groups (n=5): phosphate-buffered saline (PBS)-injected control, OVA-injected asthma, and OVA-injected plus PD1-treated (50 mg/kg) groups. The mice were sensitized by intraperitoneal injections of 10 μg OVA (A5503) and 1 mg aluminum hydroxide (239186, both Sigma-Aldrich) on day 0 and 14. From day 28 to 30, the mice were exposed to 1% nebulized OVA for 30 min for 3 consecutive days (scheme in Fig. 1B) (Lee et al., 2016). PD1 was dissolved in dimethyl sulfoxide (DMSO) and further diluted with corn oil (C8267, Sigma-Aldrich). The animals were administered PD1 or the vehicle intraperitoneally 30 min before the OVA challenge. The mice were euthanized on day 32 (Lee et al., 2017), and bronchoalveolar lavage fluid (BALF) and lung tissues were collected.

Histological examination of lungs and cell count in BALF

Lung sections from mice in the different experimental groups were prepared and stained with hematoxylin and eosin Y (H&E) to detect the infiltration of inflammatory cells, and periodic acid-Schiff (PAS) to identify mucus-secreting (goblet) cells in the airways (Aoki et al., 2014). Briefly, the left lungs were fixed in 10% formalin, embedded in optimal cutting temperature (O.C.T.) compound, and cut into 4 μm-thick sections using a Leica CM 1860 rotary microtome (Leica Microsystems Nussloch GmbH, Nussloch, Germany). For H&E staining, the sections were stained in distilled water for 5 min, counterstained with hematoxylin solution (Gill No. 3, Sigma-Aldrich) and eosin Y, and cover-slipped with permount (Fisher Scientific Inc., SP-15, Waltham, MA, USA). For PAS staining, the sections were hydrated and placed in periodic acid solution at room temperature for 5 min. After rinsing with distilled water, the sections were stained with Schiff’s reagent (3952, Sigma-Aldrich) for 15 min, rinsed with running tap water and stained with hematoxylin for 90 s. Then, they were rinsed, dehydrated, and cover-slipped.

Cells in BALF were attached to slides using a cell-spin centrifuge (5 min, 500 rpm). After fixation with methanol for 30 s, the slides were stained with May–Grünewald solution (32856, Sigma-Aldrich) for 8 min and then Giemsa solution (32884, Sigma-Aldrich) for 12 min. The slides were rinsed with an ethanol concentration gradient and then covered. The nucleuses of macrophage, eosinophil and lymphocyte stain deep violet, cytoplasm of eosinophil stains pink, but it is bluish violet for macrophage and lymphocyte, they are distinguished by cell size.

Lung inflammation was graded in a blinded fashion using a previously described reproducible scoring system (Curtis et al., 1990). The severity of the lung inflammation in tissue sections...
on separate slides was graded using four categories, as previously described (Tournoy et al., 2000; Kwak et al., 2003). The scores were assigned as follows: 0, no inflammation detected; 1, occasional cuffing and presence of inflammatory cells; 2, most bronchi or vessels were surrounded by a thin layer (one to five cells thick) of inflammatory cells; 3, most bronchi or vessels were surrounded by a thick layer (>five cells thick) of inflammatory cells. Five lung sections per mouse were scored, and the inflammation score of each group was expressed as a mean value (Tournoy et al., 2000). Mucin-secreting cells in the airways were stained with PAS. The length of the basal lamina of bronchi was measured. As at least two lung sections were detected, the average number of PAS-positive cells could be calculated in the bronchi per millimeter of basement membrane (Aoki et al., 2013).

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

The gene expression of asthmatic markers was measured using reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using TRIZOL reagent (Invitrogen, Waltham, MA, USA). RNA (1 μg) was used to synthesize cDNA using the Promega ImProm-II RT system (Promega, Madison, WI, USA) (Huang et al., 2017). The synthesized cDNA products, primers for each gene, and Promega GoTaq DNA polymerase (Promega) were used for the PCR. Specific primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), sense 5′-TTC ACC ACC ATG GAG AAG GC-3′ and antisense 5′-GAG TCA TCT TCA TCT TTG TTA-3′, interleukin (IL)-4 (sense 5′-CTA GTT GTC TTC TCA TCA TTG TTT-3′), IL-5 (sense 5′-ATG GAG ATT CCC ATG AGC AC-3′ and antisense 5′-CTT TAG GCT TTC CAG GAA GTC TTT-3′), IL-13 (sense 5′-CAT GTG ATG TGG TGT GGT-3′ and antisense 5′-GCC GCA CTC ATG AGG TCT TTT-3′), interferon (IFN)-γ (sense 5′-ACT GCC AAA AGG ATG GTC AC-3′ and antisense 5′-TGA GCT CAT TGA ATG CGT TTT-3′), were used to amplify the gene fragments. Aliquots (7 μL) were electrophoresed using a 1.2% agarose gel and stained with StaySafe™ nucleic acid gel stain (Real Biotech Corporation, Taipei, Taiwan).

**Measurement of cytokine (IL-4 and IL-13) levels**

IL-4 and IL-13 levels in the BALF isolated from five mice per group were detected using enzyme-linked immunosorbent assay (ELISA) kits. Briefly, 96-well plates were coated with capture antibodies specific for IL-4 (cat no. 14-7041-68, ebioscience, San Diego, CA, USA) or IL-13 (cat no. 14-7043-68, ebioscience) overnight at 4°C. After washing, the plates were blocked for 1 h at room temperature with the blocking buffer. Standard dilutions of the cytokines and supernatants of the BALF were added to the appropriate wells separately. Plates were incubated for 2 h at room temperature with shaking and then washed five times. A biotinylated detection antibody specific for IL-4 (cat no. 33-7042-68C) or IL-13 (cat no. 33-7135-68B, both ebioscience) was added and incubated for 1 h at room temperature with shaking. After washed five times, avidin-horseradish peroxidase (HRP) was added, followed by incubation for 30 min with shaking. The plates were then washed seven times and incubated with substrate solution for 15 min. After adding the stop solution, the optical density was measured at 450 nm (Lee et al., 2017).

**Statistical analysis**

All the results are expressed as means ± standard deviation (SD). The statistical significance of the differences was determined using a one-way analysis of variance (ANOVA) and statistical significance was accepted for p-values < 0.05.
**RESULTS**

**PD1 inhibited antigen-induced degranulation in RBL-2H3 mast cells**

Mast cell plays a vital role in the initiation and development of allergic inflammation. Secretion of histamine, leukotrienes, and prostaglandins from antigen-exposed mast cells is a key step in allergic responses (Lee et al., 2013). To investigate the anti-allergic asthma effect of PD1, we tested its activity in IgE-exposed RBL-2H3 leukemia cells, a tumor analog of mast cells with high FcεRI surface expression. As shown in Fig. 2, PD1 inhibited the β-hexosaminidase activity in RBL-2H3 cells in a concentration-dependent manner.

**Effect of PD1 on immune cells infiltration in BALF**

To further confirm the inhibitory effect of PD1 *in vitro* on antigen-induced degranulation in RBL-2H3 mast cells, we tested its activity in an OVA-induced asthma mouse model. Cell numbers and population distribution in the BALF were analyzed. As shown in Fig. 3, total cell numbers increased to 311% in the OVA-induced asthma group compared with the PBS control mice. PD1 inhibited this increase in total cell number by 78.9% at a dose of 50 mg/kg. Eosinophil and lymphocyte numbers were increased by 479 and 144 times, respectively, compared to the PBS-treated control group. PD1 treatment inhibited the increase by 66% and 65%, respectively. The number of macrophages was not significantly changed by OVA or PD1 treatment (Fig. 3).

**PD1 inhibited inflammation in lungs of OVA-induced asthmatic mice**

H&E staining was performed to show the bronchioles infiltration of eosinophils in the different groups. Eosinophils are shown as small black dots in the figures. Eosinophils densely infiltrated around the airway bronchioles and mucus secretion. (A) H&E-stained sections of lung tissues from phosphate-buffered saline (PBS), ovalbumin (OVA), and PD1-treated OVA groups. (B) PAS-stained sections of lung tissues from PBS, OVA, and PD1-treated OVA groups. (C) Severity of lung inflammation was semi-quantitatively accessed. (D) Degree of mucus production was evaluated by counting numbers of PAS-positive cells per millimeter of bronchiole length. Results are means ± standard deviation (SD, n=5). ***p<0.001 vs. PBS control group; “p<0.01 and ***p<0.001 vs. OVA-treated group.

**Fig. 4.** Histological evaluation using H&E and PAS staining. PD1 inhibited eosinophil infiltration around the airway bronchioles and mucus secretion. (A) H&E-stained sections of lung tissues from phosphate-buffered saline (PBS), ovalbumin (OVA), and PD1-treated OVA groups. Eosinophils (small black dots) are marked with an arrow. (B) PAS-stained sections of lung tissues from PBS, OVA, and PD1-treated OVA groups. (C) Severity of lung inflammation was semi-quantitatively accessed. (D) Degree of mucus production was evaluated by counting numbers of PAS-positive cells per millimeter of bronchiole length. Results are means ± standard deviation (SD, n=5). ***p<0.001 vs. PBS control group; “p<0.01 and ***p<0.001 vs. OVA-treated group.

**Fig. 5.** PD1 inhibited mRNA expression of Th2 and Th1 cytokines in lung tissue. (A) Expression levels of interleukin (IL)-4, IL-5, IL-13, interferon (IFN)-γ, and IL-2 mRNA in the lung tissues. Each lane represents one of five different mice. (B) Quantified results of mRNA expression levels of IL4, IL-5, IL-13, IFN-γ, and IL-2 as ratios of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels in lung tissue and bronchoalveolar lavage fluid (BALF) cells. Results are means ± standard deviation (SD, n=5). ***p<0.001 vs. PBS control group; **p<0.01 and ***p<0.001 vs. OVA-treated groups.
Fig. 6. PD1 inhibited mRNA expression of Th2 and Th1 cytokines in BALF cells. (A) Expression levels of interleukin (IL)-4, IL-5, IL-13, interferon (IFN)-γ, and IL-2 mRNA in BALF cells. Each lane represents one of five different mice. (B) Quantification results of mRNA expression levels of IL4, IL-5, IL-13, IFN-γ, and IL-2 as ratios of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels in lung tissue and BALF cells. Results are means ± standard deviation (SD, n=5). ***p<0.001 vs. PBS control group; **p<0.01 vs. PBS control group; *p<0.05 vs. ovalbumin (OVA)-treated group.

PD1 suppressed mucin secretion in lungs of OVA-induced asthmatic mice

PAS staining was conducted to detect mucous glycoproteins (mucins), which are produced by goblet cells. In PAS staining, secreted or stored mucin is stained as purple color. As shown in Fig. 4B, dark stained mucin was found surround bronchioles in the OVA group; however, the staining was much weaker in the lung of PD1-administered mice. The degree of mucin production was semi-quantitatively analyzed with a method described previously (Aoki et al., 2013). PAS-positive cells were rarely observed in the PBS group. There were nearly 120 stained cells/mm in the OVA-treated group, and PD1 significantly reduced the PAS-positive cells in the lung (Fig. 4D).

Fig. 7. Effects of PD1 on IL-4 and IL-13 levels in BALF. ELISA results for IL-4 and IL-13 were obtained using bronchoalveolar lavage fluid (BALF) samples from five mice per group. Results are means ± standard deviation (SD, n=5). ***p<0.001 vs. phosphate-buffered saline (PBS) control group; **p<0.01 vs. PBS control group; *p<0.05 vs. PBS control group.

PD1 inhibited gene production of Th1 and Th2 cytokines in BALF cells and lung tissue

Elevated Th2 cells are characteristic features of allergic asthma (Umetsu and DeKruyff, 1997). Th2 cytokines, in particular, IL-4, IL-5, and IL-13 play a key role in initiating and sustaining the asthmatic response by regulating the production of IgE and the growth, differentiation, and recruitment of mast cells, basophils, and eosinophils (Hams and Fallon, 2012; Lloyd and Saglani, 2013; Sun et al., 2016). In this study, we examined mRNA expression changes of IL-4, IL-5, and IL-13 in lung tissue and BALF cells, which were elevated by 537%, 419%, and 382%, respectively, in the lung tissue of OVA-induced asthma group compared to PBS control group (Fig. 5A, 5B). Administration of PD1 inhibited the elevation by 49%, 43%, and 87%, respectively. On the other hand, the mRNA levels of IL-4, IL-5, and IL-13 were elevated by 261%, 419%, and 382%, respectively in the lung tissue of OVA-induced asthma group compared to PBS control group. PD1 administration decreased their expression by 86%, 31%, and 51%, respectively (Fig. 6A, 6B).

Th1 cells have been reported to protect against allergic disease and asthma (Romagnani, 1994; Abbas et al., 1996). IFN-γ secreted by Th1 cells was shown to inhibit the synthesis of IgE (Coffman et al., 1988). The results showed that the mRNA expression of IFN-γ and IL-2 were both upregulated 403% and 281% in the lung tissue and 1239% and 447% in BALF cells in the OVA group (Fig. 5, 6), and treatment with PD1 reduced IFN-γ and IL-2 by 57% and 45%, respectively, in the lung tissues. Moreover, the increased expression levels of IFN-γ and IL-2 were suppressed by 42% and 72%, respectively in BALF cells (Fig. 5, 6).

PD1 inhibited protein production of IL-4 and IL-13 in BALF cells

To confirm the effect of PD1 on the expression of Th2 cytokines, protein levels of IL-4 and IL-13 in the BALF were measured using ELISA. The expression levels of IL-4 and IL-13 were strongly elevated in the OVA group compared to levels in the PBS control group, and this increase was inhibited in PD1-treated group by 52% and 59%, respectively (Fig. 7).
DISCUSSION

Phthalimide derivatives have been widely used and studied in medicinal chemistry because of their multiple activities including analgesic, antitumor, anticonvulsant, and anti-inflammatory (Sharma et al., 2010). Numerous synthesized phthalimides have been reported to exhibit anti-inflammatory effects both in vitro and in vivo (Lima et al., 2002; Bhat et al., 2015). The 2-[4-(1,4-thiazinan-4-ylsulfonyl)phenyl]-1,3-isoinodolinodione (LASSBio-468) phthalimide analog was shown to inhibit neutrophil recruitment and decrease tumor necrosis factor (TNF)-α levels in the BALF of LPS-stimulated mouse lungs (Alexandre-Moreira et al., 2005). Thalidomide, another phthalimide analog, has been shown to inhibit polarization of macrophages and attenuate AHR and eosinophil inflammation in allergic asthma mice (Asano et al., 2010; Lee et al., 2010). Numerous synthesized phthalimide analogs have been reported to exhibit anti-inflammatory (Sharma et al., 2002; Bhat et al., 2002; Liddle et al., 2017). In the present study, we investigated the anti-allergic asthma effects of PD1, which were displayed both in vitro and in vivo.

PD1 inhibited antigen-induced degranulation of RBL-2H3 mast cells. IL-4, IL-5, and IL-13 are important Th2 cytokines, which contribute to the pathogenesis of allergic disease. IL-4 and IL-13 induce the production of IgE. Previous studies proved that IL-4 and IL-13 promoted IgE production and the expression of adhesion molecules by endothelial cells (Umetsu and DeKruyff, 1997; Alvaro et al., 2013). IL-5 induces eosinophil differentiation, survival, and growth. Studies showed that the inhibition of the Th2 cytokines could attenuate OVA-induced allergic asthma (Duan et al., 2004). In the present study, PD1 not only inhibited the induction of Th2 cytokine mRNA levels both in lung tissue and BALF cells, but also the protein levels of IL-4 and IL-13 in the BALF. While Th2 cytokines are related to the promotion of airway inflammation in allergic asthma, IFN-γ secreted by Th1 cells is proposed to suppress the activity of Th2 effector cells (Abbas et al., 1996). However, there is evidence that Th1 cells may contribute to the pathology of asthma (Cembrzynska-Nowak et al., 1993; Hessel et al., 1997). In this study, the mRNA expression of Th1 cytokines, including IFN-γ and IL-2, were markedly increased in the OVA-induced mouse model, and PD1 administration reversed these effects. This may come from suppression of NF-κB by PD1 activation of PPAR γ. The balance of Th1/Th2 cells and cytokines needs to be further studied. Moreover, histological analysis of lung tissue showed that PD1 reduced eosinophil accumulation surrounding the bronchioles and mucin secretion by goblet cells, which suggests that it may suppress histopathological changes in the OVA-induced asthma model. In conclusion, these findings suggest that PD1 has potential in the treatment of asthmatic disease.

CONFLICT OF INTEREST

There are no conflict of interest.

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