



Immunopharmacology and inflammation

Suppressive effects of type I angiotensin receptor antagonists, candesartan and irbesartan on allergic asthma

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ABSTRACT

The effects of candesartan and irbesartan, antagonists of the type I angiotensin II receptor, were investigated on allergic asthma. The antigen-induced degranulation was measured by evaluating β -hexosaminidase activity *in vitro*. Additionally, a murine ovalbumin-induced allergic asthma model was used to test the *in vivo* efficacy. It was observed that while candesartan inhibited the antigen-induced degranulation in rat RBL-2H3 mast cells, irbesartan did not. Administration of candesartan and irbesartan decreased the number of immune cells in the bronchoalveolar lavage fluid and reduced the expression of Th2 (IL-4, IL-5, and IL-13) and Th1 cytokines (IL-2 and IFN- γ) in the lung tissues of mice with ovalbumin-induced allergic asthma. Histological studies revealed that both antagonists reduced inflammation and mucin production in the lungs. Therefore, these findings provide evidence that candesartan and irbesartan could have potential applications as anti-allergic agents.

1. Introduction

Several studies have reported on the expression of the type I angiotensin II receptor in human lung tissues (Mauzy et al., 1992; Rakugi et al., 1997). It has been reported that in severe acute attacks of asthma, the renin-angiotensin system is activated by elevated plasma concentrations of renin and angiotensin II (Millar et al., 1994). Additionally, it has been demonstrated that angiotensin II induces hypertrophy of human airway smooth muscle cells (McKay et al., 1998) and stimulates the production of peptide leukotrienes in the guinea pig airway via the type I angiotensin II receptor pathway (Kanazawa et al., 1995). Furthermore, angiotensin II potentiates methacholine-induced bronchoconstriction in patients with mild asthma (Millar et al., 1995). Therefore, the type I angiotensin II receptor is considered to play an important role in asthma.

However, the *in vivo* anti-allergic effect of type I angiotensin II receptor antagonists has been poorly evaluated. Previously, candesartan was tested in models of allergic asthma in guinea pigs. Here, reductions in airway hyperresponsiveness and airway inflammation were observed (Myou et al., 2002b, 2000c). Losartan slightly reduced the methacholine-induced airway hyperresponsiveness in patients with asthma (Myou et al., 2000a). However, when candesartan was tested on bronchial hyperresponsiveness to methacholine in Japanese patients with bronchial asthma, the observed effect was not significant (Myou

et al., 2002a; Tanaka et al., 2001).

Irbesartan, an angiotensin II receptor antagonist with selective PPAR γ -modulating activity, has anti-inflammatory and protective effects on the concentrations of inflammatory cytokines in patients with chronic glomerulonephritis (Tsuruoka et al., 2013) and on bleomycin-induced lung injury in mice (Tanaka et al., 2013). Therefore, the aim of the current study was to investigate the effects of irbesartan and candesartan in allergic asthma using female Balb/c mice and rat RBL-2H3 mast cells.

2. Materials and methods

2.1. Materials

Candesartan cilexetil (SML0245, $\geq 98\%$ HPLC, molecular weight 610.66, Pubchem ID 329825255), irbesartan (I2286, $\geq 98\%$ HPLC, molecular weight 428.53, Pubchem ID 329815365) and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Rat RBL-2H3 mast cells were obtained from the 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

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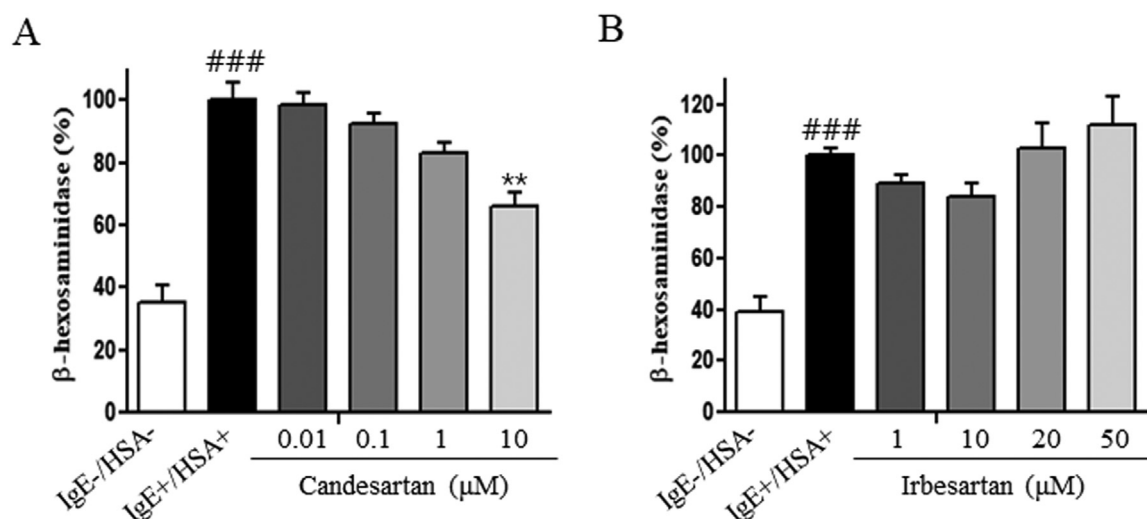


Fig. 1. Effects of candesartan and irbesartan on antigen-induced β -hexosaminidase release in RBL-2H3 mast cells. RBL-2H3 cells were sensitized for 18 h with anti-DNP IgE and then challenged with DNP human serum albumin (HSA). Candesartan or irbesartan was administered at the indicated concentrations 30 min before antigen challenge. The samples without IgE and HSA mean basal degranulation from the cells and samples with IgE and HSA mean positive control of antigen-induced degranulation. The results are presented as mean $\pm$ S.E.M. of three independent experiments. ### $P < 0.001$ vs. the HSA-treated group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the OVA-treated group.

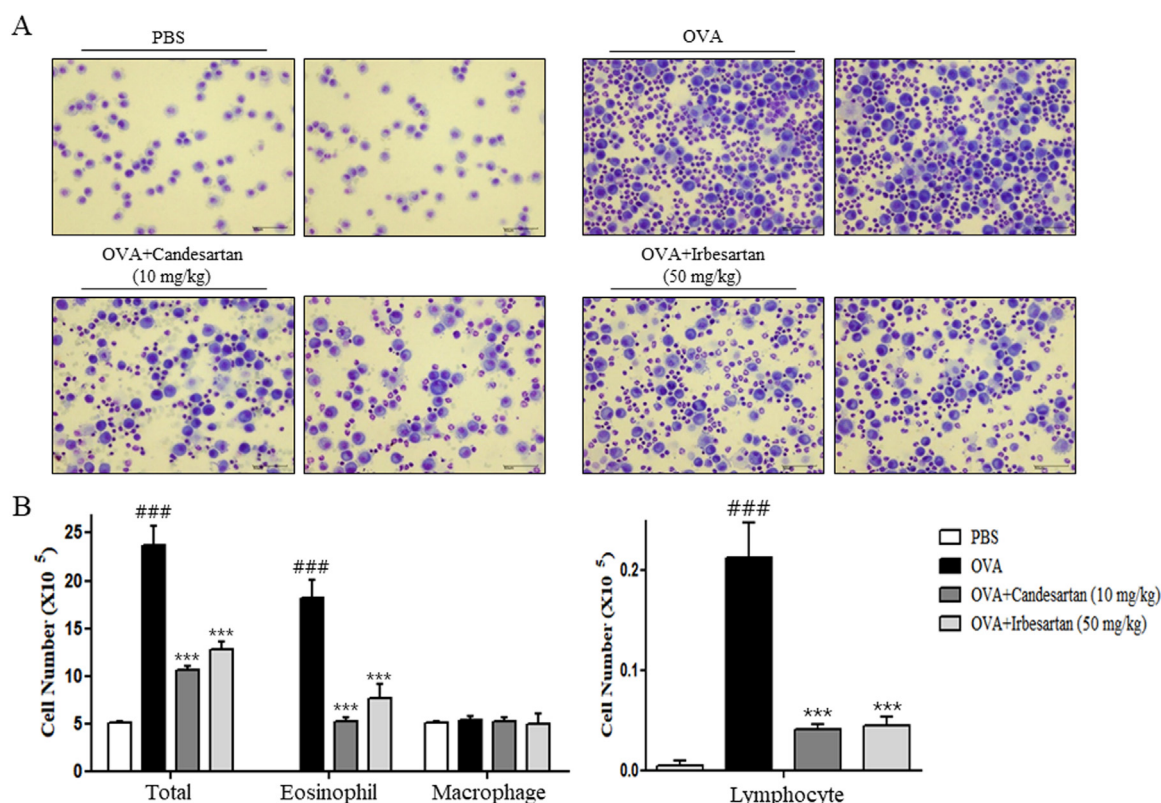


Fig. 2. Effects of candesartan and irbesartan on immune cell accumulation in BAL fluid of the OVA-induced asthmatic mice. Mice ($n = 5$ per group) were sensitized with OVA twice by intraperitoneal injection on days 0 and 14, and later challenged with nebulized OVA on days 28, 29, and 30. Candesartan and irbesartan was intraperitoneally administered 30 min before OVA challenge at 10 mg/kg and 50 mg/kg, respectively. (A and B) Cells in BAL fluid were stained using May-Grünwald stain and counted. (B) Total cell counts in BAL fluid of PBS-treated control mice, OVA-induced asthmatic mice (OVA), and candesartan-treated (10 mg/kg) OVA groups and counts of eosinophils, macrophages, and lymphocytes in BAL fluid samples obtained from these four groups. (B) Total cell counts in BAL fluid of PBS-treated control mice, OVA-induced asthmatic mice (OVA), and irbesartan-treated (50 mg/kg) OVA groups and counts of eosinophils, macrophages, and lymphocytes in BAL fluid samples obtained from these four groups. Values represent the mean $\pm$ S.E.M. ($n = 5$). ### $P < 0.001$ vs. the PBS-treated group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the OVA-treated group.

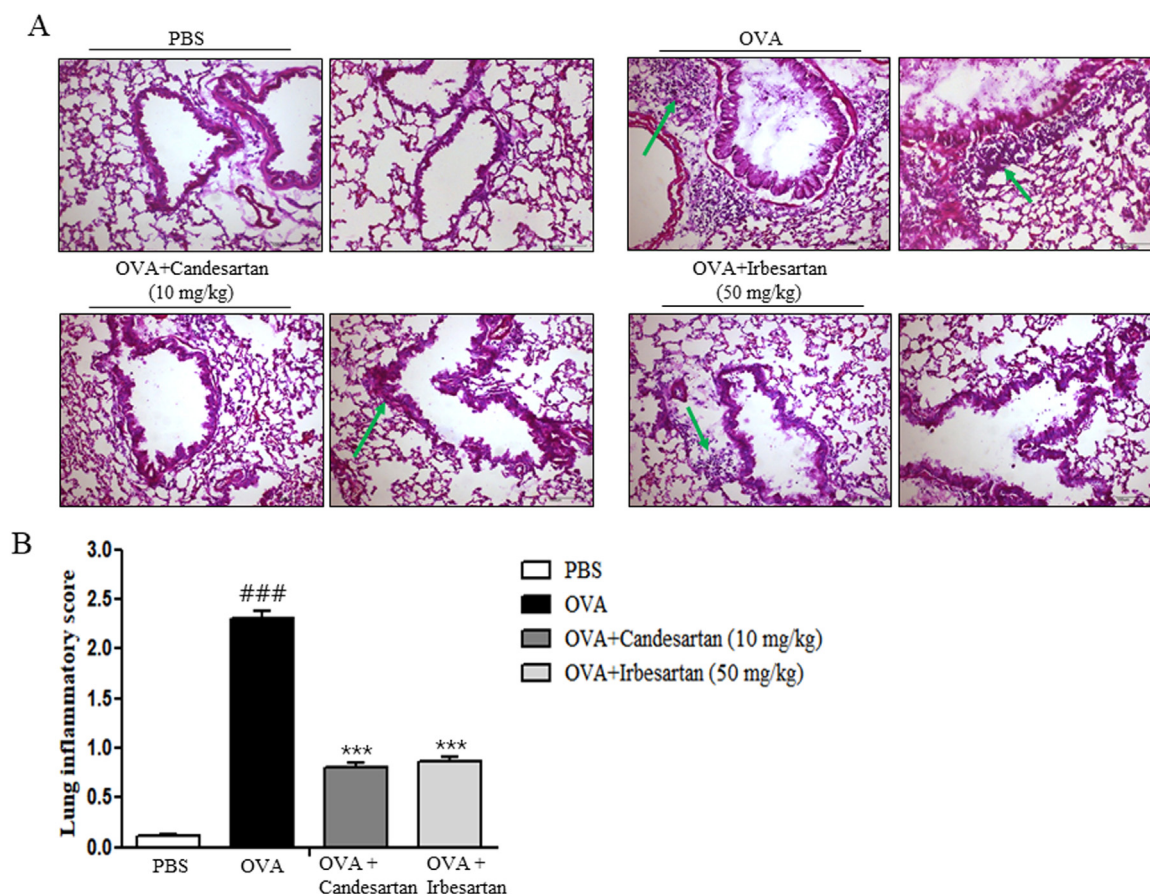


Fig. 3. Protective effects of candesartan and irbesartan against airway inflammation. (A) Panels show hematoxylin & eosin (H&E)-stained sections of lung tissues from the PBS group, OVA group, and candesartan (10 mg/kg)- or irbesartan (50 mg/kg)-treated OVA groups. Sections from mice in the OVA and candesartan or irbesartan-treated OVA groups showed accumulation of eosinophils (small navy blue dots) around the bronchioles, whereas eosinophils were rarely observed in the PBS group. Moreover, eosinophils densely accumulated around the bronchioles in the OVA group (green arrows); however, eosinophil accumulation was less obvious in the OVA + candesartan or irbesartan groups than in the OVA group, which suggested that candesartan and irbesartan reduced airway inflammation. (B) Lung inflammation was semi-quantitatively evaluated; histological findings were scored as described in the Materials and methods section and by [Tournoy et al. \(2000\)](#) and [Kwak et al. \(2003\)](#). Values represent the mean $\pm$ S.E.M. ($n = 5$). ### $P < 0.001$ vs. the PBS-treated group, ** $P < 0.01$, *** $P < 0.001$ vs. the OVA-treated group.

high-glucose Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/mL penicillin, and 50 μ g/mL streptomycin ([Lee et al., 2016](#)).

2.3. Animals

Five-week-old female Balb/c mice were purchased from Daehan Biolink (DBL; Seoul, Korea) and housed in the laboratory animal facility at Pusan National University. They were provided food and water *ad libitum*. The protocol used in this study was reviewed and approved by the Pusan National University Institutional Animal Care Committee (PNU-IACUC) with respect to ethical issues and scientific care (Approval Number, ED-PNU-2018–2029).

2.4. Assessment of degranulation

Degranulation was evaluated by measuring β -hexosaminidase release from rat RBL-2H3 mast cells ([Lee et al., 2018](#)). Briefly, RBL-2H3 cells (1.2×10^5 cells/well in 24-well plates) were sensitized by incubation with 0.2 μ g/mL monoclonal anti-dinitrophenyl mouse immunoglobulin E (DNP-IgE; D8406, Sigma-Aldrich) overnight at 37 °C in a 5% CO₂ incubator. Before stimulation, the cells were washed twice with piperazine-*N,N'*-bis-(2-ethanesulfonic acid) (PIPES) buffer (pH 7.2) containing 25 mM PIPES, 0.05 mM NaOH, 110 mM NaCl, 5 mM

KCl, 5.6 mM glucose, 0.4 mM MgCl₂, 0.1% bovine serum albumin (BSA) and 1 mM CaCl₂ to remove DNP-IgE. Cells were then incubated in 400 μ L of PIPES buffer containing different concentrations of candesartan cilexetil or irbesartan at 37 °C for 30 min. The cells were then incubated for additional 15 min at 37 °C with human dinitrophenyl albumin (1 μ g/mL; DNP-hAb; A6661, Sigma-Aldrich) to induce degranulation. Twenty-five microliters of the medium were then transferred to a 96-well microplate and incubated for 110 min with 25 μ L of 5 mM 4-nitrophenyl *N*-acetyl- β -D-glucosaminide (N9376, Sigma-Aldrich) in 0.1 M citrate buffer (pH 4.5). The reaction was terminated by addition 200 μ L of 0.05 M sodium carbonate buffer (pH 10; 0.05 M Na₂CO₃/0.05 M NaHCO₃). The absorbance (optical density; OD) of the final product was measured at 405 nm using a microplate reader.

2.5. Induction of asthma in Balb/c mice and administration of candesartan and irbesartan

Six-week-old Balb/c mice (22 g) were divided into four groups ($n = 5$) as follows: phosphate-buffered saline (PBS)-injected control group, ovalbumin (OVA)-injected asthma group, OVA-injected plus candesartan cilexetil-treated group (10 mg/kg), and OVA-injected plus irbesartan-treated group (50 mg/kg). Asthma was induced by intraperitoneal (i.p.) injection of OVA (50 μ g; A5503, Sigma-Aldrich) and aluminum hydroxide (1 mg; 239186, Sigma-Aldrich) on days 0 and 14. Mice were exposed to nebulized OVA for 30 min for three consecutive

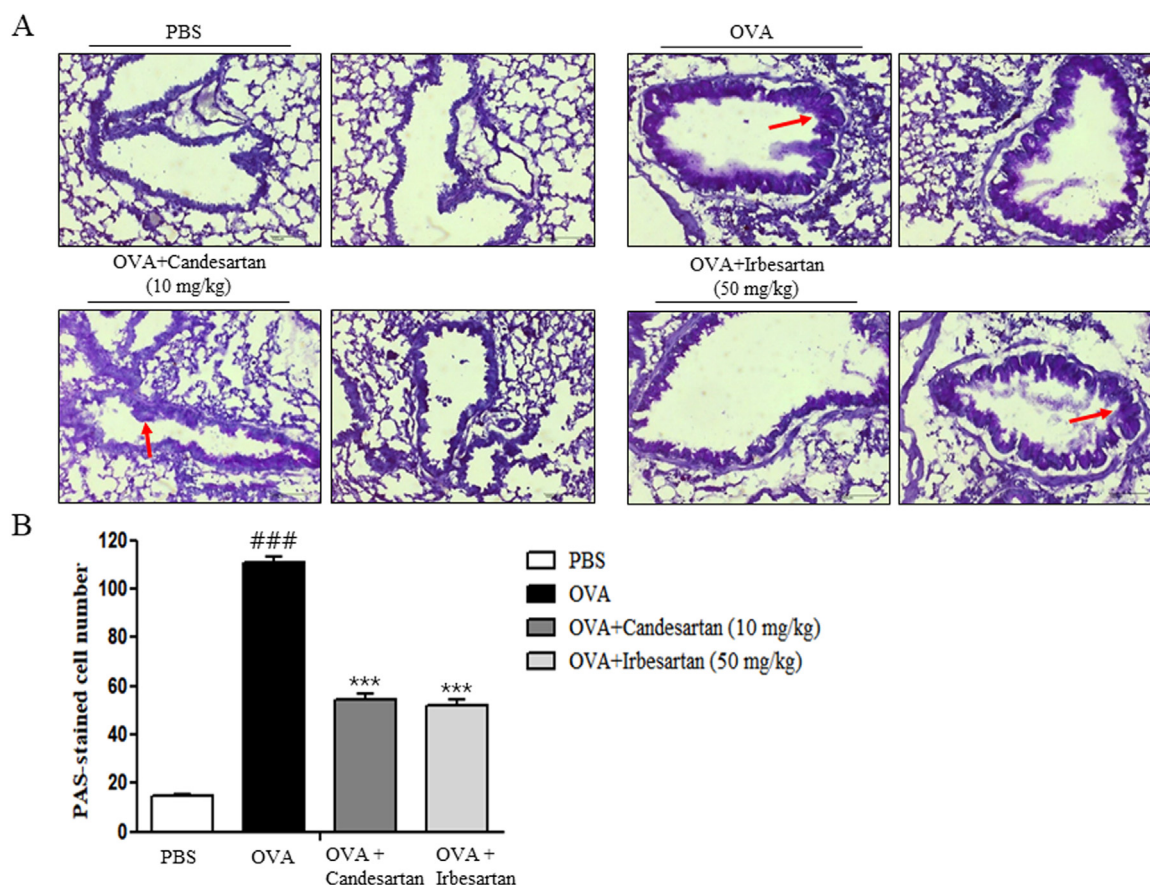


Fig. 4. Protective effects of candesartan and irbesartan against mucin production. (A) Panels show PAS/hematoxylin-stained sections of lung tissues from the PBS group, OVA group, and candesartan (10 mg/kg)- or irbesartan (50 mg/kg)-treated OVA groups. In PAS staining, mucin is stained as purple color. In OVA group, darker and thicker purple color is observed surrounding the bronchiole compare to PBS group. (B) Mucous production was evaluated by counting the number of PAS-positive cells (red arrows) per mm of bronchiole ($n = 5$ per group). Bar = 50 μm . $### p < 0.001$ vs. the PBS-treated group, $*** p < 0.001$ vs. the OVA-treated group.

days from day 28 (Aoki et al., 2010). Each constituent was administered intraperitoneally 30 min before OVA challenge. After two days (day 32), bronchoalveolar lavage (BAL) fluid was collected from the lungs, and immune cells in BAL fluid were stained and counted.

On day 32, lungs were excised and fixed with 10% formalin solution. Next, they were cryoprotected using 30% sucrose solution in PBS (pH 7.4) overnight at 4 °C. Tissues were embedded in OCT compound and sectioned at 8 μm thickness. Sections were then thaw-mounted onto micro slides (Muto pure chemicals co. LTD, Hongo, Bunkyo-ku, Tokyo, JAPAN) and stored at - 80 °C until further use.

2.6. Histological examination of the lungs and cell counting in BAL fluid

Lung sections from mice of different experimental groups were prepared and examined. Briefly, left lungs were fixed in 10% formalin and dehydrate 30% sucrose solution and embedded in O.C.T. compound. Sections (8 μm) were stained with periodic acid-Schiff (PAS) stain to identify mucus-secreting cells (goblet cells) in the airways, and Hematoxylin and Eosin (H&E) staining was performed to confirm eosinophil infiltration. Lung tissues from all five mice in each group were examined. For PAS staining, O.C.T. compound was removed from the sections; the sections were hydrated, and placed in periodic acid solution at room temperature for 5 min. After rinsing with water, sections were stained with Schiff's reagent (ab150680, Abcam) for 15 min, rinsed with warm running tap water, and stained with hematoxylin for 3 min. After rinsing with water, sections were stained with blurring reagent at room temperature for 30 s. The sections were then rinsed, dehydrated, and cover-slipped. For H&E staining, O.C.T. compound was removed from the sections; the sections were hydrated, and were stained with

hematoxylin reagent for 15 s. After rinsing with warm running tap water, sections were stained with Eosin reagent for 10 s, followed by rinsing, dehydration, and mounting the sections on the cover-slipped.

Lung inflammation was scored by a treatment-blind observer. Degree of lung inflammation was evaluated using a subjective scale of 0–3, as previously described (Kwak et al., 2003; Tournoy et al., 2000). In brief, a score of 0 indicated no inflammation, 1 indicated occasional cuffing and presence of inflammatory cells, 2 indicated that most bronchi or vessels were surrounded by a thin layer (one to five cells thick) of inflammatory cells, and 3 indicated that most bronchi or vessels were surrounded by a thick layer (> five cells thick) of inflammatory cells. Total lung inflammation was defined as the average lung inflammation score. Five lung sections per mouse were evaluated, and the average inflammation score was calculated (Braber et al., 2010). Mucin-secreting cells in the airways were stained with PAS and counted in the large-caliber preterminal bronchi in at least two lung sections per mouse. The length of the basal lamina of bronchi was measured using ImageJ software (National Institute of Health).

Cells in BAL fluid were made to adhere to a glass slide using a Cellspin® centrifuge (5 min, 15g; Hanil Electric) and fixed in methanol for 30 s. Subsequently, the cells were processed with May-Grünwald solution (32856, Sigma-Aldrich) for 8 min followed by Giemsa solution (32884, Sigma-Aldrich) for 14 min. Slides were then rinsed, dehydrated and covered.

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

Gene expression of asthmatic markers in type 2 T helper (Th2) cells and Th1 cells was measured by RT-PCR. Total RNA was isolated from

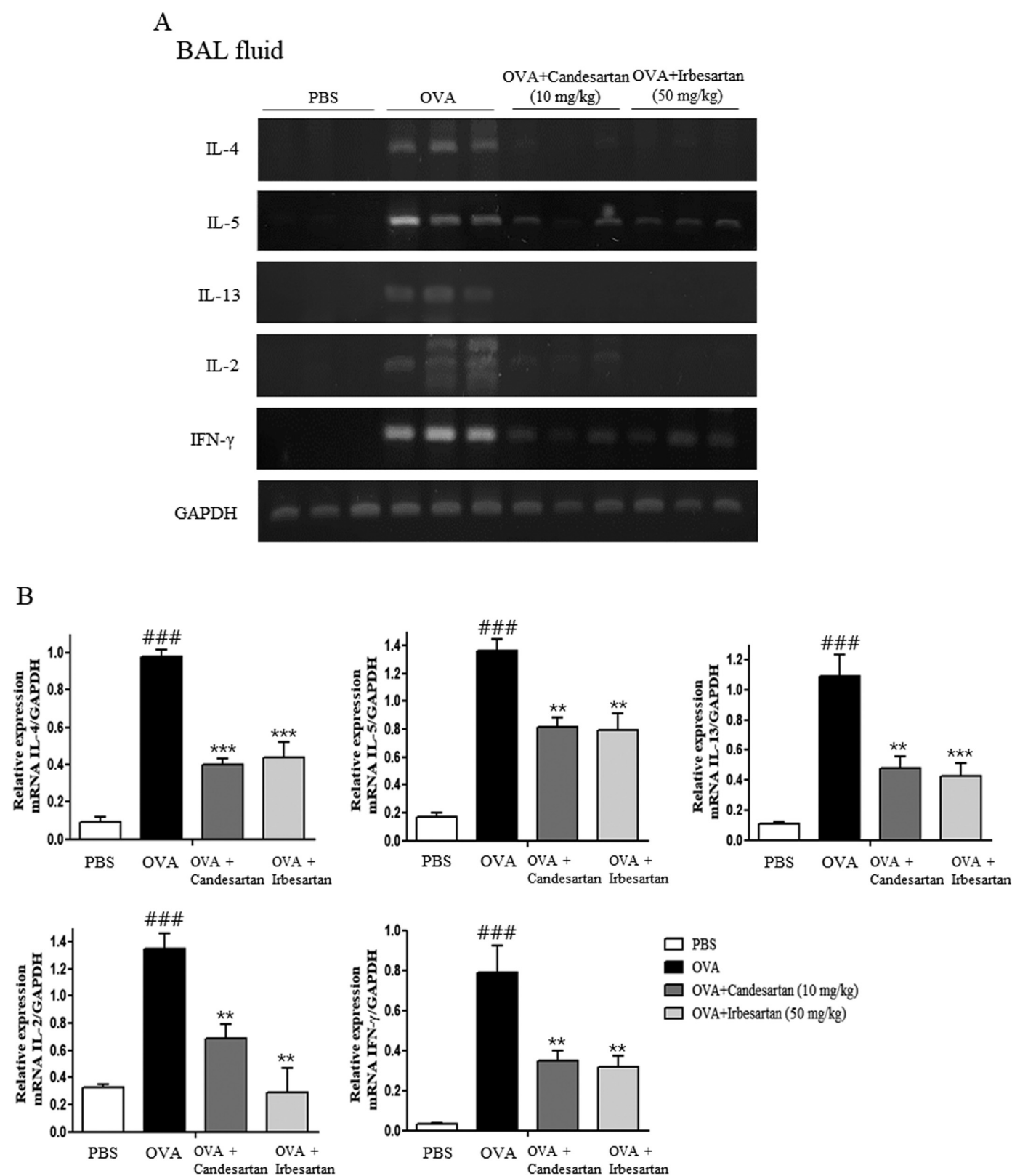
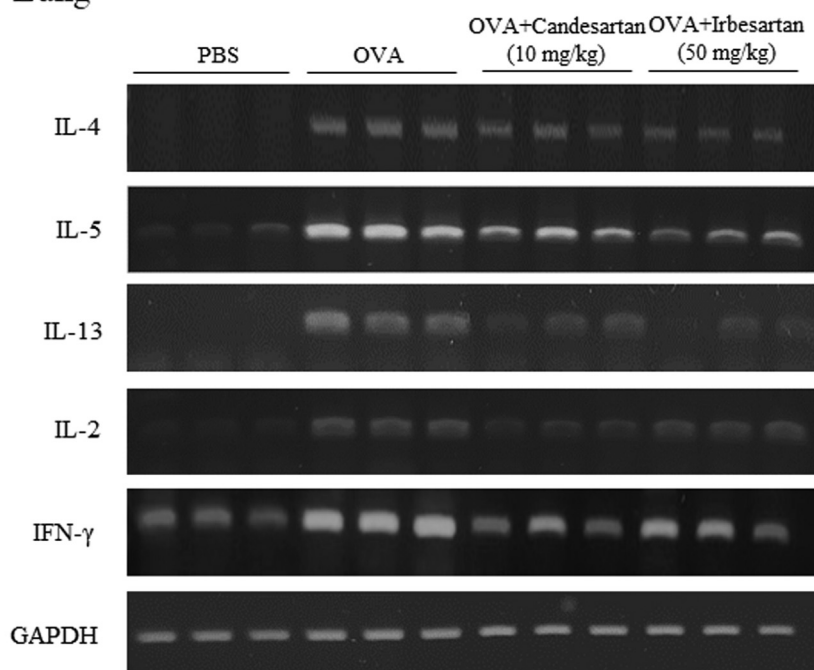


Fig. 5. Candesartan and irbesartan inhibited Th2 and Th1 cytokine expression at the mRNA level in BAL fluid-associated cells. (A) mRNA expression of Th2 cytokines (IL-4, IL-5, and IL-13) in the BAL fluid cells of candesartan or irbesartan-treated mice groups. (A) mRNA expression of Th1 cytokines (IL-2 and IFN-γ) in the BAL fluid cells of candesartan or irbesartan-treated mice groups. (B) mRNA levels of cytokines were quantified as ratios to GAPDH mRNA level. Each lane represents one of five different mice. Values represent the mean $\pm$ S.E.M. ($n = 5$). $^{***} P < 0.001$ vs. the PBS-treated group, $^* P < 0.05$, $^{**} P < 0.01$, $^{***} P < 0.001$ vs. the OVA-treated group.

A

Lung



B

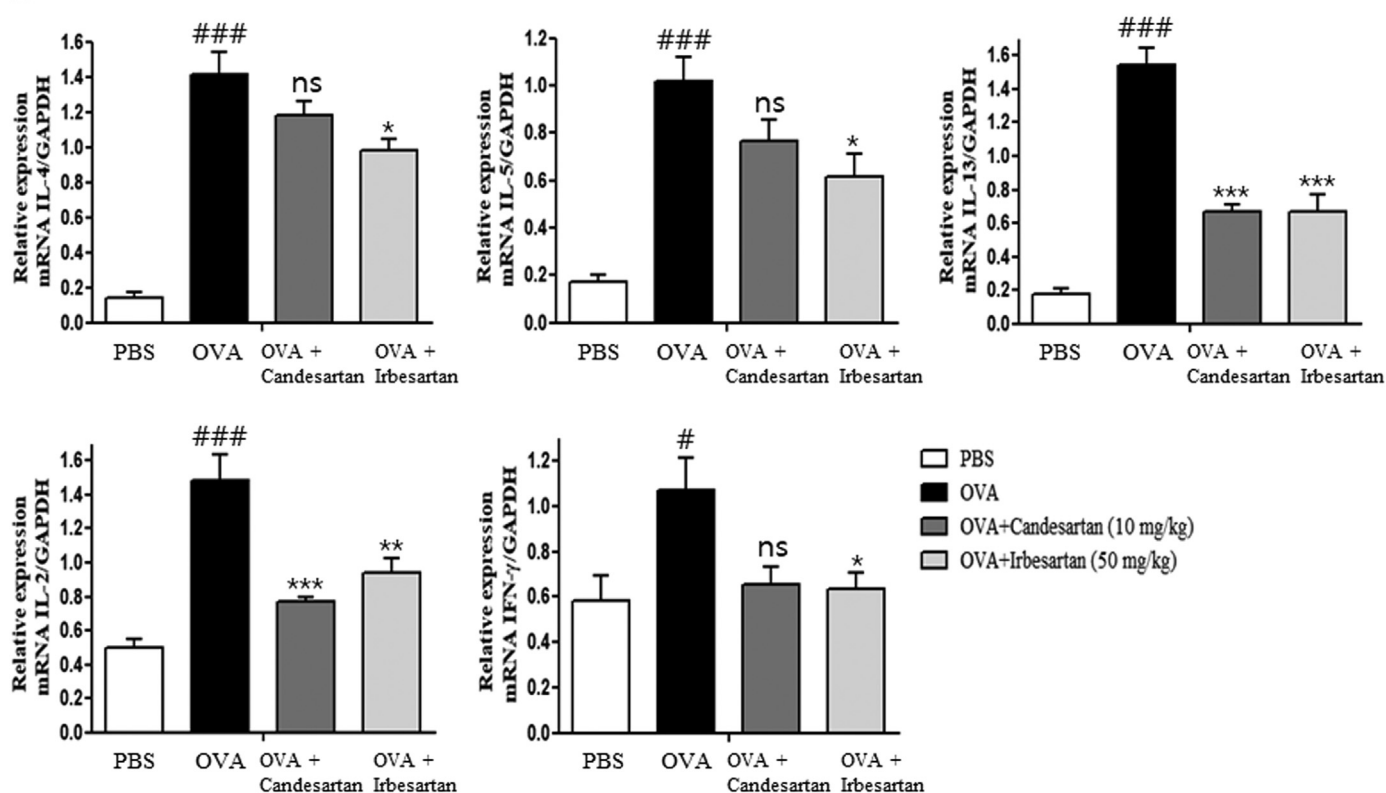


Fig. 6. Candesartan and irbesartan inhibited Th2 and Th1 cytokine expression at the mRNA level in the lung tissues of the OVA-induced asthmatic mice. (A) mRNA expression of Th2 cytokines (IL-4, IL-5, and IL-13) in the lung tissues of candesartan or irbesartan-treated mice groups. (B) mRNA expression of Th1 cytokines (IL-2 and IFN- γ) in the lung tissues of candesartan or irbesartan-treated mice groups. (B) mRNA levels of cytokines were quantified as ratios to GAPDH mRNA level. Each lane represents one of five different mice. Values represent the mean $\pm$ S.E.M. ($n = 5$). ^{###} $P < 0.001$ vs. the PBS-treated group, ^{*} $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$ vs. the OVA-treated group.

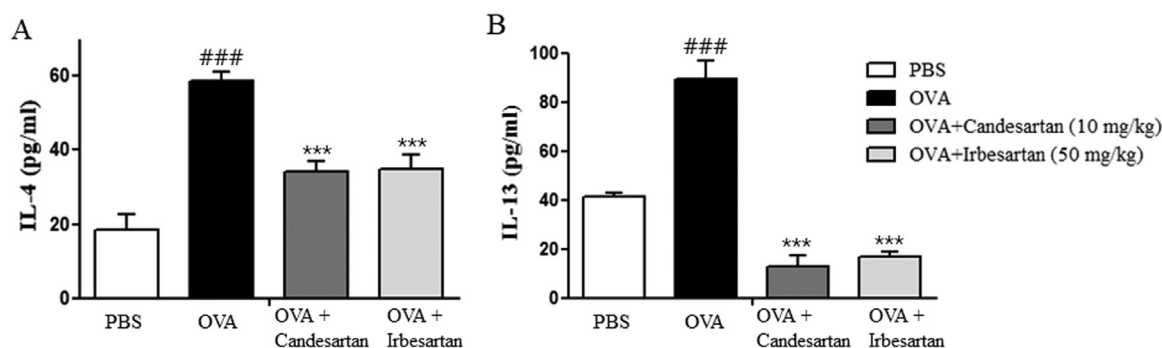


Fig. 7. Effects of candesartan and irbesartan on IL-4 and IL-13 protein levels in BAL fluid. ELISA was used to measure IL-4 and IL-13 levels in BAL fluid samples ($n = 5$ per group). Values represent the mean $\pm$ S.E.M. ($n = 5$). ^{###} $P < 0.001$ vs. the PBS-treated group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the OVA-treated group.

all five mice in each group using TRIzol reagent (Invitrogen, Waltham, MA, USA). cDNA was first synthesized from the total RNA. The synthesized cDNA products, primers for each gene, and Promega GoTaq[®] DNA polymerase (Promega Corporation, Madison, WI, USA) were used to amplify the specific genes. Specific primers for mIL-4 (sense 5'-CTA GTT GTC ATC CTG CTC TTC TTT-3', antisense 5'-CTT TAG GCT TTC CAG GAA GTC TTT-3', 360 bp), mIL-13 (sense 5'-CAG CAT GGT ATG GAG TGT GG-3', antisense 5'-TGG GCT ACT TCG ATT TTG GT-3', 168 bp) were used to amplify gene fragments. PCR was performed as follows: 32 amplification cycles of denaturation at 95 °C for 30 s, annealing at 49 °C for 30 s, and elongation at 72 °C for 30 s in a SimpliAmp Thermal Cycler PCR machine (Thermo Fisher Scientific, Life Technologies Holdings Pre Ltd., Singapore). Specific primers for mIL-5 (sense 5'-AGA ATC AAA CTG TCC GTG GG-3', antisense 5'-GTC TCT CCT CGC CAC ACT TC-3', 105 bp), mIL-2 (sense 5'-AAC CTG AAA CTC CCC AGG AT-3', antisense 5'-TCC ACC ACA GTT GCT GAC TC-3', 254 bp), mIFN- γ (sense 5'-ACT GGC AAA AGG ATG GTG AC-3', antisense 5'-TGA GCT CAT TGA ATG CTT GG-3', 237 bp) were used and annealing was performed at 53 °C (33 amplification cycles). Specific primers for mGAPDH (sense 5'-TTC ACC ATG GAG AAG GC-3', antisense 5'-GGC ATG GAC TGT GGT CAT GA-3', 237 bp) was used and annealing was performed at 60 °C (27 amplification cycles). Aliquots (7 μ L) were electrophoresed on 1.2% agarose gel and stained with StaySafe[™] nucleic acid gel stain (Real Biotech Corporation, Taipei, Taiwan) (Park et al., 2018).

2.8. Measurement of cytokine (IL-4 and IL-13) levels

Interleukin (IL)-4 and IL-13 levels in BAL fluid isolated from five mice per group were measured by using enzyme-linked immunosorbent assay (ELISA) kits. Briefly, 96-well plates (NUNC, Penfield, NY, USA) were coated overnight at 4 °C with a capture antibody specific for IL-4 (cat. 14-7041-68, eBioscience, San Diego, CA, USA) or IL-13 (cat. 14-7043-68, eBioscience). After washing, plates were blocked with a blocking buffer for 1 h at room temperature. Serial dilutions of standard cytokines were prepared and added to the appropriate wells; alternatively, supernatants of BAL fluid were added to the wells. Plates were incubated for 2 h at room temperature with shaking and then washed five times. Biotinylated detection antibody specific for IL-4 (cat. 33-7042-68 C, eBioscience) or IL-13 (cat. 33-7135-68B, eBioscience) was then added and incubated for 1 h at room temperature with shaking. Then plates were washed five times, and avidin-horseradish peroxidase (HRP) was used at room temperature for 30 min with shaking. Plates were then washed 14 times and incubated with the substrate solution at room temperature for 15 min. Then a stop solution was added, and the absorbance of final product was measured at 450 nm (Lee et al., 2016).

2.9. Statistical analysis

Results are expressed as mean $\pm$ standard error of mean (S.E.M.). Differences among groups were tested for statistical significance using analysis of variance (ANOVA) followed by Turkey's *post hoc* test. P values < 0.05 indicated statistical significance. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Candesartan inhibited antigen-induced degranulation in the RBL-2H3 mast cells while irbesartan did not

Mast cells play a critical role in the development of allergic responses. Fc epsilon RI (Fc ϵ RI) present on the mast cell membranes traps the circulating IgEs. The antigen exposure induces cross-linking of the cell-bound IgE, resulting in degranulation (Lane and Lee, 1996). Degranulation of histamine, leukotrienes, and prostaglandins from the antigen-exposed mast cells is an important step in the allergic responses. Rat basophilic RBL-2H3 leukemia cells, a tumor analog of mast cells with exhibit a high membrane expression of Fc ϵ RI, were used in this study. Degranulation was assessed by measuring the β -hexosaminidase activity in the medium after the RBL-2H3 cells were exposed to antigen. Candesartan inhibited the antigen-induced release of β -hexosaminidase in a concentration-dependent manner (Fig. 1A). Additionally, it was observed that candesartan significantly inhibited the degranulation at a concentration of 10 μ M (Fig. 1A). However, in this regard, irbesartan was ineffective (Fig. 1B).

3.2. Candesartan and irbesartan inhibited the accumulation of eosinophils in the BAL fluid of the OVA-induced asthmatic mice

Next, an OVA-induced asthma mouse model was employed to confirm the inhibitory effects of candesartan and irbesartan. Changes in the total cell number and the distribution of immune cell populations in the BAL fluid were analyzed. It was observed that the total cell number in the BAL fluid increased to 363.1% in the OVA-induced asthma group compared to that in the PBS-treated control group (Fig. 2). Candesartan and irbesartan significantly inhibited the OVA-induced increase in the total cell number by 70.6% and 58.8%, respectively (Fig. 2B). Analyses of the immune cell populations in the BAL fluid provided additional supportive evidence (Fig. 2B). In particular, the OVA-induced increase in the eosinophil number in the BAL fluid was significantly inhibited by candesartan and irbesartan by 71.1% and 57.6%, respectively (Fig. 2B). The lymphocyte counts were observed to be lower than the eosinophil counts. However, the OVA administration resulted in a significant increase in the lymphocyte counts, which were also inhibited by treatment with candesartan and irbesartan (Fig. 2B). Moreover, the

macrophage counts in the BAL fluid did not exhibit a significant increase in the OVA group. These values did change after treatment with candesartan and irbesartan (Fig. 2B).

3.3. Candesartan and irbesartan inhibited the inflammation and mucin secretion in the lungs of the OVA-induced asthmatic mice

Histological examination of the lung samples was performed two days after final antigen challenge. Fig. 3A shows the representative hematoxylin and eosin (H&E)-stained lung sections obtained from the mice in the PBS, OVA, OVA + candesartan, and OVA + irbesartan groups. The eosinophils appear as small, navy-blue dots, as indicated by the green arrows. In the PBS control group, very few eosinophils were observed (Fig. 3A). In the OVA group, however, the bronchioles were densely surrounded by the eosinophils (Fig. 3A). Treatment with candesartan and irbesartan reduced the eosinophil infiltration (Fig. 3A). Lung inflammation was semi-quantitatively evaluated using a subjective scale of 0–3, as previously described (Choi et al., 2016; Kwak et al., 2003; Lee et al., 2018; Tournoy et al., 2000). The average inflammation score in the OVA-treated group was approximately 2.3. Alternatively, the score was significantly reduced after treatment with candesartan and irbesartan (Fig. 3B).

PAS was used to stain the mucous glycoproteins (mucins), which are produced by the goblet cells. Secreted or stored mucins are stained purple with PAS. As shown in Fig. 4A, the mucins are the dark violet-stained cells surrounding the bronchioles in the OVA group, as indicated by the red arrows. However, sections from the mice in the OVA + candesartan- or irbesartan-treated groups had fewer stained cells than sections from mice in the OVA group. These results indicate that the mucin production was inhibited by candesartan and irbesartan (Fig. 4A). Further, the mucin production was semi-quantitatively analyzed by counting the PAS-positive cells per mm of the bronchioles (Fig. 4B) (Aoki et al., 2013). Rare staining was observed in the vehicle group. However, approximately 100 PAS-positive cells/mm were detected in the OVA-treated group. Candesartan or irbesartan significantly reduced the number of PAS-positive cells (Fig. 4B).

3.4. Candesartan and irbesartan inhibited the production of Th2 cytokines in the lung tissues

Th2 cytokines play key roles in the pathogenesis of asthma (Fish et al., 2005; Locksley, 2010). In particular, IL-4, IL-5, and IL-13 induce the recruitment and activation of eosinophils, mucus hypersecretion by epithelial cells, goblet cell metaplasia, and the proliferation of smooth muscle cells (Fish et al., 2005; Locksley, 2010). Therefore, in this study, the mRNA levels of IL-4, IL-5, and IL-13 in BAL fluid-associated cells and lung tissues were measured. The mRNA levels of IL-4, IL-5, and IL-13 were increased by 987.5%, 713.0% and 917.1%, respectively, in the BAL fluid of the mice from the OVA group. However, these elevations were inhibited by candesartan and irbesartan (Fig. 5). Both drugs inhibited the mRNA expression of IL-4 by 62.8% and 59.4%; of IL-5 by 45.4% and 47.4%; and of IL-13 by 62.3% and 67.4%, respectively (Fig. 5B). Furthermore, both drugs inhibited the mRNA expression of the Th1 cytokines, IL-2 and INF- γ (Fig. 5). Moreover, as shown in Fig. 6, the mRNA levels of IL-4, IL-5, and IL-13 were observed to have been increased by 922.2%, 503.1% and 772.3%, respectively, in the lung tissues of the mice from the OVA group. However, irbesartan alone inhibited the mRNA expression of IL-4 by 33.8%; of IL-5 by 47.7%; and of IL-13 by 64.1% (Fig. 6B). Additionally, the mRNA expression of the Th1 cytokines, IL-2 and INF- γ , in the lung tissues was also inhibited by irbesartan (Fig. 6). Candesartan inhibited the mRNA expression of IL-13 by 64.0% and IL-2 by 72.1%. However, the expression of IL-4, IL-5, and INF- γ was not significantly affected (Fig. 6).

To confirm the effects of candesartan and irbesartan on the expression of the Th2 cytokines, the levels of IL-4 and IL-13 in the BAL fluid were measured by ELISA. These levels were increased in the OVA

group, compared to those in the PBS control group (Fig. 7). The OVA-induced increase in the levels of IL-4 and IL-13 was significantly inhibited by candesartan and irbesartan (Fig. 7).

4. Discussion

The *in vitro* efficacy tests of candesartan and irbesartan have been investigated for the first time in the present study. In the RBL-2H3 cell-based test, it was observed that candesartan has an inhibitory effect on the mast cell degranulation. Similarly, in the mouse-based *in vivo* test, both drugs showed significant efficacy. The efficacy of irbesartan *in vivo* was studied at the first time in this study.

Since candesartan exhibited an inhibitory effect on mast cell degranulation, this effect could contribute to its *in vivo* efficacy. On the other hand, irbesartan did not show an inhibitory effect on the degranulation. Therefore, five times higher dose of irbesartan was used. The same dose of irbesartan was applied in the other *in vivo* experiments (Clasen et al., 2005; Iida et al., 2012; Iwai et al., 2011; Munoz et al., 2009; Patel et al., 2012). Nevertheless, both caused significant suppression of allergic asthma *in vivo*. Candesartan and irbesartan caused a significant inhibition of the immune cell accumulation in the BAL fluid. A similar efficacy was exhibited on lung inflammation and mucin production. Especially, PAS-stained mucin secreting cells in drug-treated groups were suppressed over 50% compared to the cell numbers in OVA-treated groups. Further, a similarity in the efficacy of both drugs was observed in the production of cytokines in the BAL fluid-associated cells and lung tissues. Only the effect of candesartan on the cytokines of IL-4, IL-5, and INF- γ in the lung tissues was not significant. This may come from distribution of candesartan in the lung was not high enough as in the BAL fluid. In particular, both candesartan and irbesartan showed inhibitory effects on the Th1 cytokines in addition to the Th2 cytokines. This may imply that the anti-allergic effects of both candesartan and irbesartan could be attributed to their PPAR γ modulating activity (Erbe et al., 2006; Schupp et al., 2005). This is because, the activation of the PPAR γ is known to exert anti-inflammatory effects (Martin et al., 2012; Zhang et al., 2013). Additionally, their inhibitory effects on the generation of epoxyeicosatrienoic acid from arachidonic acid in the liver microsome may contribute to the anti-inflammatory effects (Senda et al., 2017). Therefore, we propose three action mechanisms for inhibitory effects of candesartan and irbesartan on allergic asthma: first, antagonism on type I angiotensin receptors, second, PPAR- γ activation-mediated anti-inflammation, and third, inhibition on mast cell degranulation.

Since the effect of candesartan on the bronchial hyperresponsiveness to methacholine in Japanese patients with bronchial asthma was not significant (Myou et al., 2002a; Tanaka et al., 2001), it was necessary to be careful while translating the present results to clinical settings. In summary, it was observed that while candesartan alone has an inhibitory effect on the mast cell degranulation, both candesartan and irbesartan possess anti-allergic effects in the OVA-induced mouse asthma model. Thus, the present study provides evidence of the therapeutic potential of candesartan and irbesartan in allergic asthma.

CRedit authorship contribution statement

Mi-Jeong Kim: Conceptualization, Investigation, Resources, Data curation, Writing - original draft, Visualization. **Dong-Soon Im:** Conceptualization, Writing - original draft, Writing - review & editing, Supervision.

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Statement of contribution

MJ Kim and DS Im designed the experiments. MJ Kim performed the experiments and analyzed the data. MJ Kim and DS Im wrote manuscript.

Conflict of interest

Authors declare there is no conflict of interest.

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