

# Effect of intranasal rosiglitazone on airway inflammation and remodeling in a murine model of chronic asthma

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**Background/Aims:** Asthma is characterized by airway hyperresponsiveness, inflammation, and remodeling. Peroxisome proliferator-activated receptors have been reported to regulate inflammatory responses in many cells. In this study, we examined the effects of intranasal rosiglitazone on airway remodeling in a chronic asthma model.

**Methods:** We developed a mouse model of airway remodeling, including smooth muscle thickening, in which ovalbumin (OVA)-sensitized mice were repeatedly exposed to intranasal OVA administration twice per week for 3 months. Mice were treated intranasally with rosiglitazone with or without an antagonist during OVA challenge. We determined airway inflammation and the degree of airway remodeling by smooth muscle actin area and collagen deposition.

**Results:** Mice chronically exposed to OVA developed sustained eosinophilic airway inflammation, compared with control mice. Additionally, the mice developed features of airway remodeling, including thickening of the peribronchial smooth muscle layer. Administration of rosiglitazone intranasally inhibited the eosinophilic inflammation significantly, and, importantly, airway smooth muscle remodeling in mice chronically exposed to OVA. Expression of Toll-like receptor (TLR)-4 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) was increased in the OVA group and decreased in the rosiglitazone group. Co-treatment with GW9660 (a rosiglitazone antagonist) and rosiglitazone increased the expression of TLR-4 and NF- $\kappa$ B.

**Conclusions:** These results suggest that intranasal administration of rosiglitazone can prevent not only airway inflammation but also airway remodeling associated with chronic allergen challenge. This beneficial effect is mediated by inhibition of TLR-4 and NF- $\kappa$ B pathways.

**Keywords:** Asthma; Remodeling; Rosiglitazone; Smooth muscle

## INTRODUCTION

Asthma is defined in the Global Initiative for Asthma guidelines as a chronic inflammatory disease of the air-

ways, characterized by reversible airflow obstruction [1]. Alterations in the structure of the airway wall, referred to as airway remodeling, have also been recognized as regular features of asthma. The structural changes not-

ed in the asthmatic airway include subepithelial fibrosis, increased smooth muscle mass, angiogenesis, and an increased number of mucous glands. These changes make it difficult to treat chronic asthma patients [2]. Traditionally, airway smooth muscles (ASM) cells have been considered to be the main effector cells of airway narrowing. However, ASMs have also recently been shown to play roles in airway remodeling and inflammation in asthma [3]. ASMs can secrete immunomodulatory cytokines and chemokines [4-6] and express surface receptors that are important for cell adhesion and leukocyte activation [7-9].

Toll-like receptors (TLRs) are a class of pathogen-recognition receptors that activate innate immune responses, causing disease exacerbations in various airway inflammatory diseases, such as asthma and chronic obstructive pulmonary disease [9]. Beyond these post-infectious immune responses, recent studies have implicated TLRs in the pathobiology of asthma, not only inducing T helper lymphocyte type 2 (Th2) cytokine responses, but also the process of airway remodeling in ASMs [10,11]. Among the subtypes of TLRs, ASMs constitutively express mRNAs for TLR4, which elicits proasthmatic ASM tissue constriction or relaxation, mediated by nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) signaling [12]. It also seems that pleiotropic proinflammatory effects of TLR4 in ASMs are associated with the pathophysiology of the airway remodeling process in asthma, although the exact mechanisms remain unclear.

Peroxisome proliferator-activated receptor (PPAR)- $\gamma$  ligands are known to inhibit the release of pro-inflammatory cytokines from airway epithelial cells, as well as macrophages, vascular smooth muscle cells, and endothelial cells [13-15]. Our group demonstrated that the PPAR- $\gamma$  agonist ciglitazone inhibited the development of airway hyperresponsiveness significantly [16] in a murine chronic asthma model. Inhibitory effects on airway mucus hypersecretion [17] and inflammation in ASMs have also been reported [18]. Although potentially preventative effects of PPAR- $\gamma$  on airway remodeling have emerged [19], the molecular mechanisms remain to be investigated. Recent studies have shown that the PPAR- $\gamma$  agonist rosiglitazone exerted anti-inflammatory effects by inhibiting the TLR4 dependent IP-10/PKC/NF- $\kappa$ B signaling pathways in vascular smooth muscle cells [20]. In the present study, we sought to determine whether

intranasal rosiglitazone suppressed airway remodeling and whether its effect was associated with TLR-4 and IP-10/PKC/NF- $\kappa$ B pathways.

## METHODS

### Animals and experimental design

Female BALB/c mice (Dae-Han Experimental Animal Center, Daejeon, Korea) at 7 weeks of age were used. Mice were assigned randomly to one of four groups: (1) control, (2) ovalbumin (OVA) challenge, (3) OVA challenge plus the PPAR- $\gamma$  agonist rosiglitazone, or (4) OVA challenge plus rosiglitazone and the PPAR- $\gamma$  antagonist GW9660.

### Sensitization and antigen challenge protocol

Mice were immunized by subcutaneous injection with 25  $\mu$ g of OVA (Grade V; Sigma-Aldrich, St. Louis, MO, USA) adsorbed to 1 mg of aluminum hydroxide (Aldrich, Milwaukee, WI, USA) in 200  $\mu$ L of phosphate-buffered saline (PBS). Subcutaneous injections were performed on days 0, 7, 14, and 21 and intranasal OVA challenge (20  $\mu$ g/50  $\mu$ L in PBS) was administered on days 27, 29, and 31 under isoflurane (Vedco, St. Joseph, MO, USA) anesthesia. Intranasal OVA challenges were then repeated twice per week for 3 months. Age- and gender-matched control mice were treated in the same way with PBS but without OVA. Mice were sacrificed 24 hours after the final OVA challenge, and bronchoalveolar lavage (BAL) fluid and lung tissues were obtained.

All procedures for animal research were performed in accordance with the Laboratory Animals Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Guidelines and Policies for Rodent Experiments provided by the Institutional Animal Care and Use Committee at the School of Medicine, The Catholic University of Korea.

### Administration of rosiglitazone and GW9660

Micronized dry rosiglitazone powder (Alexis Biochemicals; Enzo Life Sciences, Philadelphia, PA, USA) was dissolved in sterile normal saline (20  $\mu$ g/25  $\mu$ L), and given daily by intranasal administration once per day starting on day 38 for 3 months. The PPAR- $\gamma$  antagonist GW9660 (0.5 mg/kg, dissolved in normal saline) was also administered intranasally once per day on days 35, 38, and

once per week, continuing for 3 months, during the ovalbumin challenge. The control mice were treated in the same way with normal saline.

### Bronchoalveolar lavage

Mice were sacrificed by CO<sub>2</sub> asphyxiation after measuring airway responsiveness. The trachea was exposed and cannulated with silicone tubing attached to a 23-gauge needle on an 800- $\mu$ L tuberculin syringe. BAL fluid was withdrawn after instillation of 1 mL of sterile PBS through the trachea into the lung. The total number of cells in BAL fluid was counted using a hemocytometer. The BAL fluid was cytospun (2,000 rpm, 7 minutes) onto microscope slides and stained with Diff-Quick (Sysmax, Kobe, Japan). The percentages of macrophages, eosinophils, lymphocytes, and neutrophils in the BAL fluid were obtained by counting 400 leukocytes on randomly selected areas of the slide using light microscopy. Supernatants were stored at  $-70^{\circ}\text{C}$ .

### Enzyme-linked immunosorbent assay

The concentration of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) was measured in the BAL fluid with an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA). The assay was performed according to the manufacturer's protocol.

### Western blotting

Separated lung tissues, frozen in liquid nitrogen, were disrupted using a Polytron homogenizer (Tissue-Tearor, Biospec Products Inc., Bartlesville, OK, USA) and centrifuged. The proteins were purified from the supernatant, and the concentration was assessed using the Bradford method [21]. The protein samples were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). After blocking with 10% skimmed milk (BD Difco, Franklin Lakes, NJ, USA), the membrane was incubated with antibodies to TLR4, IP-10, NF- $\kappa$ B (1:1,000; Santa Cruz Biotech, Santa Cruz, CA, USA), and p-Akt (1:1,000; BD Bioscience, San Diego, CA, USA) overnight. The membrane was washed three times with PBS and incubated with secondary antibody for 2 hours. The target protein was detected using the ECL Western Blotting Analysis system (Amersham Pharmacia Biotech,

Piscataway, NJ, USA) and X-ray film.

### Hydroxyproline analysis

Lung tissue (60 mg) from each mouse was used for the hydroxyproline assay. A sample of lung homogenate was subsequently added to 250  $\mu$ L of 12 N HCl for 16 hours at  $110^{\circ}\text{C}$ . After centrifugation, 25  $\mu$ L of each supernatant was assayed. To a 25- $\mu$ L sample of the digested lung, 25  $\mu$ L of citrate/acetate buffer (5% citric acid, 7.2% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid) and 500  $\mu$ L of chloramine T solution (1.41 g of chloramine T, 26 mL of *n*-propanol, 20.7 mL of distilled water, and 53.3 mL of citrate/acetate buffer) were added. The resulting samples were then incubated at room temperature for 20 minutes before 500  $\mu$ L of Ehrlich's solution (4.5 g of *p*-dimethylaminobenzaldehyde, 18.6 mL of *n*-propanol, and 7.8 mL of 70% perchloric acid) were added. These samples were incubated for 15 minutes at  $65^{\circ}\text{C}$ , and cooled samples were read at 550 nm in a spectrophotometer. Hydroxyproline concentrations were calculated from a standard curve of hydroxyproline.

### Measurement of smooth muscle actin

Immunohistochemical detection of  $\alpha$ -smooth muscle actin (Sigma-Aldrich) was performed as described previously [22]. The immunostained area of  $\alpha$ -smooth muscle actin in each paraffin wax-embedded lung was outlined and qualified using a light microscope attached to an image analysis system (BX50, Olympus, Tokyo, Japan). Results are expressed as the immunostained area of basement membrane of bronchioles (internal diameter 650 to 750  $\mu$ m). At least 10 bronchioles were counted on each slide.

### Data analysis

Results from each group were compared by analysis of variance with the nonparametric Kruskal-Wallis test, followed by *post hoc* testing with Dunn's multiple comparison of means. All statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). A  $p < 0.05$  was considered to indicate statistical significance. All results are given as mean  $\pm$  standard error of the mean.

**RESULTS**

**Effects of rosiglitazone on airway inflammation**

Numbers of total cells and different inflammatory cells in BAL fluid were compared between the treatment groups. After OVA challenge, total cells, eosinophils, and lymphocytes were increased in BAL fluid, which were reduced significantly (total cells,  $p < 0.01$ ; eosinophils,  $p < 0.05$ ; lymphocytes,  $p < 0.05$ ) in the rosiglitazone-treated group (Fig. 1). The PPAR- $\gamma$  antagonist GW9660 induced an increase in the number of total cells ( $p < 0.05$ ), eosinophils ( $p < 0.05$ ), and neutrophils ( $p < 0.01$ ) (Fig. 1).

**Effects of rosiglitazone on lung collagen levels**

Total lung collagen levels, representing pulmonary fibrosis, were measured by hydroxyproline analysis. Chronic OVA challenge for 3 months induced an increase in the level of hydroxyproline (OVA vs. control,  $1,932 \pm 200 \mu\text{g/lung}$  vs.  $578 \pm 200 \mu\text{g/lung}$ ). Rosiglitazone treatment decreased the level of hydroxyproline significantly compared with the OVA group (rosiglitazone vs. OVA,  $1,293 \pm 180 \mu\text{g/lung}$  vs.  $1,932 \pm 200 \mu\text{g/lung}$ ,  $p < 0.05$ ). Combined treatment with rosiglitazone and GW9660 increased the hydroxyproline level, similar to that of the OVA group (rosiglitazone vs. rosiglitazone + GW9660,  $1,293 \pm 180 \mu\text{g/}$

lung vs.  $1,900 \pm 150 \mu\text{g/lung}$ ,  $p < 0.05$ ) (Fig. 2).

**Effects of rosiglitazone on ASM area**

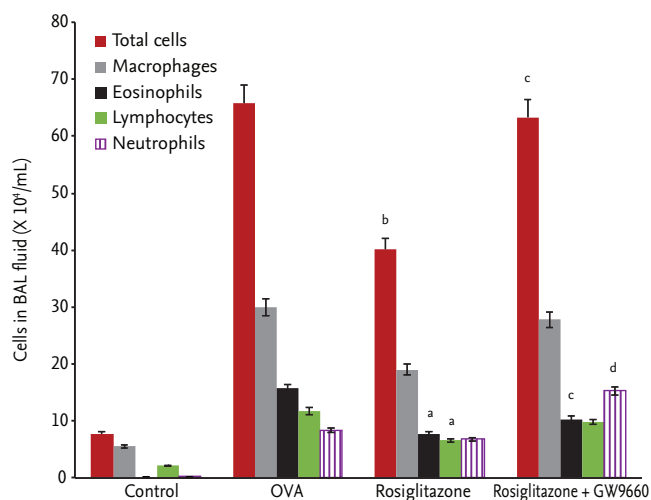
Peribronchial  $\alpha$ -smooth muscle actin immunostaining was quantified by imaging (Fig. 3). Rosiglitazone decreased the area of ASM compared with the OVA group (rosiglitazone vs. OVA,  $850 \pm 29.29 \mu\text{m}^2$  vs.  $1,339 \pm 57.46 \mu\text{m}^2$ ,  $p < 0.01$ ). Treatment with rosiglitazone and GW9660 increased the area of ASM compared with the rosiglitazone-alone treatment group (rosiglitazone vs. rosiglitazone + GW9660,  $850 \pm 29.29 \mu\text{m}^2$  vs.  $1,052 \pm 48.01 \mu\text{m}^2$ ,  $p < 0.01$ ) (Fig. 4).

**Effects of rosiglitazone on bronchoalveolar lavage fluid PGE<sub>2</sub> levels**

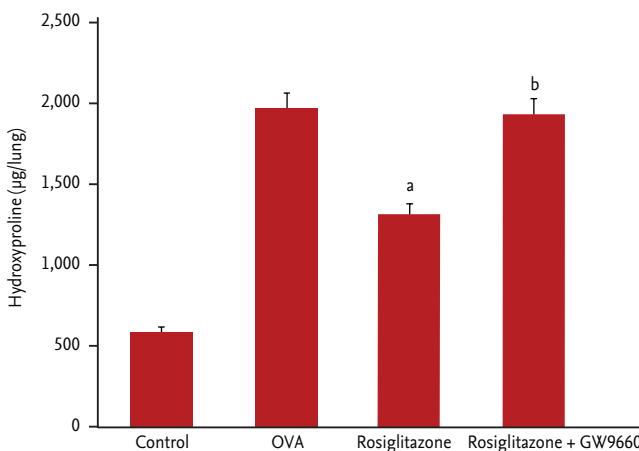
The concentration of PGE<sub>2</sub> in bronchoalveolar lavage fluid increased in the OVA challenge group. The level of PGE<sub>2</sub> decreased with rosiglitazone treatment (rosiglitazone vs. OVA,  $208,893 \pm 181,428$  vs.  $538,030 \pm 10,040$ ,  $p < 0.05$ ); however, GW9660 reversed the lowering effect of rosiglitazone (rosiglitazone vs. rosiglitazone + GW9660,  $208,893 \pm 181,428$  vs.  $412,048 \pm 71,126$ ,  $p < 0.05$ ) (Fig. 5).

**Effects of rosiglitazone on TLR4 signaling pathway proteins**

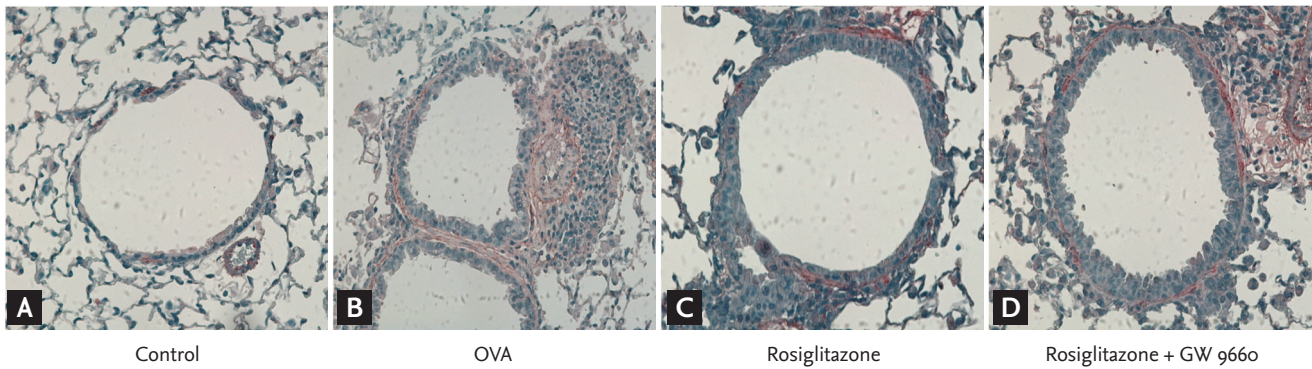
To investigate the effects of rosiglitazone in the TLR4



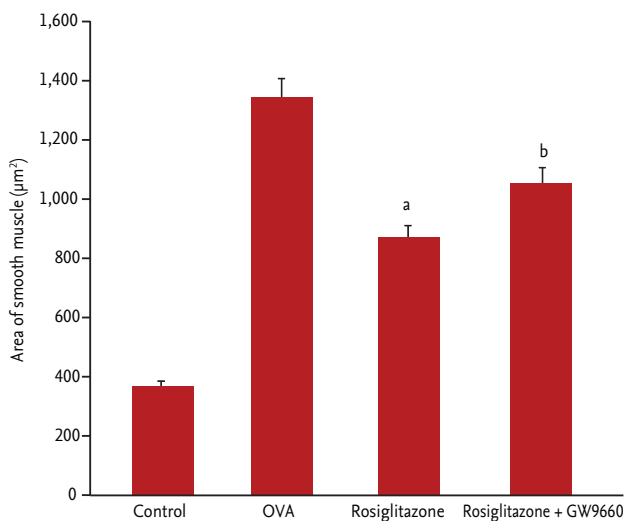
**Figure 1.** Effect of intranasal rosiglitazone on total and differential cell counts in bronchoalveolar lavage fluid. Mice were sacrificed 24 hours after the final ovalbumin (OVA) challenge, and bronchoalveolar lavage (BAL) cells were isolated. Values are expressed as mean  $\pm$  SE (n = 6 to 9 / group). <sup>a</sup> $p < 0.05$ , <sup>b</sup> $p < 0.01$  compared with the OVA group, <sup>c</sup> $p < 0.05$ , <sup>d</sup> $p < 0.01$  compared with the rosiglitazone group.



**Figure 2.** Effects of intranasal rosiglitazone on total lung collagen levels. Lung tissue (60 mg) was collected from each mouse for the hydroxyproline assay. Values are presented as mean  $\pm$  SE (n = 8 to 15/group). OVA, ovalbumin. <sup>a</sup> $p < 0.05$ , compared with the OVA group, <sup>b</sup> $p < 0.05$ , compared with the rosiglitazone group.

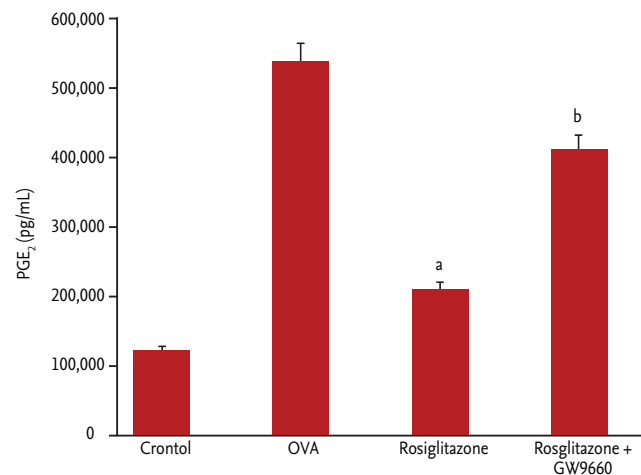


**Figure 3.** Peribronchial  $\alpha$ -smooth muscle actin immunostain was done. Ovalbumin (OVA) challenged mice (B) exhibited increased peribronchial staining (red) compared to the control mice (A) ( $\times 200$ ). Treatment of rosiglitazone (C) decreased the immunostained area which was inhibited by combination treatment of GW9660 (D).



**Figure 4.** Effects of intranasal rosiglitazone on airway smooth muscle cells area. The smooth muscle area is expressed as the immunostained area of basement membrane of the bronchioles using an image analyzer. Values are presented as mean  $\pm$  SE ( $n = 11$  to  $16$ /group). OVA, ovalbumin. <sup>a</sup> $p < 0.01$ , compared with the OVA group, <sup>b</sup> $p < 0.01$ , compared with the rosiglitazone group.

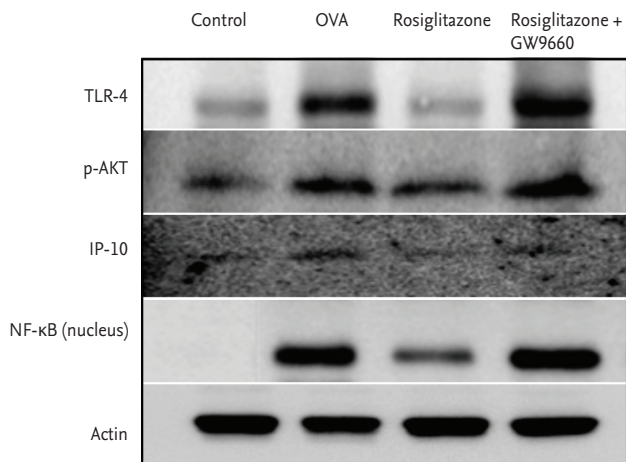
signaling pathway, expression of TLR4 and downstream signaling proteins were determined in homogenized lung tissues. After the OVA challenge, protein expression of TLR4 and the downstream p-Akt, IP-10, and NF- $\kappa$ B increased significantly, compared with the control group. Rosiglitazone effectively attenuated the levels of protein expression, which were again reversed with co-treatment with GW9660 (Fig. 6).



**Figure 5.** Effects of intranasal rosiglitazone on prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) levels in bronchoalveolar lavage fluid. Values are expressed as mean  $\pm$  SE ( $n = 5$ /group). OVA, ovalbumin. <sup>a</sup> $p < 0.05$ , compared with the OVA group, <sup>b</sup> $p < 0.05$ , compared with the rosiglitazone group.

## DISCUSSION

PPAR- $\gamma$  is highly expressed in adipose tissue, and was first identified for a role in lipid and glucose metabolism. Based on previous *in vitro* and *in vivo* studies, PPAR- $\gamma$  has recently been accepted to play a role in airway diseases, negatively regulating the expression of proinflammatory genes. This effect is mediated through inhibiting cytokines, chemoattractants, and cell survival factors. Patel et al. [19] revealed that the effect of PPAR- $\gamma$  on cell



**Figure 6.** Effect of intranasal rosiglitazone on expression of Toll-like receptor 4 (TLR4) and downstream proteins in lung tissue. Repetitive ovalbumin (OVA) challenge for 3 months induced TLR4, p-Akt, Interferon-gamma-induced protein 10 (IP-10) and nuclear factor-kappa B (NF-κB) expression which were inhibited by rosiglitazone therapy. Cotreatment of rosiglitazone with GW9660 re-induced the expression of the proteins.

growth and G-CSF was greater than that produced by a glucocorticoid, the most widely used anti-inflammatory agent. Inoue et al. [23] reported that PPAR-γ activation suppressed cyclooxygenase-2 expression by inhibiting the NF-κB signaling pathway. Also, several groups have used inhaled or oral PPAR-γ agonists in animal models of allergic asthma; ciglitazone and rosiglitazone showed inhibitory effects on inflammatory cell influx and airway hyperresponsiveness [24-26]. These anti-inflammatory effects demonstrate that PPAR-γ agonists may provide new therapeutic modalities for the treatment of allergic asthma.

In our study, daily administration of intranasal rosiglitazone during the 3-month OVA challenge reduced the influx of inflammatory cells in BAL fluid significantly (Fig. 1). Combined treatment with GW9660 reversed the effect; these results suggest that the PPAR-γ agonist had a protective effect against chronic airway inflammation. Also, the increased level of PGE<sub>2</sub> in BAL fluid after the OVA challenge decreased with rosiglitazone treatment (Fig. 5) and again increased with combined treatment with GW9660. This result is compatible with the previous study of Sun et al. [27], which showed that rosiglitazone treatment decreased urinary PGE<sub>2</sub> levels by 57%.

PGE<sub>2</sub> is generally recognized as a mediator of inflammation and Th2 immunity, key elements of asthma pathophysiology [28,29]. These results reflect the suppressive effects of rosiglitazone on the Th2-mediated immune response, airway inflammation, and development of asthmatic airways.

Our study is original in using a chronic ovalbumin-challenged asthma model instead of an acute model. Among murine allergic asthma models, the chronic model demonstrates more similar pathological features to human asthma patients than the acute model. However, until now, studies investigating the role of intranasal PPAR-γ on the chronic airway remodeling process have been lacking.

Recently, Fogli et al. [30] demonstrated that the PPAR-γ ligand rosiglitazone and β<sub>2</sub>-agonist salbutamol had synergistic interactions on ASM proliferation. Stephen et al. [31] reported that the PPAR-γ ligand ciglitazone decreased human ASM migration and extracellular matrix (ECM) synthesis. The airway remodeling process in allergic asthma is characterized by subepithelial thickening from the deposition of ECM, increased ASM mass, and angiogenesis [32]. In this study, collagen deposition, demonstrated by hydroxyproline analysis, decreased with intranasal rosiglitazone treatment (Fig. 2). The area of ASM also decreased in the rosiglitazone-treated group. The effect was reversed with GW9660 (Figs. 3 and 4) suggesting that PPAR-γ signaling is involved in collagen deposition and ASM hyperplasia, important mechanisms of the airway remodeling process.

To investigate the molecular mechanism of PPAR-γ action on airway remodeling, we hypothesized that PPAR-γ negatively regulated the TLR4 receptor pathway in ASMs. TLR4 activation with lipopolysaccharide (LPS) has been reported to induce Th2 cytokine responses in a murine asthma model [11]. Several recent studies reported that PPAR-γ agonists may inhibit TLR4 activation, when it is induced by LPS, oxyhemoglobin, or angiotensin [20,33,34]. Also, Yin et al. [35] described that the PPAR-γ agonist rosiglitazone regulated the increased expression of TLR4 and NF-κB after chronic exposure to cigarette smoke in alveolar macrophages. The effects of PPAR-γ on TLR4 activation in an OVA-challenged murine asthma model have not been reported before. This is the first report of inhibitory regulation of TLR4 signaling, induced by OVA, through a PPAR-γ agonist.

In our study, the affected signaling proteins downstream of TLR4 were AKT, IP-10, and NF- $\kappa$ B in homogenized lung tissues (Fig. 6). Activation of TLR4 stimulated both a myeloid differentiation factor 88 (Myd88)-dependent and a Myd88-independent pathway. The downstream Myd88-dependent pathway proteins are NF- $\kappa$ B, phosphatidylinositol 3-kinase/Akt, and mitogen-activated protein kinases, which regulate cell viability and inflammation [36]. Also, the Myd88-independent pathway induced expression of IFN-inducible genes, such as IP-10 and glucocorticoid-attenuated response gene 16. Through the recruitment of adaptor molecules, the Myd88-independent pathway resulted in late-phase activation of NF- $\kappa$ B [36]. Thus, the suppressed expression of NF- $\kappa$ B, Akt, and IP-10 in lung tissues of the rosiglitazone-treated group suggests that rosiglitazone could act as an inhibitor of both Myd88-dependent and -independent signaling pathways. Increased expression of NF- $\kappa$ B, Akt, and IP-10 in the combined treatment group of rosiglitazone with antagonist GW9660 reinforces the downregulating effect of TLR4 signaling by rosiglitazone.

Since the approval of rosiglitazone as an anti-diabetic agent, concerns about increased cardiovascular risks due to the agent have been suggested [37]. Although the results of a randomized study did not suggest any increased risk of major adverse cardiovascular events recently [38], questions about the systemic effects of rosiglitazone, such as hypoglycemia, fluid retention, and increased levels of low density lipoprotein cholesterol must be considered in long-term usage to gain a preventative effect against airway remodeling. As intranasal rosiglitazone had sufficient effects in mice in our study, we consider that an inhalation method may help to reduce systemic side effects of the agent. Further clinical studies about drug dosing and safety are needed.

In conclusion, we confirmed that intranasal administration of rosiglitazone effectively reduced the characteristics of asthmatic airway, such as airway inflammation, fibrosis, and smooth muscle cell hyperplasia. Moreover, decreased TLR4 receptors and downstream signaling proteins in lung tissue suggested that these effects were mediated by regulation of TLR4 signaling by rosiglitazone. The association between PPAR- $\gamma$  agonist and TLR4 signaling in ASMs should be demonstrated by *in vitro* experiments separately.

## KEY MESSAGE

1. Peroxisome proliferator-activated receptor  $\gamma$  (PPAR- $\gamma$ ) agonists are known to have anti-inflammatory effects in chronic airway disease.
2. This study demonstrated that rosiglitazone inhalation has a protective effect on developing asthmatic airways through inhibiting Toll-like receptor 4 signaling pathways in an ovalbumin-challenged chronic asthma model.
3. Intranasal administration of the PPAR- $\gamma$  agonist rosiglitazone may have therapeutic potential in asthma patients with few systemic side effects.

## Conflict of interest

No potential conflict of interest relevant to this article was reported.

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