

Modulation of Airway Remodeling and Airway Inflammation by Peroxisome Proliferator-Activated Receptor γ in a Murine Model of Toluene Diisocyanate-Induced Asthma¹

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Toluene diisocyanate (TDI) is a leading cause of occupational asthma. Although considerable controversy remains regarding its pathogenesis, TDI-induced asthma is an inflammatory disease of the airways characterized by airway remodeling. Peroxisome proliferator-activated receptor γ (PPAR γ) has been shown to play a critical role in the control of airway inflammatory responses. However, no data are available on the role of PPAR γ in TDI-induced asthma. We have used a mouse model for TDI-induced asthma to determine the effect of PPAR γ agonist, rosiglitazone, or pioglitazone, and PPAR γ on TDI-induced bronchial inflammation and airway remodeling. This study with the TDI-induced model of asthma revealed the following typical pathophysiological features: increased numbers of inflammatory cells of the airways, airway hyperresponsiveness, increased levels of Th2 cytokines (IL-4, IL-5, and IL-13), adhesion molecules (ICAM-1 and VCAM-1), chemokines (RANTES and eotaxin), TGF- β 1, and NF- κ B in nuclear protein extracts. In addition, the mice exposed to TDI developed features of airway remodeling, including thickening of the peribronchial smooth muscle layer, subepithelial collagen deposition, and increased airway mucus production. Administration of PPAR γ agonists or adenovirus carrying PPAR γ 2 cDNA reduced the pathophysiological symptoms of asthma and decreased the increased levels of Th2 cytokines, adhesion molecules, chemokines, TGF- β 1, and NF- κ B in nuclear protein extracts after TDI inhalation. In addition, inhibition of NF- κ B activation decreased the increased levels of Th2 cytokines, adhesion molecules, chemokines, and TGF- β 1 after TDI inhalation. These findings demonstrate a protective role of PPAR γ in the pathogenesis of the TDI-induced asthma phenotype. *The Journal of Immunology*, 2006, 177: 5248–5257.

Isocyanate chemicals, including toluene diisocyanate (TDI)⁴, are currently the most common causes of occupational asthma (1, 2). TDI-induced asthma is characterized by hyperresponsiveness, inflammation, and remodeling of the airways (2–4). This inflammation is associated with infiltration of lymphocytes, eosinophils, and neutrophils into the bronchial lumen (3, 5). These cellular infiltrates release various chemical mediators, which can cause airway hyperresponsiveness (6–8). Although reversibility of airway obstruction has traditionally formed part of the definition of asthma, it is apparent that airway obstruction in asthma is often not completely reversible, which is associated with development of structural changes (remodeling) in the air-

way and functional derangement. This airway remodeling has been speculated to be one of the factors that make it difficult to treat asthma patients (9).

Peroxisome proliferator-activated receptors (PPARs) are transcriptional factors belonging to the ligand-activated nuclear receptor superfamily (10). They are ubiquitously expressed throughout the body. Three major types have been identified, namely PPAR α , PPAR β δ , and PPAR γ . PPAR γ is originally known to regulate adipocyte differentiation and lipid metabolism (11). However, accumulating evidence indicates that PPAR γ affects cell cycle, differentiation, and apoptosis (12). In addition, PPAR γ activation down-regulates synthesis and release of immunomodulatory cytokines from various cell types (13, 14). Previous studies have shown that PPAR γ is involved in airway inflammation and remodeling in asthma (15–17). PPAR γ expression is increased in the airway mucosa of asthmatics as compared with healthy subjects (16). Recent studies suggest the use of PPAR γ ligands for the possible treatment of bronchial asthma (17, 18). However, there are no data on the effect of PPAR γ on the hyperresponsiveness, inflammation, and remodeling of the airways in TDI-induced asthma.

In the present study, we used a murine model of TDI-induced asthma to evaluate the effect of PPAR γ agonist, rosiglitazone, or pioglitazone, and an adenovirus gene transfer vector expressing a PPAR γ 2 cDNA (AdPPAR γ) on hyperresponsiveness, inflammation, and remodeling of the airways.

Materials and Methods

Animals and experimental protocol

Female BALB/c mice, 8–10 wk of age and free of murine-specific pathogens, were obtained from the Korean Research Institute of Chemistry

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⁴ Abbreviations used in this paper: TDI, toluene diisocyanate; PPAR, peroxisome proliferator-activated receptor; BAL, bronchoalveolar lavage; Ad, adenovirus; p-Akt, phosphorylated Akt; PAS, periodic acid-Schiff; R_L, airway resistance; AdPPAR γ , adenoviruses carrying PPAR γ 2 cDNA.

Technology. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School. Mice were sensitized twice by two courses of intranasal administration of 20 μ l of 3% TDI dissolved in ethyl acetate:olive oil (1:4) under light anesthesia (sodium pentobarbitone, 30 mg/kg, i.p.) once daily for 5 consecutive days with a 3-wk interval as previously described (19) with some modifications (Fig. 1). At 7 days after the second course of sensitization (day 38), mice were challenged via the airways with 1% TDI dissolved in ethyl acetate:olive oil (1:4) for 10 min by ultrasonic nebulization (NE-U12; Omron), and then repeated once a week for 4 wk (Fig. 1). As a control, mice were sensitized and challenged using the same protocol but using only the solvent, ethyl acetate:olive oil (1:4). Bronchoalveolar lavage (BAL) was performed at 48 h after the last challenge.

Vectors

The E1/E3-deleted replication-deficient recombinant adenovirus (Ad) was made using the AdEasy system (Quantum Biotech) described by He et al. (20).

Administration of rosiglitazone, pioglitazone, GW9662, Ad vectors, or BAY 11-7085

Rosiglitazone (1, 2.5, or 5 mg/kg body weight/day; GlaxoSmithKline) dissolved in distilled water or pioglitazone (1, 5, or 10 mg/kg body weight/day; Takeda Chemical Industries) dissolved in 0.05% DMSO and diluted with saline, was administered by oral gavage at 24-h interval on days 36–66, beginning 2 days before the first challenge as previously described (15) (Fig. 1). A selective antagonist of PPAR γ , GW9662 (0.5 mg/kg body weight/day; Cayman) dissolved in PBS was administered intratracheally six times to each animal on days 37, 40, 47, 54, 61, and 67, beginning 1 day before the first challenge as previously described (15). Ad vectors were administered intratracheally (10^9 PFU) six times to each animal on days 37, 40, 47, 54, 61, and 65, beginning 1 day before the first challenge. An inhibitor of NF- κ B activation, BAY 11-7085 (20 mg/kg body weight/day; BIOMOL) dissolved in DMSO and diluted with 0.9% NaCl, was administered by i.p. injection six times to each animal on days 37, 40, 47, 54, 61, and 67, beginning 1 day before the first challenge (21, 22).

Measurement of Th2 cytokines and TGF- β 1

Levels of IL-4, IL-5, IL-13, and TGF- β 1 were quantified in the supernatants of BAL fluids by enzyme immunoassays (IL-4 and IL-5 (Endogen); IL-13 and TGF- β 1 (R&D Systems)).

Western blot analysis

Protein expression levels were analyzed by Western blot analysis, as described previously (15). The blots were incubated with an anti-IL-4 Ab (Serotec), anti-IL-5 Ab (Santa Cruz Biotechnology), anti-IL-13 Ab (R&D Systems), anti-TGF- β 1 Ab (Santa Cruz Biotechnology), anti-ICAM-1 Ab (Santa Cruz Biotechnology), anti-VCAM-1 Ab (Santa Cruz Biotechnology), anti-RANTES Ab (Abcam), anti-eotaxin Ab (Abcam), anti-Akt Ab (Cell Signaling Technology), and anti-phosphorylated Akt (p-Akt) Ab (Cell Signaling Technology).

Cytosolic or nuclear protein extractions for analysis of NF- κ B p65 and PPAR γ

Cytosolic or nuclear extractions for analysis of NF- κ B p65 and PPAR γ were performed as described previously (15). The levels of these proteins were analyzed by Western blotting using Ab against NF- κ B p65 (Upstate Biotechnology) or PPAR γ (Santa Cruz Biotechnology).

Processing of lungs for histologic and image analysis

At 48 h after the last challenge, lungs were removed from the mice after sacrifice. The specimens were stained sequentially with H&E (Richard-Allan Scientific), periodic acid-Schiff (PAS), Masson's trichrome stain, or α -smooth muscle actin stain. Stained and immunostained slides were all quantified under identical light microscope conditions, including magnification ($\times 20$), gain, camera position, and background illumination (23).

Histology

For histological examination, 4- μ m sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica Microsystems). The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0–3, as described elsewhere (24).

Quantitation of airway mucus expression

To quantitate the level of mucus expression in the airway, the number of PAS-positive and PAS-negative epithelial cells in individual bronchioles were counted as described previously (23, 25). Results are expressed as the percentage of PAS-positive cells per bronchiole, which is calculated from the number of PAS-positive epithelial cells per bronchiole divided by the total number of epithelial cells of each bronchiole.

Quantitation of peribronchial fibrosis

Two methods (trichrome staining and total lung collagen content) were used to quantify peribronchial fibrosis.

Peribronchial trichrome staining. The area of peribronchial trichrome staining in a paraffin-embedded lung was outlined and quantified using a light microscope (Leica DM LB; Leica) attached to an image-analysis system (analySIS Pro version 3.2; Soft Imaging System). Results are expressed as the area of trichrome staining per micron length of basement membrane of bronchioles 150–200 μ m of internal diameter. At least 10 bronchioles were counted in each slide.

Determination of total lung collagen content. The total lung collagen content was determined using the Sircol Collagen Assay kit (Biocolor) according to the manufacturer's protocols.

Quantitation of peribronchial smooth muscle

For immunohistochemical detection of α -smooth muscle actin, the lung sections were incubated with either a primary mAb directed against α -smooth muscle actin (Sigma-Aldrich), or as a negative control mouse serum instead of the primary Ab. Results are expressed as the area of immunostaining per micron length of basement membrane of bronchioles 150–200 μ m of internal diameter.

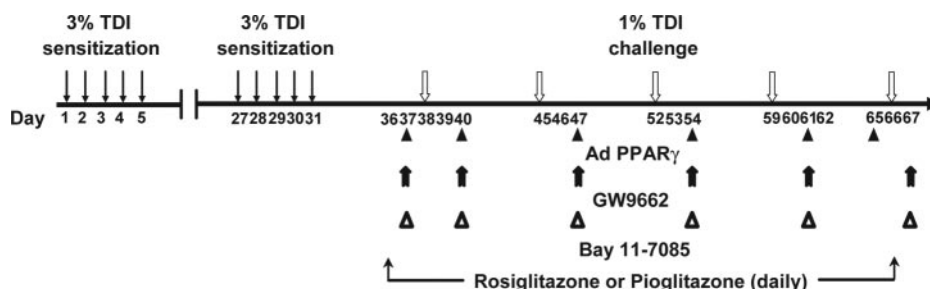


FIGURE 1. Schematic diagram of the experimental protocol. Mice were sensitized twice by two courses of intranasal administration of 3% TDI once a day for 5 consecutive days with a 3-wk interval. Seven days later, mice were challenged via the airways with 1% TDI for 10 min by ultrasonic nebulization, and then repeated once a week for 4 wk. In the case of treatment with rosiglitazone or pioglitazone, it was given by oral gavage at 24-h interval on days 36–66. In the case of treatment with GW9662, GW9662 was administered intratracheally six times to each animal on days 37, 40, 47, 54, 61, and 67. Ad vector was administered intratracheally six times to each animal on days 37, 40, 47, 54, 61, and 65. BAY 11-7085 was administered by i.p. injection six times to each animal on days 37, 40, 47, 54, 61, and 67.

Determination of airway responsiveness

Airway responsiveness was assessed as a change in airway function after challenge with aerosolized methacholine via airways, as described elsewhere (26, 27). Each mouse was challenged with methacholine aerosol in increasing concentrations (2.5–50 mg/ml in saline). After each methacholine challenge, the data of airway resistance (R_L) was continuously collected. Maximum values of R_L were selected to express changes in airway function which was represented as a percentage change from baseline after saline aerosol.

Densitometric analyses and statistics

All immunoreactive signals were analyzed by densitometric scanning (Gel Doc XR; Bio-Rad). Data were expressed as mean $\pm$ SEM. Statistical comparisons were performed using one-way ANOVA followed by the Scheffé's test. Significant differences between groups were determined using the unpaired Student *t* test. Statistical significance was set at *p* < 0.05.

Results

Effect of rosiglitazone, pioglitazone, or AdPPAR γ on PPAR γ protein levels in lung tissues of TDI-sensitized and -challenged mice

Western blot analysis revealed that the levels of PPAR γ in nuclear protein extracts of lung tissues were increased at 48 h after the last TDI inhalation compared with the levels in the control mice (Fig. 2A). The increased PPAR γ levels in nuclear protein extracts of lung tissues were further increased by the administration of rosiglitazone or pioglitazone. In contrast, the levels of PPAR γ in cytosolic protein extracts of lung tissues were decreased at 48 h after the last TDI inhalation compared with the levels in the control mice. The decreased PPAR γ levels in cytosolic protein extracts of lung tissues were further decreased by the administration of rosiglitazone or pioglitazone. In addition, the levels of PPAR γ in

nuclear protein extracts of lung tissues were increased in TDI-sensitized and -challenged mice treated with AdLacZ compared with the levels in the control mice on days 42, 49, 56, and 63 (Fig. 2B). The increased PPAR γ levels in nuclear protein extracts of lung tissues were further increased by the administration of AdPPAR γ on days 42, 49, 56, and 63.

Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on cellular changes in BAL fluids

Numbers of total cells, lymphocytes, neutrophils, and eosinophils were significantly increased in the BAL fluid at 48 h after the last TDI inhalation compared with the levels in the control mice (Fig. 3A). The increased numbers of total cells, lymphocytes, neutrophils, and eosinophils in BAL fluids at 48 h after the last TDI inhalation were significantly reduced by the administration of rosiglitazone (5 mg/kg), pioglitazone (10 mg/kg), or AdPPAR γ in a dose-dependent manner. The inhibitory effect of rosiglitazone treatment on numbers of total cells, lymphocytes, neutrophils, and eosinophils in BAL fluids was abrogated when a PPAR γ antagonist, GW9662, was administered concomitantly with the agonist. These results indicate that rosiglitazone was mainly acting through PPAR γ in this model.

Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on pathological changes of TDI-induced asthma

Histologic analyses revealed typical pathologic features of asthma in the TDI-exposed mice. Numerous inflammatory cells infiltrated around the bronchioles, the airway epithelium was thickened, and mucus and debris had accumulated in the lumen of bronchioles (Fig. 3C) as compared with the control (Fig. 3B). Mice treated with rosiglitazone (Fig. 3D) or AdPPAR γ (Fig. 3E) showed marked reductions in the thickening of airway epithelium, in the infiltration of inflammatory cells in the peribronchiolar region, in the number of inflammatory cells, and in the amount of debris in the airway lumen. In contrast, no significant changes were observed in AdLacZ-treated mice (Fig. 3F).

The scores of peribronchial, perivascular, and total lung inflammation were significantly increased at 48 h after the last TDI inhalation compared with the scores in the control mice (Fig. 3G). The increased peribronchial, perivascular, and total lung inflammation after TDI inhalation were significantly decreased by the administration of rosiglitazone, pioglitazone, or AdPPAR γ . These results suggest that rosiglitazone, pioglitazone, and AdPPAR γ inhibit Ag-induced inflammation in the lungs. Supporting the observations, the inhibitory effect of rosiglitazone treatment on scores of peribronchial, perivascular, and total lung inflammation in lung tissues was abrogated when GW9662 was administered concomitantly with the agonist (Fig. 3G).

Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on airway hyperresponsiveness

Airway responsiveness was assessed as a percent increase of R_L in response to increasing doses of methacholine. In TDI-sensitized and -challenged mice, the dose-response curve of R_L shifted to the left compared with that of control mice (Fig. 3, H and I). In addition, the R_L produced by methacholine administration (at doses from 5 to 50 mg/ml) increased significantly in the TDI-sensitized and -challenged mice compared with the controls. TDI-sensitized and -challenged mice treated with rosiglitazone, pioglitazone, or AdPPAR γ showed a dose-response curve of R_L that shifted to the right compared with that of untreated mice in a dose-dependent manner (Fig. 3I). These results indicate that rosiglitazone, pioglitazone, or AdPPAR γ treatment reduces TDI-induced airway hyperresponsiveness. The inhibitory effect of rosiglitazone treatment

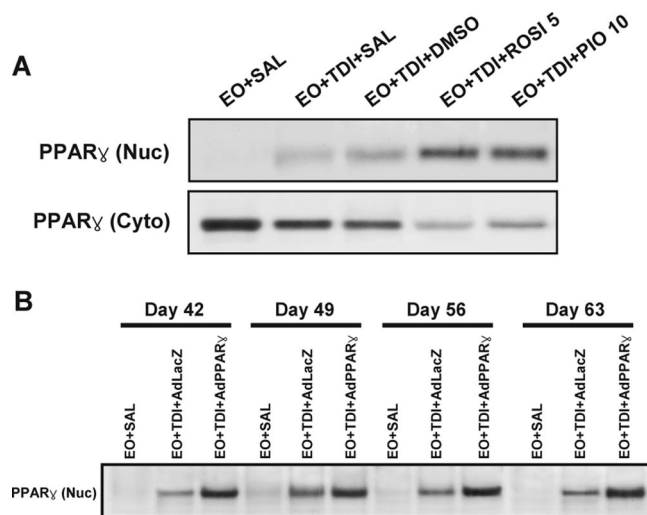


FIGURE 2. Effect of rosiglitazone, pioglitazone, or AdPPAR γ on PPAR γ expression in nuclear and cytosolic protein extracts from lung tissues. **A**, PPAR γ expression in nuclear (Nuc) and cytosolic (Cyto) protein extracts from lung. PPAR γ expression was measured at 48 h after the last challenge in vehicle control (ethyl acetate plus olive oil) mice administered saline (EO+SAL), TDI-inhaled mice administered saline (EO+TDI+SAL), TDI-inhaled mice administered drug vehicle (0.05% DMSO) (EO+TDI+DMSO), TDI-inhaled mice administered rosiglitazone 5 mg/kg (EO+TDI+ROSI 5), or TDI-inhaled mice administered pioglitazone 10 mg/kg (EO+TDI+PIO 10). **B**, PPAR γ expression in nuclear protein extracts from lung tissues was measured on days 42, 49, 56, and 63 in vehicle control mice administered saline (EO+SAL), TDI-inhaled mice administered AdLacZ (EO+TDI+AdLacZ), or TDI-inhaled mice administered AdPPAR γ (EO+TDI+AdPPAR γ). Results were similar in six mice per group.

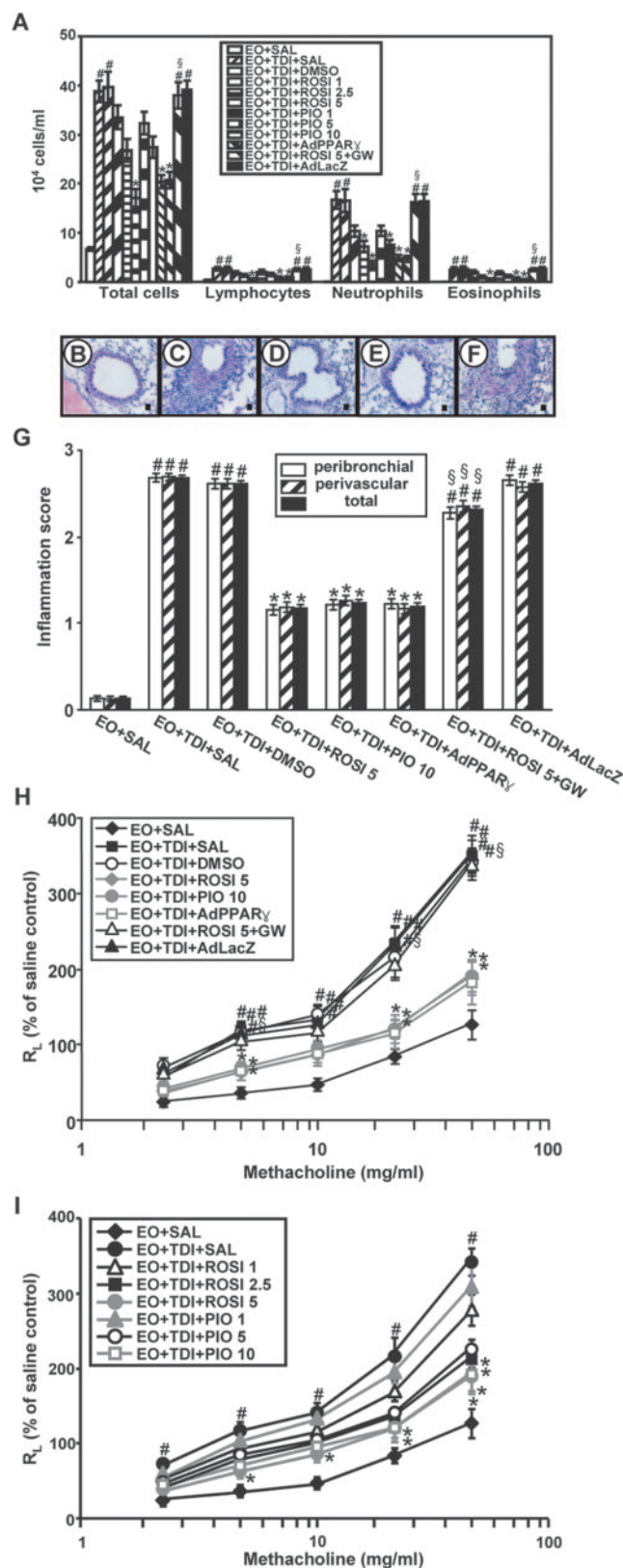


FIGURE 3. A, Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on total cells and differential cellular components in BAL fluids. Sampling was performed at 48 h after the last challenge in vehicle control mice administered saline (EO+SAL), TDI-inhaled mice administered saline (EO+TDI+SAL), TDI-inhaled mice administered drug vehicle (0.05% DMSO) (EO+TDI+DMSO), TDI-inhaled mice administered either 1 mg/kg rosiglitazone (EO+TDI+ROSI 1), 2.5 mg/kg rosiglitazone (EO+TDI+ROSI 2.5), or 5 mg/kg rosiglitazone (EO+TDI+ROSI 5), TDI-inhaled mice administered either 1 mg/kg pio-

on airway hyperresponsiveness was abrogated when GW9662 was administered concomitantly with the agonist.

Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on airway mucus expression

The percentage of airway epithelium which stained positively with PAS in mice repetitively challenged with TDI (Fig. 4, B and F) was significantly greater than in control non-TDI-challenged mice (Fig. 4, A and F). The administration of rosiglitazone (1, 2.5, and 5 mg/kg) (Fig. 4, C and F), pioglitazone (1, 5, and 10 mg/kg) (Fig. 4F), or AdPPAR γ (Fig. 4, D and F) to mice repetitively challenged with TDI significantly reduced the percentage of airway epithelium staining positively with PAS compared with untreated mice, whereas GW9662 plus rosiglitazone or AdLacZ did not (Fig. 4, E and F).

Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on peribronchial collagen deposition

Mice exposed to repetitive TDI challenge (Fig. 5, B, F, and G) had a significant increase in the levels of peribronchial fibrosis compared with non-TDI-challenged mice (Fig. 5, A, F, and G) as assessed by trichrome staining and determination of total lung collagen content. The administration of rosiglitazone (2.5 and 5 mg/kg) (Fig. 5, C, F, and G), pioglitazone (10 mg/kg) (Fig. 5, F and G), or AdPPAR γ (Fig. 5, D, F, and G) to mice repetitively challenged with TDI significantly reduced the levels of peribronchial fibrosis compared with untreated mice, whereas GW9662 plus rosiglitazone or AdLacZ did not (Fig. 5, E, F, and G).

Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on peribronchial α -smooth muscle actin expression

Mice exposed to repetitive TDI challenge (Fig. 6, B and F) had a significant increase in the area of peribronchial α -smooth muscle actin immunostaining compared with non-TDI-challenged mice (Fig. 6, A and F). The administration of rosiglitazone (1, 2.5, and 5 mg/kg) (Fig. 6, C and F), pioglitazone (5 and 10 mg/kg) (Fig. 6F), or AdPPAR γ (Fig. 6, D and F) to mice repetitively challenged with TDI significantly reduced the area of peribronchial α -smooth muscle actin immunostaining compared with untreated mice, whereas GW9662 plus rosiglitazone or AdLacZ did not (Fig. 6, E and F).

glitazone (EO+TDI+PIO 1), 5 mg/kg pioglitazone (EO+TDI+PIO 5), or 10 mg/kg pioglitazone (EO+TDI+PIO 10), TDI-inhaled mice administered AdPPAR γ (EO+TDI+AdPPAR γ), TDI-inhaled mice administered 0.5 mg/kg GW9662 plus 5 mg/kg rosiglitazone (EO+TDI+ROSI 5+GW), and TDI-inhaled mice administered AdLacZ (EO+TDI+AdLacZ). B–G, Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ in lung tissues of TDI-sensitized and -challenged mice. Representative H&E-stained sections of the lungs. Sampling was performed at 48 h after the last challenge in vehicle control mice administered saline (B), TDI-inhaled mice administered saline (C), TDI-inhaled mice administered rosiglitazone 5 mg/kg (D), TDI-inhaled mice administered AdPPAR γ (E), and TDI-inhaled mice administered AdLacZ (F). Bars, Scale of 50 μ m. G, Peribronchial and perivascular lung inflammation were measured, and total lung inflammation was defined as the average of the peribronchial and perivascular inflammation scores. H and I, Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on airway responsiveness. Bars, Mean $\pm$ SEM from six mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL; §, $p < 0.05$ vs EO+TDI+ROSI.

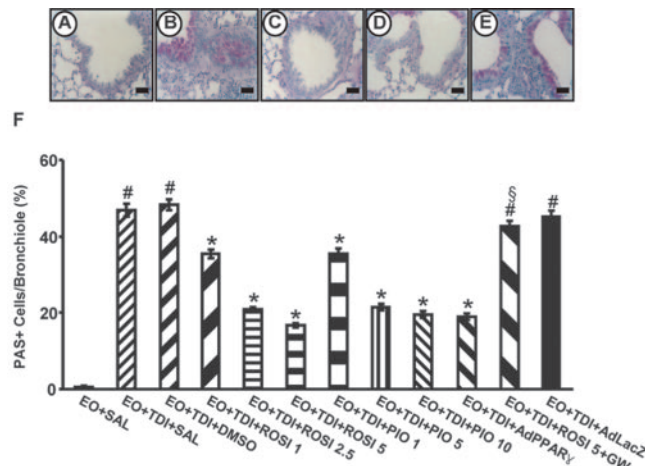


FIGURE 4. Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on airway mucus expression. A–E, Representative PAS-stained sections of the lungs. Sampling was performed at 48 h after the last challenge in vehicle control mice administered saline (A), TDI-inhaled mice administered saline (B), TDI-inhaled mice administered rosiglitazone 5 mg/kg (C), TDI-inhaled mice administered AdPPAR γ (D), and TDI-inhaled mice administered AdLacZ (E). The red color indicates PAS-positive mucus expression. Bars, Scale of 50 μ m. F, Quantitation of airway mucus expression. Bars, Mean $\pm$ SEM from six mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL; §, $p < 0.05$ vs EO+TDI+RSI.

Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on NF- κ B p65 protein levels in lung tissues

Western blot analysis showed that levels of NF- κ B p65 in nuclear protein extracts from lung tissues were increased at 48 h after the last TDI inhalation compared with the levels in the control mice (Fig. 7A). The increased NF- κ B p65 levels in nuclear protein extracts were decreased by the administration of rosiglitazone, pioglitazone, or AdPPAR γ . No significant changes were observed in TDI-sensitized and -challenged mice treated with GW9662 plus rosiglitazone or AdLacZ. In contrast, the levels of NF- κ B p65 protein in cytosol fractions from lung tissues were decreased at 48 h after the last TDI inhalation as compared with the levels in the control mice. The decreased NF- κ B p65 protein levels in cytosol fractions from lung tissues were increased by the administration of rosiglitazone, pioglitazone, or AdPPAR γ . However, no significant changes were observed in TDI-sensitized and -challenged mice treated with GW9662 plus rosiglitazone or AdLacZ.

Determination of Akt phosphorylation in lung tissues of TDI-sensitized and -challenged mice

The levels of p-Akt protein in the lung tissues were increased significantly at 48 h after the last TDI inhalation compared with the levels in the control mice (Fig. 7B). In contrast, no significant changes in total Akt protein levels were observed in any of the groups tested. The increased p-Akt but not Akt protein levels in the lung tissues at 48 h after TDI inhalation were significantly reduced by the administration of rosiglitazone, pioglitazone, or AdPPAR γ . However, no significant changes were observed in TDI-sensitized and -challenged mice treated with GW9662 plus rosiglitazone or AdLacZ.

Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on levels of IL-4, IL-5, and IL-13 expression in lung tissues and BAL fluids

Western blot analysis revealed that IL-4, IL-5, and IL-13 protein levels in lung tissues were significantly increased at 48 h after the

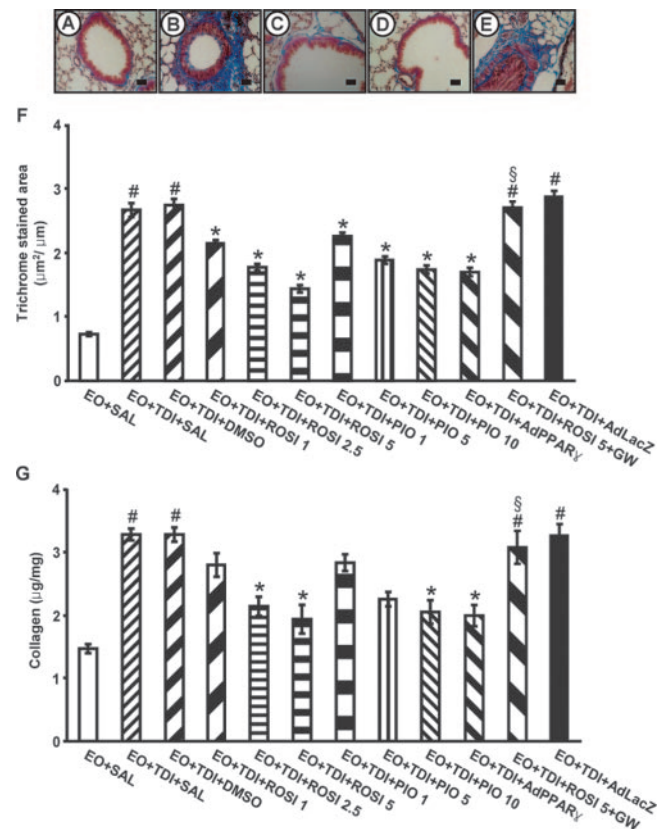


FIGURE 5. Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on peribronchial fibrosis in lung tissues. A–E, Representative peribronchial trichrome-stained sections of the lungs. Sampling was performed at 48 h after the last challenge in vehicle control mice administered saline (A), TDI-inhaled mice administered saline (B), TDI-inhaled mice administered rosiglitazone 5 mg/kg (C), TDI-inhaled mice administered AdPPAR γ (D), and TDI-inhaled mice administered AdLacZ (E). The blue color indicates peribronchial trichrome staining collagen deposition/fibrosis. Bars, Scale of 50 μ m. F, Quantitation of peribronchial fibrosis. G, Total lung collagen content. The amount of lung collagen was measured using a collagen assay kit. Bars, Mean $\pm$ SEM from six mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL; §, $p < 0.05$ vs EO+TDI+RSI.

last TDI inhalation compared with the levels in the control mice (Fig. 8, A and B). The increased levels of these cytokines were significantly reduced by the administration of rosiglitazone, pioglitazone, or AdPPAR γ . No significant changes were observed in TDI-sensitized and -challenged mice treated with GW9662 plus rosiglitazone or AdLacZ. Consistent with the results obtained from the Western blot analysis, enzyme immunoassays showed that levels of IL-4, IL-5, and IL-13 in BAL fluids were significantly increased at 48 h after the last TDI inhalation compared with the levels in the control mice (Fig. 8C). The increased levels of these cytokines were significantly reduced by the administration of rosiglitazone, pioglitazone, or AdPPAR γ .

Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on ICAM-1, VCAM-1, RANTES, and eotaxin expression in lung tissues

Western blot analysis showed that ICAM-1, VCAM-1, RANTES, and eotaxin protein levels in lung tissues were significantly increased at 48 h after the last TDI inhalation compared with the levels in the control mice (Fig. 9). The increased levels of these adhesion molecules and chemokines were significantly reduced by

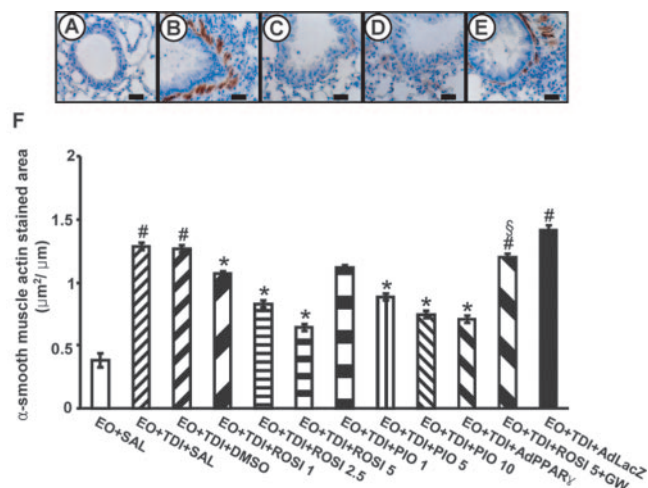


FIGURE 6. Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on peribronchial α -smooth muscle actin expression in lung tissues. *A–E*, Representative immunohistochemical-stained sections for α -smooth muscle actin of the lungs. Sampling was performed at 48 h after the last challenge in vehicle control mice administered saline (*A*), TDI-inhaled mice administered saline (*B*), TDI-inhaled mice administered rosiglitazone 5 mg/kg (*C*), TDI-inhaled mice administered AdPPAR γ (*D*), and TDI-inhaled mice administered AdLacZ (*E*). The red color indicates immunostained peribronchial α -smooth muscle actin expression. Bars, Scale of 50 μm . *F*, The area of peribronchial α -smooth muscle actin immunostaining. Bars, Mean $\pm$ SEM from six mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL; §, $p < 0.05$ vs EO+TDI+ROSI.

the administration of rosiglitazone, pioglitazone, or AdPPAR γ . In contrast, no significant changes were observed in TDI-sensitized and -challenged mice treated with GW9662 plus rosiglitazone or AdLacZ.

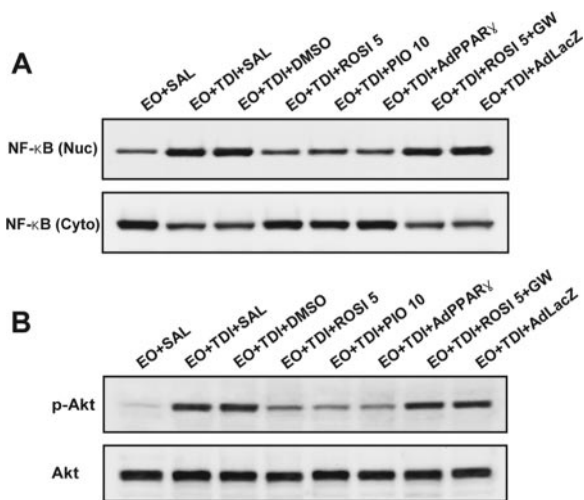


FIGURE 7. *A*, Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on NF- κ B expression in nuclear and cytosolic protein extracts from lung tissues. NF- κ B p65 level in nuclear (Nuc) and cytosolic (Cyto) protein extracts from lung tissues. NF- κ B expression was measured at 48 h after the last challenge. *B*, Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on p-Akt and Akt protein expression in lung tissues of TDI-sensitized and -challenged mice. Results were similar in six mice per group.

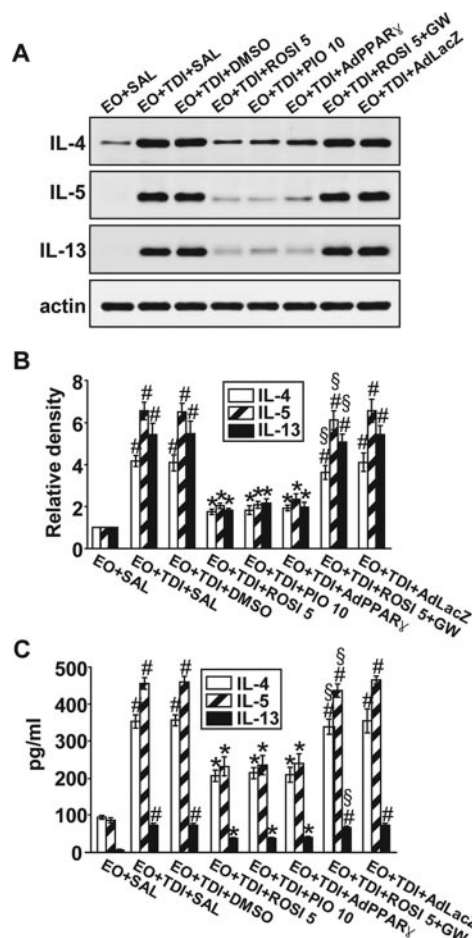


FIGURE 8. Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on IL-4, IL-5, and IL-13 protein expression in lung tissues and in BAL fluids. *A*, Western blotting of IL-4, IL-5, and IL-13. *B*, Densitometric analyses are presented as the relative ratio of each molecule to actin. The relative ratio of each molecule in the lung tissues of EO+SAL is arbitrarily presented as 1. *C*, Enzyme immunoassay of IL-4, IL-5, and IL-13 in BAL fluids. Bars, Mean $\pm$ SEM from six mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL; §, $p < 0.05$ vs EO+TDI+ROSI.

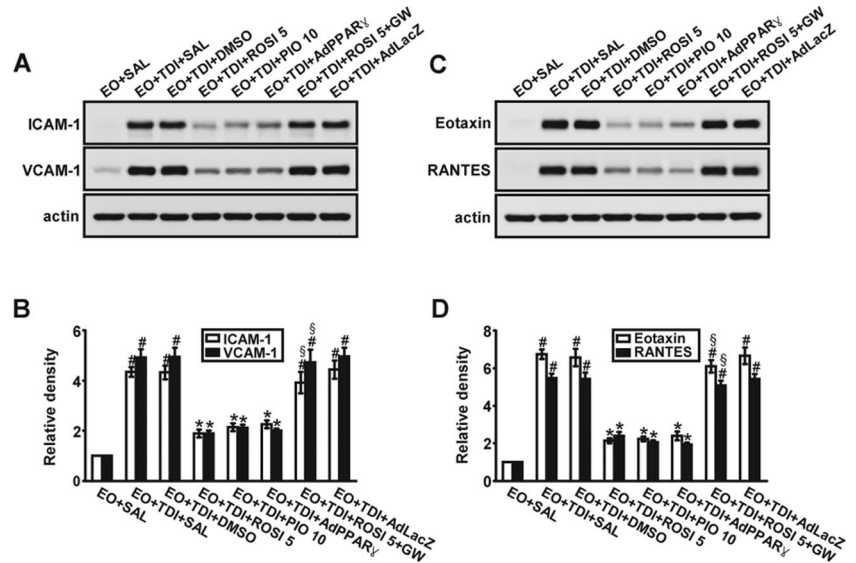
Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on TGF- β 1 expression in lung tissues and BAL fluids

Western blot analysis showed that TGF- β 1 protein levels in lung tissues were significantly increased at 48 h after the last TDI inhalation compared with the levels in the control mice (Fig. 10, *A* and *B*). The increased TGF- β 1 levels were significantly reduced by the administration of rosiglitazone, pioglitazone, or AdPPAR γ . No significant changes were observed in TDI-sensitized and -challenged mice treated with GW9662 plus rosiglitazone or AdLacZ. Consistent with the results obtained from the Western blot analysis, enzyme immunoassays revealed that levels of TGF- β 1 in BAL fluids were significantly increased at 48 h after the last TDI inhalation compared with the levels in the control mice (Fig. 10C). The increased TGF- β 1 levels were significantly reduced by the administration of rosiglitazone, pioglitazone, or AdPPAR γ .

Effect of BAY 11-7085 on Th2 cytokines (IL-4, IL-5, and IL-13), adhesion molecules (ICAM-1 and VCAM-1), chemokines (RANTES and eotaxin), and TGF- β 1 in lung tissues

Western blot analysis showed that IL-4, IL-5, IL-13, ICAM-1, VCAM-1, RANTES, eotaxin, and TGF- β 1 protein levels in the

FIGURE 9. Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on ICAM-1, VCAM-1, RANTES, and eotaxin expression in lung tissues. **A**, Western blotting of ICAM-1 and VCAM-1. **C**, Western blotting of RANTES and eotaxin. **B** and **D**, Densitometric analyses are presented as the relative ratio of each molecule to actin. Bars, Mean $\pm$ SEM from six mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL; §, $p < 0.05$ vs EO+TDI+ROSI.



lung tissues were increased significantly at 48 h after the last TDI inhalation compared with the levels in the control mice (Fig. 11). The increased levels of these molecules were significantly reduced by the administration of BAY 11-7085.

Discussion

TDI is currently one of the most common causes of occupational asthma. Although considerable controversy remains regarding its pathogenesis, TDI-induced asthma is characterized by inflammation and remodeling of the airways. PPAR γ has been shown to play a critical role in the control of airway inflammatory responses. This study with the TDI-induced model of asthma has revealed the following typical pathophysiological features in the lungs: increase in numbers of inflammatory cells of the airways, airway responsiveness, levels of Th2 cytokines (IL-4, IL-5, and IL-13), adhesion molecules (ICAM-1 and VCAM-1), chemokines (RANTES and eotaxin), TGF- β 1, and NF- κ B in nuclear protein extracts. In addition, the mice exposed to TDI developed features of airway remodeling, including thickening of the peribronchial smooth muscle layer, subepithelial collagen deposition, and increased airway mucus production. Administration of PPAR γ agonists or AdPPAR γ reduced the pathophysiological symptoms of asthma and decreased the increased levels of Th2 cytokines, adhesion molecules, chemokines, TGF- β 1, and NF- κ B in nuclear protein extracts after TDI inhalation. In addition, administration of NF- κ B inhibitor decreased the increased levels of Th2 cytokines, adhesion molecules, chemokines, and TGF- β 1 after TDI inhalation. These findings suggest that PPAR γ reduces airway inflammation, airway hyperresponsiveness, and airway remodeling.

Previous reports have demonstrated that activation of PPAR γ inhibits expression of various cytokines, airway hyperresponsiveness, airway remodeling, and activation of inflammatory cells which are increased by induction of asthma (17, 28). Consistent with these observations, our results have shown that administration of the PPAR γ agonists or AdPPAR γ substantially reduced expression of cytokines, airway hyperresponsiveness, airway inflammation, and airway remodeling in a murine model of occupational asthma. In addition, induction of asthma through TDI-challenge increased expression of PPAR γ itself, and administration of the agonists and AdPPAR γ further increased the receptor expression. Up-regulation of PPAR γ expression is also observed in human asthmatic airways (16). These findings indicate that regulation of PPAR γ expression may play a protective role in the induction and maintenance of TDI-induced asthma.

Intriguingly, our data have shown that the administration of AdPPAR γ without ligands resulted in the constitutive PPAR γ activation in TDI-induced asthma. Several studies have revealed that

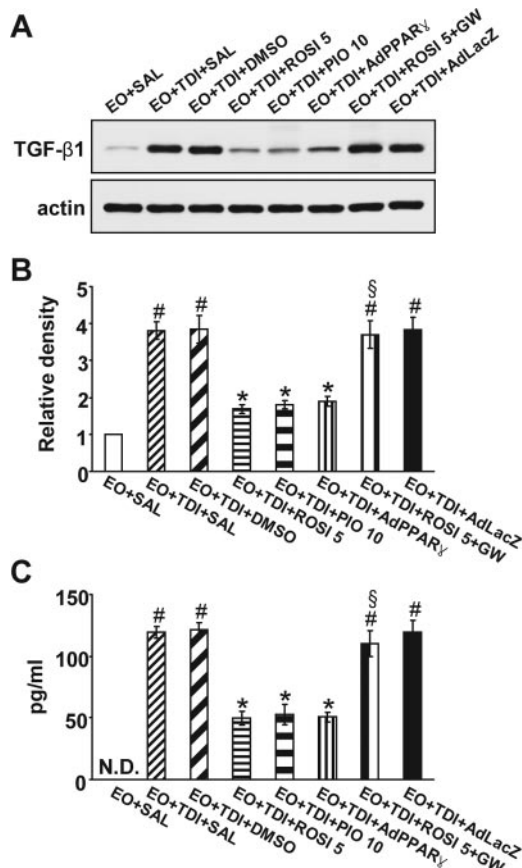
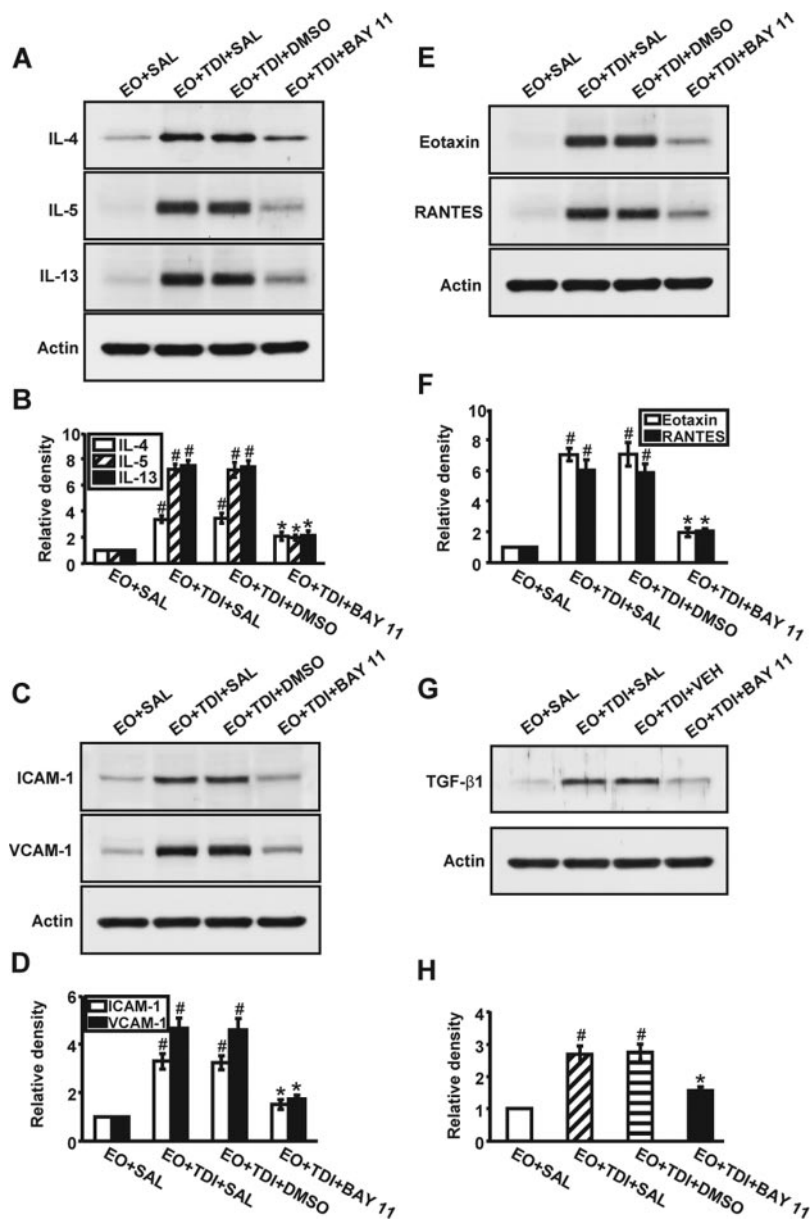


FIGURE 10. Effect of rosiglitazone, pioglitazone, GW9662 plus rosiglitazone, or AdPPAR γ on TGF- β 1 expression in lung tissues and in BAL fluids. **A**, Western blotting of TGF- β 1. **B**, Densitometric analyses are presented as the relative ratio of TGF- β 1 to actin. **C**, Enzyme immunoassay of TGF- β 1 in BAL fluids. Bars, Mean $\pm$ SEM from six mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL; §, $p < 0.05$ vs EO+TDI+ROSI; ND, not detected.

FIGURE 11. Effect of BAY 11-7085 on Th2 cytokines, adhesion molecules, chemokines, and TGF- β 1 protein expression. *A*, Western blotting of IL-4, IL-5, and IL-13. Sampling was performed at 48 h after the last challenge in vehicle control mice administered saline (EO+SAL), TDI-inhaled mice administered saline (EO+TDI+SAL), TDI-inhaled mice administered drug vehicle (0.05% DMSO) (EO+TDI+DMSO), and TDI-inhaled mice administered BAY 11-7085 (EO+TDI+BAY 11). *C*, Western blotting of ICAM-1 and VCAM-1. *E*, Western blotting of RANTES and eotaxin. *G*, Western blotting of TGF- β 1. *B*, *D*, *F*, and *H*, Densitometric analyses are presented as the relative ratio of each molecule to actin. Bars, Mean $\pm$ SEM from six mice per group. #, $p < 0.05$ vs EO+SAL; *, $p < 0.05$ vs EO+TDI+SAL.



a range of substances produced by induction of asthma, such as 15-hydroxyicosatetraenoic acid or IL-4, can activate PPAR γ and enhance its expression (13, 29–32). In addition, PPAR γ 2 used in this study possesses ligand-dependent and considerable ligand-independent transactivation potential (33–35). Therefore, we suggest that the stimulation for AdPPAR γ by the increased several substances and cytokines produced in the airways of asthmatics and the ligand-independent activation of PPAR γ 2 could be possible mechanisms for the constitutive PPAR γ activation by AdPPAR γ without ligands in our present asthmatic lungs.

Previous studies have demonstrated that the molecular mechanisms of PPAR-dependent anti-inflammatory responses are based on 1) the interaction of PPARs with various transcription factors stimulating inflammation, such as NF- κ B, AP-1, C/EBP, STAT, and NF-AT, 2) the formation of complexes between PPARs and transcriptional coactivators and corepressors, and 3) the ability of PPARs to modulate the activity of different kinases involved in various proinflammatory pathways (36–45). NF- κ B plays a critical role in immune and inflammatory responses, including asthma (46–53). In addition, several studies have also shown that PI3K

activation enhances NF- κ B signaling through production of phosphatidylinositol 3,4,5-triphosphate which leads to the stimulation of several downstream targets, including the serine/threonine protein kinase Akt (54–57). Determination of NF- κ B protein levels in nuclear extracts and p-Akt protein levels in the lung tissues has revealed that these protein levels were substantially increased in our present TDI-induced model of asthma, suggesting that NF- κ B and PI3K are activated. It is known that activation of this transcription factor induces a variety of inflammatory genes that are abnormally expressed in asthma. These genes include cytokines, chemokines, growth factor, and adhesion molecules (58, 59). We have also assessed whether these genes are up-regulated in the TDI-induced asthma model. As expected, expression of Th2 cytokines, adhesion molecules, chemokines, and TGF- β 1 was significantly increased after TDI challenge. Administration of rosiglitazone, pioglitazone, or AdPPAR γ resulted in significant reduction in NF- κ B activity as well as in expression of these Th2 cytokines, adhesion molecules, chemokines, and TGF- β 1. We have also shown that the increased p-Akt but not Akt protein levels in lung tissues after TDI inhalation were significantly reduced by the

administration of rosiglitazone, pioglitazone, or AdPPAR γ . In addition, we have demonstrated that blocking of NF- κ B activation by BAY 11-7085 decreased the increased levels of Th2 cytokines, adhesion molecules, chemokines, and TGF- β 1 after TDI inhalation in our murine model. These findings suggest that a protective role of PPAR γ in the pathogenesis of the TDI-induced asthma is at least mediated through an NF- κ B-dependent mechanism.

IL-4, IL-5, and IL-13 are cytokines produced primarily by activated Th2 cells and promote airway inflammation, mucus metaplasia, subepithelial fibrosis, airway obstruction, and airway hyperresponsiveness (60–63). TGF- β is a profibrotic cytokine, and the TGF- β isoforms are implicated in the extracellular matrix changes observed in fibrosis. In vitro, TGF- β has been shown to secrete a number of extracellular matrix proteins, including collagen types I and III, fibronectin, tenascin, and proteoglycans via stimulation of fibroblasts (64–70). In addition, expression of TGF- β is increased in the airways of patients with asthma and seems to correlate with disease severity and degree of subepithelial fibrosis (71). Consistent with these previous findings, in the present TDI-induced model of asthma, our results have shown that expression of cytokines and growth factor was increased after TDI challenge. Administration of PPAR γ agonists or AdPPAR γ to TDI-induced mice decreased the increased expression of Th2 cytokines and TGF- β 1.

In conclusion, our results have demonstrated that PPAR γ agonists or AdPPAR γ reversed all pathophysiological symptoms of TDI-induced asthma examined and reduced the increased levels of various Th2 cytokines, adhesion molecules, chemokines, and TGF- β 1 in TDI-induced asthma. Hence, the PPAR γ agonist may have therapeutic potential for the treatment of occupational asthma.

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Disclosures

The authors have no financial conflict of interest.

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