# PPARγ Ligand Ciglitazone Inhibits TNFα -induced ICAM-1 in Human Airway Smooth Muscle Cells

Chien-Da Huang*[1], Te-Chih Hsiung*[2], Shu-Chuan Ho[1,3], Kang-Yun Lee[1], Yao-Fei Chan[1], Li-Wei Kuo[1], Shu-Min Lin[1], Chun-Hua Wang[1], Horng-Chyuan Lin[1], Han-Pin Kuo[1]

**Background:** Modification of human airway smooth muscle (ASM) function by proinflammatory cytokines has been regarded as a potential mechanism underlying bronchial hyperresponsiveness in asthma. Human ASM cells express intercellular adhesion molecule (ICAM)-1 in response to cytokines. Synthetic ligands for peroxisome proliferator-activated receptor (PPAR)γ reportedly possess anti-inflammatory and immunomodulatory properties. In this study, we examined whether ciglitazone, a synthetic PPARγ ligand, can modulate the basal and tumor necrosis factor (TNF) α-induced *ICAM1* gene expression in human ASM cells.

**Methods:** Human ASM cells were treated with TNFα. ICAM-1 expression was assessed by flow cytometry and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. PPARγ activity was inhibited by target-specific small interfering (si) RNA targeting PPARγ and GW9662, a PPARγ antagonist. Activity of nuclear factor (NF)-κB was assessed by using immunoblot analysis, immune-confocal images, and electrophoretic mobility shift assay (EMSA).

**Results:** By flow cytometry, ciglitazone alone had no effect on ICAM-1 expression in ASM cells, but inhibited ICAM-1 expression in response to TNFα (10 ng/ml) in a dose-dependent manner (1–10 μM). It also inhibited TNFα-induced *ICAM1* gene expression by RT-PCR analysis. Knockdown of *PPARγ* gene by target-specific siRNA targeting *PPARγ* enhanced ICAM-1 expression and the inhibitory effect of ciglitazone on TNFα-induced ICAM-1 expression was reversed by PPARγ siRNA and GW9662. SN-50 (10 μg/ml), an inhibitor for nuclear translocation of NF-κB, inhibited TNFα-induced ICAM-1 expression. Ciglitazone did not prevent TNFα-induced degradation of the cytosolic inhibitor of NF-κB (IκB), but inhibited the nuclear translocation of p65 induced by TNFα and suppressed the NF-κB/DNA binding activity.

**Conclusion:** These findings suggest that ciglitazone inhibits TNFα-induced *ICAM1* gene expression in human ASM cells through the ligand-dependent PPARγ activation and NF-κB–dependent pathway. *(Biomed J 2014;37:191-198)*

**Key words: airway inflammation, airway smooth muscle, ICAM-1, NF-κB, PPARγ**

**At a Glance Commentary**

**Scientific background of the subject**

Asthma is a chronic inflammatory disease of the airways, characterized by reversible airflow obstruction and hyperresponsiveness attributed to inflammation. Modification of human airway smooth muscle (ASM) function by proinflammatory cytokines has been regarded as a potential mechanism underlying bronchial hyperresponsiveness in asthma. Synthetic ligands for peroxisome proliferator-activated receptor (PPAR)γ reportedly possess anti-inflammatory and immunomodulatory properties.

**What this study adds to the field**

These findings suggest that ciglitazone, a synthetic PPARγ ligand, inhibits TNFα-induced *ICAM1* gene expression in human ASM cells through the ligand-dependent PPARγ activation and NF-κB–dependent pathway.

*Chien-Da Huang and Te-Chih Hsiung contributed equally as first authors.

From the [1]Department of Thoracic Medicine, Chang Gung Memorial Hospital at Linkou, Chang Gung University College of Medicine, Taoyuan, Taiwan; [2]Department of Thoracic Medicine, St. Paul's Hospital, Taoyuan, Taiwan; [3]School of Respiratory Therapy, College of Medicine, Taipei Medical University, Taipei, Taiwan

Received: Jun. 04, 2013; Accepted: Aug. 20, 2013

Correspondence to: Dr. Chien-Da Huang, Department of Thoracic Medicine, Chang Gung Memorial Hospital at Linkou. 5 Fusing St., Gueishan, Taoyuan 333, Taiwan (R.O.C.). Tel: 886-3-3281200 ext. 8467; Fax: 886-3-3272474; E-mail: cdhuang@adm.cgmh.org.tw

**DOI:** 10.4103/2319-4170.132890

Asthma is a chronic inflammatory disease of the airways, characterized by reversible airflow obstruction and hyperresponsiveness attributed to inflammation. Modification of human airway smooth muscle (ASM) function by proinflammatory cytokines has been regarded as a potential mechanism underlying bronchial hyperresponsiveness in asthma. The role of ASM in asthma has been conventionally thought of as a passive tissue-regulating bronchoconstriction. However, ASM not only modulates bronchomotor tone but also plays an important immunomodulatory role in orchestrating and perpetuating airway inflammation in asthma.[1,2] Increased levels of intercellular adhesion molecule (ICAM)-1 have been detected in asthmatic patients after allergen exposure[3,4] or during asthma attacks.[5] ASM cells express ICAM-1 and vascular cell adhesion molecule (VCAM)-1 constitutively, which are up-regulated by cytokines such as tumor necrosis factor (TNF)α.[6,7] The induction of ICAM-1 expression requires *de novo* protein and mRNA synthesis, and *ICAM1* gene expression is predominantly transcriptionally regulated by nuclear factor (NF)-κB and activator protein (AP)-1.[8,9] Proinflammatory cytokines up-regulate ASM expression of ICAM-1 and VCAM-1, which promotes lymphocyte adhesion and induces smooth muscle cell deoxyribonucleic acid (DNA) synthesis.[6] Wegner *et al*,[10] had demonstrated that antibodies against ICAM-1, a counter-receptor for lymphocyte function associated antigen (LFA)-1, decreased eosinophil infiltration, and attenuated bronchial hyperresponsiveness in a primate model of asthma.

Even though there are potent anti-inflammatory drugs available for treatment, such as glucocorticoids and anti-leukotrienes, these drugs exhibit limited efficacy in treating certain subgroups of asthma.[11,12] Thus, the search for novel drug targets leading to novel therapies for airway inflammation is important. Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor superfamily of steroids, retinoids, thyroid hormones, and vitamin D.[13] Three different PPAR subtypes have been identified to date, including α, β, and γ. PPARγ is activated by several ligands, including 15-deoxy-delta 12, 14-prostaglandin J2 ($15dPGJ_2$, a natural ligand) and thiazolidinedione (TZD) derivatives (such as ciglitazone, pioglitazone, and rosiglitazone).[14] PPARγ is highly expressed in adipose tissue and plays a key role in differentiating adipose, lipid metabolism, and glucose homeostasis,[15] and is also expressed in a number of cell types, including ASM cells,[16] epithelial cells,[17] colonic epithelial cells,[18] and leukocytes.[19] Previous studies suggest that PPARγ may be involved in modulating the expression of various cytokines by activated macrophages, airway epithelial cells, and ASM cells via inhibition of transcription factors such as NF-κB, signal transducers and activators of transcription (STAT), and AP-1.[16,19,20]

Agents that modulate regulatory mechanisms for ICAM-1 expression on human ASM cells may become new therapeutic approaches in treating airway inflammation in chronic airway diseases such as asthma and chronic obstructive pulmonary disease (COPD). This study examined the transcription regulation of ciglitazone, a synthetic PPARγ ligand, on basal and TNFα-induced *ICAM1* gene expression, as well as the implicated signal pathway in human ASM cells.

## METHODS

### Human ASM cell culture

Human ASM cells were obtained from Clonetics (San Diego, CA, USA) and prepared according to the manufacturer's instructions. They were maintained in Falcon culture flasks and incubated (37° C; 5% CO2) until monolayer confluence was reached, as described previously.[21] ASM cells in the subculture from the fifth to the seventh cell passages were studied.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to measure the viability of ASM cells. Apoptosis/cell death did not occur with ciglitazone.

### Flow cytometry analysis for ICAM-1 expression

ASM cells were pre-treated with ciglitazone (10 μM) and SN-50 (10 μg/ml) for 30 min before TNFα (10 ng/ml) was added for another 4 h [Figures 1 and 2]. SN-50,

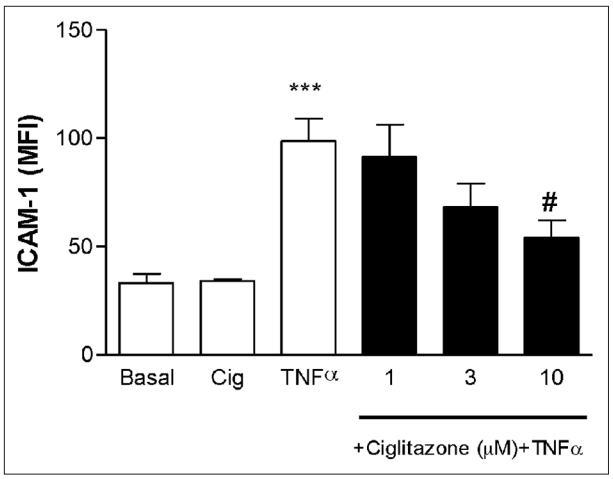


**Figure 1:** Ciglitazone inhibited TNFα-induced ICAM-1 expression. Ciglitazone (10 μM) exerted an inhibitory effect on TNF -induced ICAM-1 expression when compared to TNFα (10 ng/ml) alone. ICAM-1 expression was increased after the cells were exposed to TNFα (10 ng/ml) for 4 h compared to basal level ($n = 8$, $p < 0.001$). Ciglitazone inhibited the induction of ICAM-1 in a dose-dependent manner, significantly at concentrations of 10 μM ($n = 5$, $p < 0.001$). Results are expressed as mean ± SEM ($n$ = 3-8) [Cig, ciglitazone (10 μM); ***$p < 0.001$ compared to untreated cells (basal); #$p < 0.05$ compared to TNFα alone].

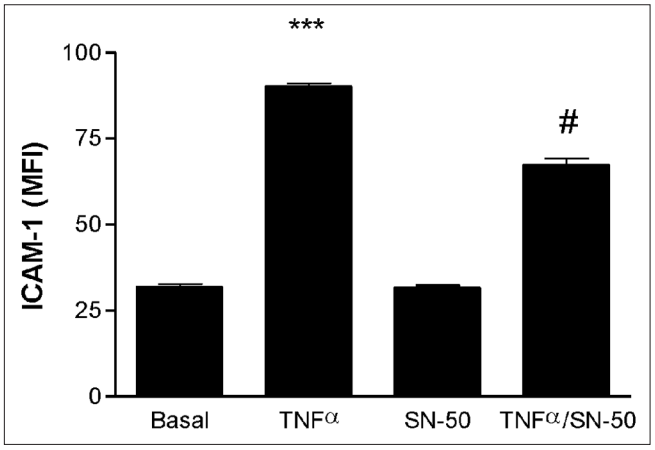


**Figure 2:** ICAM-1 expression was regulated by NF-κB. SN-50 (10 μg/ml) significantly reduced 10 ng/ml TNFα-induced ICAM-1 expression (by 25%) on ASM cells. The levels decreased from 90.3 ± 1.5 to 68.8 ± 2.0 MFI ($n = 3$, $p < 0.05$). Results are expressed as mean ± SEM of two to three separate experiments (***$p < 0.001$ compared to untreated cells; #$p < 0.05$ compared to TNFα alone).

a cell-permeable peptide carrying a functional cargo representing the nuclear localization sequence of NF-κB p50, was used to inhibit nuclear translocation of NF-κB. Flow cytometry was performed with slight modifications for ICAM-1 expression.[7] Following incubation with monoclonal mouse anti-ICAM-1 Ab (1:100 dilution, M7063, Dako, Glostrup, Denmark) for 1 h at 4ºC, the ASM cells were centrifuged and re-suspended in cold phosphate-buffered saline (PBS) in microfuge tubes. Subsequently, Fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (1:50 dilution, F0313, Dako, Demark) was incubated for 30 min at 4ºC in the dark. ICAM-1 expression was measured by a FACScan flow cytometer equipped with an argon ion laser (Becton Dickinson, Mountain View, CA, USA) and expressed as the mean fluorescence intensity (MFI).

## RNA isolation and reverse transcriptase-polymerase chain reaction for ICAM-1 mRNA

ASM cells were pre-treated with ciglitazone (10 μM) [Figure 3] and GW9662 (2 μM) [Figure 4C] for 30 min before TNFα (10 ng/ml) was added for another 2 h. Total RNA was isolated from the ASM cells using Trizol Reagent (Life Technologies, Rockville, MD, USA) according to the manufacturer's instructions. The cDNA was reverse transcribed from the isolated RNA using established techniques. Polymerase chain reactions (PCR) were performed with a specific primer for ICAM-1 (obtained from R and D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. GAPDH PCR primer was synthesized by Gibco-BRL (Gaithersburg, MD, U.S.A.).

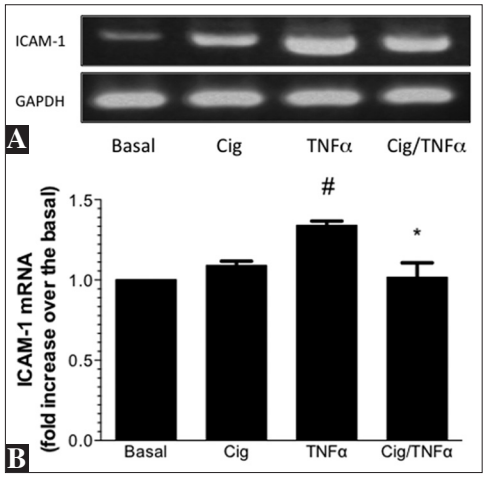


**Figure 3:** Ciglitazone inhibited TNFα-induced ICAM-1 mRNA. (A) ICAM-1 mRNA expression increased 2 h after addition of 10 ng/ml TNFα (1.34-fold increase, $n = 4$, $p < 0.05$), and TNFα induction of ICAM-1 mRNA was inhibited by 10 μM ciglitazone ($n = 4$, $p < 0.05$). Ciglitazone alone had no effect on ICAM-1 expression. The images are representative of four independent experiments with similar results. (B) Bar graphs show quantitative analysis of scanning densitometric values of ICAM-1 mRNA with each value normalized over the mean density of the corresponding GAPDH PCR products. Data are expressed as fold increase (mean ± SEM) over the basal and are representative of three independent experiments [Cig, ciglitazone (10 μM); #$p < 0.05$ compared to untreated cells (basal); *$p < 0.05$ compared to TNFα alone].

## PPARγ-specific small interfering RNA and transfection of ASM cells

PPARγ small interfering RNA (siRNA), a target-specific 20-25 nt siRNA, was designed to knock down gene expression (sc-29455, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Control siRNA (sc-36869, Santa Cruz Biotechnology, Inc.), a non-targeting 20-25 nt siRNA, was designed as a transfection control. Transfection of PPARγ siRNA was conducted with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 6-well plates according to the manufacturer's instruction. The ASM cells were incubated for another 24, 48, and 72 h without replacing the medium. Proof of transient PPARγ knockdown was confirmed by showing decrease of PPARγ protein by western blot analysis at 24 h [Figure 4A]. Pre-transfection of PPARγ siRNA and control siRNA was performed for 24 h before TNFα (10 ng/ml) was added for another 4 h [Figure 4B].

## SDS-polyacrylamide gel electrophoresis and western blot analysis for the cytoplasmic inhibitor of NF-κB

ASM cells were pre-treated with or without ciglitazone (10 μM) for 30 min before TNFα (10 ng/ml) was

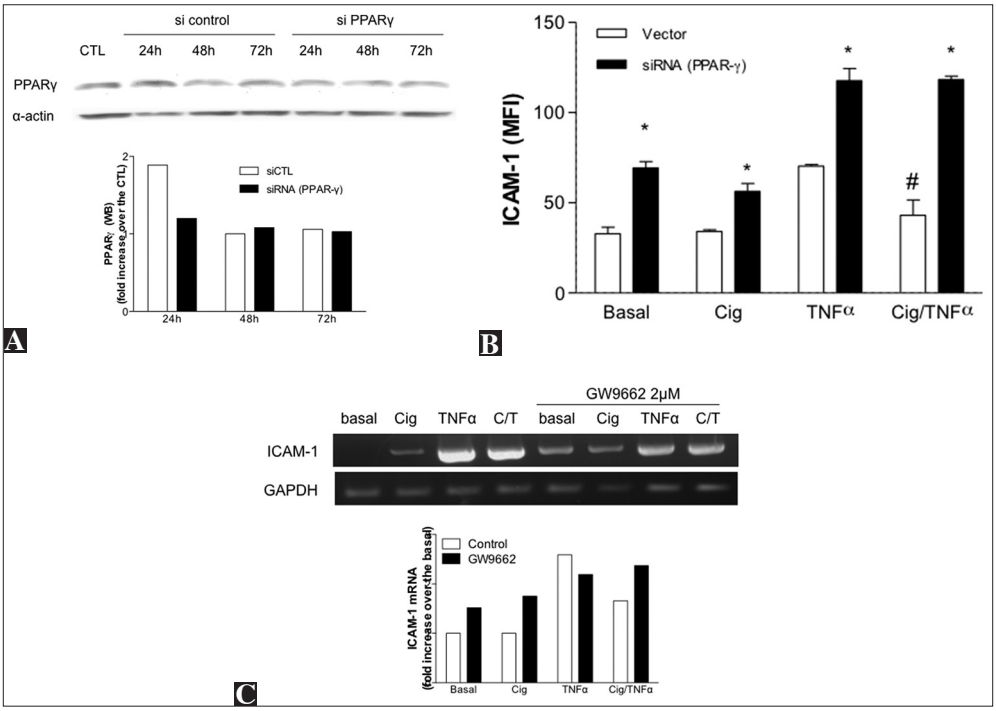


**Figure 4:** The PPARγ-dependent pathway was involved in the modulation of ciglitazone on ICAM-1 expression. (A) Proof of PPARγ knockdown was confirmed by showing decrease of PPARγ protein by western blot analysis at 24 h (CTL, control). (B) PPARγ-specific siRNA alone enhanced basal and 10 μM ciglitazone-treated ICAM-1 expression compared with that of control siRNA ($n = 3$, $p < 0.05$). Furthermore, PPARγ-specific siRNA alone augmented 10 ng/ml TNF$\alpha$-induced ICAM-1 expression ($n = 3$, $p < 0.05$) and reversed the inhibitory effect of ciglitazone on TNF$\alpha$-induced ICAM-1 expression ($n = 3$, $p > 0.05$). Results are expressed as mean ± SEM of three separate experiments [Cig, ciglitazone (10 μM); *$p < 0.05$ compared to corresponding control siRNA; #$p < 0.05$ compared to cells treated with TNF$\alpha$]. (C) GW9662 (2 μM), a PPAR antagonist, also reversed the inhibitory effect of ciglitazone on TNF$\alpha$-induced ICAM-1 mRNA expression. Data are representative of two independent experiments [Cig, ciglitazone (10 μM); C/T, ciglitazone (10 μM)/TNF (10 ng/ml)].

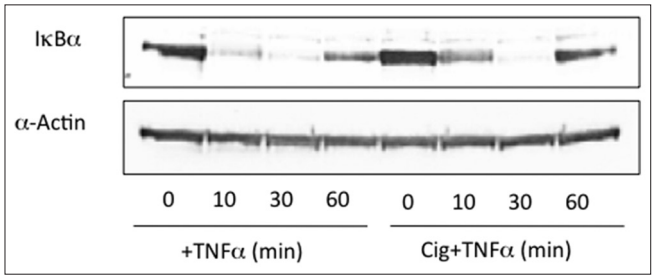


**Figure 5:** TNF$\alpha$ enhanced IκB degradation maximally at 30 min. Ciglitazone (10 μM) did not prevent TNF$\alpha$-induced IκB degradation compared to TNF$\alpha$ (10 ng/ml) alone. Three identical experiments performed independently gave similar results.

added for another 0–1 h [Figure 5]. Immunoblot analysis for the cytosolic inhibitor of NF-κB (IκB) was performed as described previously.[7,22,23] Immunoblot was performed with rabbit polyclonal antibodies against IκB (New England Biolabs, Beverly, MA, U.S.A.) or PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were visualized using anti-rabbit IgG (Sigma Chemical, St. Louis, MO, USA) and enhanced chemiluminescence solution (Amersham Life Science, Piscataway, NJ, USA) for 1 min at room temperature and exposed on KODAK XAR film. To ensure equal loading, the membranes were stripped and re-probed with anti-α-actin antibody.

## Immuno-confocal study for p65 nuclear translocation

ASM cells were pre-treated with or without ciglitazone (10 μM) for 30 min before TNFα (10 ng/ml) was added for another 1 h [Figure 6]. ASM cells on 25-mm cover slips were fixed in methanol at 20 C for 5 min and then washed three times in PBS. They were blocked in PBS containing 2% bovine serum albumin (BSA), 0.1% Triton X-100, and fetal bovine serum (FBS) at room temperature for 30 min. Cells were exposed to rabbit polyclonal anti-p65 antibody (Upstate, Millipore, Temecula, CA, USA) for 60 min, washed three times in PBS, and incubated for 60 min at room temperature with secondary antibodies diluted in blocker, including Cy3 goat anti-rabbit IgG (Chemicon, Temecula, CA, USA). Cells were incubated in Hoechst dye (0.5 μg/ml) staining solution for 5 min and washed in PBS before they were mounted in anti-fade mounting solution. The fluorescence-labeled slides were then examined with a Leica TCS 4D confocal laser scanning microscopy system as previously described.[24]

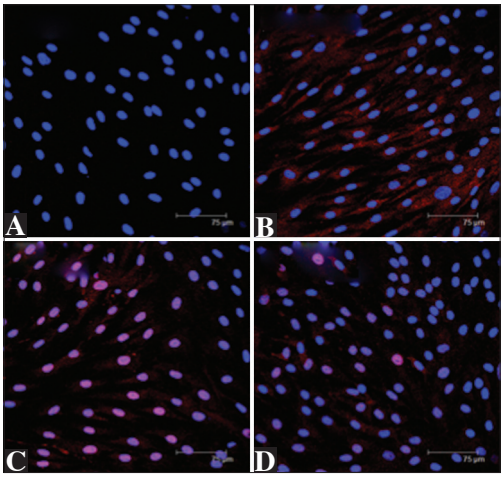


**Figure 6:** Ciglitazone attenuated TNF$\alpha$-induced p65 nuclear translocation at 60 min using confocal imaging studies. Simultaneous immunocytochemistry for p65 (red) and nuclei (blue) in ASM cells, and confocal images of (A) IgG control, (B) basal, (C) TNF$\alpha$ (10 ng/ml) for 1 h, and (d) those pre-treated with ciglitazone (10 μM) for 30 min before TNF$\alpha$ (10 ng/ml) was added for another 1 h. The merged pictures show double immunostaining of p65 and the nucleus.

### Electrophoretic mobility shift assay for DNA binding activity of NF-κB

In the presence of TNFα (10 ng/ml) for 10-60 min, NF-κB activation of ASM cells was examined by electrophoretic mobility shift assay (EMSA). ASM cells were pre-treated with or without ciglitazone (10 μM) for 30 min before TNFα (10 ng/ml) was added for another 1 h. The fluorescence EMSA method using digoxigenin 3′ end-labeled oligonucleotides with DIG-11-ddUTP (DIG Gel Shift Kit, Boehringer Mannheim GmbH, Mannheim, Germany) was applied as described previously.[23] The NF-κB oligonucleotide containing DNA binding sites for NF-κB transcription factors was obtained from Promega (Madison, WI, USA), and 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (Promega) was the oligonucleotide sequence used. The nuclear extract (10 μg) was incubated with digoxigenin-labeled NF-κB probe in binding buffer (Boehringer Mannheim GmbH, Mannheim, Germany). DNA/nuclear protein complexes were separated from the DNA probe by polyacrylamide gel electrophoresis, and then the gel was vacuum-dried and auto-radiographed.

### Statistical analysis

Data were expressed as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to compare the mean values of more than two experimental groups. If there was variance among groups, the Bonferroni test was used to determine significant differences between specific points within groups. Student's *t*-test was used to analyze paired or unpaired data. A *p* value less than 0.05 was considered statistically significant.

## RESULTS

### Ciglitazone inhibited TNFα-induced ICAM-1 expression in human ASM cells

Ciglitazone exerted an inhibitory effect on TNFα-induced ICAM-1 expression compared to TNFα alone [Figure 1]. The level of ICAM-1 expression increased after the cells had been exposed to TNFα for 4 h (98.6 ± 10.5 MFI, $n$ = 8, $p < 0.001$) compared with basal level (33.1 ± 4.3 MFI, $n = 8$). The induction of ICAM-1 was inhibited by ciglitazone in a dose-dependent manner, significantly at a concentration of 10 μM (54.0 ± 8.1 MFI, $n = 5$, $p < 0.05$). However, ciglitazone alone had no effect on basal ICAM-1 expression. ICAM-1 mRNA expression increased after the cells were exposed to TNFα (10 ng/ml) for 2 h (1.34-fold increase, $n = 4$, $p < 0.05$), and TNFα induction of ICAM-1 mRNA was inhibited by 10 μM ciglitazone ($n = 4$, $p < 0.05$) [Figure 3]. Ciglitazone alone had no effect on ICAM-1 mRNA expression.

### Ligand-dependent PPARγ activation mediated inhibitory effect of ciglitazone on ICAM-1 expression

PPARγ siRNA, a target-specific 20-25 nt siRNA, was introduced to knock down gene expression. Proof of PPARγ knockdown was confirmed by the decrease in PPARγ protein by western blot analysis at 24 h [Figure 4A]. PPARγ siRNA enhanced basal and ciglitazone-treated ICAM-1 expression compared to that of control siRNA, from 32.8 ± 3.7 to 69.3 ± 3.5 MFI and from 34.0 ± 1.0 to 56.3 ± 4.3 MFI, respectively ($n$ = 3, $p < 0.05$) [Figure 4B]. Furthermore, PPARγ-specific siRNA alone augmented TNFα-induced ICAM-1 expression from 69.7 ± 0.3 to 117.7 ± 6.7 MFI ($n = 3$, $p < 0.05$) and reversed the inhibitory effect of ciglitazone on TNFα-induced ICAM-1 expression (117.7 ± 6.7 vs. 118.3 ± 1.9 MFI, $n$ = 3, $p > 0.05$) [Figure 4B]. GW9662 (2 μM, Cayman Chemicals, Ann Arbor, MI, USA), a PPAR antagonist, also reversed the inhibitory effect of ciglitazone on TNFα-induced ICAM-1 mRNA expression [Figure 4C].

### Ciglitazone inhibited TNFα-induced ICAM-1 in a NF-κB–dependent pathway

SN-50 (10 μg/ml) significantly reduced 10 ng/ml TNFα-induced ICAM-1 expression (by 25%) in ASM cells, with levels decreasing from 90.2 ± 0.9 to 67.5 ± 1.7 MFI ($n = 3$, $p < 0.05$) [Figure 2]. TNFα (10 ng/ml) enhanced IκB degradation maximally at 30 min, which regenerated after 60 min [Figure 5]. However, ciglitazone (10 μM) did not prevent TNFα-induced IκB degradation, compared to TNFα alone. Ciglitazone (10 μM) attenuated TNFa-induced p65 nuclear translocation at 60 min as observed in confocal images [Figure 6]. EMSA showed that the binding activity

of NF-κB on the DNA binding site increased from 10 to 60 min after stimulation with TNFα (10 ng/ml) [Figure 7A]. Ciglitazone (10 μM) attenuated TNFα-induced NF-κB activation at 60 min [Figure 7B].

## DISCUSSION

The present study demonstrates that PPARγ activation attenuates TNFα-enhanced ICAM-1 expression in human ASM cells. Ciglitazone, a synthetic PPARγ ligand, inhibited TNFα-stimulated, but not basal, ICAM-1 expression. Knocking down PPARγ by PPARγ siRNA and PPARγ antagonist reversed the inhibitory effect of ciglitazone on TNFα-enhanced ICAM-1 expression. Ciglitazone did not prevent TNFα-induced degradation of cytosolic IκB, but did suppress p65 nuclear translocation and NF-κB/DNA binding activity. Taken together, this study suggests that ciglitazone inhibits TNFα-induced *ICAM1* gene expression in human ASM cells through ligand-dependent PPARγ activation and NF-κB–dependent pathway.

PPARγ expression, originally described in adipocytes, has an essential role in adipogenesis and glucose homeostasis.[15] However, some studies demonstrate its presence in the structural cells of the lungs and in inflammatory cells.[16,25] PPARγ ligands also inhibit the release of proinflammatory cytokines and matrix metalloproteinase (MMP)-9 from airway epithelial cells.[20,26] In a mouse model, PPARγ activation is shown to be expressed in the airways and inhibited features of airway remodeling.[27] PPARγ also modulated airway remodeling and inflammation in a murine model of toluene diisocyanate-induced asthma.[28] PPARγ activation decreased antigen-induced airway hyperresponsiveness, lung inflammation, eosinophilia, cytokine production, and GATA-3 expression, as well as serum levels of antigen-specific IgE in a murine model of human asthma.[29] Using a murine model of allergic asthma, pioglitazone was shown to be as effective as dexamethasone in cockroach allergen-induced asthma.[30] The administration of ciglitazone intranasally significantly inhibits the development of airway hyperresponsiveness, eosinophilic inflammation, and importantly, ASM remodeling in mice chronically exposed to ovalbumin.[31] Our data are consistent with a similar conclusion that the anti-inflammatory actions of PPARγ agonists could possibly be explained in part by their suppressive effect on ICAM-1 expression.

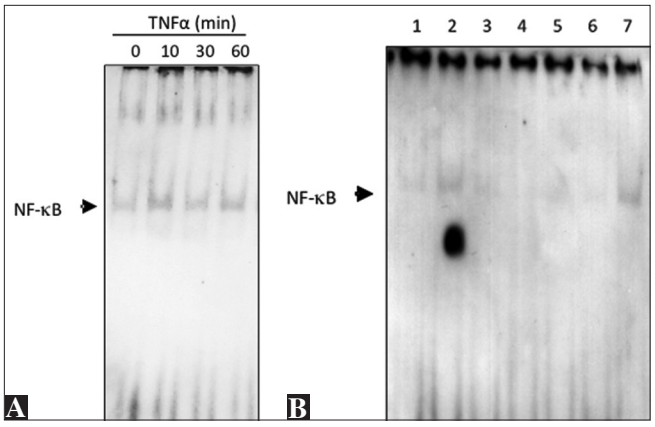


**Figure 7:** DNA binding activity of NF-κB was examined by gel mobility shift assay. (A) In the presence of TNF$\alpha$ (10 ng/ml) for 10-60 min, NF-κB activation was noted. (B) Ciglitazone (10 μM) attenuated TNF$\alpha$-induced NF-κB activation at 60 min. Three identical experiments performed independently gave similar results (1, basal; 2, TNF$\alpha$ for 60 min; 3, ciglitazone alone; 4, pre-treated with ciglitazone for 30 min before TNF$\alpha$ was added for another 1 h; 5, unlabeled NF-κB probe was added; 6, unlabeled oligonucleotides were added; 7, anti-NF-κB antibody was added to confirm that the indicated band was NF-kB).

In light of previous observations,[1,32] ASM may be an important cellular target for asthma management. In ASM cells, activation of PPARγ by natural and synthetic ligands inhibits serum-induced cell growth more effectively than dexamethasone and induces apoptosis and inhibits the release of granulocyte-macrophage colony stimulating factor (GM-CSF) and granulocyte colony stimulating factor (G-CSF).[16] More recent data suggest that chemokine expression in human ASM cells is differentially regulated by PPARγ agonists and that the interaction between PPARγ and glucocorticoid receptor (GR) may be responsible for the additive and synergistic inhibition of chemokine expression by PPARγ agonists, glucocorticoids, and β2-agonists.[33] In contrast, Pang *et al*., demonstrated that the PPARγ activator ciglitazone significantly increased $PGE_2$ accumulation, either alone or in combination with interleukin (IL)-1β, in line with the increase in cyclooxygenase (COX)-2 expression. Nonsteroidal anti-inflammatory drugs (NSAIDs) induced COX-2 expression in human ASM cells through PPARγ activation and the peroxisome proliferator response element (PPRE) in the promoter was required for NSAID-induced COX-2 expression in human ASM cells.[34] Our results suggested that ciglitazone, a PPARγ ligand, inhibited TNFα-induced ICAM-1 expression in ASM cells and might play a negative regulatory role on airway inflammation in chronic airway diseases such as asthma or COPD.

Our study demonstrates that the knockdown of *PPAR*γ gene by siRNA transfection and PPAR antagonist, GW9662, reverses the inhibitory effect of ciglitazone on TNFα-induced ICAM-1 expression, which indicates that ciglitazone is acting through the PPAR-dependent pathway. In endothelial cells, the ability of PPARγ ligands to stimulate endothelial cell nitric oxide release was PPARγ dependent because it was inhibited by treatment with either siRNA or the PPARγ antagonist, GW9662.[35] In U937 cells, PPARγ siRNA transfection reversed the inhibitory effects of troglitazone on heat shock protein (Hsp) 70 translation, indicating that troglitazone inhibits Hsp70 protein expression via a

PPARγ-dependent mechanism.[36] PPARγ is also required for ciglitazone-mediated increases in androgen receptor (AR) transcriptional activity. siRNA-based experiments demonstrate that the ciglitazone-induced regulation of AR activity observed in C4-2 cells is dependent on the presence of PPARγ.[37] However, 15dPGJ$_2$, a natural PPARγ ligand, can induce caspase-dependent granulocyte apoptosis via inhibition of IκB degradation through PPARγ-independent pathways.[38] Although both rosiglitazone and 15dPGJ2 can inhibit the proliferation of human ASM cells irrespective of the mitogen used, only the antiproliferative effects of rosiglitazone appear to be PPARγ dependent.[39] Recent study indicates that TZDs inhibit the release of a variety of inflammatory mediators from human ASM cells. Nevertheless, the effects of TZDs are not mediated by PPARγ.[40] The "off-target" mechanisms underlying the antitumor effects of TZDs have also been noted.[41] The discrepancy could be specific to cell, stimulator, or target gene. It is, therefore, critical to determine the relative contributions of PPARγ-dependent versus PPARγ-independent pathways in mediating anti-inflammatory effects in the future.

ICAM-1 promoter is known to be differentially regulated by AP-1 and NF-κB with different stimulators.[42] In ASM cells, previous data have shown that NF-kB activation, together with IkB degradation, increases ICAM-1 expression. NF-kB activation is critically important for TNFa-induced ICAM-1 expression.[7] The modulatory role of PPARγ ligands in TNFα-induced *ICAM1* gene response has also been noted by the down-regulation of NF-κB activity on human vascular endothelial cells.[43] The inhibition of NF-κB activation by PPARγ activators has also been noted in human airway epithelial cells.[26] Our study explores a new therapeutic intervention by ciglitazone through PPARγ to prevent NF-κB activation and DNA binding activity, resulting in the inhibition of ICAM-1 expression. In this study, ciglitazone prevented the activation of NF-κB by preventing p65 nuclear translocation and attenuating NF-κB DNA binding activity in response to TNFα. These findings show a dominant role of NF-κB activation by TNFα in modulating the activation of *ICAM1* gene expression. This result suggests that PPARγ ligand ciglitazone inhibits *ICAM1* gene expression by decreasing TNFα-induced NF-κB activation. However, the cross-talk between PPARγ and NF-κB is needed to be studied in the future.

In conclusion, ciglitazone plays a negative role on TNFα-induced *ICAM1* gene expression through ligand-dependent PPARγ activation and NF-κB–dependent signaling pathway. Endogenous PPARγ expression may act as a braking factor in regulating basal *ICAM1* gene expression in human ASM cells. Understanding the signaling mechanisms by which PPARγ regulates gene expression in ASM cells may lead to the design of new therapeutic approaches for treating airway inflammation in chronic airway diseases such as asthma and COPD.

## Acknowledgments

This work was supported by St. Paul's Hospital (Grant SPMRP-U 1-2001-1), Chang Gung Memorial Hospital (Grant CMRPG 371221), and in part by the National Science Council of Taiwan (Grant NSC 98-2314-B-182A-087).

## REFERENCES

1. Panettieri RA, Jr. Airway smooth muscle: Immunomodulatory cells that modulate airway remodeling? Respir Physiol Neurobiol 2003;137:277-93.
2. Huang CD, Ammit AJ, Tliba O, Kuo HP, Penn RB, Panettieri RA Jr, *et al*. G-protein-coupled receptor agonists differentially regulate basal or tumor necrosis factor-alpha-stimulated activation of interleukin-6 and RANTES in human airway smooth muscle cells. J Biomed Sci 2005;12:763-76.
3. Gosset P, Tillie-Leblond I, Janin A, Marquette CH, Copin MC, Wallaert B, *et al*. Expression of E-selectin, ICAM-1 and VCAM-1 on bronchial biopsies from allergic and non-allergic asthmatic patients. Int Arch Allergy Immunol 1995;106:69-77.
4. Ohkawara Y, Yamauchi K, Maruyama N, Hoshi H, Ohno I, Honma M, *et al*. *In situ* expression of the cell adhesion molecules in bronchial tissues from asthmatics with air flow limitation: *In vivo* evidence of VCAM-1/VLA-4 interaction in selective eosinophil infiltration. Am J Respir Cell Mol Biol 1995;12:4-12.
5. Shiota Y, Wilson JG, Marukawa M, Ono T, Kaji M. Soluble intercellular adhesion molecule 1 (ICAM-1) antigen in sera of bronchial asthmatics. Chest 1996;109:94-9.
6. Lazaar AL, Albelda SM, Pilewski JM, Brennan B, Pure E, Panettieri RA Jr. T lymphocytes adhere to airway smooth muscle cells via integrins and CD44 and induce smooth muscle cell DNA synthesis. J Exp Med 1994;180:807-16.
7. Amrani Y, Lazaar AL, Panettieri RA Jr. Up-regulation of ICAM-1 by cytokines in human tracheal smooth muscle cells involves an NF-kappa B-dependent signaling pathway that is only partially sensitive to dexamethasone. J Immunol 1999;163:2128-34.
8. Voraberger G, Schafer R, Stratowa C. Cloning of the human gene for intercellular adhesion molecule 1 and analysis of its 5´regulatory region. Induction by cytokines and phorbol ester. J Immunol 1991;147:2777-86.
9. Farina AR, Cappabianca L, Mackay AR, Tiberio A, Tacconelli A, Tessitore A, *et al*. Transcriptional regulation of intercellular adhesion molecule 1 by phorbol ester in human neuroblastoma cell line SK-N-SH involves jun- and fos-containing activator protein 1 site binding complex(es). Cell Growth Differ 1997;8:789-800.
10. Wegner CD, Gundel RH, Reilly P, Haynes N, Letts LG, Rothlein R. Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma. Science 1990;247:456-9.
11. Chung KF, Godard P, Adelroth E, Ayres J, Barnes N, Barnes P, *et al*. Difficult/therapy-resistant asthma: The need for an integrated approach to define clinical phenotypes, evaluate risk factors, understand pathophysiology and find novel therapies. ERS Task Force on Difficult/Therapy-Resistant Asthma. European Respiratory Society. Eur Respir J 1999;13:1198-208.

12. ATS. Proceedings of the ATS workshop on refractory asthma: Current understanding, recommendations, and unanswered questions. American Thoracic Society. Am J Respir Crit Care Med 2000;162:2341-51.

13. Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. Cell 1995;83:841-50.

14. Michalik L, Wahli W. Peroxisome proliferator-activated receptors: Three isotypes for a multitude of functions. Curr Opin Biotechnol 1999;10:564-70.

15. Spiegelman BM, Flier JS. Adipogenesis and obesity: Rounding out the big picture. Cell 1996;87:377-89.

16. Patel HJ, Belvisi MG, Bishop-Bailey D, Yacoub MH, Mitchell JA. Activation of peroxisome proliferator-activated receptors in human airway smooth muscle cells has a superior anti-inflammatory profile to corticosteroids: Relevance for chronic obstructive pulmonary disease therapy. J Immunol 2003;170:2663-9.

17. Pawliczak R, Han C, Huang XL, Demetris AJ, Shelhamer JH, Wu T. 85-kDa cytosolic phospholipase A2 mediates peroxisome proliferator-activated receptor gamma activation in human lung epithelial cells. J Biol Chem 2002;277:33153-63.

18. Su CG, Wen X, Bailey ST, Jiang W, Rangwala SM, Keilbaugh SA, *et al*. A novel therapy for colitis utilizing PPAR-gamma ligands to inhibit the epithelial inflammatory response. J Clin Invest 1999;104:383-9.

19. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature 1998;391:82-6.

20. Wang AC, Dai X, Luu B, Conrad DJ. Peroxisome proliferator-activated receptor-gamma regulates airway epithelial cell activation. Am J Respir Cell Mol Biol 2001;24:688-93.

21. Huang CD, Chen HH, Wang CH, Chou CL, Lin SM, Lin HC, *et al*. Human neutrophil-derived elastase induces airway smooth muscle cell proliferation. Life Sci 2004;74:2479-92.

22. Huang CD, Tliba O, Panettieri RA Jr., Amrani Y. Bradykinin induces interleukin-6 production in human airway smooth muscle cells: Modulation by Th2 cytokines and dexamethasone. Am J Respir Cell Mol Biol 2003;28:330-8.

23. Chen HC, Lin HC, Liu CY, Wang CH, Hwang T, Huang TT, *et al*. Neutrophil elastase induces IL-8 synthesis by lung epithelial cells via the mitogen-activated protein kinase pathway. J Biomed Sci 2004;11:49-58.

24. Ko YS, Yeh HI, Ko YL, Hsu YC, Chen CF, Wu S, *et al*. Three-dimensional reconstruction of the rabbit atrioventricular conduction axis by combining histological, desmin, and connexin mapping data. Circulation 2004;109:1172-9.

25. Benayoun L, Letuve S, Druilhe A, Boczkowski J, Dombret MC, Mechighel P, *et al*. Regulation of peroxisome proliferator-activated receptor gamma expression in human asthmatic airways: Relationship with proliferation, apoptosis, and airway remodeling. Am J Respir Crit Care Med 2001;164:1487-94.

26. Hetzel M, Walcher D, Grub M, Bach H, Hombach V, Marx N. Inhibition of MMP-9 expression by PPARgamma activators in human bronchial epithelial cells. Thorax 2003;58:778-83.

27. Honda K, Marquillies P, Capron M, Dombrowicz D. Peroxisome proliferator-activated receptor gamma is expressed in airways and inhibits features of airway remodeling in a mouse asthma model. J Allergy Clin Immunol 2004;113:882-8.

28. Lee KS, Park SJ, Kim SR, Min KH, Jin SM, Lee HK, *et al*. Modulation of airway remodeling and airway inflammation by peroxisome proliferator-activated receptor gamma in a murine model of toluene diisocyanate-induced asthma. J Immunol 2006;177:5248-57.

29. Woerly G, Honda K, Loyens M, Papin JP, Auwerx J, Staels B, *et al*. Peroxisome proliferator-activated receptors alpha and gamma down-regulate allergic inflammation and eosinophil activation. J Exp Med 2003;198:411-21.

30. Narala VR, Ranga R, Smith MR, Berlin AA, Standiford TJ, Lukacs NW, *et al*. Pioglitazone is as effective as dexamethasone in a cockroach allergen-induced murine model of asthma. Respir Res 2007;8:90.

31. Rhee CK, Lee SY, Kang JY, Kim SJ, Kwon SS, Kim YK, *et al*. Effect of peroxisome proliferator-activated receptor-gamma on airway smooth muscle thickening in a murine model of chronic asthma. Int Arch Allergy Immunol 2009;148:289-96.

32. Hirst SJ. Regulation of airway smooth muscle cell immunomodulatory function: Role in asthma. Respir Physiol Neurobiol 2003;137:309-26.

33. Nie M, Corbett L, Knox AJ, Pang L. Differential regulation of chemokine expression by peroxisome proliferator-activated receptor gamma agonists: Interactions with glucocorticoids and beta2-agonists. J Biol Chem 2005;280:2550-61.

34. Pang L, Nie M, Corbett L, Knox AJ. Cyclooxygenase-2 expression by nonsteroidal anti-inflammatory drugs in human airway smooth muscle cells: Role of peroxisome proliferator-activated receptors. J Immunol 2003;170:1043-51.

35. Polikandriotis JA, Mazzella LJ, Rupnow HL, Hart CM. Peroxisome proliferator-activated receptor gamma ligands stimulate endothelial nitric oxide production through distinct peroxisome proliferator-activated receptor gamma-dependent mechanisms. Arterioscler Thromb Vasc Biol 2005;25:1810-6.

36. Lee KJ, Ha ES, Kim MK, Lee SH, Suh JS, Park KH, *et al*. CD36 signaling inhibits the translation of heat shock protein 70 induced by oxidized low density lipoprotein through activation of peroxisome proliferators-activated receptor gamma. Exp Mol Med 2008;40:658-68.

37. Moss PE, Lyles BE, Stewart LV. The PPARgamma ligand ciglitazone regulates androgen receptor activation differently in androgen-dependent versus androgen-independent human prostate cancer cells. Exp Cell Res 2010;316:3478-88.

38. Ward C, Dransfield I, Murray J, Farrow SN, Haslett C, Rossi AG. Prostaglandin D2 and its metabolites induce caspase-dependent granulocyte apoptosis that is mediated via inhibition of I kappa B alpha degradation using a peroxisome proliferator-activated receptor-gamma-independent mechanism. J Immunol 2002;168:6232-43.

39. Ward JE, Gould H, Harris T, Bonacci JV, Stewart AG. PPAR gamma ligands, 15-deoxy-delta12,14-prostaglandin J2 and rosiglitazone regulate human cultured airway smooth muscle proliferation through different mechanisms. Br J Pharmacol 2004;141:517-25.

40. Zhu M, Flynt L, Ghosh S, Mellema M, Banerjee A, Williams E, *et al*. Anti-inflammatory effects of thiazolidinediones in human airway smooth muscle cells. Am J Respir Cell Mol Biol 2011;45:111-9.

41. Wei S, Yang J, Lee SL, Kulp SK, Chen CS. PPARgamma-independent antitumor effects of thiazolidinediones. Cancer Lett 2009;276:119-24.

42. Roebuck KA, Rahman A, Lakshminarayanan V, Janakidevi K, Malik AB. H2O2 and tumor necrosis factor-alpha activate intercellular adhesion molecule 1 (ICAM-1) gene transcription through distinct cis-regulatory elements within the ICAM-1 promoter. J Biol Chem 1995;270:18966-74.

43. Chen NG, Han X. Dual function of troglitazone in ICAM-1 gene expression in human vascular endothelium. Biochem Biophys Res Commun 2001;282:717-22.