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Peroxisome proliferator-activated receptor ligands decrease human airway smooth muscle cell migration and extra-cellular matrix synthesis

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Short title: PPAR-ligands and human airway smooth muscle migration

Key words: PPAR, airway smooth muscle migration, extracellular matrix, PGE_2

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Abstract

Airway smooth muscle cells produce extracellular matrix proteins, which in turn can promote smooth muscle survival, proliferation and migration. Currently available therapies have little effect on airway smooth muscle matrix production and migration. Peroxisome-proliferator-activated receptor (PPAR) ligands are reported to decrease migration and matrix production in various cell lines. In this study, we examined the effect of PPAR ligands on human airway smooth muscle matrix production and migration. PPAR expression was examined by RT-PCR and Western blotting. Endogenous PPAR activity was examined by transfecting cells with a PPRE-luciferase reporter plasmid. We observed that human airway smooth muscle cells express α, β, and γ PPAR. A 6-fold induction of luciferase activity was observed by stimulating cells with a pan-agonist indicating endogenous PPAR activity. The PPAR-ligands ciglitazone, 15d-PGJ2, and WY-14643 decreased migration towards PDGF. This was not mediated by inhibiting Akt phosphorylation or promoting PTEN activity, but partly through COX-2 induction and PGE_2 production that increased c-AMP levels in the cells. All three ligands also caused an inhibition of collagen and fibronectin secretion by cultured smooth muscle cells. We conclude that PPAR-ligands decrease human airway smooth muscle migration and matrix production and are therefore potentially useful to modulate airway remodelling.

Introduction

Two conspicuous features in the airway walls of patients with severe asthma are increased quantity of smooth muscle cells and extra cellular matrix components (1). The increased smooth muscle mass is more likely to be due to increased number (hyperplasia) than size of the cells (hypertrophy) (2,3). One of the mechanisms of smooth muscle accumulation in the remodelled airway may be by migration from deeper smooth muscle bundles in the submucosa (4,5), which, in turn, is partly regulated by extra cellular matrix proteins such as collagen and fibronectin (6). Both these structural changes, which contribute to airflow limitation in asthma (7), are not effectively reversed by currently available asthma medications. Thus, it would be attractive to identify mechanisms or strategies that would inhibit both smooth muscle migration and matrix production.

Peroxisome proliferator-activated receptors (PPAR) are a family of hormone receptors that belong to the steroid receptor super family (8). PPAR α and γ isoforms are reported to be expressed on airway smooth muscle cells (9,10) and their activation by ligands such as the thiazoledinedione, rosiglitazone, have anti-inflammatory properties that are superior to corticosteroids (11). PPAR ligands can decrease human vascular smooth muscle migration (12) and they can decrease matrix production by renal mesangial cells (13). Proposed mechanisms include direct transcriptional activation or suppression of target genes (14), antagonism of other signal-dependent transcription factors such as nuclear factor κB, activator protein-1 or CAAT/enhancer binding protein, activation of signaling pathways that involve Phosphoinositol 3-kinase (PI3K), Src-kinase or MAP-kinase (15), inhibiting signals such as phosphatase and tensin homolog (PTEN), by modulating activation of cyclooxygenase and increasing intracellular

cyclic AMP (c-AMP) (16), or by decreasing or interrupting TGF-β1 level (17) or signaling (18). Activation of other nuclear hormone receptors such as the Liver X receptor (LXR) has been demonstrated to decrease human airway smooth muscle migration towards chemotactic stimuli (19,20).

In this study, we investigated the expression of PPAR isoforms on human airway smooth muscle cells (HASM cells), the effects of their ligands on smooth muscle migration, production of collagen and fibronectin, and some mechanisms of these effects. Specifically, we examined the role of Akt, Src kinase, PTEN, and COX signaling to offer some explanations for the phenomena that we set out to explore. We used four different agonists (WY14643 for the α-isoform, GW501516 for the β-isoform, ciglitazone/troglitazone and 15-d PGJ2 for the γ-isoform) and 3 antagonists (MK866 against α-isoform, Sulindac against the β-isoform, GW9662 against the γ-isoform) to study the role of specific PPAR isoforms. We demonstrate that PPAR ligands, similar to other nuclear hormone receptor ligands, inhibit the migration of HASM cells towards platelet-derived growth factor (PDGF) and attenuate collagen secretion from HASM cells

Methods

Airway smooth muscle culture

Portions of human lungs that were resected at St. Joseph's Healthcare, Hamilton were obtained from 16 patients (table 1) undergoing surgery for lung cancer with their informed consent and approval from the hospital Research Ethics Board. Smooth muscle tissue was isolated from macroscopically disease-free areas of human bronchi. Airway smooth muscle (HASM) cells were grown to confluence as described before (4,6). The cells were passaged between 2-5 times and used for the experiments.

Detection of PPAR transcripts

Total RNA was isolated from ASM cells and 1 ug was reverse-transcribed using Quantitect Reverse Transcription according to the manufacturer's instruction (QIAGEN). PPAR isoforms were then amplified using gene specific primers in an Applied Biosystems 7900HT machine. Briefly, reactions contained 12.5µl of SYBR-green PCR supermix (Invitrogen), 10.5µl of H_2O, 1µl of primer sets (10µM each) and 1µl of cDNA. PCR amplification was carried out for 28 cycles with the following parameters: denaturation at 95^{o}C for 15 minutes; 30 cycles at 95C for 30 sec and 60C for 1 minute. Products were separated on 2% agarose gels, stained with SYBR green and imaged on a Typhoon 9200 Variable Mode Imager (Molecular Dynamics, Amersham Biosciences, Baie D'Urfe, QU, CA).

The primer sequences were as follows: PPARα: Forward Primer (5'-3') - AGCCTAAGGAAACCGTTCTG; Reverse - Primer (5'-3') ACGATCTCCACAGCAAATGA; PPARβ/δ: Forward primer -TCACACAACGCTATCCGTTT; Reverse primer – GGCATTGTAGATGTGCTTGG; PPARγ: Forward primer - ACCAGCTGAATCCAGAGTCC; Reverse primer – CGAATGGTGATTTGTCTGTTG

PPAR Transfections and Reporter Gene Assay

HASM cells were transfected with 0.4 µg of PPRE-luciferase reporter gene in 6-well dishes at approximately 75% confluency using Effectene reagent (QIAGEN) according to the manufacturer's instructions. Briefly, per well, 0.4ug of a PPRE-luciferase reporter plasmid, which contains three copies of the murine PPAR-response element from acyl co-A oxidase, was incubated for 5 minutes at room temperature with 95ul of enhancer reagent and 3.2ul of

enhancer. 10ul of Effectene was then added for an additional 10 minutes followed by 600ul of RPMI media supplemented with 10% FBS and incubated overnight. Following transfection, plates were incubated overnight in DMEM lacking phenol red and supplemented with 10% charcoal-stripped FBS, 1% L-glutamine and 1% penicillin/streptomycin with 1 μM of a Pan Agonist (Full 2, obtained from Joel Berger, Merck Frosst, Montreal, PQ) alone or in combination with PPARα antagonist (MK866; 10μM), PPARβ/δ antagonist (Sulindac; 10μM), PPARγ antagonist (GW9662; 10μM) or all three together for an additional 48hrs (fresh ligand was added after 24hrs). Control cells received an equivalent amount of vehicle. Luciferase activity was assayed as described previously (19).

Migration assay

Migration experiments were performed using 6.5mm Transwell culture plates with a 8.0μM pore, collagen-I coated, polycarbonate membrane separating the inner and the outer chambers (Fisher Scientific Limited, Nepean, ON), as previously described (4,6). Assays were done in duplicate using tissues from six different lung specimens. Migration was studied by adding PDGF-BB (20 ng/ml) (Invitrogen Canada Inc, Burlington, ON) to the outer well. The effects of PPAR-ligands were studied by treating the cells with various concentrations (0.1-100 ng/ml) of the ligands for 30 minutes prior to adding PDGF. The viability of cells treated with various agonists was checked by confirming that the cell numbers under the various conditions were between 93-151% of the untreated controls using an MTT-assay.

Enzyme-linked Immunosorbent Assay

Collagen, fibronectin

HASM cells were plated at 10^4/well in 96 well plates. On the 4^{th} day, they were growth arrested for 48 hours with 0.3% BSA in RPMI and treated with varyious concentrations of the PPAR ligands for 48 hours. Extra-cellular matrix was harvested from the plates by incubating with 0.02M NH_4OH for 5 minutes at room temperature. The plates were stored at -70^0C until the assays were run. Primary antibodies to fibronectin (1:5000 dilution, BD Biosciences, Oakville ON) and collagen (1:2000 dilution, Sigma, Oakville ON) were added to the plates and then incubated with secondary antibody goat anti-mouse in 200ul blocking buffer. The colour was developed with 100ul p-nitrophenyl phosphate solution (Sigma, Oakville, ON) and the adsorbance was read at 405nm.

PGE_2

Prostaglandin E_2 (PGE_2) levels in smooth muscle culture supernatants were measured by a sensitive enzyme immunoassay according to manufacturer's instructions (Assay Designs, Ann Arbor, MI). The limit of detection for PGE_2 was 13.4 pg/ml.

Cyclic AMP

HASM cells were grown to confluence, growth arrested for 24 hours, treated with the various agonists for the same duration as the migration assay (5 hours), and cyclic AMP was assayed in the supernatant using a sensitive immunoassay according to the manufacturer's instruction (R&D Systems Inc, Minneapolis, MN, Catalogue number DE0450).

Western blotting (Src, Akt, COX, PPAR)

HASM cells were cultured in 6-well plates and grown to approximately 90% confluency. The cells were then serum-starved in 0.2% RPMI-BSA for 24 hours. The cells were then pre-treated with the PPAR-agonists for 1 hour followed by PDGF (20 ng/ml) for the indicated times. Cells were then lysed in 0.1% Trition X-100 detergent containing protease and phosphatase inhibitors and extracts (20 μg) were separated by SDS-PAGE and blotted on nitrocellulose. Membranes were blocked with 5% milk-TBST for 1 hour and were first probed with antibodies for phosphoSrcY416 (1:1000), phosphoAktS473 (1:1000), PTEN (1:1000) (Cell Signaling, Danvers, MA) or COX-1 and -2 (1:2000) (Millipore, Billerica, MA) in 5% Bovine Serum Albumin-Trisbuffer Saline Tween 20 (BSA-TBST) overnight followed by secondary anti-rabbit antibody (1:2000) (anti-goat, 1:2000 for COX-2) in 5% milk-TBST for 1 hour. Membranes were washed extensively and proteins were detected with a standard chemiluminescence kit. The blots were then stripped in a low pH glycine solution and re-probed with total Src kinase (1:1000), total Akt (1:1000) antibodies. PPAR isoforms on untreated HASM cells were detected by using anti-mouse polyclonal antibodies (1:1000) (R&D Systems, Burlingon, ON) and sheep anti-mouse horse radish peroxidase labelled secondary antibody (1:2000).

Phosphatase assay

PTEN activation was also assessed by a phosphatase assay. PTEN was immunoprecipitated from 500ug protein lysate using 1ug mouse anti-PTEN (Cell Signaling Technology, Danvers MA) , protein G agarose (Invitrogen, Burlington ON) in immunoprecipitation buffer (50mM Tris pH 7.5, 100mM NaCl, 15mM EGTA, 0.1% Triton X-100). The beads were rocked overnight at 4C, washed with 100mM Tris (pH 8.0), resuspended in phosphatase buffer (100mM Tris pH 8.0, 10mM DTT) in 96 well plate, and incubated with 5ul diC8-PIP3 (Echelon Biosciences Inc., Salt

Lake City UT), 15ul phosphatase buffer, and 100ul Biomol Green (Biomol International, Plymouth Meeting PA). The free phosphate was measured at 630nm.

Statistical analysis

Statistical analysis was performed by ANOVA using the different time points or experimental conditions as within-subject factors. The source of significant variation was identified by predefined contrasts. $P < 0.05$ was considered significant. All analyses were performed using SPSS-version 13.0 (Statistical Package for Social Sciences, Chicago, IL).

Results

PPAR expression and activation

Human airway smooth muscle cells express α, β, and γ PPAR message (figure 1a) and protein (figure 1b). A 6-fold induction of luciferase activity was observed by stimulating cells with a pan-agonist indicating endogenous PPAR activity (figure 1c). This was attenuated by specific inhibitors of the three isoforms (figure 1d).

Migration

All four PPAR-ligands, ciglitazone, 15d-PGJ2, WY-14643, and GW501516, decreased migration towards PDGF (figures 2a-d). The specificity of the isoforms is suggested by the ability of the specific PPAR antagonists to reverse the inhibitory effects.

Matrix production

All three PPAR ligands that we tested (ciglitazone, 15d-PGJ2, WY-14643) inhibited collagen and fibronectin production by cultured smooth muscle cells (figures 3a-c). The effect on

fibronectin was statistically significant only with 15d PGJ2. The effect on collagen was significant for all 3 concentrations of all the three agonists.*Signalling*

PPAR ligands, except for 15d-PGJ2, did not inhibit PDGF-induced increase in Akt or Src phosphorylation (figure 4a,b) or increase PTEN activity (figure 4c) in airway smooth muscle cells. 15d-PGJ2 attenuated Src kinase phosphorylation (figure 4a). In the presence of indomethacin, the inhibitory effects of 15d-PGJ2, ciglitazone and GW501516 on smooth muscle migration towards PDGF were attenuated. This was not observed on the inhibitory effect of WY-14643 (figures 2a-d). We also observed that 15d-PGJ2 and ciglitazone increased PGE2 (figure 5a) and cyclic AMP levels (by 1.6 and 1.2 fold respectively) in cultured smooth muscle supernatant. Consistent with this, we observed activation of COX-2, not COX-1, at 4 and 24h after stimulation with 15d-PGJ2 and ciglitazone.(figure 5b). WY-14643 did not increase PGE2 levels or induce COX-1 or COX-2 (data not shown).

Discussion

We confirm previous observations of PPAR expression on human airway smooth muscle cells and we report three novel observations. First, PPAR agonists decrease airway smooth muscle chemotaxis towards PDGF. Second, PPAR agonists also decrease collagen and fibronectin secretion by airway smooth muscle cells. Third, the inhibition of migration seems to be mediated partly through the secretion of PGE2 and increase in intracellular c-AMP. These results suggest that PPAR agonists may be able to reverse some of the structural changes seen in the airway sub-mucosa of patients with chronic severe asthma.

We confirm previous observations that human airway smooth muscle cells express PPAR α and γ isoforms (9-11). In addition, they also express the β isoform. We have confirmed this by demonstrating the message and the protein. It is not clear why previous experiments using cultured smooth muscle cells did not demonstrate the expression of the β/δ isoforms. It may be related to the polyclonal nature of the antibody used (Santa Cruz) or the loss of expression with advanced passage of the cells (2-5 passages in our experiments vs >6 passages in previous experiments). PPARs are endogenously active as indicated by the PPAR responsive element-luciferase reporter assay. They are known to have potent anti-inflammatory effects on HASM cells. Here we report two additional biologically relevant effects of the molecule. Similar to the observations in vascular tissue (12), we demonstrate that PPAR ligands are able to decrease airway myocyte migration towards PDGF. We examined relevant signalling mechanisms to understand this phenomenon. We did not observe inhibition of Src-kinase (except for 15d-PGJ2) or PI3-kinase activity or an increase in PTEN activity as potential mechanisms. However, we did observe that the inhibitory effect was attenuated by indomethacin. PPAR γ agonists also caused an increase in PGE2 release from airway smooth muscle cells and an induction of COX-2 and this is consistent with reports of COX induction by PPAR γ agonists (16). Thus, the decrease in muscle migration by PPAR-γ is most likely due to an increase in intracellular cyclic AMP facilitated by PGE2. This is consistent with observations with other agents such as beta-agonists that increase intracellular c-AMP and decrease airway (21) and vascular (22) smooth muscle and fibroblast migration (23). It is likely that cAMP modulates the morphology of smooth muscle cells by inhibiting a Rac-dependent signaling pathway resulting in disassembly of actin stress fibers and lamellipodia, loss of focal adhesions, and the formation of small F-actin rings (24), thus decreasing the ability of cells to migrate. It is likely that PPAR-β isoform also exerts its

inhibitory effect through the same pathway as the effect was inhibited by indomethacin. We are unable to explain the mechanism of inhibitory effect of the PPAR-α agonist. This is unlikely to be due to the same pathway as we did not observe either COX induction or an increase in PGE2 levels. This requires further investigation.

Although a PPAR response element (PPRE) has not been found upstream of ECM genes, there is evidence suggesting that PPAR may participate in regulation of ECM genes, especially type I collagen (25,26). We observed a small, but statistically significant, decrease in collagen and fibronectin secretion from airway smooth muscle cells. The precise mechanism needs further investigation. Unlike the effect on muscle migration, this does not appear to depend on intra-cellular c-AMP levels as the observed phenomenon was not susceptible to inhibition by indomethacin. PPARγ agonists are reported to decrease TGF-β expression in mesangial cells (13) and since TGF-β increases fibronectin and collagen secretion from airway smooth muscle cells (27), it is likely that PPAR ligands may decrease TGF-β secretion by HASM cells. This needs further exploration.

These observations are likely to have important clinical relevance. Assuming that the chronic structural changes are detrimental to asthma pathophysiology (28), treatment with thiazoledinediones are likely to limit or even potentially reverse the two components of the remodelling processes that are currently not amenable to pharmacological therapy. This needs further evaluation in a clinical trial. In conclusion, PPAR-ligands decrease human airway smooth muscle migration and matrix production and are therefore potentially useful to modulate airway remodelling.

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Table 1: clinical characteristics of subjects who donated airways

Subjects, n	16
Male, n	12
Age, y (mean, SD)	52 (22)
Smokers, n	11
Atopy, n	6
FEV1, L (mean, SD)	1.9 (1.5)
FEV1, % (mean, SD)	68 (23)
FEV1/VC, % (mean, SD)	64 (33)

Legends

Figure 1a: A representative PCR blot demonstrating expression of PPAR α, γ and β (δ) expression on cultured human airway smooth muscle cells.

Figure 1a

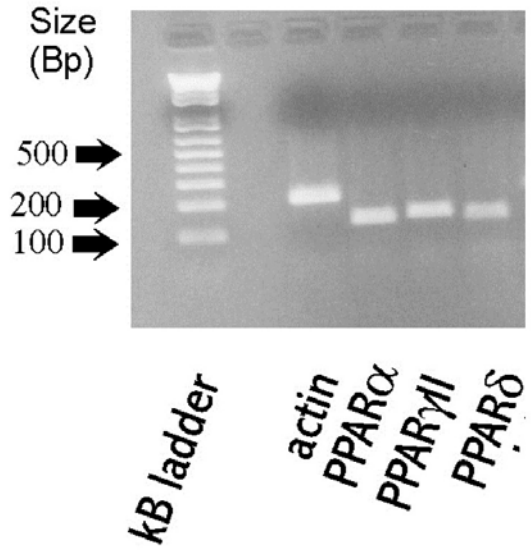

Figure 1b: Western blotting demonstrating that human airway smooth muscle cells from four different donors express PPARα, β, and γ isoforms

Figure 1b

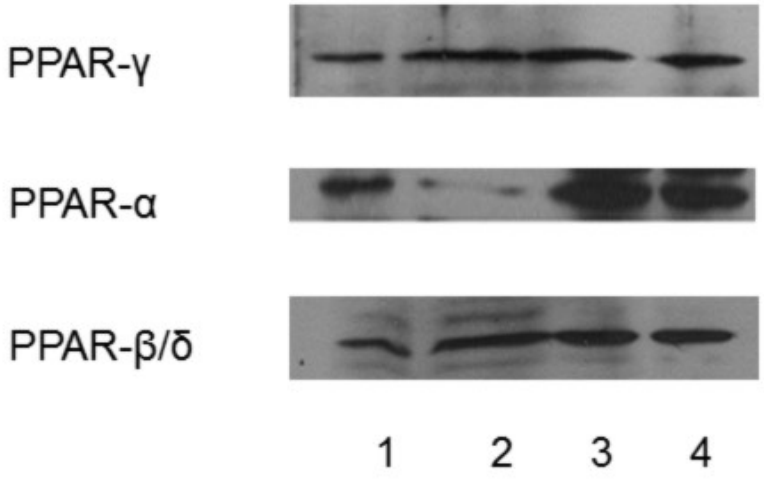

Figure 1c: Airway smooth muscle cells transfected with PPRE-luciferase reporter gene demonstrates endogenous PPAR-activity when stimulated with a pan-PPAR agonist (6-fold induction compared to control, $p<0.05$).

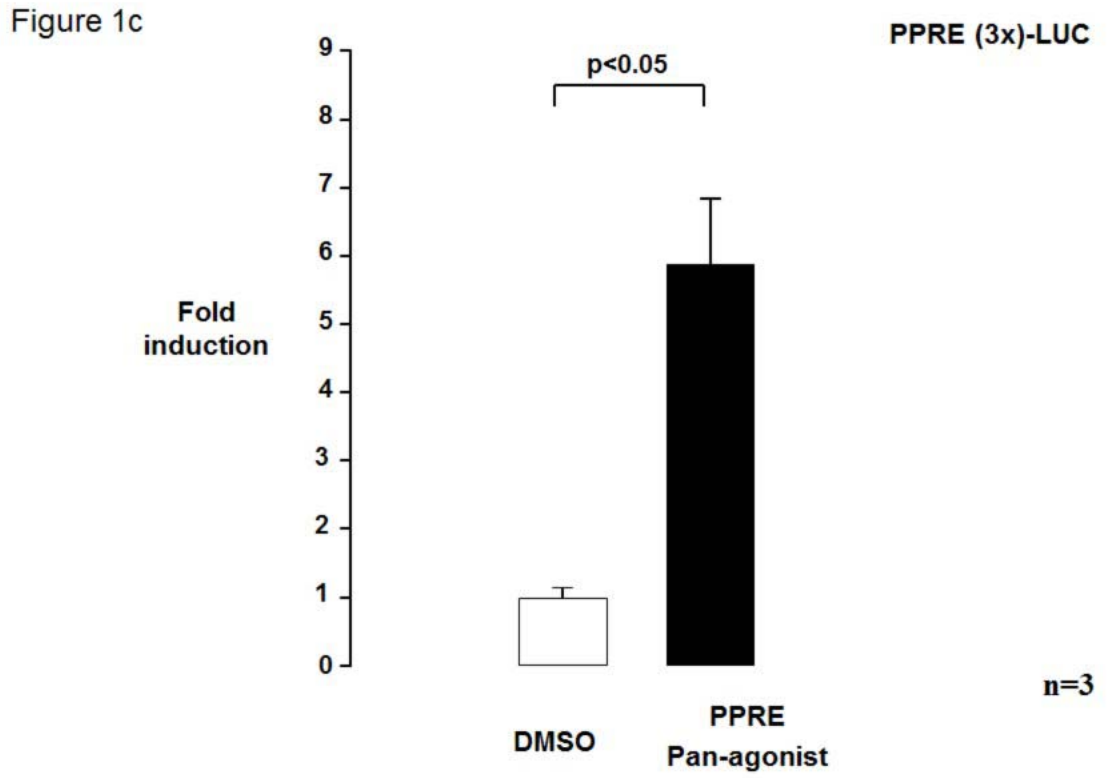

Figure 1d: PPAR antagonists repress PPRE reporter genes in the presence of a PPAR Pan-agonist. Human ASM cells were transfected with a PPRE-luc reporter gene and treated with Pan agonist A (PanA; 1μM)) alone or in combination with PPARα antagonist (MK866; 10μM), PPARβ/δ antagonist (Sulindac; 10μM), PPARγ antagonist (GW9662; 10μM) or all three together, as indicated. Cells were incubated for 24hrs and luciferase activity was measured. Values represent the average of triplicates (+/- S.D.) with PanA alone taken as 100% (dashed line). Compared to the PanA, all the three specific antagonists and the three antagonists in combination significantly reduced PPAE-luc gene expression ($p<0.05$ for all the assays).

Figure 1d

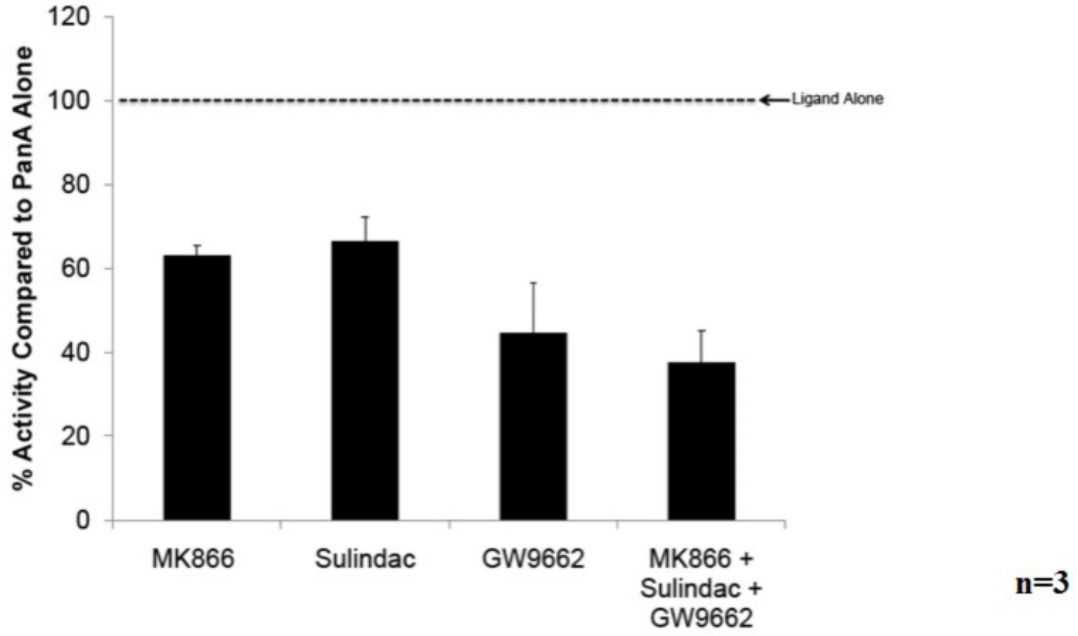

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Figure 2a-d: Migration of human airway smooth muscle cells towards PDGF was inhibited by PPAR agonists. This inhibitory effect was abolished by indomethacin (for PPAR γ and β agonists) and specific PPAR isoform antagonists (GW9662 for γ-isoform, MK866 for α-isoform, and sulindac for the β-isoform).. Data are shown as mean and SD. *$p<0.05$ compared to PDGF.

Figure 2a

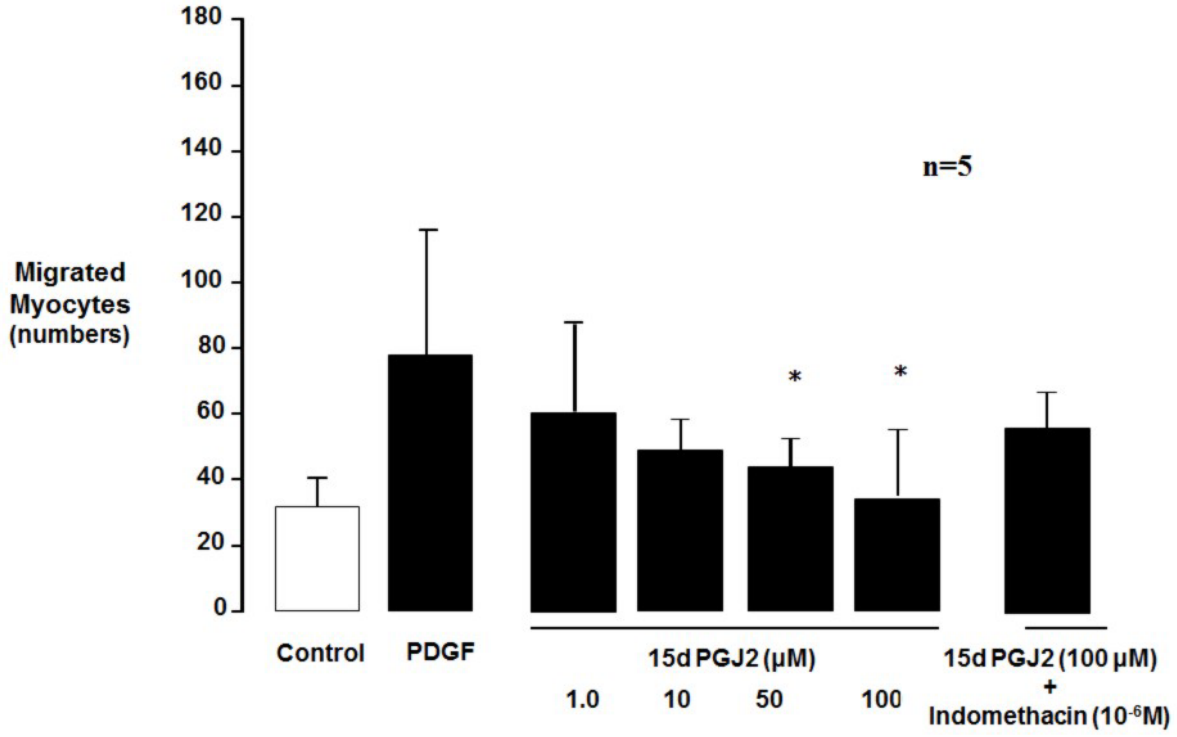

Migrated Myocytes (numbers)
180
160
140
120
100
80
60
40
20
0
n=5
*
*
Control
PDGF
15d PGJ2 (μM)
1.0
10
50
100
15d PGJ2 (100 μM) + Indomethacin (10-6M)

Figure 2b

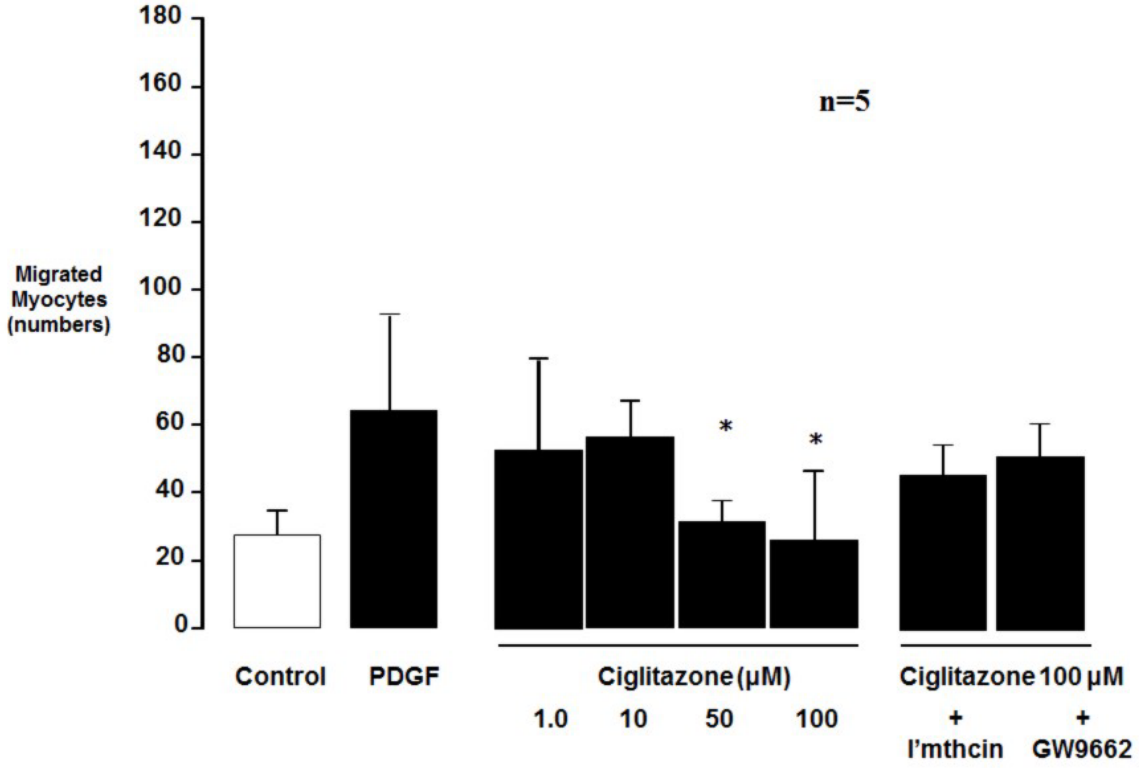
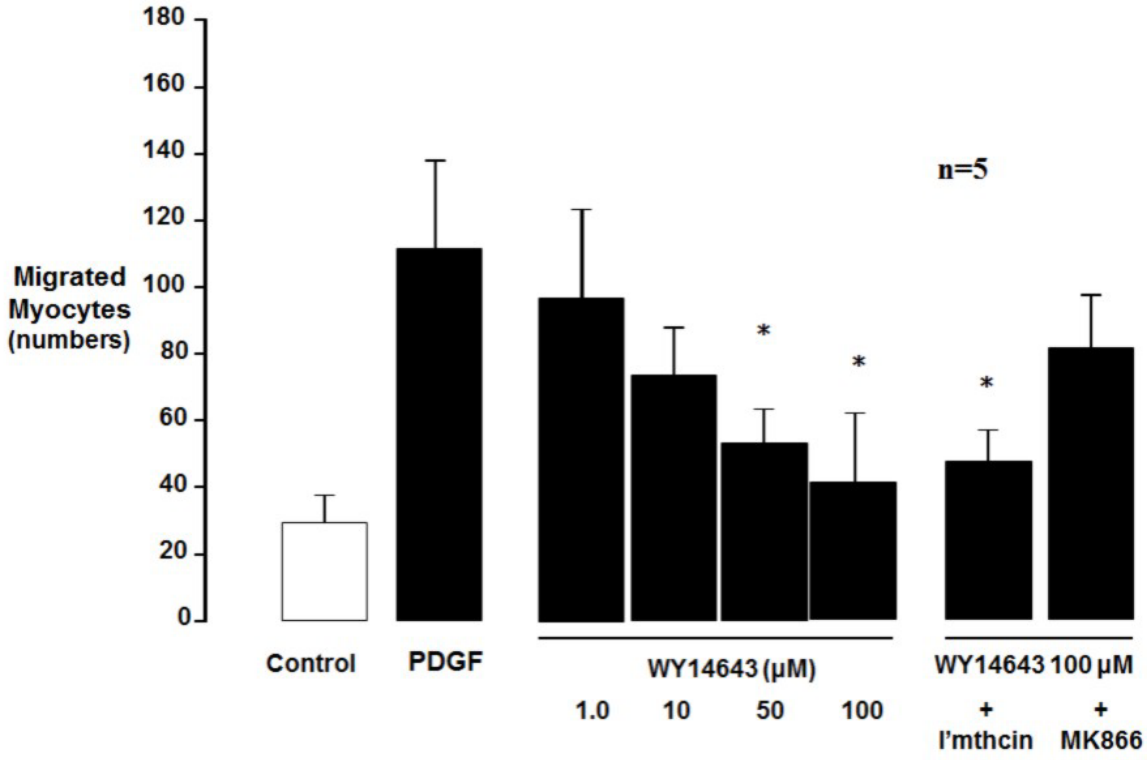

180
160
140
120
100
80
60
40
20
0
Migrated Myocytes (numbers)
n=5
*
*
Control
PDGF
Ciglitazone (μM)
1.0
10
50
100
Ciglitazone 100 μM
+ l'mthcin
+ GW9662

Figure 2c


180
160
140
120
100
80
60
40
20
0
Migrated Myocytes (numbers)
n=5
*
*
*
Control
PDGF
WY14643 (μM)
1.0
10
50
100
WY14643 100 μM
+ l'mthcin
+ MK866

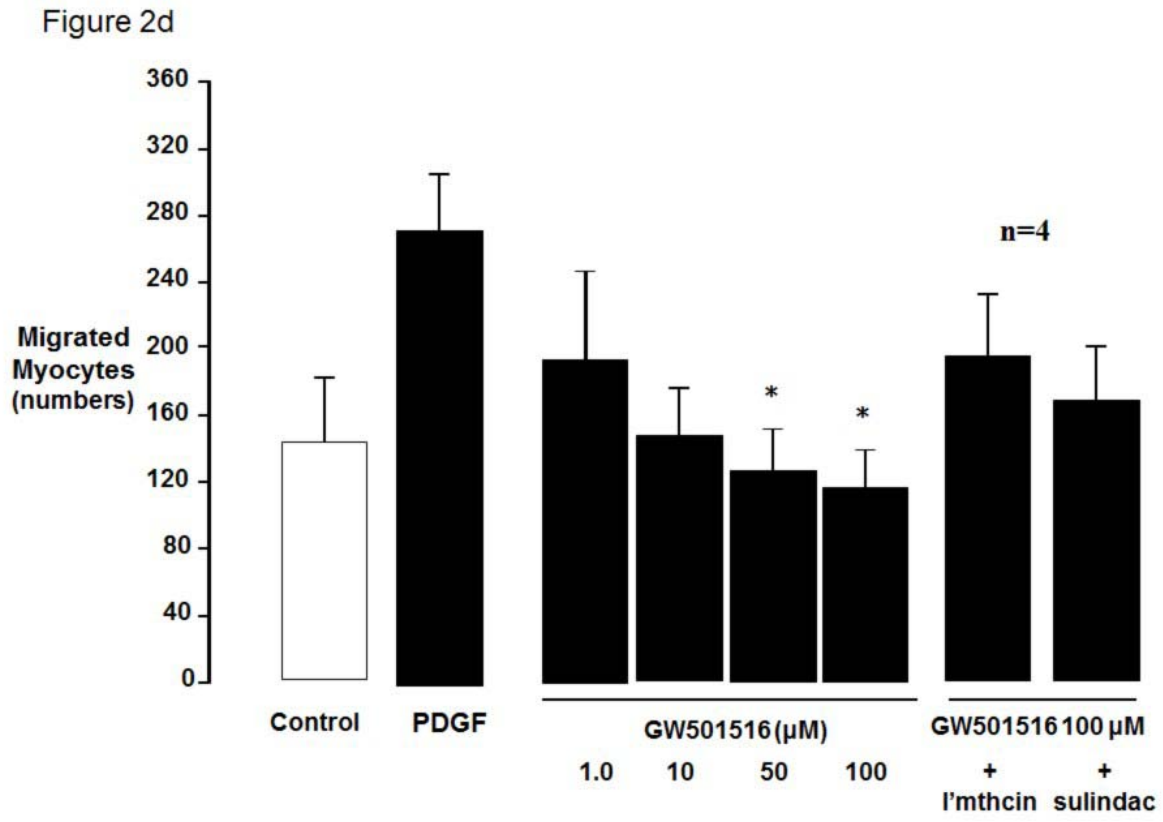


Figure 3a-c: Collagen and fibronectin synthesis by human airway smooth muscle cells were inhibited by PPAR agonists. This was unaffected by indomethacin. Data are shown as mean and SD compared to control.

Figure 3a

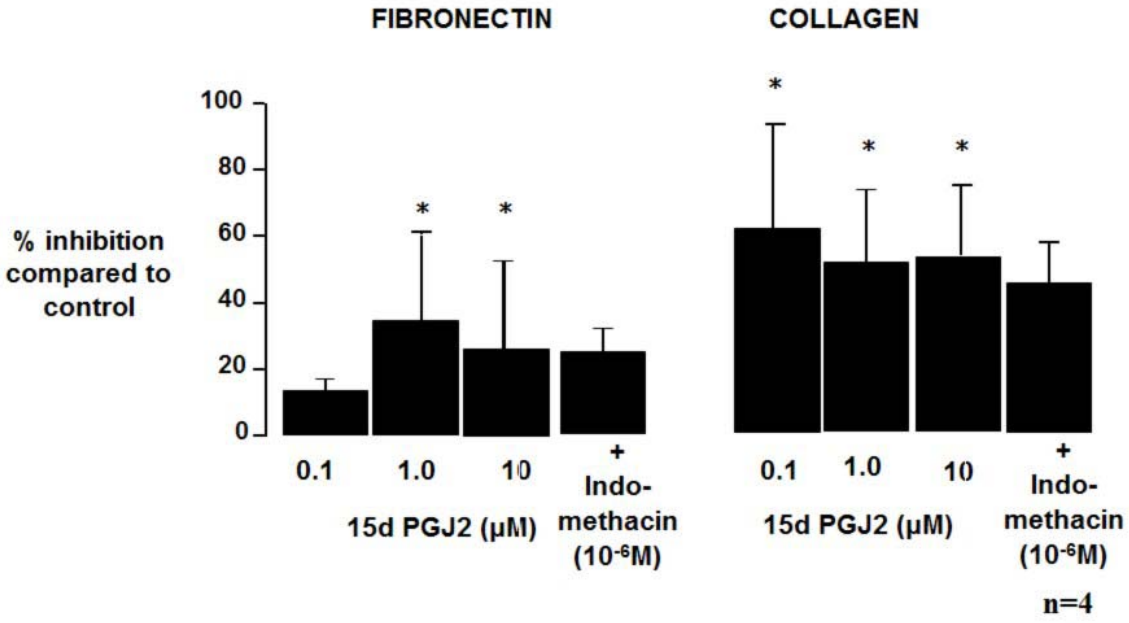

FIBRONECTIN
COLLAGEN
% inhibition compared to control
100
80
60
40
20
0
*
*
*
*
*
0.1
1.0
10
+ Indo-methacin (10-6M)
15d PGJ2 (μM)
0.1
1.0
10
+ Indo-methacin (10-6M)
15d PGJ2 (μM)
n=4

Figure 3b

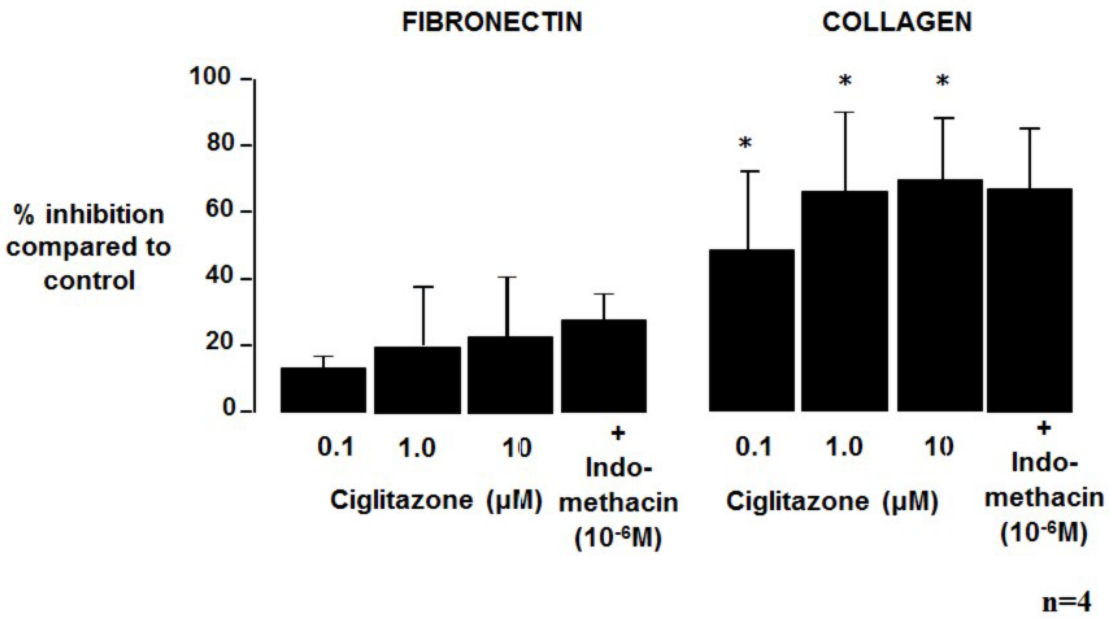

FIBRONECTIN
COLLAGEN
100
80
60
40
20
0
% inhibition compared to control
*
*
*
0.1
1.0
10
+ Indo-methacin (10-6M)
Ciglitazone (μM)
0.1
1.0
10
+ Indo-methacin (10-6M)
Ciglitazone (μM)
n=4

Figure 3c

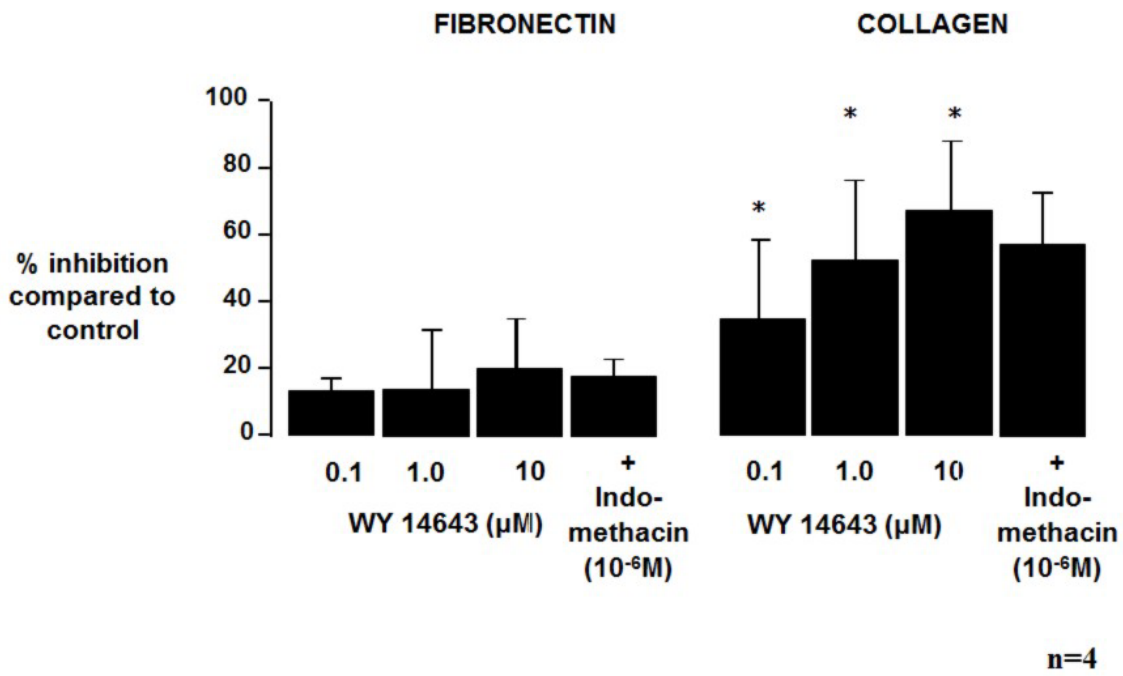


Figure 4a: Total and phosphorylated Src Kinase were examined on cultured human airway smooth muscle cells at 2 and 5 minutes after treating with PDGF in the presence of DMSO control, 100 µg/ml WY14643, 10µg/ml 15-d PGJ2, 50 µg/ml Ciglitazone and 10 µg/ml Troglitazone, A representative western blot showing that PPAR agonists, except for PGJ2, do not increase total or phosphorylated Src kinase on human airway smooth muscle cells.

Figure 4a

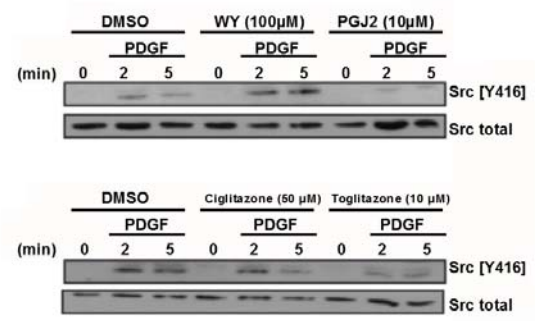


Figure 4b: Total and phosphorylated Akt Kinase were examined on cultured human airway smooth muscle cells at 2 and 5 minutes after treating with PDGF in the presence of DMSO control, 100 µg/ml WY14643, 10µg/ml 15-d PGJ2, 50 µg/ml Ciglitazone and 10 µg.ml Troglitazone. A representative western blot showing that PPAR agonists do not increase total or phosphorylated Akt kinase on human airway smooth muscle cells.

Figure 4b

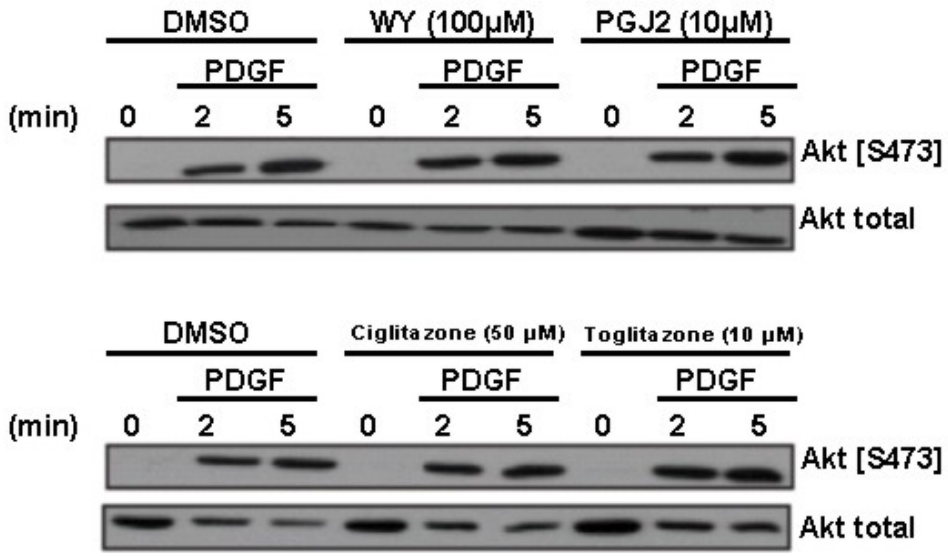


Figure 4c: PTEN activity was assayed using a phosphatase assay and the results are expressed a normalized for the control. There was no statisitically significant difference in PTEN activity between PDGF (10 ng/ml), WY14643 (100 µg/ml) and Ciglitaxone 50 µg/ml. Data are shown as mean and SD.

Figure 4c

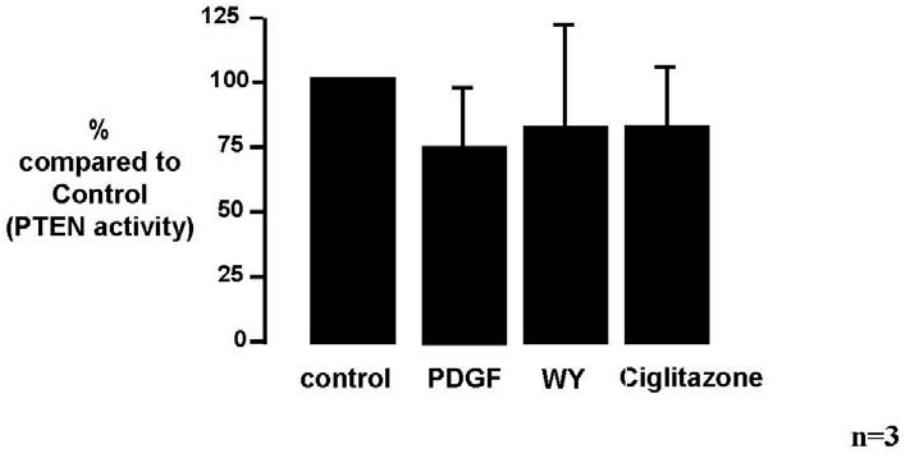


Figure 5a: PPAR agonists, Ciglitazone and 15d PGJ2 not WY14643, increased PGE2 synthesis (EIA) by human airway smooth muscle cells. Data are shown as mean and SD. *$p<0.05$ compared to control.

Figure 5a

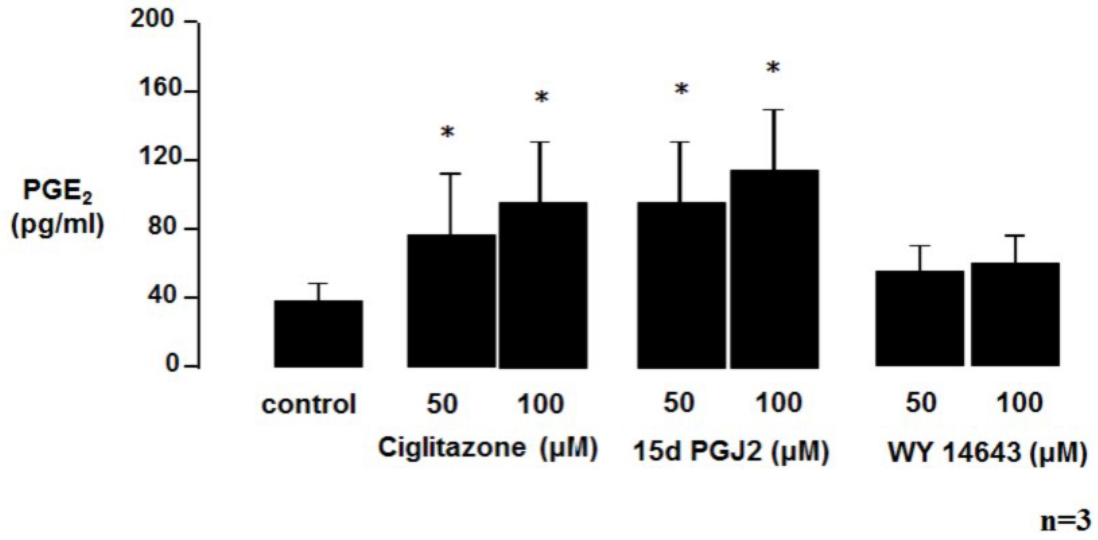
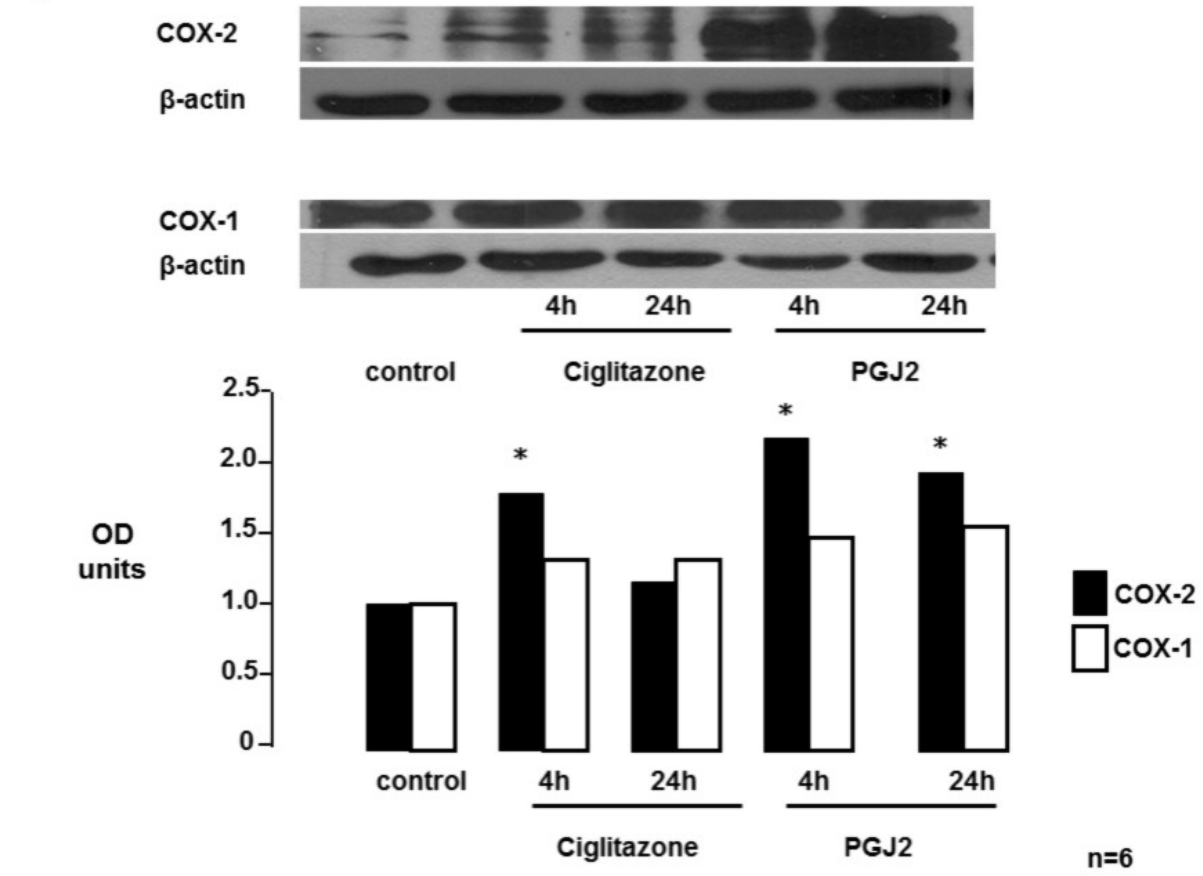


Figure 5b: PPAR-agonists Ciglitaxone and 15d PGJ2 induced COX-2, not COX-1, expression at 4 and 24h after stimulation (*$p<0.05$ compared to control)

Figure 5b


COX-2
β-actin
COX-1
β-actin
4h
24h
4h
24h
control
Ciglitazone
PGJ2
2.5
2.0
1.5
1.0
0.5
0
OD units
*
*
*
control
4h
24h
4h
24h
Ciglitazone
PGJ2
COX-2
COX-1
n=6