Recent reports demonstrate that peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily, acts as a repressor of type I collagen synthesis. Our data demonstrate that exogenously expressed PPARγ down-regulates collagen expression in a dose-responsive manner in human lung fibroblast cells. Silencing PPARγ using lentivirus expressing short hairpin RNAs partially reverses interferon-γ (IFN-γ)-induced repression and activates collagen mRNA levels. Previous studies indicate that IFN-γ represses collagen gene expression and induces major histocompatibility complex II (MHC II) expression by activating the formation of a regulatory factor for X-box 5 (RFX5) complex with class II transactivator (CIITA). This report demonstrates that PPARγ is within the RFX5-CIITA complex as judged by co-immunoprecipitation and DNA affinity precipitation studies. Most importantly, occupancy of PPARγ on the collagen transcription start site and MHC II promoter increases with IFN-γ treatment. The PPARγ agonist, troglitazone, sensitizes the cells to IFN-γ treatment by increasing recruitment of PPARγ to collagen gene while repressing collagen expression, and these effects are blocked by the PPARγ antagonist T0070907. PPARγ may mediate IFN-γ-stimulated collagen transcription down-regulation and MHC II up-regulation by interacting with CIITA as well as regulating CIITA expression. Therefore, PPARγ is a critical target for investigations into therapeutics of diseases involving extracellular matrix remodeling and the immune response.

The process of wound healing has been traditionally divided into several stages, including hemostasis, inflammation, cellular proliferation, and remodeling, when collagen is synthesized and fibers are deposited (1). These stages proceed by a series of overlapping, yet coordinated and highly regulated events highlighted by the interplay of cells, extracellular matrix, and an array of soluble mediators that are necessary to regenerate and remodel tissue. The extent of injury and the subsequent repair depend on the balanced control of matrix remodeling and inflammation. When this process is not regulated properly, there is either delayed healing with destruction of tissues or a fibroproliferative process with overproduction of collagen resulting in scarring, fibrosis, or cirrhosis depending on the organ.

Peroxisome proliferator-activated receptor-γ (PPARγ) is a member of the nuclear hormone receptor superfamily that regulates the expression of genes involved in a variety of biological processes, including lipid metabolism, insulin sensitivity, and inflammation (2–4). Ligands such as fatty acids, eicosanoids and oxidized lipids, have been identified that bind to PPARγ and alter its transcriptional activity. The thiazolidinediones class of drugs used in diabetes and dyslipidemias are synthetic ligands that activate PPARγ. Synthetic antagonists of PPARγ are also available to inhibit PPARγ activity (5–7).

Activation of PPARγ by agonists attenuates fibrosis in several organs, including kidneys (8, 9), liver (10, 11), heart (13), and lungs (14, 15), and arteries in atherosclerosis (12). Several studies suggest that PPARγ inhibits extracellular matrix synthesis, including collagen (16–18). PPARγ may alter connective tissue target genes by blocking TGF-β signaling (16, 19, 20), an important profibrotic cytokine. Alternatively, PPARγ may be directly involved in constitutive expression of collagen as part of transcriptional regulation complexes (17). Recent results suggest that PPARγ represses collagen transcription on the minimal COL1A1 promoter (17). Usually PPARγ heterodimerizes with retinoid-X-receptor and binds to a specific DNA response element with a direct repeat separated by a single intervening nucleotide (21). There are no PPARγ response elements in either collagen type I proximal promoter. On the other hand, PPARγ interacts with many proteins in different combinations to alter transcription of several other genes (4).

We have demonstrated that interferon-γ (IFN-γ), an important inflammatory cytokine, represses collagen transcription, in part, through the induction of regulatory factor for X-box 5 (RFX5) complex proteins and Class II transactivator (CIITA), and their recruitment to the collagen transcription start site (22–24). RFX5 and CIITA are important activators of the
immune response through transcriptional activation of major histocompatibility complex II (MHC II). In fact, mutations in these proteins cause bare lymphocyte syndrome. Because PPARγ may regulate a subset of IFN-γ target genes (25), we decided to investigate whether collagen was a target of PPARγ especially during IFN-γ treatment. In this report, we demonstrate that PPARγ is recruited to a complex with RFX5 and CIITA at the collagen transcription start site and the MHC II promoter during IFN-γ treatment. The IFN-γ-induced repression of collagen and activation of MHC II transcription is enhanced in the presence of PPARγ agonists and reversed by PPARγ antagonists. Finally, silencing PPARγ blocks the IFN-γ-induced collagen repression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Maintenance and Treatment Protocols**—Human lung fibroblasts, IMR-90 (IMR, NJ), and human embryonic kidney cells 293FT (Invitrogen) were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin G-streptomycin (Sigma). In several studies IMR-90 cells were treated with IFN-γ (Roche Applied Science), troglitazone (Trog, Biomol, Plymouth Meeting, PA), and/or T0070907 (Biomol). IMR-90 fibroblasts were plated in p150 tissue culture dishes at 4 × 10⁵ cells/dish, or 6-well 35-mm culture dishes at 3 × 10⁵ cells/dish, and maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum for 16–24 h. Cells were pretreated in Dulbecco’s modified Eagle’s medium with 0.4% fetal bovine serum for 16 h prior to IFN-γ treatment (100 units/ml), Trog treatment (5 μM), and/or T0070907 (1 μM) treatment.

**DNA Affinity Pulldown Assay**—The collagen sequence (COL1A2 -25/+30, GenBank™ accession number AF004877) with a HindIII overhang was synthesized as complementary strands and annealed as previously described (26). Double-stranded collagen DNA was biotin-labeled by incubating with Klenow fragment (New England Biolabs, Ipswich, MA) and biotin-14-dATP (Invitrogen) supplemented with regular dCTP, dTTP, and dGTP at room temperature for 30 min. The reaction mixture was phenol-chloroform-extracted and alcohol-purified to remove unincorporated biotin.

Nuclear protein extracts from IMR-90 cells were obtained as previously described using 450 mM sodium chloride (22, 27). The streptavidin beads (Promega, Madison, WI) were washed three times with ice-cold phosphate-buffered saline supplemented with 1 mM phenylmethylsulfonyl fluoride. Nuclear proteins (100–200 μg) were pre-cleared by incubating with the washed beads for 30 min at 4 °C on a shaking platform as described previously (23). Pre-cleared nuclear proteins were prepared by capturing the beads on a magnetic stand and removing the supernatant. The supernatant was then incubated with biotin-labeled collagen DNA probe (-25/+30) for 1 h at room temperature in binding buffer (60 mM NaCl, 20 mM Hepes, pH 7.9, 0.1 mM EDTA, 4% glycerol, 2 mM dithiothreitol) supplemented with bovine serum albumin, poly(dI-dC), and sonicated salmon sperm DNA to remove nonspecific binding. DNA-protein complex formed was captured by the magnetic beads and washed extensively with binding buffer supplemented with 0.01% Triton X and 100 mM KCl. The bound proteins were eluted with 1× electrophoresis sample buffer by incubating at 90 °C for 10 min and analyzed by SDS-PAGE gels.

**Plasmids, Transfections, and Luciferase Assays**—The col1α2-luciferase construct (pH20) (28) contains sequences from −221 to +5 bp of mouse col1α2 promoter fused to the luciferase reporter gene. A larger col1α2-luciferase construct (pGL3-Col-Luc) containing sequences from −357 to +55 bp of mouse col1α2 promoter fused to the luciferase reporter gene was a gift from Dr. Jenny Ting (29). One human COL1A1 promoter plasmids were all cloned into a pGL2-luciferase vector from the −804-hCOL1-luc plasmid described previously (24). Full-length PPARγ construct was generated as previously described (30). Full-length FLAG-RFX5 and three different isoforms of FLAG-CIITA were kindly provided by Dr. Jenny Ting. Cells were plated at the density of 3 × 10⁵ cells/well in 6-well tissue culture dishes for (IMR-90 cells) or 5 × 10⁶ cells per 100 tissue culture dish for (293FT cells). Transfections were performed with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol. Cells were harvested 48 h post transfections, and luciferase assays were performed with a luciferase reporter assay system (Promega).

**Immunoprecipitations**—To investigate whether factors interact in vivo, co-immunoprecipitations were performed. Whole cell lysates (IMR-90 or 293FT with transfected constructs as indicated where applicable) were obtained by re-suspending cell pellets in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100) with freshly added protease inhibitor (Roche Applied Science) and phenylmethylsulfonyl fluoride (100 μg/ml RIPA). Anti-RFX5 (194, Rockland), anti-PPARγ (H-100 and E8, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-CIITA (7–1H, Santa Cruz Biotechnology) antibodies were added to and incubated with IMR-90 cell lysate overnight before being absorbed by Protein A/G-plus agarose beads (Santa Cruz Biotechnology). Precipitated immune complex was released by boiling with 1× SDS-electrophoresis sample buffer. Alternatively, FLAG-conjugated beads (M2, Sigma) were added to and incubated with 293FT cell lysate overnight. Precipitated immune complex was eluted with 3× FLAG peptide (Sigma).

**Western Blots**—Proteins were quantified with the BCA reagent (Pierce) according to the manufacturer’s protocol, and separated by 10% PAGE with pre-stained markers (Bio-Rad) for estimating molecular weight and efficiency of transfer to blots. Proteins were transferred to nitrocellulose membranes (Bio-Rad) in a Mini-Trans-Blot Cell (Bio-Rad). The membranes were blocked with 5% milk powder in Tris-buffered saline (TBST) (0.05% Tween 20, 150 mM NaCl, 100 mM Tris-HCl, pH 7.4) buffer at 4 °C for several hours and incubated with monoclonal anti-FLAG (1:5000, Sigma), polyclonal anti-RFX5 (194, 1:1000, Rockland), monoclonal anti-CIITA (7–1H, 1:200) (Santa Cruz Biotechnology), monoclonal anti-β-actin (1:5000, Sigma), or monoclonal anti-PPARγ (E8, 1:200, Santa Cruz Biotechnology) antibody overnight. After three washes with TBST, the membranes were incubated with appropriate secondary antibodies, either anti-goat IgG (Sigma), anti-mouse IgG, or anti-rabbit IgG (Pharmacia Biotech, Piscataway, NJ) conjugated to horseradish peroxidase, for another hour at room temperature. Then protein blots were visualized using the...
Supersignal ECL reagent (Pierce) on a Kodak image station (PerkinElmer Life Sciences).

**Chromatin Immunoprecipitation**—Chromatin in control and treated cells were cross-linked with 1% formaldehyde for 8 min at room temperature, sequentially washed with phosphate-buffered saline, Solution I (10 mM HEPES, pH 7.5, 10 mM EDTA, 0.5 mM EGTA, 0.75% Triton X-100), and Solution II (10 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA). Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with protease inhibitor tablet (Roche Applied Science) and phenylmethylsulfonyl fluoride. DNA was fragmented into ~500 bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 µg of protein were used for each immunoprecipitation reaction with anti-RFX5 (194, Rockland), anti-PPARγ (H-100, Santa Cruz Biotechnology), anti-CIITA (7–1H, Santa Cruz Biotechnology), and anti-p300 (C-20, Santa Cruz Biotechnology) antibodies followed by adsorption to Protein A/G plus agarose beads (Santa Cruz Biotechnology). Precipitated DNA-protein complexes were washed sequentially with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM EDTA), high salt buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM EDTA), LiCl buffer (50 mM Tris, pH 8.0, 250 mM LiCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM EDTA), and TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), respectively. DNA-protein cross-linking was reversed by heating the samples to 65 °C overnight. Proteins were digested with proteinase K (Sigma), and DNA was phenol-chloroform-extracted and precipitated by 100% ethanol. Dried DNA was dissolved in 50 µl of deionized distilled water, and 10 µl was used for each real-time PCR reaction. The primers surrounding the collagen start site for real-time PCR have been described previously (22).

**RNA Isolation and Real-time PCR**—Cells were harvested, and RNA was extracted using an RNaseasy RNA isolation kit (Qiagen) according to the manufacturer’s protocol. Reverse transcriptase reactions were performed using a SuperScript First-strand synthesis system (Invitrogen) according to the manufacturer’s protocol. Real-time PCR reactions were performed on an ABI-Prism 7700 sequence detection PCR machine (Applied Biosystems, Foster City, CA) according to the manufacturer’s protocol. The oligonucleotide forward and reverse PCR primers and fluorescent probes are described in previous studies (22, 23, 31).

**PPARγ RNA Interference**—Five PPARγ-silencing clones were purchased from Sigma. Upon testing, two were determined to knock down PPARγ expression efficiently (clone #2 and clone #4, data not shown). These two clones, along with a non-functional clone (#5) as a control, were transfected into 293FT cells using Lipofectamine 2000 reagent according to the manufacturer’s protocol (Invitrogen). Viruses were harvested from different sources of anti-PPARγ antibodies (C), or anti-pre-immune IgG (5 µg) as indicated. Eluates were separated by 10% SDS gel, and Western blots were performed with antibodies as indicated. The original lysate (10%) was also loaded and labeled as input. A representative picture from three independent experiments is shown. M, protein marker; H.C., heavy chain.
PPARγ Interacts with RFX5:CIITA Complex

48 h post-transfection and used to infect IMR-90 cells. 24 h after infection, IMR-90 cells were treated with 100 units/ml IFN-γ or left untreated for additional 48 h before harvesting. Proteins were extracted using RIPA buffer (1× phosphate-buffered saline, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) and analyzed by SDS-PAGE gel followed by Western blot. RNAs were extracted and analyzed as described before.

Statistical Analysis—One-way analysis of variance with post-hoc Scheffe analyses were performed using an SPSS package. p values smaller than 0.05 were considered statistically significant.

RESULTS

PPARγ Co-immunoprecipitates with RFX5 and CIITA IV—PPARγ represses COL1A1 gene transcription even on the minimal promoter (17) raising the possibility that there might be an interaction between PPARγ and RFX5, because the latter binds to the collagen transcription start site and represses collagen synthesis (22). First, protein extracts from cells transfected with FLAG-RFX5 were immunoprecipitated with a FLAG antibody (M2) or IgG antibody-conjugated beads. PPARγ was co-immunoprecipitated with FLAG-RFX5 using specific epitope antibody but not control IgG (Fig. 1A). Next, proteins from human lung fibroblasts were immunoprecipitated by anti-RFX5 antibodies to examine endogenous PPARγ interactions with RFX5. PPARγ was co-immunoprecipitated with RFX5 (Fig. 1B). The secondary anti-rabbit IgG reacts with a nonspecific band in the non-immune control and the RFX5 serum. In the reciprocal experiment, RFX5 was co-immunoprecipitated using two different sources of PPARγ antibody (Fig. 1C) suggesting that PPARγ and RFX5 are in a similar complex.

CIITA is difficult to detect in un-stimulated mesenchymal cells. Yet, CIITA is largely responsible for the IFN-γ-induced repression of collagen transcription, because IFN-γ treatment stimulates CIITA expression, nuclear localization, and occupation on the collagen start site (23). Most importantly, silencing CIITA blocks IFN-γ-induced collagen repression (23). Two isoforms, CIITA III and CIITA IV, are inducible by IFN-γ in human smooth muscle cells (32) and fibroblasts (data not shown). To determine whether individual isoforms might co-immunoprecipitate with PPARγ, these two isoforms were overexpressed in 293FT cells and immunoprecipitated with epitope-specific antibody (M2). PPARγ was co-immunoprecipitated with CIITA IV isoform, but not with CIITA III isoforms (Fig. 2A). Experiments were also performed with CIITA I, which was not expressed in these cells as CIITA III or IV. There was no detectable association of CIITA I with PPARγ (data not shown).

To examine endogenous interactions, human lung fibroblasts were treated with IFN-γ and proteins were immunoprecipitated with either an anti-CIITA or an anti-PPARγ antibody. CIITA was detected in IFN-γ-treated extracts along with PPARγ using CIITA antibody that recognizes all CIITA isoforms (Fig. 3A). In a reciprocal experiment with PPARγ antibody, CIITA was co-immunoprecipitated only from IFN-γ-treated extracts (Fig. 2C) suggesting that when CIITA is expressed it interacts with PPARγ in the same complex.

PPARγ Represses Collagen Promoter Activity in a Dose-responsive Manner and Enhances CIITA IV Transcription Activity—PPARγ suppresses COL1A1 proximal promoter activity in activated hepatic stellate cells (17). In dermal fibroblasts, PPARγ transient transfection had a small inhibitory effect on COL1A2 activity (16). Our data demonstrate that col1a2 promoter activity is repressed by increasing amounts of PPARγ expression in human lung fibroblasts (Fig. 3A). Because PPARγ interacts with RFX5 and CIITA, we addressed whether RFX5 or the isoforms of CIITA might function to repress collagen promoter activity in an additive fashion with PPARγ. PPARγ (0.1 µg) enhanced the repression of col1a2 promoter activity most prominently with CIITA IV (Fig. 3B). This exact pattern was observed using a larger promoter of col1a2 (data not shown) and COL1A1 (Fig. 3C). The collagen genes have RFX5:CIITA binding sites at the transcription start site within a potential initiator site not in the promoter. Mutations in this region may block transcription as well as blocking RFX binding, so using mutations as a method to determine whether PPARγ-dependent transcription is mediated through the RFX5 binding site was not performed.

Most importantly, CIITA IV in combination with PPARγ was the best activator of the MHC II promoter (Fig. 3D) as well as the best repressing combination for collagen promoter activi-
PPARY Interacts with RFX5-CIITA Complex

FIGURE 3. PPARγ represses collagen promoter activity. A, PPARγ represses col1a2 promoter activity in a dose response manner. A col1a2 promoter construct (pH20, 0.5 μg) was co-transfected with different concentrations of PPARγ as indicated, along with GFP (0.1 μg) for normalization, into IMR-90 cells in triplicate wells as described under “Experimental Procedures.” Average luciferase activities were normalized by both protein concentration and GFP fluorescence and were expressed as relative percentage activity compared with the control group in which an empty vector was transfected. This representative experiment was repeated at least three times. At each dose tested, PPARγ repressed collagen promoter activity significantly (**, p < 0.01). B and C, PPARγ enhances the repression of collagen promoter activities by CIITA. A col1a2 promoter construct (pH20, 0.5 μg) (B) or a COL1A1 promoter construct (COL1A1–311, 0.5 μg) (C) was co-transfected with PPARγ (0.1 μg), RXFS (0.1 μg), CIITA III (0.1 μg), or CIITA IV (0.1 μg) construct as indicated, along with GFP (0.1 μg) for normalization, into IMR-90 cells in triplicate wells described under “Experimental Procedures.” Average luciferase activities were normalized by both protein concentration and GFP fluorescence and were expressed as relative percentage activity compared with the control group in which an empty vector was transfected. This representative experiment was repeated at least three times. Co-transfection of PPARγ rendered significant (**, p < 0.01) additional repression of col1a2 promoter activity by CIITA IV. D, PPARγ enhances the activation of MHC II promoter activity by CIITA. An MHC promoter construct (pGL3-DRA300, 0.5 μg) was co-transfected with PPARγ (0.1 μg), RXFS (0.1 μg), CIITA III (0.1 μg), or CIITA IV (0.1 μg) construct as indicated, along with GFP (0.1 μg) for normalization, into IMR-90 cells in triplicate wells as described under “Experimental Procedures.” Average luciferase activities were normalized by both protein concentration and GFP fluorescence and were expressed as relative percentage activity compared with the control group in which an empty vector was transfected. This representative experiment was repeated at least three times. Co-transfection of PPARγ renders significant (**, p < 0.01) additional activation of HLA-DRA promoter activity by CIITA IV.

PPARY Agonists Enhance the IFN-γ-induced Repression of Collagen—PPARY agonists disrupt TGF-β signaling in normal human dermal fibroblasts (16) and in pulmonary myofibroblasts (19). Because IFN-γ induces CIITA expression and abrogates TGF-β activity, we next used the agonist, Trog (0.5 μm), in the presence or absence of IFN-γ. Without exogenously added PPARγ, Trog had minimal effect on collagen promoter activity (Fig. 4, A and B). However, Trog enhanced IFN-γ-induced collagen repression for all collagen type I promoters tested. To determine if the agonist effect was through PPARγ, cells were pretreated with the antagonist T0070907 for 2 h before IFN-γ and/or Trog treatment. The antagonist blocked the action of Trog on all collagen promoters in the presence of IFN-γ suggesting there is a PPARγ effect (Fig. 4, A and B). Interestingly, without IFN-γ treatment the antagonist activated collagen promoter activity in the smaller (Fig. 4A) but not the larger col1a2 promoter (Fig. 4B). All collagen promoter activity changes were similar with a second antagonist GW9662 (data not shown), and the COL1A1 promoter (311 bp) was similar to the larger col1a2 promoter (357 bp) (data not shown).

PPARY Agonists Enhance the IFN-γ-induced Repression of Collagen and Activation of MHC II and CIITA mRNA Steady-state Levels—Human lung fibroblasts were treated with PPARγ agonists and antagonists either in the presence or absence of IFN-γ to examine mRNA steady-state levels. Clearly PPARγ agonist and antagonists altered collagen steady-state levels only in the presence of IFN-γ (Fig. 5, A and B). Trog enhanced collagen repression, and T0070907 antagonized this repression suggesting that PPARγ was involved.

CIITA steady-state mRNA was not detectable in the absence of IFN-γ and, neither the agonist nor the antagonist altered the CIITA mRNA level (Fig. 5C). IFN-γ stimulated the CIITA mRNA, and Trog slightly enhanced the stimulation, which was blocked by the antagonist, T007. MHC II expression followed a similar pattern to CIITA, indicating that PPARγ might directly impact on CIITA expression and hence MHC II and the inflammatory response.

Agonist and Antagonist Alter the Association of Proteins at the RFX5 Site on the Collagen Transcription Start Site by DNA Affinity Chromatography—RFX5 recruits CIITA to the collagen start site to repress transcription during IFN-γ treatment (22, 23). Because PPARγ is associated with both RFX5 and CIITA in the same complex, we next determined whether PPARγ could also be recruited by RFX5 and, if so, how the recruitment would be altered by different treatments. The biotinylated DNA oligonucleotide spanning the col1a2 start site (−25/+30) was incubated with nuclear proteins extracted from lung fibroblasts with different treatments as described earlier; eluates were separated by SDS gels and Western blots were performed to detect recruitment of RFX5, PPARγ, and CIITA as described under “Experimental Procedures” (22, 23). IFN-γ enhanced the recruitment of all three proteins to the collagen transcriptional start site (Fig. 6, compare lane 2 to lane 1), whereas Trog decreased the binding of both RFX5 and PPARγ.

moter activity in the smaller (Fig. 4A) but not the larger col1a2 promoter (Fig. 4B). All collagen promoter activity changes were similar with a second antagonist GW9662 (data not shown), and the COL1A1 promoter (311 bp) was similar to the larger col1a2 promoter (357 bp) (data not shown).

PPARY Agonists Enhance the IFN-γ-induced Repression of Collagen and Activation of MHC II and CIITA mRNA Steady-state Levels—Human lung fibroblasts were treated with PPARγ agonists and antagonists either in the presence or absence of IFN-γ to examine mRNA steady-state levels. Clearly PPARγ agonist and antagonists altered collagen steady-state levels only in the presence of IFN-γ (Fig. 5, A and B). Trog enhanced collagen repression, and T0070907 antagonized this repression suggesting that PPARγ was involved.

CIITA steady-state mRNA was not detectable in the absence of IFN-γ and, neither the agonist nor the antagonist altered the CIITA mRNA level (Fig. 5C). IFN-γ stimulated the CIITA mRNA, and Trog slightly enhanced the stimulation, which was blocked by the antagonist, T007. MHC II expression followed a similar pattern to CIITA, indicating that PPARγ might directly impact on CIITA expression and hence MHC II and the inflammatory response.

Agonist and Antagonist Alter the Association of Proteins at the RFX5 Site on the Collagen Transcription Start Site by DNA Affinity Chromatography—RFX5 recruits CIITA to the collagen start site to repress transcription during IFN-γ treatment (22, 23). Because PPARγ is associated with both RFX5 and CIITA in the same complex, we next determined whether PPARγ could also be recruited by RFX5 and, if so, how the recruitment would be altered by different treatments. The biotinylated DNA oligonucleotide spanning the col1a2 start site (−25/+30) was incubated with nuclear proteins extracted from lung fibroblasts with different treatments as described earlier; eluates were separated by SDS gels and Western blots were performed to detect recruitment of RFX5, PPARγ, and CIITA as described under “Experimental Procedures” (22, 23). IFN-γ enhanced the recruitment of all three proteins to the collagen transcriptional start site (Fig. 6, compare lane 2 to lane 1), whereas Trog decreased the binding of both RFX5 and PPARγ.

moter activity in the smaller (Fig. 4A) but not the larger col1a2 promoter (Fig. 4B). All collagen promoter activity changes were similar with a second antagonist GW9662 (data not shown), and the COL1A1 promoter (311 bp) was similar to the larger col1a2 promoter (357 bp) (data not shown).

PPARY Agonists Enhance the IFN-γ-induced Repression of Collagen and Activation of MHC II and CIITA mRNA Steady-state Levels—Human lung fibroblasts were treated with PPARγ agonists and antagonists either in the presence or absence of IFN-γ to examine mRNA steady-state levels. Clearly PPARγ agonist and antagonists altered collagen steady-state levels only in the presence of IFN-γ (Fig. 5, A and B). Trog enhanced collagen repression, and T0070907 antagonized this repression suggesting that PPARγ was involved.

CIITA steady-state mRNA was not detectable in the absence of IFN-γ and, neither the agonist nor the antagonist altered the CIITA mRNA level (Fig. 5C). IFN-γ stimulated the CIITA mRNA, and Trog slightly enhanced the stimulation, which was blocked by the antagonist, T007. MHC II expression followed a similar pattern to CIITA, indicating that PPARγ might directly impact on CIITA expression and hence MHC II and the inflammatory response.

Agonist and Antagonist Alter the Association of Proteins at the RFX5 Site on the Collagen Transcription Start Site by DNA Affinity Chromatography—RFX5 recruits CIITA to the collagen start site to repress transcription during IFN-γ treatment (22, 23). Because PPARγ is associated with both RFX5 and CIITA in the same complex, we next determined whether PPARγ could also be recruited by RFX5 and, if so, how the recruitment would be altered by different treatments. The biotinylated DNA oligonucleotide spanning the col1a2 start site (−25/+30) was incubated with nuclear proteins extracted from lung fibroblasts with different treatments as described earlier; eluates were separated by SDS gels and Western blots were performed to detect recruitment of RFX5, PPARγ, and CIITA as described under “Experimental Procedures” (22, 23). IFN-γ enhanced the recruitment of all three proteins to the collagen transcriptional start site (Fig. 6, compare lane 2 to lane 1), whereas Trog decreased the binding of both RFX5 and PPARγ.
PPARγ Interacts with RFX5·CIITA Complex

PPARγ agonists enhance the IFN-γ-induced messages. A and B, PPARγ agonists enhance the IFN-γ-mediated repression of collagen mRNA levels. IMR-90 cells were treated with IFN-γ (100 units/ml), Trog (5 μM), or T0070907 (1 μM) as described under “Experimental Procedures.” Total RNAs were prepared, transcribed to form cDNA, and real-time PCR reactions were performed with the cDNA samples using primers to detect COL1A1 mRNA (A) or COL1A2 mRNA (B). Each experiment was repeated at least three times in triplicate wells. Data are expressed as relative RNA levels compared with control levels, normalized to 18 S RNA and presented as average ± S.D. Trog significantly enhanced the IFN-γ-mediated repression of both COL1A1 mRNA (**, p < 0.01) and COL1A2 mRNA (*, p < 0.05). C and D, PPARγ agonists enhance the IFN-γ stimulation of CIITA and MHC II messages. IMR-90 cells were treated with IFN-γ (100 units/ml), Trog (5 μM), or T0070907 (1 μM) as described under “Experimental Procedures.” Total RNAs were prepared, transcribed to form cDNA, and real-time PCR reactions were performed with the cDNA samples using primers to detect CIITA mRNA (C) or HLA-DRA mRNA (D). Each experiment was repeated at least three times in triplicate wells. Data are expressed as relative RNA levels compared with control levels, normalized to 18 S RNA, and presented as average ± S.D. Trog significantly enhanced the IFN-γ-activated messages of both CIITA and HLA-DRA (**, p < 0.01).
pparg interacts with rfx5-cita complex

**FIGURE 7.** Agonist enhances the ifn-γ-induced recruitment of repressors on the collagen transcription start site and on the MHC II promoter. A–F, chromatin immunoprecipitation assays were performed with IMR-90 cells treated with IFN-γ (100 units/ml), Trog (5 μM), or t0070907 (1 μM) as indicated using anti-pparg (A and B), anti-rfx5 (C and D), or anti-cita (E and F) antibodies as described under “Experimental Procedures.” Precipitated genomic DNA was amplified by primers surrounding the collagen transcription start site (A, C, and E) or the MHC II X-box (B, D, and F). These experiments were repeated three times in triplicate wells and plotted as average ± S.D. Data were expressed as picogram DNA precipitated by indicated antibody per nanogram total genomic DNA. In each case, IFN-γ significantly enhanced the recruitment of repressors by these factors and Trog significantly enhanced the IFN-γ- induced repression. Infection by clone #5 did not cause any significant change in collagen expression either with or without IFN-γ treatment. Expression of col1a2 mRNA exhibited a similar pattern when cells were infected with pparg-silencing virus (Fig. 8B, second graph). On the other hand, pparg clone #2 and #4 infection blocked the IFN-γ induction of CITA mRNA, but #5 did not cause any change in ifn-γ-induced CITA expression (Fig. 8B, third graph). Because, surprisingly, IFN-γ-induced CITA expression was also greatly reduced by pparg short hairpin RNA at both the mRNA and protein levels (Fig. 8, A and B), pparg might be a direct regulator of CITA expression. Therefore, the changes in collagen gene expression could be caused by changes in CITA expression.

**DISCUSSION**

Recent reports demonstrate that pparg, a member of the nuclear receptor superfamily, acts as a repressor of type I collagen synthesis (16, 17, 33). Our data confirm that exogenously expressed pparg down-regulates collagen expression in a dose-responsive manner in human lung fibroblast cells as well as human aortic smooth muscle cells.3 pparg interacts with CITA (Figs. 1 and 2) and is recruited to the collagen start site and to the MHC II promoter site (Figs. 6 and 7). Silencing of pparg expression activated collagen gene expression and alleviated IFN-γ-induced repression of collagen mRNAs (**, p < 0.01). D, silencing clones partially inhibits IFN-γ-induced activation of CITA messages. RNA interference experiments were performed as described under “Experimental Procedures.” Total RNAs were prepared from cells with different infections and IFN-γ (100 units/ml) treatment as indicated, transcribed to form cDNA, and real-time PCR reactions were performed with the cDNA samples using primers to detect Col1a1 mRNA (B) or Col1a2 mRNA (C). pparg silencing clones significantly up-regulated basal-level collagen messages and partially alleviated IFN-γ-repression of collagen mRNAs (**, p < 0.01). D, silencing clones partially inhibits IFN-γ-induced activation of CITA messages. RNA interference experiments were performed as described under “Experimental Procedures.” Total RNAs were prepared from cells with different infections and IFN-γ (100 units/ml) treatment as indicated, transcribed to form cDNA, and real-time PCR reactions were performed with the cDNA samples using primers to detect CITA mRNA. PPARG silencing clones significantly down-regulated IFN-γ-activation of CITA mRNA (**, p < 0.01).

3 Y. Xu, S. R. Farmer, and B. D. Smith, unpublished data.

**FIGURE 8.** Silencing pparg partially reverses IFN-γ-induced repression of collagen messages. A, silencing clones decreased the expression of pparg RNA interference experiments were performed as described under “Experimental Procedures.” Cell lysates with different infections as indicated were analyzed for expression of pparg (top panel), CITA (middle panel), or Sin3B (bottom panel). Each experiment was repeated at least twice and a representative Western blot is shown. B, silencing clones partially reverses IFN-γ-induced repression of collagen messages. RNA interference experiments were performed as described under “Experimental Procedures.” Total RNAs were prepared from cells with different infections and IFN-γ (100 units/ml) treatment as indicated, transcribed to form cDNA, and real-time PCR reactions were performed with the cDNA samples using primers to detect COL1A1 mRNA (B) or COL1A2 mRNA (C). PPARG silencing clones significantly up-regulated basal-level collagen messages and partially alleviated IFN-γ-repression of collagen mRNAs (**, p < 0.01). D, silencing clones partially inhibits IFN-γ-induced activation of CITA messages. RNA interference experiments were performed as described under “Experimental Procedures.” Total RNAs were prepared from cells with different infections and IFN-γ (100 units/ml) treatment as indicated, transcribed to form cDNA, and real-time PCR reactions were performed with the cDNA samples using primers to detect CITA mRNA. PPARG silencing clones significantly down-regulated IFN-γ-activation of CITA mRNA (**, p < 0.01).
down regulating the profibrotic process. Interestingly, PPARγ levels are also dramatically reduced with age in several tissues (37). Therefore, the gene expression of PPARγ may be an important mechanism for modulating extracellular matrix production and inflammation through MHC II expression.

PPARγ agonists inhibit TGF-β-induced myofibroblast differentiation and signaling of collagen gene expression (16, 19, 36). IFN-γ has been recognized as an antagonist of TGF-β signaling (38–40). Our studies demonstrate that PPARγ agonist, Trog, sensitizes the cells to IFN-γ treatment by further lowering collagen expression levels (Figs. 4 and 5). Combined with the finding that TGF-β attenuates CIITA expression (41, 42), we propose that CIITA is a key protein, when facilitated by other factors such as RFX5 (22, 24), which determines the ultimate consequences of collagen transcription in response to different signaling pathways. Therefore, we investigated whether PPARγ agonists alter the IFN-γ response through the CIITA-RFX5 complex.

Previous studies in this laboratory have identified the RFX family of transcription factors that play vital roles in fine-tuning collagen expression and activating MHC II (43) during different physiological and/or pathophysiological scenarios (26, 31, 44). The RFX5-CIITA complex, which mediates IFN-γ-induced collagen repression (22, 23), binds to the transcription start site of type I collagen genes and represses collagen gene expression (22, 44). PPARγ exists in the same complex with RFX5 (Fig. 1) and type IV CIITA (Fig. 2) in both 293FT cells and IMR-90 cells. Moreover, recruitment of PPARγ to the collagen transcription start site was, like that of CIITA, dependent on RFX5 as judged by the removal of RFX5 binding that prevented PPARγ from interacting with the collagen DNA (Fig. 6, lane 9).

It should be noted that one of the unique features of the RFX proteins in regulating collagen transcription is that they bind to the first exon of the collagen gene where transcription starts, which coincidentally also serves as the binding site for the pre-initiation complex. Our published results indicate that there is a competition of occupancy on the collagen start site between the RFX5-CIITA complex and the pre-initiation complex (23). In addition, a +7 C to T mutation mimicking a methylated C at that position increases RFX1 binding and decreases activity of the promoter by 80% (45). Therefore, additional mutations at the RFX site would also deny the access of RNA polymerase II to the promoter, making data interpretation difficult. Therefore, we chose instead to use DNA competitors to demonstrate the specificity and recruitment of RFX binding in DNA affinity pulldown assays. It should be pointed out that this method has been exploited before and has satisfactorily served the same purpose in demonstrating the specificity and transcriptional activity of the RFX5-CIITA complex to this site (22–24). Further experiments extending this line of investigation are currently underway and will eventually provide extra evidence to our hypothesis.

More importantly, the physical association correlates with the function in which PPARγ enhanced the repression of collagen and activation of MHC II promoter activities by type IV CIITA (Fig. 3, B and C). Interestingly, although it is clear that PPARγ and RFX5 co-immunoprecipitate, there was no functional synergism between them on either promoter (Fig. 3, B and C). One reason could be that the interaction between PPARγ and RFX5 is not sufficient for transcriptional activity, but rather necessary to recruit CIITA, which then co-operates with PPARγ to determine the ultimate transcriptional outcome. Alternatively, this repression may require post-translational modification of the factors through IFN-γ signaling.

Our data presented in this report indicates that Trog enhances the IFN-γ repression of collagen and activation of MHC II (Figs. 4B, 4C, and 5). Most importantly, the recruitment of PPARγ at the collagen start site and the MHC II promoter is increased with IFN-γ treatment followed by a further increase in the presence of Trog, which is neutralized by an antagonist (Fig. 7, A and B). There is a strikingly similar pattern of RFX5 and CIITA recruitment on both genes suggesting that IFN-γ stimulates PPARγ recruitment within a complex with CIITA and RFX5. On the other hand, CBP/p300 decreases slightly on the collagen start site with IFN-γ treatment. PPARγ binds to the COL1A2 transcription start site both in vitro (Fig. 6) and ex vivo (Fig. 7A). It has become clear that combinatorial interactions among promoter-bound proteins determine transcriptional outcome in different cellular and experimental contexts on COL1A2 gene (46). Our results in this report demonstrate a unique association of the nuclear receptor, PPARγ, with transcription factors, RFX5 and CIITA type IV, at the transcriptional start site of both type I collagen genes (Figs. 6 and 7A) and at the MHC II promoter (Fig. 7B) especially during IFN-γ signaling.

CIITA has been termed a scaffold protein that interacts with several proteins, including NF-Y/CBF, RFX5, CREB (47), and CBP/p300 (29). The collagen type I promoters have a reverse CAAT box where a heterotrimeric CAAT binding factor NF-Y/CBF interacts (48). On the other hand, this site in the COL1A1 promoter has been identified as a nuclear factor-1 (NF-1) binding site in hepatic stellate cells (49). Interestingly, exogenously transfected PPARγ blocks the function of NF-1 and its interaction with the COL1A1 promoter in hepatic stellate cells (17). It is possible that the NF-1 is replaced by NF-Y-RFX5 complex (50).

Two collagen promoters of different sizes were compared to determine whether a 136-bp region alters the PPARγ response. PPARγ antagonists activate the shorter promoter but not the longer promoter with the additional 136 bases (Fig. 4). As shown in the model (Fig. 9), the 136 bases contain multiple binding sites for known activators of collagen transcription that interact with CBP/p300. The action of antagonist-bound PPARγ may be blocked by these upstream binding proteins. The larger promoter pattern mimics the mRNA pattern (Fig. 5). Studies are ongoing to determine the upstream interactions of proteins with CIITA and PPARγ.

Earlier results indicate that two CIITA domains (N-terminal and PST domain) are required for repression on the larger promoter, whereas the PST domain alone represses the small basal promoter (23). Others demonstrated that the N-terminal CIITA is required for repression through CBP/p300 (29). In addition, CBP/p300 counteracts PPARγ repression (17) and CIITA repression of collagen transcription (29). Our results suggest that PPARγ and CIITA are in the same complex and CIITA IV, which has a different N-terminal domain than the
PPARγ Interacts with RFX5-CIITA Complex

FIGURE 9. Model of collagen repression by PPARγ, CIITA, and RFX5 during IFN-γ treatment. This model is a schematic representation showing PPARγ interactions with both RFX5 and CIITA IV. RFX5 can associate NF-Y/CBF components without DNA (50). IFN-γ treatment increases expression of CIITA and RFX5 complex formation (RFX5, RFXAP, and RFXB) with CIITA on collagen transcription start site. The drawing at the bottom represents the possible interactions showing proteins such as TAF32, TAF70, and NF-Y that are known to interact with CIITA. The curved line represents DNA at the collagen transcription start site from about −357 to +55. The flash arrow indicates approximately the position of the short promoter at −221. The collagen promoter cis-acting consensus sites are shown as boxes (TATA, TATA box or TFIIID binding site; CCAAT, CBP/NF-Y binding site (68); Sp1, Sp1 binding sites (69); IFNRE, IFN-γ response element (70); BFCOL1, binding factor for type-I collagen consensus site (71); Krox, krox consensus site (72); EBS, Ets binding site (73); NF-kB (74); AP1 (74); Smad3 (75, 76); and Sp1 (77, 78) sites). The PPAR-γ-RFX5-CIITA complex with co-repressors may replace NF-1-CBP complex as represented in the bottom left of the model.

other isoforms, is necessary for transcriptional repression possibly by inactivating CBP/p300. CBP/p300 occupancy is decreased in the presence of IFN-γ (Fig. 7G). A model (Fig. 9) shows the interactions of PPARγ with both RFX5 and CIITA. On the collagen genes, this complex containing NF-Y may replace the NF-1-CBP/p300 complex.

To our surprise, IFN-γ-induced expression of CIITA dramatically decreased in the absence of PPARγ when the cells were infected by PPARγ short hairpin RNAs. However, expression of several early response genes in the IFN-γ signaling pathway was not affected (data not shown), suggesting that PPARγ might directly bind to the CIITA promoter and activate its transcription. Because CIITA is the critical factor in IFN-γ-mediated connective tissue remodeling (down-regulation of collagen expression) and inflammatory response (up-regulation of MHC II expression), this observation further highlights the importance of PPARγ during the regulation of these two events, because it is able to not only functionally interact with CIITA, but also control the expression level of CIITA.

Nuclear receptors are certainly among the proteins that can interact with multiple families of transcription factors, co-activators, and co-repressors (4). PPARγ forms a heterodimer with retinoic acid X receptor when interacting with its peroxisome proliferator response element consisting of direct repeats of hexanucleotides separated by a single nucleotide spacer (see reviews (4, 51, 52)). There is no peroxisome proliferator response element in the proximal collagen promoters used in this study, although there are putative untested upstream sites in col1α2 promoter (−581 to −530 and from −1364 to −1352) (18). Usually PPARγ interacts with co-activators (SRC1 and CBP/p300) when ligands are available. Without ligand, PPARγ interacts with co-repressor complexes containing NCoR-SMRT and HDAC3 and represses genes. In addition, PPARγ when it is sumoylated interacts with NCoR-SMRT and HDAC3 without interacting with DNA in a transrepression mechanism (3, 53). Although PPARγ interacts with the collagen gene and the MHC II gene both in vitro (Fig. 6) and ex vivo (Fig. 7) in the presence of IFN-γ, this is not accompanied by HDAC3 as indicated in our previous report, which demonstrates that Sin3B with HDAC2, not HDAC3, dramatically interacts with collagen promoters in the presence of IFN-γ (31). In addition, HDAC2 deacetylates RFX5 as well as histones (31). Our unpublished results indicate that no NCoR-SMRT is detected in DNA affinity precipitations (data not shown) and that Sin3B and HDAC2 interact with CIITA in a phosphorylation-dependent manner (data not shown). It is not clear yet which proteins interact directly with PPARγ in this complex and whether other known PPARγ-interacting proteins, such as retinoic-X-receptor, are in this complex. Our results demonstrate a hitherto un-described complex with PPARγ containing CIITA-RFX5-HDAC2-Sin3B that assembles on the collagen gene to repress collagen gene transcription by transrepression.

CIITA, RFX5, and PPARγ are recruited to the MHC II promoter during IFN-γ treatment, but this gene is activated not repressed. More experiments are required to determine why similar complexes on two different genes can repress or activate. This could be through promoter context or by recruitment of different co-activator/co-repressor complexes on different promoters. We have preliminary data, gained by chromatin immunoprecipitation analysis, that CIITA-RFX5-PPARγ on the MHC II promoter recruits less Sin3B and HDAC2 in the presence of IFN-γ for 24 h (data not shown). However, the activation of MHC II may be down-regulated by similar molecules at a later time (54).

In addition to sumoylation and acetylation, PPARγ (55) and RFX5 (56) are also phosphorylated, which could alter their transcriptional outcome if IFN-γ causes post-translational modifications. Therefore, it is quite possible that combinations of different cytokine and/or growth factor treatments result in variations in modifications, which in turn, determine different transcriptional outcomes. This strongly suggests that the interactions demonstrated with CIITA-RFX5 are unique with repression of collagen being caused by Sin3-HDAC2 associated co-repressor.

Structurally diverse molecules bind to and activate PPARγ (2). The thiazolidinedione compounds and certain prostaglan-
dins have been used to demonstrate that activation of PPARγ inhibits collagen synthesis (18) and fibrosis in lung (14, 15, 19, 57), kidney (8, 9, 58), liver (10, 11, 33, 36, 59), cardiac fibroblasts (60), and skin (61). The issue of endogenous ligand for PPARγ in cells has been controversial. PPARγ ligands, such as fatty acids, eicosanoids, and oxidized lipids, have been identified that bind to and alter PPARγ transcriptional activity. Prostaglandin J2 derivative, often considered the endogenous ligand, has a highly specific natural ligand or collagen gene expression (Fig. 5) by Trog alone in lung fibroblasts similar to dermal fibroblasts (16). It is possible that the cells produce an endogenous ligand so that exogenously added Trog does not alter collagen gene expression. Antagonists might be able to reverse the effects of an endogenous ligand. The antagonist T0070907 and GW9662 (data not shown) activated the minimal col1a2 promoter activity (Fig. 4A) but had no effect on the larger col1a2 (Fig. 4B) or COL1A1 (data not shown) promoters or collagen mRNA levels (Fig. 5). The difference in the long and minimal promoter might suggest that PPARγ interacts with a protein recruited to an upstream region between −350 and −221 independent of ligand.

Alternatively, part of the mechanism of collagen transcriptional regulation by PPARγ might not depend on ligand, but rather through interactions with other factors such as the RFX5- CIITA complex in a facilitative fashion. Decreased levels of PPARγ in these cells could lead to impaired transrepression complex formation on the collagen promoter and partially relieve the repression of collagen transcription, which is irrelevant to the presence or absence of ligand. Another probable explanation is that endogenous ligand might exist in a wide range of concentrations in different cell types. Therefore, one given synthetic ligand at certain concentrations may or may not cause any change to a particular PPARγ target gene. Our observation that Trog represses collagen transcription only in the presence of IFN-γ (Figs. 4 and 5) raises yet another possibility that PPARγ activity partially depends on its post-translational modification (such as sumoylation), which is controlled by its ligands in conjugure with IFN-γ (and/or other cytokines); increasing the ligand level alone would not change PPARγ modification or alter its activity, hence no effect of its target genes could be detected.

PPARγ is involved with inflammatory response (4, 51, 52) and in atherosclerosis (66). Activation by agonists makes it an anti-inflammatory molecule in part by down-regulation of cytokines like IFN-γ. PPARγ activation by agonist negatively regulates a specific subset of IFN-γ target genes in macrophages (25). This negative repression is most likely through sumoylation and transrepression (53). CIITA and MHC II, investigated in this report, are clearly IFN-γ target genes involved in inflammation, because MHC II is critical for activation of T cells and CIITA-RFX5 are critical for activation of MHC II. However, these genes are not negatively regulated in fibroblasts by PPARγ agonists. In fact, agonists enhance the IFN-γ induction of CIITA and MHC II expression, which contrasts to a previous report using atheroma cells (67). One explanation is that cell type differences exist or the agonists have PPARγ-independent effects (67). In our studies, antagonists reversed the activation by agonist (Figs. 5 and 7) suggesting agonist-induced activation is through PPARγ.

In summary, we demonstrate that PPARγ associates with RFX5 and type IV CIITA in the same complex. PPARγ is recruited to the collagen transcription start site by RFX5-CIITA complex, where it represses collagen expression, and more importantly, is involved in mediating and enhancing the IFN-γ-stimulated collagen transcription down-regulation and MHC II up-regulation. Without IFN-γ treatment, there is little recruitment of RFX5-PPARγ to the collagen start site or to MHC II promoter. In addition, silencing PPARγ lowers IFN-γ induction of CIITA and attenuates repression of collagen expression. Therefore, PPARγ may be involved in a regulatory loop by regulating the expression of a protein that it interacts with. This report highlights the importance of both connective tissue remodeling and inflammatory response by bringing the transcription factors, RFX5-CIITA/PPARγ complex, which regulate transcriptional events in a coordinated and cooperative manner. Therefore, PPARγ is a critical target for investigations into therapeutics of diseases to whose etiologies both extra matrix remodeling and immune response contribute.

REFERENCES

1. Diegelmann, R. F., and Evans, M. C. (2004) *Front. Biosci.* 9, 283–289
2. Standiford, T. J., Keshamouni, V. G., and Reddy, R. C. (2005) *Proc. Am. Thorac. Soc.* 2, 226–231
3. Pascual, G., and Glass, C. K. (2006) *Trends Endocrinol. Metab.* 17, 321–327
4. Glass, C. K., and Ogawa, S. (2006) *Nat. Rev. Immunol.* 6, 44–55
5. Schaefer, K. L., Wada, K., Takahashi, H., Matsuhashi, N., Ohnishi, S., Wolfe, M. M., Turner, J. R., Nakajima, A., Borkan, S. C., and Saubermann, L. J. (2005) *Cancer Res.* 65, 2251–2259
6. Collin, M., Murch, O., and Thiemermann, C. (2006) *Crit. Care Med.* 34, 1131–1138
7. Lee, G., Elwood, F., McNally, I., Weissmann, J., Lindstrom, M., Amaral, K., Nakamura, M., Miao, S., Cao, P., Learned, R. M., Chen, J. L., and Li, Y. (2002) *J. Biol. Chem.* 277, 19649–19657
8. Panchapakesan, U., Sumual, C., Pollock, C. A., and Chen, X. (2005) *Am. J. Physiol.* 289, F1153–F1158
9. Zafiriou, S., Stanners, S. R., Saad, S., Polhill, T. S., Poronnin, P., and Pollock, C. A. (2005) *J. Am. Soc. Nephrol.* 16, 638–645
10. Kon, K., Bejima, K., Hirose, M., Yoshikawa, M., Enomoto, N., Kitamura, T., Takei, Y., and Sato, N. (2002) *Biochem. Biophys. Res. Commun.* 291, 55–61
11. Yang, L., Chan, C. C., Kwon, O. S., Liu, S., McGhee, J., Stimpson, S., Chen, L., Harrington, W. W., Symonds, W., and Rockey, D. C. (2006) *Am. J. Physiol.* 291, G902–G911
12. Li, A. C., Brown, K. K., Silvestre, M. J., Willson, T. M., Palinski, W., and Glass, C. K. (2000) *J. Clin. Invest.* 106, 523–531
13. Ogata, T., Miyauci, T., Sakai, S., Takanashi, M., Irukayama-Tomobe, Y., Nakamura, M., Miao, S., Cao, P., Learned, R. M., Chen, J. L., and Li, Y. (2002) *J. Biol. Chem.* 277, 19649–19657
14. Genovese, T., Cuzzocrea, S., Di Paola, R., Mazzon, E., Mastruzzo, C., Catania, P., Sortino, M., Crimi, N., Caputi, A. P., Thiemermann, C., and Vancheri, C. (2005) *Eur. Respir. J.* 25, 225–234
15. Belvisi, M. G., Hele, D. J., and Birrell, M. A. (2006) *Eur. J. Pharmacol.* 533, 101–109
