Hepatocyte Growth Factor Is a Downstream Effector that Mediates the Antifibrotic Action of Peroxisome Proliferator–Activated Receptor-γ Agonists

Yingjian Li, Xiaoyan Wen, Bradley C. Spataro, Kebin Hu, Chunsun Dai, and Youhua Liu

Division of Cellular and Molecular Pathology, Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania

Peroxisome proliferator–activated receptor-γ (PPAR-γ) is a ligand-dependent transcription factor that plays an important role in the regulation of insulin sensitivity and lipid metabolism. Evidence shows that PPAR-γ agonists also ameliorate renal fibrotic lesions in both diabetic nephropathy and nondiabetic chronic kidney disease. However, little is known about the mechanism underlying their antifibrotic action. This study demonstrated that PPAR-γ agonists could exert their actions by inducing antifibrotic hepatocyte growth factor (HGF) expression. Incubation of mesangial cells with natural or synthetic PPAR-γ agonists 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) or troglitazone and ciglitazone suppressed TGF-β1-mediated α-smooth muscle actin, fibronectin, and plasminogen activator inhibitor-1 expression. PPAR-γ agonists also induced HGF mRNA expression and protein secretion. Transfection studies revealed that 15d-PGJ2 stimulated HGF gene promoter activity, which was dependent on the presence of a novel peroxisome proliferator response element. Treatment of mesangial cells with 15d-PGJ2 induced the binding of PPAR-γ to the peroxisome proliferator response element in the HGF promoter region. PPAR-γ agonists also activated c-met receptor tyrosine phosphorylation, induced Smad transcriptional co-repressor TGF-β1–mediated factor expression, and blocked TGF-β1/Smad-mediated gene transcription in mesangial cells. Furthermore, ablation of c-met receptor through the LoxP-Cre system in mesangial cells abolished the antifibrotic effect of 15d-PGJ2. PPAR-γ activation also induced HGF expression in renal interstitial fibroblasts and repressed TGF-β1–mediated myofibroblast activation. Both HGF and 15d-PGJ2 attenuated Smad nuclear translocation in response to TGF-β1 stimulation in renal fibroblasts. Together, these findings suggest that HGF may act as a downstream effector that mediates the antifibrotic action of PPAR-γ agonists.

In addition to their ability to improve insulin sensitivity, increasing evidence indicates that PPAR-γ agonists possess antifibrotic potential that results in an attenuation of renal fibrosis after chronic injury. Of particular interest, studies show that PPAR-γ agonists not only are able to ameliorate glomerulosclerosis and kidney dysfunctions in diabetic nephropathy (5–7) but also exert beneficial actions in nondiabetic chronic kidney disease (8,9). In rat remnant kidney model of renal fibrosis, administration of PPAR-γ agonist troglitazone is associated with a reduction of proteinuria, serum creatinine, and glomerulosclerosis (8). PPAR-γ activation also decreases glomerular cell proliferation and suppresses plasminogen activator inhibitor-1 (PAI-1) and TGF-β expression (8). Similarly, in anti–glomerular basement membrane nephritic rats, troglitazone suppresses urinary protein excretion and crescent formation and inhibits glomerular infiltration of monocytes/macrophages (9). In vitro investigations have also revealed that PPAR-γ activators are capable of inhibiting cell proliferation and suppressing the expression of extracellular matrix (ECM) components such as type I collagen and fibronectin (10–13). These observations underline that the activation of PPAR-γ by its ligands may have a direct effect on the processes of renal fibrogenesis, primarily through a mechanism independent of the insulin/glucose regulation. However, little is known about

JASN Express. Published on November 9, 2005 as doi: 10.1681/ASN.2005030257
the molecular mechanism underlying the antifibrotic action of PPAR-γ agonists. The pathogenesis of kidney fibrosis is characterized by relentless overproduction and deposition of ECM, which ultimately lead to fibrotic lesions and tissue scarring. Extensive studies have indicated that the myofibroblastic activation of glomerular mesangial cells and interstitial fibroblasts, as manifested by α-smooth muscle actin (α-SMA) induction, plays a crucial role in ECM overproduction. In this context, it is conceivable that the regulation of myofibroblastic activation may provide an effective means to modulate fibrotic processes under pathologic conditions. Indeed, many fibrogenic factors, including TGF-β, are known to promote the myofibroblastic activation (14–16), whereas antifibrotic hepatocyte growth factor (HGF) inhibits this process by counteracting TGF-β1 activation (17,18).

To elucidate the molecular mechanism by which PPAR-γ agonist exerts its antifibrotic activity, we have investigated the effects of PPAR-γ activation on TGF-β1–induced mesangial cell activation. The results presented herein suggest that the antifibrotic activity of PPAR-γ agonist is mediated primarily by HGF, an endogenous cytokine that has emerged as a key antifibrotic factor in vivo. We show that PPAR-γ activation induces HGF expression, activates c-met receptor, upregulates Smad transcriptional co-repressor TG-interacting factor (TGIF) expression, and suppresses Smad-mediated gene transcription. Our data define a novel molecular pathway that couples PPAR-γ, HGF, and TGF-β/Smad signaling.

Materials and Methods

Cell Culture and Treatment

Human renal mesangial cells (HMC) was purchased from ScienCell Research Laboratories (San Diego, CA). Rat mesangial cells (RMC) were provided by Dr. C. Wu (University of Pittsburgh, Pittsburgh, PA). Normal rat kidney interstitial fibroblast cells (NRK-49F) were maintained in DMEM/F12 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA). HMC were cultured in mesangial cell medium (ScienCell Research Laboratories) plus 10% FBS. The cells were seeded onto six-well culture plates to 60 to 70% confluence in the complete medium for 16 h and then changed to serum-free medium. Recombinant human TGF-β1 (R & D Systems, Minneapolis, MN) was added to the cultures at a final concentration of 1 ng/ml. 15-Deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2; Cayman Chemical, Ann Arbor, MI) and troglitazone and ciglitazone (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) were also added at the concentrations as indicated. Recombinant human HGF protein was provided by Genentech Inc. (South San Francisco, CA). The cells typically were incubated for 24 to 72 h after addition of cytokotines, before being subjected to Western blot or immunofluorescence staining, respectively.

Western Blot Analysis

Western blot analysis for specific protein expression was performed essentially according to an established procedure (19). The primary antibodies used were as follows: Anti-α-SMA (clone IA4) and anti-α-tubulin (Sigma, St. Louis, MO); antifibronectin (clone 10; BD Pharmin- gen, San Jose, CA); anti–PAI-1 (sc-5297), anti-TGIF (sc-17800), antifibronectin (sc-9068), and anti–PPAR-γ (sc-7196; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); antibodies against phospho-Met (Tyr1234/1235) and total c-met receptor (Cell Signaling Technology Inc., Beverly, MA); anti–phospho-specific Smad-2 (Upstate, Charlottes- ville, VA); and anti-Cre recombinase (EMD Biosciences, Inc., San Diego, CA) and anti-actin (Chemicon International, Inc., Temecula, CA). Monoclonal anti-HGF antibody (clone H8) was prepared and charac- terized as described previously (20,21). For detecting HGF secretion, equal amounts of supernatants that were derived from mesangial cells and NRK-49F cells after various treatments were concentrated with Microcon YM-100 centrifugal filter (Millipore, Billerica, MA), followed by Western blot analysis.

Immunofluorescence Staining

Indirect immunofluorescence staining was performed using an es- tablished procedure (22). Briefly, cells that were cultured on coverslips were washed with cold PBS twice and fixed with cold methanol:acetone (1:1) for 10 min at −20°C. After extensive washing three times with PBS that contained 0.5% BSA, the cells were blocked with 20% normal donkey serum in PBS buffer and then incubated with the specific primary antibodies against α-SMA, fibronectin, and Cre recombinase as described above, as well as anti-Smad2/3 antibody (sc-6032; Santa Cruz Biotechnology). For visualizing primary antibodies, cells were stained with cyanine Cy2-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were double stained with DAPI (4′,6-diamidino-2-phenylindole, HCl) to visualize the nu- clei. Stained cells were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) and viewed under Nikon Eclipse E600 Fluorescence microscope equipped with a digital cam- era (Melville, NY).

Plasmid Construction, Transfection, and Reporter Gene Assay

The reporter constructs 0.2HGF-Luc and 0.3HGF-Luc, which contain 0.2 and 0.3 kb of the 5′ flanking region of mouse HGF gene and the coding sequence for firefly luciferase, were described previously (23). The reporter construct p3TP-Lux, Smad2 and Smad3 expression vec- tors, and TGIF expression vector (pHA-TGIF) were provided by Dr. J. Massague (Memorial Sloan-Kettering Cancer Center, New York, NY). RMC were transfected with various plasmids as indicated. A fixed amount (0.1 μg) of internal control reporter Renilla reniformis luciferase driven under thymidine kinase (TK) promoter (pRL-TK; Promega, Madison, WI) was co-transfected for normalizing the transfection effi- ciency. Luciferase assay was performed using the Dual Luciferase Reporter Assay Kit according to the manufacturer’s protocols (Promega). Relative luciferase activity (arbitrary unit) was reported as fold induc- tion after normalization for transfection efficiency.

Reverse Transcription–PCR and Quantitative Real-Time PCR

Total RNA was extracted using TRIzol RNA isolation system (In- vitrogen). The first strand of cDNA was synthesized using 2 μg of RNA in 20 μl of reaction buffer by reverse transcription using AMV-RT (Promega) and random primers at 42°C for 30 min. PCR was carried out using a standard PCR kit and 1-μl aliquot of cDNA, HotStarTaq polymerase (Qiagen Inc., Valencia, CA) with specific primer pairs. The se- quences of primer pairs were as follows: HGF, 5′-AAGGTGACTCT- GAATGAGTC-3′ (sense) and 5′-GGCACTTCAGCCACGAAACAA- TG-3′ (antisense); β-actin, 5′-TCAGACTATGCTCCTCGAGAC-3′ (sense) and 5′-TGGTGTGACCTTCCACACCGACG-3′ (antisense). For quantitative determination of HGF mRNA level, a real-time reverse transcription–PCR (RT-PCR) was performed on ABI PRISM 7000 Se- quence Detection System (Applied Biosystems, Foster City, CA). The
PCR reaction mixture in a 25-μl volume contained 12.5 μl of 2× SYBR Green PCR Master Mix (Applied Biosystems), 5 μl of diluted RT product (1:10), and 0.5 μM sense and antisense primer sets. The primer pairs for HGF were the same as that used in regular RT-PCR. The sequences of β-actin primer pairs used in real-time PCR were described previously (24). PCR reaction was run by using standard conditions. After sequential incubations at 50°C for 2 min and 95°C for 10 min, respectively, the amplification protocol consisted of 50 cycles of denaturing at 95°C for 15 s, annealing, and extension at 60°C for 60 s. The standard curve was made from series dilutions of template cDNA. Expression levels of HGF mRNA were calculated after normalization with β-actin.

DNA Affinity Precipitation Assay
DNA–protein interaction studies were carried out by a simple DNA affinity precipitation assay (25). Briefly, the 5′-biotinylated, double-stranded oligonucleotides (0.2 nM) that contained two tandem copies of peroxisome proliferator response element (PPRE) at the nucleotide position −244 to −231 of mouse HGF promoter (5′-CTCGGGCCAGGTGACCTTT-CGGGCCAGGTGACCTTT-3′) (23,26) were mixed with 200 μg of whole cell extracts from the control and 15d-PGJ2–treated mesangial cells for 3 h at 4°C. After streptavidin-agarose (10 μl of packed beads) was added, the incubation was continued for an additional 3 h at 4°C. The beads were washed four times with ice-cold PBS that contained 0.5% Triton X-100 and 5 mM EDTA. Proteins were eluted from the beads by the addition of 2× SDS sample loading buffer, followed by boiling for 5 min. Eluted proteins were analyzed by Western blot analysis with specific anti–PPAR-γ antibody.

Mouse Mesangial Cell Culture and Adenovirus Infection
The c-met floxed mice in which the exon 16 of the c-met gene was flanked by loxP sites were provided by Dr. S. Thorgeirsson (National Cancer Institute, National Institutes of Health, Bethesda, MD) (27). Mouse mesangial cells (MMC) from outgrowths of isolated whole glomeruli were prepared essentially as described previously (28). The cells showed the typical smooth muscle cell–like morphology and positive staining for Thy1.1 and α-SMA and negative for cytokeratin. The fourth through 10th passages of the subcultured MMC were infected with adenoviral vectors (Ad.LacZ or Ad.Cre; provided by Dr. A. Gambotto, Vector Core Facility, University of Pittsburgh, Pittsburgh, PA) in serum-free medium (10^7 particles/ml). Infected cells were incubated for 4 h and then restored to complete medium. After 24 h, infected cells were treated with or without TGF-β1 (1 ng/ml) and/or 15d-PGJ2 (2.5 μM), respectively, for 48 h. Whole-cell lysates then were collected for analyses.

Figure 1. Both natural and synthetic peroxisome proliferator–activated receptor-γ (PPAR-γ) agonists inhibit TGF-β1–mediated α-smooth muscle actin (α-SMA) expression in glomerular mesangial cells. (A through D) Human mesangial cells (HMC) were incubated with or without TGF-β1 (1 ng/ml) and/or a fixed amount (2.5 μM) of 15-deoxy-A13,14-prostaglandin J2 (15d-PGJ2) (A) or troglitazone and ciglitazone (C), respectively, for different periods of time as indicated, or with different amounts of 15d-PGJ2 (B), and troglitazone and ciglitazone (D) for 48 h. Whole-cell lysates were immunoblotted with specific antibodies against α-SMA and actin or α-tubulin, respectively. (E through H) Indirect immunofluorescence staining shows α-SMA expression in mesangial cells after various treatments. HMC were treated without or with TGF-β1 (1 ng/ml) and/or 15d-PGJ2 (2.5 μM) for 48 h. (E) Control. (F) TGF-β1. (G) 15d-PGJ2. (H) TGF-β1 plus 15d-PGJ2. Bar = 10 μm.
Nuclear Protein Extraction
NRK-49F cells were pretreated with 15d-PGJ2 (2.5 μM) for 12 h or HGF (40 ng/ml) for 0.5 h, followed by incubation with TGF-β1 (1 ng/ml) for 30 min. Cell nuclei were isolated by procedures described previously (18). The nuclei were lysed with SDS sample buffer and subjected to Western blot analysis as described above.

Statistical Analyses
All data examined were expressed as mean ± SEM. Statistical analyses of the data were performed using SigmaStat software (Jandel Scientific Software, San Rafael CA). Comparison between groups was made using one-way ANOVA, followed by Student-Newman-Keuls test. P < 0.05 was considered significant.

Results
Suppression of TGF-β1–Mediated Mesangial Cell Activation by PPAR-γ Agonists
The induction of α-SMA is a hallmark for mesangial cell activation in many types of primary glomerular diseases. Therefore, we first examined the effect of PPAR-γ activation on α-SMA expression in cultured HMC. As shown in Figure 1, 15d-PGJ2 dramatically suppressed TGF-β1–mediated α-SMA expression in a time- and dose-dependent manner. 15d-PGJ2 had little effect on basal α-SMA expression in mesangial cells, but it abolished TGF-β1–stimulated α-SMA induction (Figure 1A). At the concentration of 2.5 μM, 15d-PGJ2 completely inhibited the α-SMA expression induced by TGF-β1 (Figure 1B). Similarly, synthetic PPAR-γ agonist troglitazone and ciglitazone also effectively inhibited TGF-β1–mediated α-SMA expression in HMC (Figure 1, C and D). Comparable results were obtained when the HMC were stained with a specific antibody against α-SMA (Figure 1, E through H). Of note, 15d-PGJ2 also inhibited TGF-β1–mediated α-SMA expression in RMC (data not shown).

Figure 2. PPAR-γ agonists suppress TGF-β1–induced fibronectin expression and deposition. (A through C) Western blot demonstrates that PPAR-γ agonists suppressed the TGF-β1–stimulated fibronectin expression. HMC were treated with or without TGF-β1 (1 ng/ml) in the absence or presence of a fixed amount (2.5 μM) of 15d-PGJ2 (A) or troglitazone and ciglitazone (B) for 48 and 72 h, respectively, or with different amounts of troglitazone and ciglitazone (C) as indicated for 48 h. Whole-cell lysates were immunoblotted with specific antibodies against fibronectin, actin, or α-tubulin, respectively. (D through G) Indirect immunofluorescence staining shows fibronectin deposition after various treatments. HMC were treated without or with TGF-β1 (1 ng/ml) and/or 15d-PGJ2 (2.5 μM) for 48 h. (D) Control. (E) TGF-β1. (F) 15d-PGJ2. (G) TGF-β1 plus 15d-PGJ2.
PPAR-γ Agonists Inhibit TGF-β-Mediated Fibronectin and PAI-1 Expression

Figure 2 demonstrates that 15d-PGJ2, troglitazone, and ciglitazone suppressed TGF-β1–mediated fibronectin expression in mesangial cells. HMC expressed considerable amount of fibronectin at basal conditions, and TGF-β1 significantly induced its expression. However, treatment of HMC with PPAR-γ agonists abolished the TGF-β1–stimulated overproduction of fibronectin. Immunofluorescence staining also revealed that TGF-β1 increased the deposition of fibronectin in extracellular compartments, which was suppressed substantially by incubation with 15d-PGJ2. Similar results were observed in RMC (data not shown).

ECM accumulation is a dynamic process that results from the delicate balance between matrix synthesis and degradation. PAI-1 plays an important role in the regulation of ECM degradation and renal fibrogenesis (29). As shown in Figure 3, TGF-β1 markedly induced PAI-1 expression in HMC. 15d-PGJ2, troglitazone, and ciglitazone significantly abolished TGF-β1-stimulated PAI-1 induction, although it had no effect on PAI-1 expression in resting states (Figure 3).

PPAR-γ Agonists Induce HGF mRNA Expression and Protein Secretion

The activity of PPAR-γ agonists in mesangial cells in many aspects mimics that of HGF (17), an endogenous antifibrotic cytokine (30). This prompted us to explore any potential connection between PPAR-γ activation and HGF. We reasoned that PPAR-γ agonists may exert their antifibrotic action via an HGF-dependent mechanism. As shown in Figure 4, incubation of HMC with 15d-PGJ2, troglitazone, or ciglitazone rapidly induced HGF mRNA expression. Quantitative real-time RT-PCR

Figure 4. PPAR-γ agonists induce hepatocyte growth factor (HGF) mRNA expression and protein secretion in mesangial cells. (A through C) Reverse transcription–PCR (RT-PCR) shows that PPAR-γ agonists induced HGF mRNA expression in mesangial cells. HMC were treated with a fixed amount (2.5 μM) of 15d-PGJ2 (A), troglitazone (B), or ciglitazone (C) for different periods of time as indicated. (D) Quantitative real-time RT-PCR demonstrates the relative abundance of HGF mRNA in HMC after treatment with 2.5 μM 15d-PGJ2 for different periods of time as indicated. *P < 0.05 versus control (n = 3). (E and F) 15d-PGJ2 induced HGF mRNA expression in the presence of TGF-β1. HMC were treated without or with 15d-PGJ2 (2.5 μM) in the presence or absence of TGF-β1 (1 ng/ml) for 3 h. Representative picture (E) and graphic presentation (F) are given. *P < 0.05 versus control; †P < 0.05 versus TGF-β1 alone (n = 3). (G and H) 15d-PGJ2 induced HGF protein secretion in mesangial cells. HMC were incubated with or without a fixed amount of 15d-PGJ2 (2.5 μM) for different periods of time as indicated (G) or with different amounts of 15d-PGJ2 as indicated for 24 h (H). The supernatants were collected and concentrated, followed by Western blot analysis with monoclonal anti-HGF antibody (clone H8). The authenticity of HGF was confirmed by loading the purified human recombinant HGF (10 ng) on an adjacent lane (data not shown). Cell lysates were probed with actin to ensure that the same number of cells was used. (I) Western blot analysis demonstrates an increased HGF protein secretion by rat mesangial cells (RMC) at 12 h after incubation with different amounts of 15d-PGJ2 as indicated.
analysis showed that the steady-state levels of HGF mRNA increased by more than three-fold at 3 h after 15d-PGJ2 stimulation in HMC (Figure 4D). Similarly, 15d-PGJ2 caused a rapid induction of HGF mRNA in RMC (data not shown). 15d-PGJ2 was also able to induce HGF expression even in the presence of TGF-β1 (Figure 4, E and F).

15d-PGJ2 also induced HGF protein secretion by HMC, as demonstrated by Western blot analysis (Figure 4). The accumulated HGF protein in the supernatants of cultured HMC was increased in a time- and dose-dependent manner after 15d-PGJ2 stimulation (Figure 4, G and H). Likewise, HGF protein secretion was induced by PPAR-γ agonist in RMC (Figure 4I).

PPAR-γ Activation Stimulates HGF Promoter Activity via a Novel PPRE

To unravel the mechanism underlying the regulation of HGF expression, we investigated the effect of PPAR-γ agonist on HGF gene promoter activity. Sequence analysis revealed a novel PPRE in the promoter region of HGF gene (23), which consists of an inverted repeat of the core recognition element (AGGTCA) separated by two nucleotides and is conserved among different species (Figure 5A). A reporter construct 0.3HGF-Luc, which contained the putative PPRE, was transiently transfected into RMC. As shown in Figure 5B, 15d-PGJ2 could significantly stimulate HGF promoter activity. However, when the reporter plasmid 0.2HGF-Luc, in which the PPRE was deleted, was transfected, 15d-PGJ2 failed to activate the reporter activity. These results suggest that PPAR-γ activation stimulates HGF promoter activity, which likely is dependent on the presence of PPRE.

Western blot analyses confirmed the presence of PPAR-γ in mouse, rat, and human mesangial cells, as well as in rat renal interstitial fibroblast NRK-49F cells (Figure 5C). A doublet that represents two different splice variants of PPAR-γ was revealed (31). To examine whether 15d-PGJ2 induces PPAR-γ to bind to the putative PPRE of HGF promoter, we performed DNA–protein interaction assay. As shown in Figure 5D, 15d-PGJ2 induced the binding of PPAR-γ to the PPRE in a time-dependent manner. Marked increase in the binding of PPAR-γ to PPRE was observed as early as 15 min after 15d-PGJ2 stimulation, a time point that significantly preceded the HGF mRNA induction.

PPAR-γ Activation Stimulates HGF Receptor Phosphorylation, Induces TGIF Expression, and Blocks Smad-Mediated Gene Transcription

We next examined whether PPAR-γ agonist can activate HGF receptor, a receptor tyrosine kinase encoded by c-met gene. As shown in Figure 6A, 15d-PGJ2 was able to induce c-met receptor activation, as illustrated by the phosphorylation of c-met at tyrosine residues (Tyr1234/1235). These results...
indicate that, similar to HGF, 15d-PGJ2 can induce c-met receptor activation in mesangial cells.

One of the downstream consequences of c-met activation in mesangial cells is the upregulation of Smad transcriptional co-repressor TGIF via protein stabilization (17). Hence, we further investigated the TGIF abundance in mesangial cells after 15d-PGJ2 treatment. As presented in Figure 6, 15d-PGJ2 and troglitazone induced Smad functional antagonist TGIF protein abundance. Similar TGIF induction was also observed in HMC after ciglitzone treatment (data not shown). Kinetic studies revealed that the induction pattern of TGIF was largely overlapped with that of c-met receptor activation after 15d-PGJ2 treatment (Figure 6B).

To provide evidence for a critical role of TGIF induction in mediating the antifibrotic action of PPAR-γ agonists, we investigated the effects of 15d-PGJ2 and TGIF on TGF-β/Smad-mediated gene transcription. A TGF-β-responsive reporter construct, p3TP-Lux, was transfected into mesangial cells. As shown in Figure 6D, TGF-β1 could induce the luciferase activity of the reporter construct. In addition, ectopic expression of Smad2/3 increased the reporter activity at both basal and TGF-β-stimulated conditions (Figure 6D). It is interesting that either overexpression of exogenous TGIF or incubation with 15d-PGJ2 markedly suppressed the reporter activity of the TGF-β1-responsive genes (Figure 6D). Similar to HGF, we found that 15d-PGJ2 did not affect the TGF-β1-mediated Smad nuclear translocation in mesangial cells (Figure 6E).

**Conditional Ablation of c-met Receptor Abolishes the Antifibrotic Action of PPAR-γ Agonist**

To obtain direct evidence that HGF mediates the antifibrotic activity of PPAR-γ agonists, we investigated the action of 15d-PGJ2 in the mesangial cells that were conditionally deficient in c-met receptor. For this purpose, mesangial cells that were isolated from the c-met–floxed mice in which the endogenous c-met gene was flanked by two LoxP sites were infected with recombinant adenovirus-containing Cre recombinase (Ad.Cre) or control vector (Ad.LacZ). Western blot analysis revealed that in the MMC that were infected with Ad.Cre, c-met receptor protein was largely ablated (Figure 7A). Immunofluorescence staining illustrated that >95% of the MMC population expr-
pressed Cre recombinase after adenovirus infection (Figure 7, B and C), and c-met receptor expression in those cells was presumably disrupted.

In MMC that were infected with control Ad.LacZ adenovirus, 15d-PGJ2 was able to abolish partially TGF-β1–mediated fibronectin induction (Figure 7D). However, in MMC that were deficient in c-met receptor after infection with Ad.Cre adenovirus, 15d-PGJ2 totally failed to antagonize TGF-β1’s action (Figure 7D). Therefore, selective ablation of c-met receptor in mesangial cells abolishes the antifibrotic action of 15d-PGJ2.

Likewise, ablation of c-met receptor also abolished TGIF induction by 15d-PGJ2 in mesangial cells (Figure 7E).

**PPAR-γ Activation also Induces HGF Expression and Blocks TGF-β1–Mediated Myofibroblast Activation**

To extend our findings to other cell types, we examined the effect of PPAR-γ agonist on myofibroblast activation from quiescent fibroblasts, one of the central events during renal interstitial fibrogenesis. As shown in Figure 8A, normal rat renal interstitial fibroblasts (NRK-49F) also expressed and secreted an increasing amount of HGF in response to 15d-PGJ2 stimulation. Hence, it seems clear that PPAR-γ activation also induces HGF expression in interstitial fibroblasts.

Figure 8B shows that 15d-PGJ2 effectively suppressed myofibroblast activation from quiescent fibroblasts induced by TGF-β1. Similar to HGF (18), 15d-PGJ2 was able to inhibit the expression of α-SMA, a hallmark for myofibroblast activation, in NRK-49F cells in a dose-dependent manner (Figure 8B).

To explore the molecular mechanism by which PPAR-γ agonist inhibits TGF-β1–mediated myofibroblast activation, we studied the effect of 15d-PGJ2 on Smad signal transduction. As shown in Figure 8, C through E, preincubation of NRK-49F fibroblasts with 15d-PGJ2 was able to block Smad nuclear translocation and presumably prevented Smad-mediated gene transcription, an outcome that resembles HGF treatment (18).

**Discussion**

It has been recognized increasingly that PPAR-γ agonists possess a potent antifibrotic activity that effectively prevents glomerulosclerosis and kidney dysfunction in many types of chronic kidney diseases (3,5–9), possibly by a mechanism independent of their insulin-sensitizing and metabolic activities. It remains elusive, however, exactly how PPAR-γ activation leads to renal protection. Several different scenarios can be envisioned. One possibility is that PPAR-γ, a ligand-dependent transcription factor, may directly control the transcriptional activation of numerous fibrosis-related genes, such as fibronectin and PAI-1. Alternatively, PPAR-γ may regulate fibrotic processes by dictating the expression of an intermediate mediator, which in turn modulates the production and deposition of matrix components. The results presented in this article provide evidence arguing for HGF as an intermediate effector that mediates the antifibrotic actions of PPAR-γ agonists. As illustrated in Figure 9, we have shown that PPAR-γ binds to the putative PPRE in the promoter region of HGF gene after its ligand stimulation, which leads to an increased HGF gene expression.
transcription, mRNA expression, and protein secretion (Figures 4 and 5). Increased HGF protein then binds to its specific cell membrane receptor, c-met, triggering its downstream signaling. The PPAR-γ agonist–activated HGF signaling seems to block fibrogenic TGF-β/Smad signaling in a cell type–specific manner. Similar to HGF, PPAR-γ activation fails to interfere with Smad nuclear translocation but upregulates the Smad transcriptional co-repressor TGFIF expression in glomerular mesangial cells (Figure 6), presumably via protein stabilization (17). Accumulated TGFIF in turn binds to activated Smads and sequesters TGF-β/Smad-mediated gene transcription (17). In renal interstitial fibroblasts, PPAR-γ activation prevents the Smads from undergoing nuclear translocation (Figure 8), thereby intercepting TGF-β–mediated signal transduction (18). Therefore, our studies define a novel molecular pathway that couples PPAR-γ activation, HGF, and TGF-β/Smad signaling and provide a rational explanation for the therapeutic efficacy of the PPAR-γ agonists in the treatment of kidney fibrotic diseases.

Phenotypic activation of glomerular mesangial cells from a quiescent to activated state is one of the major pathologic features of many primary glomerular diseases (32). Such activation of mesangial cells is accompanied by an increased matrix synthesis and decreased matrix degradation, which leads to an excess deposition of the interstitial matrix that contributes directly to the pathogenesis of glomerulosclerosis. In this regard, it is of significance to find out that PPAR-γ agonists are able to suppress the expression of α-SMA, fibronectin, and PAI-1. These observations, together with several recent reports (10–12,33), are consistent with the notion that PPAR-γ activation suppresses the expression of numerous important fibrosis-related genes in mesangial cells. However, because of the absence of a putative PPRE in many of these genes (11), it seems unlikely that the effect of PPAR-γ agonists is mediated through a direct control of the transcriptional regulation of the fibrosis-related genes. In this context, our finding that HGF is a direct target of PPAR-γ agonists sheds new light on elucidation of the molecular mechanism by which PPAR-γ agonists elicit their antifibrotic activity in kidney cells. The importance of HGF in mediating PPAR-γ action is not only supported by the observation that PPAR-γ activation induces HGF expression but also illustrated by the facts that PPAR-γ agonist activates c-met receptor phosphorylation and ablation of c-met receptor abolishes the action of PPAR-γ agonist.

The induction of HGF by PPAR-γ activation is presumably mediated at the gene transcriptional level, as 15d-PGJ2 induces PPAR-γ to bind directly to the cis-acting PPRE in the HGF promoter region and trans-activates HGF promoter activity. It
Besides mesangial cells, PPAR-γ agonists, which is in harmony with the observation that TGF-β is widely expressed in many types of kidney cells in vivo (13,35–40). Besides mesangial cells, PPAR-γ activation induces HGF expression in kidney fibroblasts and blocks TGF-β-mediated myofibroblast activation (Figure 8). This suggests that the antifibrotic effect of PPAR-γ agonists is not limited to primary glomerular diseases such as diabetic nephropathy but can extend to other chronic kidney diseases as well. Because myofibroblast activation from quiescent fibroblasts plays a central role in the pathogenesis of interstitial fibrosis (41), inhibition of this process likely renders PPAR-γ activation an effective way to ameliorate interstitial fibrotic lesions, presumably through induction of HGF expression. In addition, as HGF is a secreted protein, tubular epithelial cells that are adjacent to the fibroblasts express c-met receptor and may be able to respond to HGF stimulation. Along this line, HGF has been shown to block tubular epithelial to mesenchymal transition (22,42), a phenotypic conversion that is important in the generation of the matrix-producing cells in fibrotic kidney (43,44). Although at this stage the therapeutic efficacy of PPAR-γ agonists largely has been focused on primary glomerular diseases such as glomerulonephritis, glomerulosclerosis, and diabetic nephropathy (5–9), it is conceivable that PPAR-γ agonists may also be able to mitigate the interstitial fibrotic lesions after chronic injury.

PPAR-γ plays an essential role in such diverse cellular processes as adipocyte differentiation, immune response, insulin sensitivity, and glucose homeostasis. That HGF is a direct target of PPAR-γ activation insinuates that HGF may be responsible for mediating some of these functions of PPAR-γ. Indeed, HGF has been shown to improve β cell survival, growth, and function and play a role in glucose homeostasis (45–47). Recent studies also show that HGF has anti-inflammatory activity that leads to the suppression of proinflammatory cytokine expression in diseased kidney (48), which is consistent with an inflammation-inhibitory activity of PPAR-γ agonists. It therefore is conceivable that the intrinsic connection between PPAR-γ and HGF goes beyond the inhibition of renal fibrosis. In this regard, it is worthwhile to point out that both HGF and PPAR-γ are expressed in placenta, and their deficiency causes similar placental dysfunction and embryonic lethality (49,50).

The availability of selective PPAR-γ agonists provides a feasible approach to manipulate the fibrotic processes in patients with chronic kidney insufficiency. This class of agents, through their ability to trans-activate directly the antifibrotic HGF gene expression, may hold great promise in the treatment of both diabetic nephropathy and nondiabetic kidney fibrotic diseases. Our studies also suggest that induction of the endogenous HGF expression by small molecule drugs such as PPAR-γ agonists, as an alternative to direct administration of exogenous HGF protein, may represent a practical strategy to the therapy of chronic renal fibrosis in clinical settings.

Acknowledgments
This study was supported by National Institutes of Health Grants DK054922, DK061408, DK064005, and DK071040 and American Diabetes Association Grant 7-03-RA-54. This work won the International Society of Nephrology 2004 Hong Kong Conference Best Poster Award. C.D. was supported by postdoctoral fellowships from the American Heart Association Pennsylvania-Delaware Affiliate.

References


21. Parameswaran N, Hall CS, Bomberger JM, Sparks HV, Jump DB, Spielman WS: Negative growth effects of cigli-
tazone on kidney interstitial fibroblasts: Role of PPAR-
38. Hsueh WA, Nicholas SB: Peroxisome proliferator-activated
receptor-gamma in the renal mesangium. Curr Opin Neph-
39. Yang T, Michele DE, Park J, Smart AM, Lin Z, Brosius FC
3rd, Schnermann JB, Briggs JP: Expression of peroxisomal
proliferator-activated receptors and retinoid X receptors in
40. Guan Y, Zhang Y, Davis L, Breyer MD: Expression of
peroxisome proliferator-activated receptors in urinary tract
41. Eddy AA: Molecular basis of renal fibrosis. Pediatr Nephrol
15: 290–301, 2000
42. Yang J, Dai C, Liu Y: A novel mechanism by which hepa-
tocyte growth factor blocks tubular epithelial to mesenchy-
43. Liu Y: Epithelial to mesenchymal transition in renal fibro-
genesis: Pathologic significance, molecular mechanism,
and therapeutic intervention. J Am Soc Nephrol 15: 1–12,
2004
44. Kalluri R, Neilson EG: Epithelial-mesenchymal transition
1784, 2003
45. Garcia-Ocana A, Takane KK, Reddy VT, Lopez-Talavera
JC, Vasavada RC, Stewart AF: Adenovirus-mediated hepa-
tocyte growth factor expression in mouse islets improves
pancreatic islet transplant performance and reduces beta
preserves beta cell mass and mitigates hyperglycemia in
streptozotocin-induced diabetic mice. J Biol Chem 278:
27080–27087, 2003
47. Dai C, Huh CG, Thorgerisson SS, Liu Y: Beta-cell-specific
ablation of the hepatocyte growth factor receptor results in
reduced islet size, impaired insulin secretion, and glucose
48. Gong R, Rifai A, Tolbert EM, Biswas P, Centracchio JN,
Dworkin LD: Hepatocyte growth factor ameliorates renal
interstitial inflammation in rat remnant kidney by modu-
lating tubular expression of macrophage chemoattractant
protein-1 and RANTES. J Am Soc Nephrol 15: 2868–2881,
2004
49. Uehara Y, Minowa O, Mori C, Shiota K, Kuno J, Noda T,
Kitamura N: Placental defect and embryonic lethality in
mice lacking hepatocyte growth factor/scatter factor. Na-
ture 373: 702–705, 1995
50. Barak Y, Nelson MC, Ong ES, Jones YZ, Ruiz-Lozano P,
Chien KR, Koder A, Evans RM: PPAR gamma is required
for placental, cardiac, and adipose tissue development. Mol
Cell 4: 585–595, 1999