Activation of peroxisome proliferator-activated receptor γ (PPARγ) after balloon injury significantly inhibits VSMC proliferation and neointima formation. However, the precise mechanisms of this inhibition have not been determined. We hypothesized that activation of PPARγ in vascular injury could attenuate VSMC growth and matrix production during vascular lesion formation. Since connective tissue growth factor (CTGF) is a key factor regulating extracellular matrix deposition, cytokines and growth factors such as transforming growth factor β (TGF-β) participate in these processes as evidenced by TGF-β-induced overproduction of extracellular matrix proteins in intimal vascular smooth muscle cells (VSMCs) (1, 2). Connective tissue growth factor (CTGF) is a novel cysteine-rich secreted peptide that is a key regulator of extracellular matrix production and plays an important role in atherosclerosis and restenosis (3). It was reported that CTGF could mediate the effects of TGF-β on stimulation of extracellular matrix production in atherosclerosis (4). In addition, CTGF is implicated in fibrotic disorders as systemic sclerosis (5, 6).

TGF-β signaling is initiated upon its binding to two cell membrane receptors termed type I (TβRI) and type II (TβRII). Both receptors are serine/threonine kinases, and binding by TGF-β results in phosphorylation of TβRI and TβRII. Smad proteins are the primary substrates known to date for phosphorylated TGF-β receptors. The phosphorylation of Smad2 or Smad3 by TGF-β receptor causes their association with Smad4. The Smad complexes translocate into the nucleus and bind to cofactors that determine the choice of the target gene (7). In addition to Smad proteins, c-Jun NH2-terminal kinase and mitogen-activated protein kinases are reported to be involved in TGF-β signaling (8, 9).

Peroxisome proliferator-activated receptors (PPARs) including α, γ, and δ/β are a family of ligand-activated nuclear transcriptional factors that are emerging as important determinants of vascular function and structure (10–12). Recent studies have documented that PPARγ is present in all critical vascular cells as follows: endothelial cells (13), VSMCs (14), and monocytes/macrophages (15). It was reported that thiazolidinediones (TZD), a class of anti-diabetic drugs that are specific ligands of PPARγ, inhibit neointima formation after balloon injury (16) and the development of atherosclerosis in low density lipoprotein receptor-deficient mice (17). Based on the studies above, we hypothesized that the up-regulation of inflammatory cells and intimal smooth muscle cell proliferation and migration, as well as extracellular matrix deposition. Cytokines and growth factors such as transforming growth factor β (TGF-β) participate in these processes as evidenced by TGF-β-induced overproduction of extracellular matrix proteins in intimal vascular smooth muscle cells (VSMCs) (1, 2). Connective tissue growth factor (CTGF) is a novel cysteine-rich secreted peptide that is a key regulator of extracellular matrix production and plays an important role in atherosclerosis and restenosis (3). It was reported that CTGF could mediate the effects of TGF-β on stimulation of extracellular matrix production in atherosclerosis (4). In addition, CTGF is implicated in fibrotic disorders as systemic sclerosis (5, 6).

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PPARγ gene expression induced by cytokines and growth factors in response to vascular injury may function as a counter-regulating influence that attenuates VSMC growth and matrix production during lesion formation.

To understand the role of PPARγ in VSMC, we used commercially available filter-based microarrays from Research Genetics (GF-211, Huntsville, AL) to quantitate changes in expression of mRNAs in human aortic smooth muscle cells (HASMCs). Over 50 genes are either up- or down-regulated by at least 2-fold after treatment with PPARγ ligands. Connective tissue growth factor (CTGF) was one of the genes most down-regulated by PPARγ activators (~5.6-, ~6.5-, and ~8-fold by ciglitazone, GW7845, and 15-d-PGJ2, respectively). Therefore, we postulated that CTGF down-regulation by PPARγ activation might be one of the mechanisms by which PPARγ agonists inhibit neointima formation.

In the present study, we demonstrate that activation of PPARγ inhibits TGF-β-induced CTGF expression in HASMCs by interfering with the Smad3 signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant TGF-β1 and collagenase (type VII, 10 units/ml) were purchased from Sigma. 15-Deoxyprostaglandin J2 was purchased from BioMol Research Laboratories (Plymouth Meeting, PA). GW7845 and GW9662 (18) were obtained from GlaxoSmithKline. L-[3H]Proline and [32P]dCTP were purchased from PerkinElmer Life Sciences. 15-Deoxyprostaglandin J2, GW7845, and 15-d-PGJ2, respectively). Therefore, we postulated that CTGF down-regulation by PPARγ activation might be one of the mechanisms by which PPARγ agonists inhibit neointima formation.

**RESULTS**

**Activation of PPARγ Inhibits TGF-β-Induced CTGF Expression in HASMCs**—To understand the role of PPARγ in VSMC,
we compared the expression of ~4100 genes in human aortic smooth muscle cells (HASMCs) in response to PPARγ activators using a human known gene filter (Research Genetics, GF-211, Huntsville, AL). Passage 7 HASMCs were grown to 80–90% confluence, made quiescent by serum starvation for 24 h, and then stimulated with 10 μM/liter ciglitazone, 1 μM/liter GW7845, or 5 μM/liter 15-d-PGJ2 for 16 h. Connective tissue growth factor (CTGF), which is a key regulator of extracellular matrix production, was down-regulated about −5.6-, −8.0-, and −8.0-fold by ciglitazone, GW7845, and 15-d-PGJ2 respectively (data not shown). This led us to postulate that CTGF is a PPARγ target gene in VSMCs.

To understand the biological relevance of CTGF regulation by PPARγ, we investigated whether PPARγ activators regulate TGF-β-induced CTGF mRNA expression in HASMCs. Cells were pretreated with an increasing concentration of 15-d-PGJ2 (1, 5, 10 μM/liter) or GW7845 (0.1, 0.5, and 1 μM/liter) for 1 h and subsequently stimulated with TGF-β1 (4 ng/ml) for 6 h. As shown in Fig. 1, A and B, both PPARγ activators inhibit TGF-β1-induced CTGF mRNA expression in HASMCs in a dose-dependent manner.

The effects of 15-d-PGJ2 and GW7845 on CTGF protein levels in the culture medium of TGF-β-stimulated cells were next examined. Consistent with previous work (4), we found that CTGF protein was undetectable in untreated HASMCs by Western blotting analysis, whereas TGF-β significantly induced CTGF protein production and secretion (Fig. 1, C and D). Interestingly, both 15-d-PGJ2 (5 μM/liter) and GW7845 (1 μM/liter) dramatically inhibited TGF-β1-induced CTGF secretion. Taken together, the results showed that the inhibition of CTGF expression by PPARγ ligands is more profound at the protein level than at the mRNA level, suggesting that PPARγ ligands might be exerting some translational or post-translational effects on CTGF expression. In addition, the inhibition of CTGF expression by PPARγ ligands was not due to cell death because neither 15-d-PGJ2 (10 μM/liter) nor GW7845 (1 μM/liter) was toxic to HASMCs (viability = 100%, data not shown). Taken together, the data indicate that PPARγ activation inhibits TGF-β-induced CTGF production at both the mRNA and protein levels.

**Suppression of CTGF Expression Is Mediated by PPARγ**—If the suppression of CTGF expression is mediated by PPARγ, we would expect that the PPARγ-specific antagonist would negate this effect. GW9662 from GlaxoSmithKline has been shown to be a PPARγ-specific antagonist (18, 22). To test this hypothesis, HASMCs were pretreated with or without GW9662 (1 μM/liter) for 30 min prior to the addition of GW7845 (1 μM/liter) (A) or 15-d-PGJ2 (5 μM/liter) (B). HASMCs were stimulated with or without TGF-β1 (4 ng/ml) for 6 h after treatment with GW7845 or 15-d-PGJ2 for 30 min. Equal loading was confirmed by GAPDH. The relative CTGF mRNA levels normalized by GAPDH are shown on the tops of each panel. The results are representative of three independent experiments.
The data suggest that 15-d-PGJ₂ activates a PPAR₇/H9253-independent signaling pathway to repress CTGF expression in addition to the activation of PPAR₇/H9253.

Cloning of Human CTGF Promoter—To study the molecular mechanisms by which PPAR₇/H9253 activation inhibited CTGF expression, we cloned the human CTGF promoter using the human genome database as described under “Experimental Procedures.” As shown in Fig. 3, the TATA box (TATAAAA) is located at nt —33 to nt —27 of the human CTGF promoter. Sequence analysis of this CTGF promoter revealed that there are two putative NF-κB sites and two putative AP-1 sites. In addition, a putative Smads-binding site (SBE) was reported at nt —175 to nt —167 (CAGACGGAG) (6). Surprisingly, we did not find any putative PPAR₇-like elements. Cloning of this 2-kb human CTGF promoter has provided a powerful tool with which to study the mechanism of the regulation of CTGF gene expression.

PPAR₇ Inhibits the Transcriptional Activity of the CTGF Promoter—To understand the transcriptional regulation of CTGF gene expression, we transiently transfected pCTGF-Luc with Smad3, Smad4, Smad3-Smad4, p65, or c-Jun/c-Fos expression plasmids into the HepG2 cell line, which is a well established cell model for testing TGF-β signaling. As shown in Fig. 4, CTGF promoter activity was increased about 1.9-, 3.2-, and 1.8-fold by overexpression of Smad3, Smad3-Smad4, or c-Jun/c-Fos, respectively. However, overexpression of Smad4 or p65 did not affect CTGF promoter activity. Taken together, these results indicate that the Smad3, Smad3-Smad4 complex, and AP1 regulate the transcriptional activity of CTGF promoter.

To examine further the mechanisms by which PPAR₇ activation inhibits TGF-β-induced CTGF expression, we investigated the regulation of the CTGF promoter in HepG2 cells. Since PPAR₇ is not expressed in the HepG2 cell (Fig. 5C), it is a good model to test the role of PPAR₇ in mediating the inhibitory effects of PPAR₇ ligands on TGF-β-induced CTGF expression. Without transfecting the PPAR₇ expression plasmid, treatment of HepG2 cells with GW7845 (1 μM/liter) had no effect on CTGF promoter activation induced by TGF-β (Fig. 5A). When an increasing concentration of PPAR₇ expression plasmid (50, 100, and 150 ng/well) was transfected into these cells, GW7845 strongly inhibited TGF-β-induced CTGF promoter activation in a PPAR₇ dose-dependent manner. In addition, we transfected the constant PPAR₇ expression plasmid (150 ng/well) to HepG2 cells to test the effect of GW7845 at 0.01, 0.1, and 1 μM/liter on CTGF promoter activation. We also found that PPAR₇ inhibited TGF-β-induced CTGF promoter activation in a GW7845 dose-dependent manner (data not shown). These results suggest that GW7845 inhibits TGF-β-induced CTGF expression at the transcriptional level in a PPAR₇-dependent mechanism.

Because activation of the CTGF promoter depended on a combinatorial interaction between Smad3, Smad4, and AP1,
we investigated the minimal promoter containing the binding sites only for Smad3 and Smad4 to determine whether they were targets for negative regulation by PPARγ ligands. Co-transfection of a luciferase reporter construct containing the minimal promoter with bacterially generated GST-Smad3 and GST-Smad4 did not retain PPARγ interaction. In addition, we demonstrated that both GST-Smad3 and GST-Smad4 physically interacts with Smad3 but not Smad4.

**Effect of PPARγ Activation on Collagen Synthesis Rate in HASMCs—**We examined the effect of PPARγ activation on the rate of collagen synthesis in HASMCs by metabolically labeling cells with L-[3H]proline. As illustrated in Fig. 8 , the relative rate of collagen synthesis was increased ~2.5-fold by TGF-β (4 ng/ml) stimulation. GW7845 (0.1–1 μmol/liter) inhibited TGF-β-induced collagen synthesis in a dose-dependent manner, whereas GW7845 had no effect on the basal level of collagen synthesis in HASMCs. Similar results were observed when using the PPARγ natural ligand, 15-d-PGJ2 (data not shown). Taken together, our results suggest that PPARγ activation inhibits TGF-β-induced collagen synthesis that is likely to be mediated by inhibition of CTGF expression.

**DISCUSSION**

The results of these studies demonstrate that PPARγ activation inhibits TGF-β-induced CTGF expression in HASMCs by directly interfering with the Smad3 signaling pathway. Because CTGF is a key factor in the regulation of extracellular matrix production, this repression of CTGF expression by PPARγ may be one of the mechanisms through which PPARγ agonists inhibit neointimal formation after vascular lesion.

It is postulated that pathological changes in vessel structure are induced in part by transcription factors that govern cell growth, death, differentiation, inflammation, and matrix production. PPARs are a family of ligand-activated nuclear transcriptional factors that are emerging as important determinants of vascular function and structure (10–12). Recent studies have documented that the expression of PPARγ is up-regulated in intimal VSMC (23). Moreover, it has been reported that thiazolidinediones (TZD), a class of anti-diabetic drugs that function as synthetic ligands of PPARγ, inhibit neointima formation after balloon injury in association with decreased DNA synthesis (23, 24). In this study, we have extended these observations by documenting that both synthetic and natural
GW7845, had the effect of inhibiting TGF-β-treated with the combination of TGF-β and TGF-β ligands of PPARγ/H9253, were made quiescent in serum-deprived medium for 24 h and then transfection efficiency. Twenty four hours after transfection, cells of each sample. GFP reporter plasmid (50 ng/well) was used as the control for transfection efficiency. pcDNA3 plasmid was used to ensure an equal amount of DNA in p3xSBE-Luc (A) with or without PPARγ/H9253 or p3xSBE-Luc (B) without PPARγ/H9253, was rescued by overexpression of Smad3 or Smad3/Smad4.

B

![Diagram A](Image 315x584 to 547x729)

**FIG. 6.** Inhibition of PPARγ on TGF-β-induced CTGF promoter was rescued by overexpression of Smad3 or Smad3/Smad4. HepG2 cells were co-transfected with pCTGF-Luc (A) or p3xSBE-Luc (B) with or without PPARγ expression plasmid (100 ng/well). The Smad3 (200 ng/ml), Smad4 (200 ng/ml), or Smad3/Smad4 (100 ng/well of each) expression plasmids were also transfected into cells as indicated. pcDNA3 plasmid was used to ensure an equal amount of DNA in each sample. GFP reporter plasmid (50 ng/well) was used as the control for transfection efficiency. Twenty four hours after transfection, cells were made quiescent in serum-deprived medium for 24 h and then treated with the combination of TGF-β1 (4 ng/ml) and GW7845 (1 μmol/liter), as indicated, for 24 h. The luciferase activities, normalized by GFP activity, were expressed in relative units, with no PPARγ, no TGF-β, and no GW7845 as 1 (mean ± S.E., n = 6).

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ligands of PPARγ promote a decrease in the expression of CTGF as well as collagen III in VSMC. In addition, previous studies (3, 4) have well documented that CTGF plays an important role in atherosclerosis and restenosis by mediating the effects of TGF-β-induced extracellular matrix production and by stimulating collagen and fibronectin production in fibroblasts (25). Taken together, our data suggest a novel anti-fibrotic mechanism by which PPARγ activation may reduce vascular lesion formation via decreasing CTGF expression.

Recently, it was reported (26–28) that there are PPARγ-independent effects of PPARγ ligands (i.e. TZD) at high doses. In addition, the PPARγ natural ligand 15-d-PGJ2 has many functions other than that of a PPARγ activator (29). In this study, we documented that the high affinity PPARγ ligand, GW7845, had the effect of inhibiting TGF-β-induced CTGF expression, beside TZD and 15-d-PGJ2 in HASMCs. By using the PPARγ-specific antagonist, GW9662 (18, 22), from GlaxoSmithKline, we demonstrated that the effect of GW7845 on TGF-β-induced CTGF expression was essentially mediated by PPARγ. However, the inhibition of 15-d-PGJ2 on TGF-β-induced CTGF expression was partly due to PPARγ activation, suggesting that 15-d-PGJ2 can activate other PPARγ-independent signaling pathways to repress CTGF expression. Interestingly, it was recently reported that 15-d-PGJ2 inhibits cyclooxygenase 2 expression, not only by activation of PPARγ but also by directly inhibiting NF-kB as well as by modification of IκB (29).

Cloning of this ~2-kb human CTGF promoter provided a powerful tool with which to study the mechanisms of regulation of CTGF gene expression. Sequence analysis of this CTGF promoter revealed that there were two putative NF-kB sites, two putative AP-1 sites, and a putative SBE. It is interesting to document that Smad3 and Smad4 have a synergistic effect on activating the CTGF promoter. This may represent the fact that overexpression of Smad4 can translocate more Smad3 into the nucleus (7). In this study, our data document that activation of the CTGF promoter depends on Smad3/Smad4 and c-Jun/c-Fos but not on NF-kB. At present, we cannot completely rule out the possibility of NF-kB in the regulation of CTGF expression. To understand better the transcriptional regulation of CTGF, we are currently performing a systematic deletion mapping analysis of this promoter, which is beyond the scope of this study.

![Diagram B](Image 56x376 to 290x728)

**FIG. 8.** PPARγ activation inhibits TGF-β-induced collagen synthesis in HASMCs. HASMCs were treated with GW7845 (0.1, 0.5, and 1 μmol/liter) for 1 h and subsequently stimulated with TGF-β1 (4 ng/ml) for 48 h. Cells were pulsed with L-[3H]proline during the last 24 h. L-[3H]Proline incorporation into collagenase-digestible and collagenase-indigestible protein from cultured medium was determined as described under “Experimental Procedures.” Values are expressed as mean ± S.E. (n = 6).

![Diagram C](Image 311x349 to 551x492)

**FIG. 7.** Smad3 but not Smad4 interacts with PPARγ in vitro. [35S]Methionine-labeled human PPARγ1, generated by in vitro transcription and translation, was incubated with glutathione-Sepharose beads bound with purified E. coli-expressed GST, GST-Smad3 (upper panel), or GST-Smad4 (lower panel) in the presence (+) or absence (−) of GW7845. The bound proteins were eluted and separated by using 10% SDS-polyacrylamide gel electrophoresis. The samples wereanalyzed by autoradiography. Ten μl of in vitro translated PPARγ1 protein was used as control in lanes labeled Input.
Smad proteins are the primary TGF-β receptor substrates capable of signal transduction. The Smad complex translates into the nucleus and binds to cofactors that determine the choice of target genes. Smad proteins consist of two conserved globular domains known as the MH1 (Mad homology 1) and the MH2 domains, coupled by a linker region. The MH1 domain recognizes the Smad-binding site in the target gene promoter, by phosphorylation of the corepressors such as Ski and SnoN (7). To test our hypothesis that PPARγ directly interacts with Smad proteins, we performed a series of transfection/reporter experiments. Overexpression of Smad3 or Smad3/Smad4 but not Smad4 completely abrogates the PPARγ inhibition on TGF-β-induced CTGF promoter activation. In addition, using an in vitro binding assay, we demonstrate that PPARγ physically interacts with Smad3 but not Smad4. Taken together, our data suggest that PPARγ can inhibit TGF-β-induced CTGF expression in HASMCs by directly interacting with Smad3.

Although we have documented that PPARγ activation inhibits TGF-β-induced activation of CTGF through a Smad3-dependent mechanism, it will be interesting to know whether TGF-β directly regulates PPARγ gene expression in VSMCs. Recently, it was reported (30) that TGF-β could decrease the expression of CD36, the macrophage type B receptor that is a well characterized PPARγ target gene, by phosphorylation of PPARγ through the mitogen-activated protein kinase. Although we have not detected CD36 in HASMCs, using both reverse transcriptase-polymerase chain reaction and Western blotting analyses (data not shown), this provocative paper stimulated us to examine the effect of TGF-β on PPARγ gene expression. We found that TGF-β inhibits PPARγ gene expression in a dose-dependent manner in VSMCs.2 Taken together, our data suggest that the inhibition of CD36 expression by TGF-β stimulation may be mediated by PPARγ at multiple levels including phosphorylation of PPARγ, repression of PPARγ gene expression, and the interaction of PPARγ and Smad3. Studies are underway to determine the mechanisms of TGF-β-induced PPARγ inhibition.

In conclusion, we show for the first time that CTGF, which is a key regulator of extracellular matrix production and plays an important role in atherosclerosis and restenosis, is a PPARγ-regulated gene. We have documented that activation of PPARγ abrogates TGF-β-induced CTGF expression by directly interfering with the Smad3 signaling pathway. Taken together, our data suggest that abrogation of TGF-β-induced CTGF production by PPARγ activation may be one of the mechanisms through which PPARγ agonists inhibit neointimal formation after vascular lesions.

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