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Transforming growth factor-β inhibits myocardial PPARγ expression in pressure overload-induced cardiac fibrosis and remodeling in mice

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Abstract

Objectives—Pharmacological activation of peroxisome proliferator-activated receptor gamma (PPARγ) has been shown to attenuate pressure overload-induced cardiac fibrosis, suggesting that PPARγ has an antifibrotic effect. This study tested the hypothesis that there is a functional interaction between transforming growth factor-β (TGF-β) signaling and endogenous PPARγ expression in cardiac fibroblasts and pressure overloaded heart.

Methods and results—We observed that, in response to pressure overload induced by transverse aortic constriction, left-ventricular PPARγ protein levels were decreased in wild-type mice, but increased in mice with an inducible overexpression of dominant negative mutation of the human TGF-β type II receptor (DnTGFβRII), in which TGF-β signaling is blocked. In isolated mouse cardiac fibroblasts, we demonstrated that TGF-β1 treatment decreased steady state PPARγ mRNA (−34%) and protein (−52%) levels, as well as PPARγ transcriptional activity (−53%). Chromatin immunoprecipitation analysis showed that TGF-β1 treatment increased binding of Smad2/3, Smad4 and histone deacetylase 1, and decreased binding of acetylated histone 3 to the *PPARγ* promoter in cardiac fibroblasts. Both pharmacological activation and overexpression of PPARγ significantly inhibited TGF-β1-induced extracellular matrix molecule expression in isolated cardiac fibroblasts, whereas treatment with the PPARγ agonist rosiglitazone inhibited, and treatment with the PPARγ antagonist T0070907 exacerbated chronic pressure overload-induced cardiac fibrosis and remodeling in wild-type mice *in vivo*.

Conclusion—These data provide strong evidence that TGF-β1 directly suppresses PPARγ expression in cardiac fibroblasts via a transcriptional mechanism and suggest that the down-regulation of endogenous PPARγ expression by TGF-β may be involved in pressure overload-induced cardiac fibrosis.

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Conflicts of interest
There are no conflicts of interest.

Keywords

cardiac fibroblast; cardiac fibrosis; peroxisome proliferator-activated receptor gamma; Smad; transforming growth factor-β

Introduction

Peroxisome proliferator-activated receptor gamma (PPARγ) has been implicated in the regulation of a variety of biological processes, including fibrotic, hypertrophic, and inflammatory responses of the heart to hemodynamic stress [1,2]. For example, pressure overload-induced cardiac hypertrophy is more prominent in heterozygous PPAR$\gamma^{+/-}$ mice than that in wild-type mice [3]. PPARγ agonist (pioglitazone, rosiglitazone, or ciglitazone) treatment has been shown to attenuate pressure overload-induced left-ventricular fibrosis and hypertrophy in stroke-prone spontaneously hypertensive rats [4], Dahl salt-sensitive hypertensive rats [5], and in rats and mice with abdominal aortic constriction [6,7]. PPARγ agonists also inhibit myofibroblast transformation, proliferation and production of extracellular matrix (ECM) in cardiac fibroblasts *in vitro* in response to transforming growth factor-β (TGF-β) or angiotensin II treatment [1,8-10]. Loss of PPARγ from isolated mouse embryonic fibroblasts is associated with the up-regulation of collagen synthesis in the absence of exogenous added ligand [11]. Collectively, these data suggest that PPARγ has an antifibrotic effect. Whereas most studies of PPARγ in the heart have focused on cardiomyocytes (i.e. cardiomyocyte-specific *PPARγ* deletion or overexpression) [12-14], less attention has been devoted to the role of endogenous PPARγ in cardiac fibroblasts and in the pathogenesis of cardiac fibrosis.

The pro-fibrogenic factor TGF-β is secreted by cardiac fibroblasts and cardiomyocytes in response to pressure overload stress. TGF-β signals through the membrane bound TGF-β receptors type I and II. When the receptors are activated, downstream signaling molecules Smad2 and Smad3 are phosphorylated, bind to Smad4, and translocate to the nucleus [15]. The Smad2/3/4 complex then binds to response elements in the promoter regions of the ECM genes and activates pro-fibrogenic factors by up-regulating gene transcription. Many studies, including ours, have shown that TGF-β signaling plays a dominant role in stimulating ECM synthesis and fibrosis in the pressure overloaded mouse heart [16,17]. The pro-fibrotic role of TGF-β/Smad signaling in pressure overload-induced cardiac remodeling has been well defined, but the direct effects of TGF-β/Smad signaling on antifibrotic systems, such as PPARγ, remain unclear.

In the present study, we demonstrated that in mouse heart, disruption of TGF-β signaling reverses the down-regulation of PPARγ expression induced by chronic pressure overload and that TGF-β1 directly suppresses PPARγ expression at the transcriptional level in isolated cardiac fibroblasts. Both pharmacological activation of PPARγ by agonists and PPARγ overexpression abolished TGF-β1-induced ECM molecule expression in isolated cardiac fibroblasts, whereas treatment with the PPARγ agonist rosiglitazone attenuated, and treatment with T0070907, an antagonist of endogenous PPARγ, exacerbated chronic pressure overload-induced cardiac fibrosis. These findings provide strong evidence for functionally significant counter-regulation by TGF-β and PPARγ signaling pathways in the pathogenesis of pressure overload-induced cardiac fibrosis and remodeling.

Materials and methods

Animal preparation and surgical procedures

All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the *Guide for Care and Use of Laboratory Animals* published by the US National Institutes of Health (DHEW publication No. 96–01, revised in 2002).

Protocol 1

To determine the effect of TGF-β signaling on PPARγ expression in the pressure overloaded left ventricle (LV), DnTGFβRII and wild-type C57BL/6 mice were used. The DnTGFβRII mouse expresses a cytoplasmic truncated TGFβRII receptor that lacks the cytoplasmic kinase domain and thus is a dominant-negative mutant [17-19]. Genotypes were identified by PCR assay of genomic DNA from tail snips after weaning. Overexpression of DnTGFβRII is under the control of a metallothionein-derived promoter and was induced in this study by giving 25 mmol/l $ZnSO_4$ in the drinking H_2O to DnTGFβRII mice beginning 1 week prior to transverse aortic constriction (TAC) or sham surgery and continuing throughout the study [17]. Adult (8–10 weeks) male DnTGFβRII mice drinking double distilled H_2O and wild-type C57BL/6 mice drinking either $ZnSO_4$ or double distilled H_2O served as controls. Mice were anesthetized with an intraperiotoneally administered mixture of ketamine (80 mg/kg) and xylazine (12 mg/kg), and TAC or sham was performed as described previously [17,20]. Pressure gradients across the TAC were 50–60 mmHg as described previously [20]. Mice were fed a standard diet (Harlan-Teklad) and were housed in rooms maintained at a constant humidity (60 ± 5%), temperature 24±1°C), and light cycle (0600 to 1800 h).

Protocol 2

To test the effect of the PPARγ agonist rosiglitazone and the PPARγ antagonist T0070907 on pressure over-load-induced cardiac fibrosis, male C57BL/6 mice were treated with rosiglitazone (Rosi, 10 mg/kg, Cayman), T0070907 (T007, 1.5 mg/kg; Sigma–Aldrich, St Louis, Missouri, USA), or vehicle by gavage daily for 3 days, and then subjected to TAC. Treatments were continued for 3 weeks after TAC. Sham-operated and vehicle-treated mice served as controls. Left ventricular myocardial interstitial collagen deposition was measured by picrosirius red staining and left ventricular end-diastolic pressure (LVEDP, mmHg) and left ventricular systolic pressure (LVSP, mmHg) were measured using a high-fidelity blood pressure transducer (BIOPAC Systems Inc., Goleta, California, USA) that was connected to an eight-channel polygraph (Grass Technologies, West Warwick, Rhode Island, USA) as previously described [21,22].

Histological analysis

Cardiomyocyte area and perimeter were measured using a computer-based morphometric system (Motic Image Plus 2.0; Motic, Xiamen, China) in hematoxylin-eosin stained, paraffin-embedded left-ventricular sections. Cardiac fibrosis was assessed by picrosirius red (0.1%) staining as described previously [17]. Myocardial interstitial collagen volume was analyzed using light microscopy with a Qimaging QiCam digital camera (Qimaging, Surrey, British Columbia, Canada) interfaced with a computer system running Metamorph 6.2v4 software (Universal Imaging, Ypsilanti, Michigan, USA). Cardiomyocyte perimeters were measured with a computer-based morphometric system (Motic Image Plus 2.0). At least 10 randomly selected images (×400) from each slide were analyzed.

Cardiac fibroblast preparation

Cardiac fibroblasts were isolated from hearts of adult male C57BL/6 mice as described previously [16]. Briefly, hearts were excised, minced, and digested with collagenase type 4 (100 U/ml) and trypsin (0.6 mg/ml) at 37°C for 30 min. The collagenase medium containing the cardiac fibroblasts was centrifuged for 10 min at 180*g* and resuspended in Dulbecco's Modified Eagle's Medium (DMEM) with 15% fetal bovine serum (FBS). The digestion was repeated for 5–6 times. Cells were plated in laminin-coated 100 mm dishes (BD Biocoat; BD Bioscience, Franklin Lakes, New Jersey, USA) and allowed to attach for 45 min prior to the first media change, which removed weakly adherent cells, including myocytes and endothelial cells. Cultures were assessed for admixture of endothelial cells, macrophages and myofibroblasts using immunofluorescence staining for CD31, F4/80 and α-smooth muscle actin (α-SMA), respectively. Over 95% of cultured cells were negative for these markers and had the typical appearance of fibroblasts. Passage 2 cardiac fibroblasts were used for the experiments. The cells were serum-starved for 24 h prior to treatment with TGF-β1 (Sigma–Aldrich), rosiglitazone, pioglitazone (Cayman Chemical, Ann Arbor, Michigan, USA) or vehicle.

Adenovirus-mediated gene transfer

To overexpress PPARγ, cardiac fibroblasts were infected with an adenoviral vector containing a full-length human *PPARγ* gene (50 MOI; Vector Biolabs, Philadelphia, Pennsylvania, USA) or a control empty adenoviral vector for 6 h in 10% FBS-DMEM. Cardiac fibroblasts were washed and incubated in 10% FBS-DMEM for 48 h for PPARγ expression.

Western blotting analysis

Protein samples extracted from LVs or cultured cardiac fibroblasts were separated by 10% SDS–PAGE and transferred to polyvinylidene difluoride membrane as described previously [16,19]. Blots were probed with anti-PPARγ (Millipore-Upstate), anti-CTGF (Abcam), anti-Periostin (Abcam), anti-α–SMA (Sigma–Aldrich), anti-pSmad3 (Cell Signaling Technology Inc., Danvers, Massachusetts, USA) and anti-GAPDH primary antibodies (Santa Cruz Biotech Inc., Santa Cruz, California, USA) and a horseradish peroxidase-conjugated secondary antibody, respectively. Bands were visualized by use of a Super Western Sensitivity Chemiluminescence Detection System (Pierce). Autoradiographs were quantitated by densitometry (NIH Image J).

Quantitative real-time RT-PCR analysis

Total RNA was extracted from cardiac fibroblasts using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, California, USA) and reverse transcribed to cDNA as described previously [23]. cDNA was amplified by real-time quantitative PCR using the SYBR Green RT-PCR kit (Applied Biosystems, Carlsbad, California, USA) in a Bio-Rad iCycler with specific primers of mouse (PPARγ: 5′-GAT GGA AGA CCA CTC GCA TT-3′ and 5′-AAC CAT TGG GTC AGC TCT TG-3′; connective tissue growth factor (CTGF): 5′-GCA CTT GCC TGG ATG GGG-3′, 5′-CGG TCC TTG GGC TCG TCA-3′; Periostin: 5′-TGA GCT ACT GAA TGC CTT AC-3′ and 5′-CAA TGA CAT GGA CGA CAC-3′; α-SMA: 5′-GGA GAA GCC CAG CCA GTC GC-3′ and 5′-AGC CGG CCT TAC AGA GCC-CA-3′; or GAPDH: 5′-GTT GTC TCC TGC GAC TTC A-3′ and 5′-GTG GTC CAG GGT TTC TTA CT-3′). CTGF, periostin, α-SMA and PPARγ mRNA levels were normalized using GAPDH mRNA and then standardized to the mRNA level of vehicle-treated cardiac fibroblasts.

PPARγ promoter activity analysis

Quiescent cardiac fibroblasts were transiently co-transfected with a pGL3-Luc-h*PPARγ* promoter plasmid and a pRL-TK plasmid using the Lipofectamine Plus Transfection Reagent (Invitrogen Life Technologies) [24]. PPARγ promoter activity was quantified using the Dual-Luciferase Reporter Assay System (Promega Corp., Madison, Wisconsin, USA).

Chromatin immunoprecipitation analysis

Quiescent cardiac fibroblasts were treated with TGF-β1 (1 ng/ml) or vehicle. After 1 h, cells were fixed and nuclear protein–DNA complexes were extracted and subjected to chromatin immunoprecipitation (ChIP) analysis using anti-Smad2/3 (sc-8332; Santa Cruz Biotech Inc.), anti-Smad4 (sc-7154; Santa Cruz Biotech Inc.), anti-HDAC1 (sc-7872, Santa Cruz Biotech Inc.), anti-AcH3 (06–911, Millipore-Upstate) antibodies and normal rabbit IgG (sc-2027, Santa Cruz Biotech Inc.) as previously described [25]. A pair of primers (5′-ACA TCG GTC TGA GGG ACA CGG-G-3′ and 5′-TAC CTG GCC GCC TTG CTC CT-3′) was used to amplify a 72-bp fragment (−74 to −146 bp) of the promoter region of the mouse *PPARγ* gene for detection of binding of Smad2/3, Smad4, HDAC1 and acetylated histone3. PCR was run at 29 to 31 cycles, and selective PCR product levels were measured using densitometry and normalized by respective input values.

Statistical analysis

Results were expressed as mean ± SEM. Analyses were carried out using the SigmaStat statistical package (Jandel Scientific software; Jandel Scientific, San Rafael, California, USA). Our primary statistical test was ANOVA; one-way ANOVA to evaluate the differences in mean values due to main effects (genotype, Zn^{2+}, TAC, TGF-β1, PPARγ agonists or PPARγ antagonists), and two-way ANOVA to test their interactions. If ANOVA results were significant, a post-hoc comparison among groups was performed with the Newman–Keuls test. Changes in PPARγ protein and mRNA expression, promoter activity and transcription factor binding in response to TGF-β1 or vehicle treatments were compared by using Student's *t* test. A P value less than 0.05 was considered statistically significant.

Results

TGF-β signaling is required for the down-regulation of PPARγ expression in the pressure overloaded heart

To test the specific hypothesis that PPARγ expression is reduced in the pressure overloaded heart and that TGF-β signaling is required for pressure overload-induced down-regulation of PPARγ expression, DnTGFβRII transgenic and control wild-type mice were subjected to TAC or sham surgery and followed for 1 week. We have previously observed that TAC led to significant cardiac hypertrophy and fibrosis in sham-treated mice and that blockade of TGF-β signaling in DnTGFβRII mice with Zn^{2+} significantly attenuated TAC-induced cardiac fibroblast proliferation and myocardial fibrosis [17]. In the current study, we used samples from the same groups of mice to assess PPARγ protein expression in LV. We observed that TAC led to a significant increase in myocardial PPARγ protein levels in the DnTGFβRII mice with Zn^{2+} in the drinking H_2O compared with sham ($P < 0.05$) (Fig. 1). In contrast, TAC led to significant decreases in PPARγ protein levels in both wild-type groups and in DnTGFβRII mice drinking distilled H_2O without Zn^{2+}, in which TGF-β signaling was preserved. These results suggest that TGF-β signaling is necessary for pressure overload-induced down-regulation of PPARγ expression in LV of wild-type mice and that there is a functionally significant inhibitory effect of TGF-β signaling on PPARγ, a putative endogenous antifibrogenic transcription factor, during the development of pressure overload-induced cardiac fibrosis and remodeling.

TGF-β1 down-regulates PPARγ protein expression in isolated mouse cardiac fibroblasts

Chronic pressure overload induced by TAC increases TGF-β activation in heart and leads to cardiac fibrosis and remodeling in mice [16,26]. Cardiac fibroblasts play a critical role in mediating cardiac fibrosis [27]. We therefore used isolated cardiac fibroblasts to test the hypothesis that TGF-β1 can directly inhibit PPARγ expression in this critical cell type. Consistent with the results of the in-vivo study, TGF-β1 (1 ng/ml) treatment for 24, 48 and 72 h inhibited the expression of PPARγ protein (Fig. 2a). Further, removal of TGF-β1 after 24 h of incubation restored PPARγ expression to near the basal level within 24 h (Fig. 2b), indicating that TGF-β1-induced inhibition of PPARγ expression in cardiac fibroblasts is reversible.

TGF-β1 inhibits PPARγ gene expression in cardiac fibroblasts at the transcriptional level

To determine whether TGF-β1-induced down-regulation of PPARγ expression in cardiac fibroblasts occurs at the transcriptional level, quiescent cardiac fibroblasts were exposed to TGF-β1 (1 ng/ml) for 24 h and steady-state PPARγ mRNA was measured using real-time quantitative RT-PCR analysis. Consistent with its effect on protein expression, TGF-β1 treatment significantly decreased PPARγ mRNA expression (Fig. 3a). Further, to determine whether TGF-β1 treatment alters the stability of PPARγ mRNA, cardiac fibroblasts were pretreated with TGF-β1 (1 ng/ml) or vehicle for 24 h, and then actinomycin D (5 μg/ml) was added to stop new RNA synthesis. Cellular PPARγ mRNA levels were measured at 1, 2 and 4 h post actinomycin D treatment. The half lives of PPARγ mRNA in vehicle and TGF-β1 treatment groups were 1.58 and 1.55 h, respectively, suggesting that TGF-β1 treatment did not alter the degradation rate of PPARγ mRNA (Fig. 3b).

To determine the effect of TGF-β1 on the transcriptional activity of the *PPARγ* gene, cardiac fibroblasts were transfected with a plasmid containing a human *PPARγ* promoter and then incubated with TGF-β1 (1 ng/ml) for 24 h. Measurement of luciferase activities demonstrated that TGF-β1 treatment decreased *PPARγ* promoter activity by 53% (Fig. 3c). Together, these results suggest that TGF-β1 inhibits *PPARγ* gene expression in cardiac fibroblasts at the transcriptional level.

TGF-β1 treatment increases binding of Smad2/3/4 proteins to the PPARγ promoter in cardiac fibroblasts

To provide direct evidence that activation of TGF-β1 signaling can transcriptionally suppress *PPARγ* gene expression though Smads in cardiac fibroblasts, we next tested whether Smad2/3/4 proteins bind to the *PPARγ* promoter. ChIP analysis showed that in the absence of TGF-β1, moderate levels of Smad2 and/or Smad3 and Smad4 were evident at the *PPARγ* promoter, and activation of TGF-β signaling enhanced the levels of Smad2 and/or Smad3 and Smad4 at the promoter region of the *PPARγ* gene (Fig. 4).

Histones are acetylated at promoters that are undergoing active transcription, and thus the level of histone acetylation on the promoter is a marker of gene transcriptional competence [28]. To further assess the mechanism of the TGF-β-Smad activation-induced inhibition of *PPARγ* transcription, protein–DNA complexes were immunoprecipitated using antibodies against HDAC1, a transcriptional co-repressor, and acetylated histone3, a marker of transcriptional activation. ChIP assay determined that in the absence of TGF-β1, moderate levels of HDAC1 and high levels of acetylated histone3 were present at the *PPARγ* promoter, and TGF-β1 treatment significantly increased the binding of HDAC1 and decreased the levels of acetylated histone3 (AcH3) at the *PPARγ* promoter (Fig. 4), providing evidence that the *PPARγ* gene is transcriptionally inactive in the presence of TGF-β1.

Overexpression or activation of PPARγ inhibits the profibrogenic effects of TGF-β1 in cardiac fibroblasts

To directly assess the functional significance of TGF-β-induced PPARγ suppression, we tested whether increasing PPARγ expression antagonizes the pro-fibrogenic action of TGF-β1 in cardiac fibroblasts. TGF-β1 treatment significantly increased expression of ECM molecules (CTGF and periostin) and α-SMA (a marker of myofibroblast transformation) protein (Fig. 5a-d) and mRNA (Supplementary Figure 1a–c, http://links.lww.com/HJH/A110) in control uninfected cardiac fibroblasts and cardiac fibroblasts infected with the empty adenoviral vector. Adenovirus-mediated *PPARγ* gene transfection significantly increased PPARγ protein expression by 4.7-fold (Fig. 5e). Overexpression of PPARγ completely blocked TGF-β1-stimulated CTGF, periostin and α-SMA protein and mRNA expression in cardiac fibroblasts infected with adenovirus carrying the *PPARγ* gene. Similarly, pretreatment with the PPARγ agonists rosiglitazone (1.0 μmol/l) and pioglitazone (10 μmol/l) for 24 h completely blocked TGF-β1-induced CTGF and periostin mRNA expression (Supplementary Figure 2a and b, http://links.lww.com/HJH/A110) and inhibited TGF-β1-induced Smad3 phosphorylation (Supplementary Figure 2c and d, http://links.lww.com/HJH/A110). These data provide further evidence that PPARγ acts as an antifibrogenic factor in cardiac fibroblasts, suggesting that activation of PPARγ signaling may antagonize the pro-fibrogenic action of TGF-β in the pressure overloaded heart.

PPARγ agonist inhibits, and PPARγ antagonist exacerbates, pressure overload-induced cardiac fibrosis and remodeling in mice

T0070907, a highly selective PPARγ antagonist, has been utilized to study the function of endogenous PPARγ *in vitro* and *in vivo* [29,30]. To test the hypothesis that endogenous PPARγ attenuates pressure overload-induced cardiac fibrosis and remodeling, we administrated the PPARγ antagonist T0070907 and, as a positive control, the PPARγ agonist rosiglitazone to wild-type mice that were subjected to TAC. Consistent with our previous studies, TAC significantly increased myocardial collagen volume (Fig. 6a and b), the ratio of left-ventricular weight-to-body weight/tibia length and cardiomyocyte size in vehicle-treated mice compared with sham-operated controls (Fig. 6c–e). The TAC-induced increase in left-ventricular collagen volume and LVEDP (Fig. 6b and f), but not left-ventricular peak systolic pressure (Supplementary Figure 3, http://links.lww.com/HJH/A110), was significantly amplified in T0070907-treated mice and attenuated in rosiglitazone-treated animals, suggesting that endogenous PPARγ has a protective role in preventing cardiac fibrosis and remodeling during pressure overload stress.

Discussion

The current study has demonstrated the following: TGF-β1 directly inhibits expression of PPARγ, an endogenous antifibrogenic transcription factor, in isolated cardiac fibroblasts at the transcriptional level; pressure overload stress suppresses myocardial PPARγ expression via a TGF-β-dependent mechanism; pharmacologic activation of PPARγ by the agonist rosiglitazone potently inhibits, and inactivation of endogenous PPARγ by the antagonist T0070907 exacerbates chronic pressure overload-induced myocardial fibrosis and remodeling.

PPARγ acts as a functional antifibrogenic factor and maintaining an appropriate expression level in heart is essential for assuring that cardiac structure and function adapt to pressure overload stress. PPARγ agonist (rosiglitazone or ciglitazone) treatment has been shown to increase PPARγ protein expression and inhibit chronic pressure overload-induced cardiac hypertrophy, collagen accumulation, and interstitial and perivascular fibrosis in rat and

mouse models of abdominal aortic constriction [6,7]. Cardiomyocyte-specific *PPARγ* knockout mice have been shown to develop age-progressive cardiac hypertrophy [13] and the decrease in myocardial PPARγ in these mice is associated with increasing severity of cardiac pathology in response to stress [12]. Further, mice carrying a human dominant-negative point mutation in PPARγ developed significantly more severe cardiac fibrosis in response to subcutaneous angiotensin II infusion compared with their wild-type littermates [31]. However, the effect of chronic pressure overload stress on the regulation of endogenous PPARγ expression in heart has not been examined. Using the DnTGFβRII model to define the contribution of TGF-β signaling to the expression of endogenous PPARγ in the pressure overloaded heart, we have demonstrated that disruption of TGF-β signaling prevented the down-regulation of PPARγ expression seen in wild-type murine heart under chronic pressure overload conditions. In isolated cardiac fibroblasts, we demonstrated that TGF-β1 treatment decreased *PPARγ* promoter activity, as well as PPARγ mRNA and protein levels. Consistent with our results, Fu *et al.* [32] have demonstrated that exposure to TGF-β1 for periods more than 12 h led to significant suppression of PPARγ mRNA expression in human aortic smooth muscle cells.

TGF-β-mediated transcriptional modulation requires Smad protein binding to the promoters of target genes. Our ChIP analysis indicated that the *PPARγ* promoter is constitutively bound by Smad2/3 and Smad4 proteins, and that the binding of Smad2/3/4 proteins to the *PPARγ* promoter increases in response to TGF-β1 stimulation. Future studies will map the precise region of the *PPARγ* promoter that mediates inhibition of *PPARγ* gene expression by TGF-β1. Also, we have observed that TGF-β1-stimulated binding of Smad2/3/4 enhances the recruitment of HDAC1 and reduces the levels of histone 3 acetylation at the *PPARγ* promoter. These results provide additional evidence that TGF-β1 down-regulates *PPARγ* gene expression through a transcriptional mechanism.

To test the functional significance of PPARγ down-regulation in TGF-β-mediated cardiac remodeling, we firstly examined the effect of PPARγ activation on TGF-β-stimulated ECM molecule expression in isolated cardiac fibroblasts *in vitro*. We demonstrated that both PPARγ agonists (rosiglitazone and pioglitazone) and overexpression of PPARγ completely blocked TGF-β1-stimulated CTGF and periostin gene expression. We then tested the effects of pharmacologic antagonism and activation of PPARγ in hearts subjected to pressure overload stress with TAC. We chose T0070907, a potent and selective (>800-fold preference for PPARγ over PPARα and PPARδ) PPARγ antagonist for these studies. This widely used pharmacologic antagonist has been shown to bind covalently to the Cys^{313} residue of PPARγ and induce conformational changes that block the recruitment of transcriptional cofactors to the PPARγ/retinoid X receptor heterodimer [29]. We demonstrated that inhibition of PPARγ with T0070907 exacerbated and activation of PPARγ by rosiglitazone suppressed chronic pressure overload-induced cardiac fibrosis.

TGF-β signaling is essential for the embryonic development of the heart, and homozygous deletion of the TGF-β gene leads to embryonic lethality, which has prevented the successful development of a TGF-β knockout model. The inducible DnTGFβRII transgenic mouse model does not disrupt critical TGF-β signaling pathways in the developing heart, and thus offers important advantages for studying the contribution of TGF-β signaling to pressure overload-induced cardiac fibrosis and remodeling. Previous studies have shown that global blockade of TGF-β signaling in DNTGFβRII mice was associated with progressive skeletal degeneration in aged animals [18]. However, we have observed that DnTGFβRII mice with TAC for 4 months appear to grow normally and have no obvious phenotypic abnormalities. We also observed that systemic blockade of TGF-β signaling in DnTGFβRII mice did not attenuate the increases in left-ventricular mass and cardiomyocyte size seen in pressure overloaded hearts [17]. Although we can not fully exclude the effect of systemic blockade of

TGF-β signaling on PPARγ expression in cardiomyocytes, our results do not suggest that disruption of TGF-β signaling in the current transgenic model has significant effects on cardiomyocytes under conditions of chronic pressure overload.

In summary, the present study provides the first evidence that TGF-β signaling is required for pressure overload induced down-regulation of PPARγ in heart and that TGF-β directly suppresses PPARγ expression at the transcriptional level by enhancing Smad2/3/4 and HDAC1 binding to the PPARγ promoter in cardiac fibroblasts. Since we have demonstrated that increasing extrinsic PPARγ expression has ability to inhibit, and blockade of PPARγ by antagonists exacerbates the fibrogenic response induced by TGF-β or pressure overload, the findings support the notion that down-regulation of the endogenous antifibrogenic factor PPARγ expression by TGF-β may be involved in pressure overload-induced cardiac fibrosis.

Clinical perspectives

In humans, cardiac hypertrophy and fibrosis in response to chronic pressure overload frequently lead to heart failure with preserved left-ventricular ejection fraction. The characteristic pressure overload-induced cardiac lesions are related to an imbalance in the dynamic regulation of ECM generation by pro-fibrogenic (principally, TGF-β-Smads) and antifibrogenic (e.g. PPARγ) signaling pathways in cardiac fibroblasts. The functionally significant inhibitory interaction between TGF-β signaling and PPARγ during the development of pressure overload-induced cardiac remodeling is underinvestigated, and is potentially very important for clinical medicine. Since currently available PPARγ agonists have been associated with increased risks of cardiac events, and are therefore limited in their clinical utility, there is a need to develop novel approaches to maximizing the potential benefits of endogenous PPARγ activation in heart. The fundamental research described in this study provides a conceptual basis for the search for new therapeutic approaches to the prevention and treatment of these difficult to manage forms of heart disease.

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Abbreviations

α-SMA	α-smooth muscle actin
AcH3	acetylated histone3
CFs	cardiac fibroblasts
ChIP	chromatin immunoprecipitation
CTGF	connective tissue growth factor
DnTGFβRII	dominant negative TGF-β receptor type II
ECM	extracellular matrix
FBS	fetal bovine serum
HDAC1	histone deacetylase 1
LV	left ventricle
LVEDP	left ventricular end-diastolic pressure
PPARγ	peroxisome proliferator-activated receptor gamma

TAC	transverse aortic constriction
TGF-β	transforming growth factor-β

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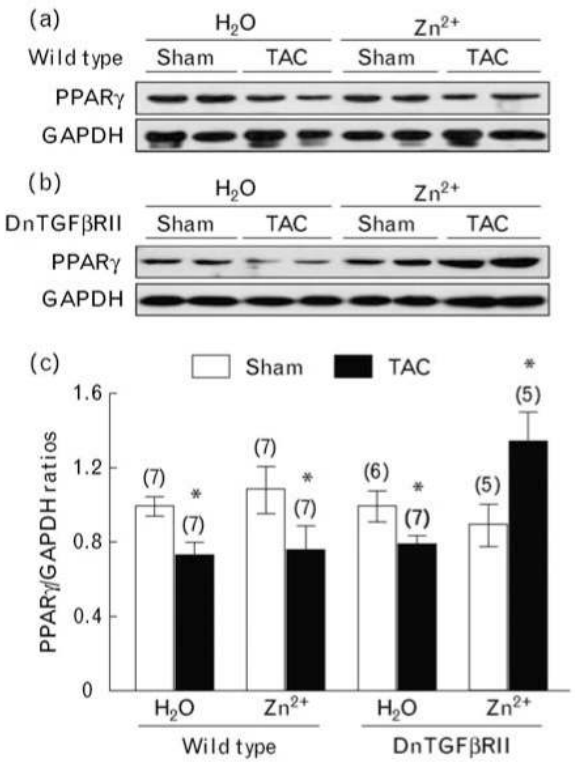

Fig. 1.

Effects of 7 days of transverse aortic constriction or sham operation on peroxisome proliferator-activated receptor gamma protein levels in left ventricle of DnTGFβRII and wild-type mice drinking 25 mmol/l $ZnSO_4$ water (Zn^{2+}) or distilled water (H_2O). PPARγ protein levels were normalized using GAPDH protein levels as an internal standard. *n* = number of mice per group. **P*<0.05 compared with respective Sham groups. LV, left ventricle; PPARγ, peroxisome proliferator-activated receptor gamma; TAC, transverse aortic constriction.

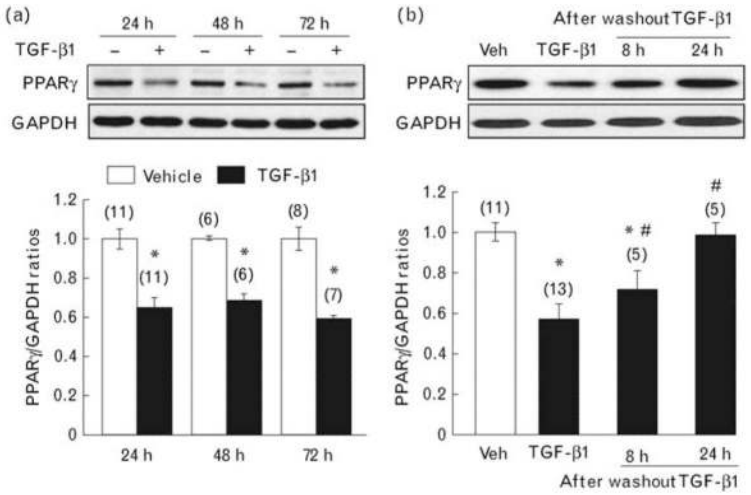

Fig. 2.

Effects of transforming growth factor-β1 on peroxisome proliferator-activated receptor gamma protein expression in isolated mouse cardiac fibroblasts. (a) CFs were treated with TGF-β1 (1 ng/ml) or vehicle for 24–72 h; (b) after TGF-β1 treatment for 24 h, the medium was replaced with fresh TGF-β1-free DMEM for an additional 8–24 h. Cellular PPARγ protein levels were normalized using GAPDH protein levels as an internal standard. *n*=number of samples. **P*<0.05 compared with respective vehicle control groups; #*P*<0.05 compared with TGF-β1 treated for 24 h. CF, cardiac fibroblast; PPARγ, peroxisome proliferator-activated receptor gamma; TGF-β1, transforming growth factor-β1.

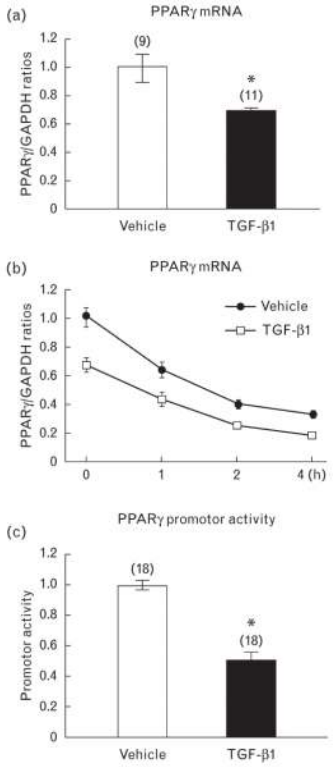

Fig. 3.

Effects of transforming growth factor-β1 on peroxisome proliferator-activated receptor gamma mRNA expression and stability, as well as promoter activity. (a) CFs were treated with TGF-β1 (1 ng/ml) or vehicle for 24 h. PPARγ mRNA levels were determined by real-time RT-PCR. (b) After CFs were pretreated with TGF-β1 or vehicle for 24 h, actinomycin D (5 μg/ml) was added to the media (time 0). The PPARγ mRNA levels at 1, 2, and 4 h after actinomycin D treatment were determined and normalized by GAPDH mRNA levels ($n = 4$ per group per time point). (c) CFs were co-transfected with pGL3-Luc-*PPARγ* and pRL-TK plasmids for 24 h and then exposed to TGF-β1 for an additional 24 h. Luciferase activity was measured by a luminometer. *n*=number of samples. **P*<0.05 compared with respective vehicle control groups. CF, cardiac fibroblast; PPARγ, peroxisome proliferator-activated receptor gamma; TGF-β1, transforming growth factor-β1.

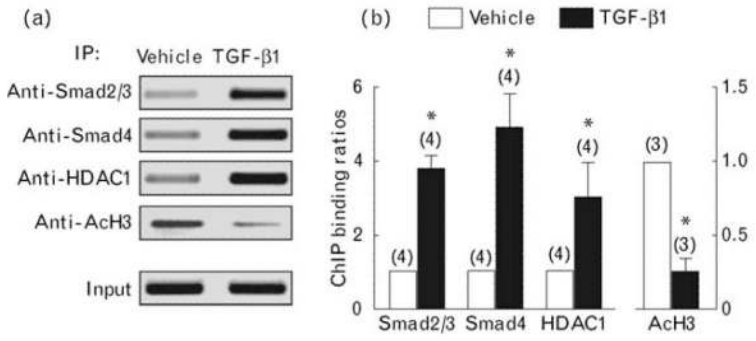

Fig. 4.

Chromatin immunoprecipitation analyses for detection of Smad2/3, Smad4, HDAC1, and AcH3 at the peroxisome proliferator-activated receptor gamma promoter. CFs were treated with TGF-β1 (1 ng/ml) or vehicle for 1 h. Smad2/3/4 and HDAC1 binding, as well as acetylated histone (AcH3) level at the *PPARγ* promoter were determined by specifically amplifying the *PPARγ* promoter fragment with PCR. A representative set of results from at least three independent assays is shown. **P*<0.05 compared with respective vehicle control groups. CF, cardiac fibroblast; PPARγ, peroxisome proliferator-activated receptor gamma.

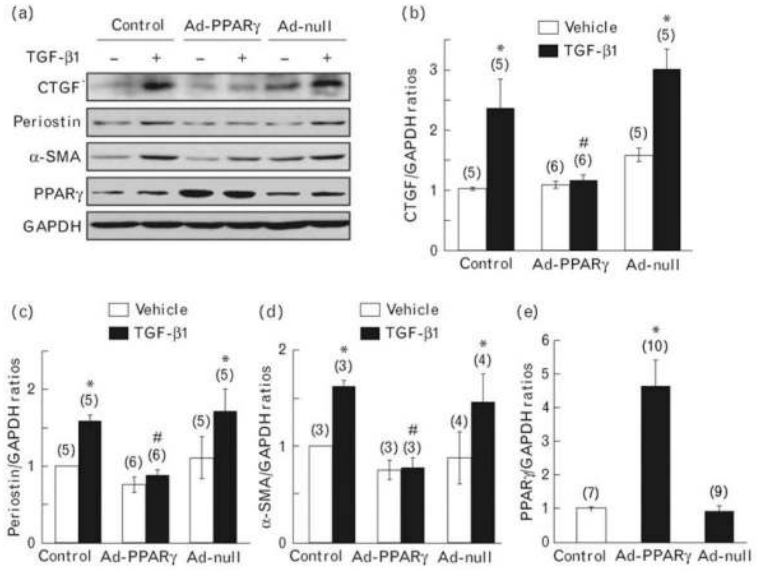

Fig. 5.

Overexpression of peroxisome proliferator-activated receptor gamma inhibits transforming growth factor-β1-stimulated ECM molecule protein expression in mouse cardiac fibroblasts. CFs were infected with PPARγ adenovirus (Ad-PPARγ), empty vector adenovirus (Ad-null) or vehicle (control) for 72 h and then stimulated with TGF-β1 (1 ng/ml) for an additional 24 h. Protein levels of CTGF (b), periostin (c), α-SMA (d), and PPARγ (e) were determined by western blot analysis. *n*=number of samples. **P*<0.05 compared with respective vehicle groups; $^{\#}P < 0.05$ compared with respective TGF-β1 in control groups. α-SMA, α-smooth muscle actin; CF, cardiac fibroblast; CTGF, connective tissue growth factor; ECM, extracellular matrix; PPARγ, peroxisome proliferator-activated receptor gamma; TGF-β1, transforming growth factor-β1.

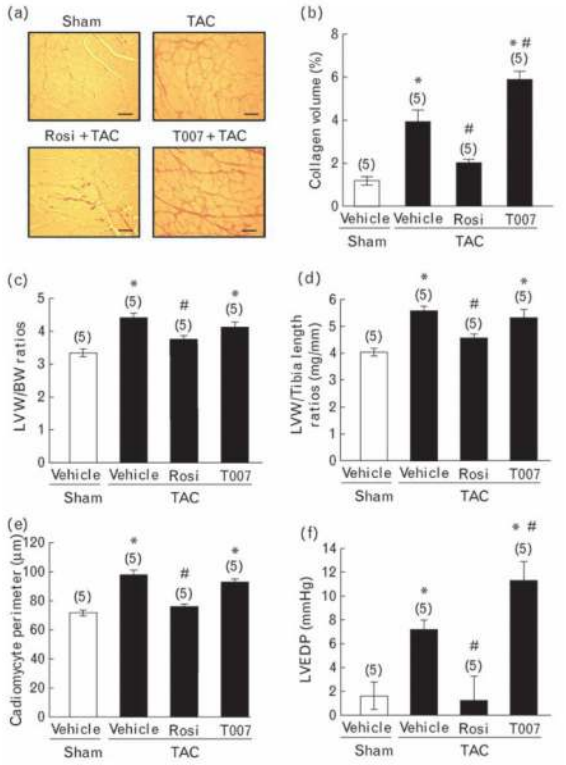

Fig. 6.

Effects of rosiglitazone and T0070907 on pressure overload-induced cardiac fibrosis. Representative photomicrographs of left-ventricular sections from four groups are shown, scale bar = 50 μm, (a). Left ventricular myocardial collagen volume (picrosirius red-stained areas, (b) ratios of left ventricular weight to body weight (c) or tibia length (d), cardiomyocyte perimeter (e) and LVEDP (f) were measured. *n*=number of mice per group. **P*<0.05 compared with Sham groups treated with vehicle. $^{\#}P$<0.05 compared with TAC mice treated with vehicle. LVEDP, left ventricular end-diastolic pressure; TAC, transverse aortic constriction.