Peroxisome Proliferator–Activated Receptor γ Plays a Critical Role in Inhibition of Cardiac Hypertrophy In Vitro and In Vivo

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Background—Peroxisome proliferator-activated receptors (PPARs) are transcription factors of the nuclear receptor superfamily. It has been reported that the thiazolidinediones, which are antidiabetic agents and high-affinity ligands for PPARγ, regulate growth of vascular cells. In the present study, we examined the role of PPARγ in angiotensin II (Ang II)–induced hypertrophy of neonatal rat cardiac myocytes and in pressure overload–induced cardiac hypertrophy of mice.

Methods and Results—Treatment of cultured cardiac myocytes with PPARγ ligands such as troglitazone, pioglitazone, and rosiglitazone inhibited Ang II–induced upregulation of skeletal α-actin and atrial natriuretic peptide genes and an increase in cell surface area. Treatment of mice with a PPARγ ligand, pioglitazone, inhibited pressure overload–induced increases in the heart weight–to–body weight ratio, wall thickness, and myocyte diameter in wild-type mice and an increase in the heart weight–to–body weight ratio in heterozygous PPARγ-deficient mice. In contrast, pressure overload–induced increases in the heart weight–to–body weight ratio and wall thickness were more prominent in heterozygous PPARγ-deficient mice than in wild-type mice.

Conclusions—These results suggest that the PPARγ-dependent pathway is critically involved in the inhibition of cardiac hypertrophy. (Circulation. 2002;105:1240-1246.)

Key Words: angiotensin ■ hypertrophy ■ myocytes ■ pressure

Cardiac hypertrophy is observed in various cardiovascular diseases such as hypertension, myocardial infarction, valvular heart disease, and hypertrophic cardiomyopathy. Clinical studies have demonstrated that cardiac hypertrophy is not only an adaptational state before heart failure but is an independent risk factor for ischemia, arrhythmia, and sudden death. Therefore, it has become even more important to prevent the development of cardiac hypertrophy.

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Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily. PPARs have 3 isoforms: α, β, and γ. It has been reported that the thiazolidinediones, novel insulin-sensitizing agents and high-affinity ligands for PPARγ, prevent growth factor–induced proliferation and migration of vascular smooth muscle cells. The thiazolidinediones have also been reported to inhibit cytokine-mediated endothelial cell proliferation and suppress ET-1 secretion from vascular endothelial cells. These observations suggest that PPARγ ligands may influence growth of vascular cells.

Recently, many reports indicate that insulin resistance and hyperinsulinemia are closely related to cardiac hypertrophy. If insulin resistance and hyperinsulinemia contribute to cardiac hypertrophy, the thiazolidinediones may prevent the development of cardiac hypertrophy. In this study, we examined the effects of the thiazolidinediones such as troglitazone, pioglitazone, and rosiglitazone on angiotensin II (Ang II)–induced hypertrophy in neonatal rat cardiac myocytes and on pressure overload–induced cardiac hypertrophy in mice. To further elucidate the role of PPARγ in the development of cardiac hypertrophy, we examined pressure overload–induced cardiac hypertrophy with the use of heterozygous PPARγ-deficient (PPARγ-/-) mice. Our results suggest that the PPARγ-dependent pathway inhibits the development of cardiac hypertrophy.

Methods

Materials
Troglitazone, pioglitazone, and rosiglitazone were generous gifts from Sankyo Co, Takeda Co, and SmithKline Beecham, respectively. Ang II was purchased from Sigma.

Materials

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**Cell Culture**

Primary cultures of cardiac myocytes were prepared from ventricles of 1-day-old Wistar rats as described previously, basically according to the method of Simpson and Savion. In brief, cardiomyocytes were plated at a field density of $1 \times 10^4$ cells/cm² on 35-mm or 100-mm culture dishes (Falcon Primaria) in culture medium (MEM with 5% bovine calf serum). Twenty-four hours after seeding, the culture medium was changed to serum-free MEM and the cells were cultured for 48 hours before stimulation. The cells were then pretreated with troglitazone, pioglitazone, or rosiglitazone for 30 minutes and subsequently stimulated with Ang II (1 μmol/L) for 24 hours.

**Isolation of RNA and Northern Blot Analysis In Vitro**

Total RNA was isolated by the guanidinium thiocyanate–phenol chloroform method. Ten micrograms of total RNA was subjected to electrophoresis in 1.0% agarose/formaldehyde gel and transferred to a Hybond-N membrane (Amersham Co). Skeletal α-actin and atrial natriuretic peptide (ANP) cDNA were used as probes. cDNA was labeled by the random priming method with $^{32}$P-dCTP. The expression of each mRNA was determined with FUJIX Bio-Imaging Analyzer BAS 2000 (Fuji Film Co).

**Measurement of Surface Area of Cardiac Myocytes**

The cells were pretreated with troglitazone, pioglitazone, or rosiglitazone for 30 minutes and then stimulated with Ang II (1 μmol/L) for 48 hours. The cells were fixed with 3.7% formaldehyde in PBS, permeabilized in 0.1% Triton X-100 in PBS, and stained with rhodamine phalloidin (Molecular Probes, Inc) at a dilution of 1:50 by standard immunocytochemical techniques. For measurements of cell surface area, 50 random cells were measured by planimetry.

**Animals**

PPARγ-deficient mice (PPARγ⁻/⁻) were generated as described elsewhere. Twenty-week-old male wild-type mice (WT) and PPARγ⁻/⁻ mice from the same genetic backgrounds were used in the present study. WT mice and PPARγ⁻/⁻ mice were divided into 4 groups as follows: (1) sham-operated mice, (2) sham-operated mice with pioglitazone, (3) pressure-overloaded mice, and (4) pressure-overloaded mice with pioglitazone (n=4, each group). Pressure overload was produced by constriction of the abdominal aorta as described previously. Briefly, mice were anesthetized by injection of sodium pentobarbital (30 mg/kg IP). The abdominal aorta was constricted at the suprarenal level with 7–0 nylon strings by ligation with a blunted 24-gauge needle, which was then pulled out. Treatment with pioglitazone was initiated 1 week before operation and continued for 4 weeks as food admixture at a concentration of 0.01% (wt/wt). At 21 days after operation, hearts were excised, weighed, and subjected to further analysis. Animal care and procedures were approved by the Animal Care Committee of the Universities of Tokyo and Chiba.

**Hemodynamic Measurement In Vivo**

To measure hemodynamic effects of aortic constriction, the right carotid artery was cannulated with a 24-gauge polyethylene catheter. The transducer (Baxter, model MP 5100) was connected to a Mac Laboratory system (model 400/s, Adinstruments), and the blood pressure was measured as described previously.
Echocardiographic Analysis
Transthoracic echocardiography was performed with HP Sonos 100 (Hewlett-Packard Co) with a 10-MHz imaging transducer as described previously. Mice were anesthetized with ketamine (10 mg/kg IP) and xylazine (15 mg/kg IP). After a good-quality 2-dimensional image was obtained, M-mode images of the left ventricle were recorded. Interventricular septum thickness and left ventricular posterior wall thickness were measured.

Histological Analysis
For histological analysis, all the hearts were arrested in diastole with KCl (30 mmol/L), followed by perfusion fixation with 10% formalin. Fixed hearts were embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin and eosin for overall morphology. Mean myocyte diameter was calculated by measuring 100 cells from sections stained with hematoxylin and eosin.

Isolation of RNA and Northern Blot Analysis In Vivo
Total RNA was isolated from the left ventricular samples with RNAzol (Tel-text), and 7.5 μg of total RNA was electrophoresed in 1.0% agarose/formaldehyde gel. Skeletal α-actin cDNA was used as probe.

Statistical Analysis
Data are presented as mean±SEM. Mean difference among 3 or more groups was tested by 1-way ANOVA, and Scheffé’s F test was used for multiple comparisons. A value of P<0.05 was considered to be statistically significant.

Results
Thiazolidinediones Inhibit Ang II–Induced Upregulation of Skeletal α-Actin and ANP Genes in Cardiac Myocytes of Neonatal Rats
It is well known that cardiac hypertrophy is accompanied by changes in the muscle phenotype characterized by the expression of fetal-type genes such as skeletal α-actin and ANP genes. Therefore, we first examined the effects of thiazolidinediones on Ang II–induced skeletal α-actin and ANP gene expression in myocytes. The cells were pretreated with troglitazone, pioglitazone, or rosiglitazone for 30 minutes and subsequently stimulated with Ang II (1 μmol/L) for 24 hours. Northern blot analysis revealed that Ang II induced the expression of skeletal α-actin and ANP genes. All three thiazolidinediones inhibited Ang II–induced upregulation of skeletal α-actin and ANP genes (Figure 1, A and B).

Thiazolidinediones Inhibit Ang II–Induced Increase in Surface Area of Cardiac Myocytes
To assess cellular hypertrophy, surface area of cardiac myocytes was quantified. The cells were pretreated with troglitazone (10 μmol/L) for 30 minutes, and subsequently stimulated with Ang II (1 μmol/L) for 48 hours. Ang II increased surface area of cardiac myocytes by 1.4-fold as compared with control. Troglitazone inhibited Ang II–induced increase in cell surface area (Figure 2, A and B). Pioglitazone and rosiglitazone also inhibited Ang II–induced increase in cell surface area (Figure 2C). The rank order of their inhibitory potencies was correlated with that of their binding affinities for PPARγ (rosiglitazone>piglitazone>troglitazone).

Pioglitazone Inhibits Pressure Overload–Induced Cardiac Hypertrophy
We next examined whether pioglitazone prevents pressure overload–induced cardiac hypertrophy. Blood pressure was...
monitored at the right carotid arteries before and after constriction of the abdominal aorta as described in Methods. The baseline blood pressure was not significantly different between WT and WT treated with pioglitazone (WT, 100.5 ± 2.7 mm Hg; WT treated with pioglitazone, 99.4 ± 2.7 mm Hg). The blood pressure after aortic constriction was not also significantly different between WT and WT treated with pioglitazone (WT, 120.5 ± 2.7 mm Hg; WT treated with pioglitazone, 119.4 ± 2.9 mm Hg). Pressure overload for 3 weeks increased heart weight (HW)/body weight (BW) ratio (banded mice, 5.52 ± 0.08 mg/g versus sham, 4.43 ± 0.02 mg/g). Treatment with pioglitazone inhibited the pressure overload–induced increase in the HW/BW ratio (banded mice treated with pioglitazone, 4.99 ± 0.07 mg/g) (Figure 3). Echocardiographic analysis revealed that pressure overload increased the left ventricular wall thickness (Figure 4A, a and c) and mean myocyte diameter (banded mice, 18.4 ± 3.0 μm versus sham, 13.9 ± 2.1 μm) (Figure 4B, a and c, and Figure 4C). Treatment with pioglitazone inhibited the pressure overload–induced increase in left ventricular wall thickness (Figure 4A, c and d) and the mean myocyte diameter (banded mice treated with pioglitazone, 13.2 ± 2.5 μm) (Figure 4B, c and d, and Figure 4C). Northern blot analysis revealed that pressure overload for 3 weeks induced the expression of skeletal α-actin gene. Treatment with pioglitazone significantly inhibited the pressure overload–induced upregulation of skeletal α-actin gene (Figure 5). In contrast, pioglitazone by itself did not induce cardiac hypertrophy in both sham-operated WT mice and sham-operated PPARγ−/− mice (Figures 3 to 5, Table).

Pressure overload induced more marked cardiac hypertrophy in PPARγ−/− mice than in WT mice. To examine whether PPARγ is involved in the inhibition of pressure overload–induced cardiac hypertrophy, pressure overload was produced in the heart of PPARγ−/− mice. The baseline blood pressure was not significantly different between PPARγ−/− and WT mice (PPARγ−/−, 101.5 ± 2.9 mm Hg versus WT, 100.5 ± 2.7 mm Hg). The blood pressure after constriction of the abdominal aorta was not also significantly different between PPARγ−/− and WT mice (PPARγ−/−, 121.4 ± 3.5 mm Hg versus WT, 120.5 ± 2.7 mm Hg). The pressure overload–

### Echocardiographic Parameters in WT and PPARγ−/− Mice

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<thead>
<tr>
<th>Parameter</th>
<th>Sham</th>
<th>Pio</th>
<th>Banding</th>
<th>Pio + Banding</th>
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<td>WS, mm</td>
<td>0.74 ± 0.02</td>
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<tr>
<td>PW, mm</td>
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<tr>
<td>%FS</td>
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<td>43.3 ± 1.7</td>
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Data are mean ± SEM (n = 4 each). Pio indicates pioglitazone.

*P < 0.05 vs WT sham-operated mice and †P < 0.05 vs WT banded mice.

Treatment with pioglitazone inhibits the pressure overload–induced increase in the thickness of IVS and PW.
induced increase in the HW/BW ratio was more prominent in PPARγ+/− mice than in WT mice (PPARγ+/−, 6.18±0.09 mg/g versus WT, 5.52±0.08 mg/g) (Figure 3). Echocardiographic analysis revealed that the pressure overload–induced increase in the thickness of IVS and PW was also more striking in PPARγ+/− mice than in WT mice (PPARγ+/−, IVS, 1.12±0.05 mm, PW, 1.14±0.04 mm versus WT, IVS, 0.96±0.03 mm, PW, 0.98±0.03 mm) (Table). There was no difference in fractional shortening (%FS) between WT and PPARγ+/− mice irrespective of treatment with pioglitazone. Histological analysis revealed that the pressure overload–induced increase in left ventricular wall thickness (Figure 4A, c and g) and mean myocyte diameter were more prominent in PPARγ+/− mice than in WT mice (PPARγ+/−, 22.5±3.1 μm versus WT, 18.4±3.0 μm) (Figure 4B, c and g, and Figure 4C). Northern blot analysis also revealed that the pressure overload–induced upregulation of skeletal α-actin was more prominent in PPARγ+/− mice than in WT mice (Figure 5). Although pressure overload–induced increases in the thickness of IVS and PW and mean myocyte diameter were not significantly different between PPARγ+/− mice without pioglitazone and PPARγ+/− mice treated with pioglitazone (Table, Figure 4), pioglitazone significantly inhibited the pressure overload–induced increase in the HW/BW ratio in PPARγ+/− mice (Figure 3). The suppression by pioglitazone in PPARγ+/− mice was approximately half of that in WT mice. These results suggest that pioglitazone inhibits pressure overload–induced cardiac hypertrophy through PPARγ in WT mice and PPARγ+/− mice.

Discussion
In this study, we demonstrated that PPARγ ligands troglitazone, pioglitazone, and rosiglitazone inhibit Ang II–induced hypertrophy of neonatal rat cardiac myocytes. The pressure overload–induced cardiac hypertrophy was more prominent in PPARγ+/− mice than in WT mice. Treatment with a PPARγ ligand, pioglitazone, inhibited the pressure overload–induced cardiac hypertrophy strongly in WT mice and moderately in PPARγ+/− mice. These results suggest that PPARγ–dependent pathway inhibits the development of cardiac hypertrophy.

PPARγ is a transcription factor belonging to the nuclear receptor superfamily. PPARγ is highly expressed in adipose tissue, where it plays a major regulatory role in adipocyte differentiation and the expression of adipocyte specific genes involved in lipid metabolism.16 Recently, the thiazolidinediones, a new class of antidiabetic agents, have been identified as high-affinity ligands for PPARγ.16 It has been reported that activation of PPARγ inhibits the expression of tumor necrosis factor-α (TNF-α), interleukin-1β and -6, inducible nitric oxide synthase, and scavenger receptor A in monocytes and monocyte-derived macrophages.17,18 It has been reported that PPARγ ligands inhibit growth factor–induced proliferation and migration of vascular smooth muscle cells.2,3 PPARγ ligands have also been demonstrated to inhibit cytokine-mediated endothelial cell proliferation and suppress endothelin-1 (ET-1) secretion from vascular endothelial cells.4,5 Therefore, the effects of PPARγ on macrophages, vascular smooth muscle cells and vascular endothelial cells are thought to be beneficial in preventing the process of atherosclerosis. We and others have identified PPARγ in the heart.19,20 We have recently reported that PPARγ activators inhibit lipopolysaccharide–induced TNF-α expression in neonatal rat cardiac myocytes and that this effect of PPARγ may be beneficial in preventing the development of congestive...
heart failure. In the present study, we demonstrate that the PPARγ-dependent pathway suppresses the development of cardiac hypertrophy. However, the molecular mechanism of how PPARγ suppresses cardiac hypertrophy remains to be determined. A variety of transcription factors may be implicated in the development of cardiac hypertrophy. c-Fos and c-Jun make the heterodimeric complex called activator protein-1 (AP-1), which transactivates many genes that have a TPA responsible element (TRE) in their promoter such as ANP and ET-1 genes. Signal transducers and activators of transcription (STATs) are known to play important roles in cytokine signaling pathways. Recently, it has been reported that STAT3 is activated in cardiac myocytes by the IL-6 family of cytokines and plays a crucial role in generating cardiac hypertrophy through gp130. The cardiac-restricted zinc finger transcription factor GATA4 has also been shown to be required for transcriptional activation of the genes for Ang II type 1a receptor, β-myosin heavy chain, and ET-1 during cardiac hypertrophy. The calcium-dependent phosphatase calcineurin dephosphorylates the transcription factor NF-AT3 and NF-AT3 translocates to the nucleus and phosphatase calcineurin dephosphorylates the transcription factor NF-AT3 and NF-AT3 translocates to the nucleus and interacts with GATA4, resulting in the development of cardiac hypertrophy. It has been recently reported that PPARγ ligands could downregulate inflammatory responses in monocytes by interfering with AP-1, STAT, and NF-κB signaling pathways through competition for essential cofactors. It has also been reported that PPARγ activators inhibit ET-1 production in human vascular endothelial cells by inhibiting the AP-1 signaling pathway. Therefore, it is conceivable that PPARγ may suppress the development of cardiac hypertrophy by antagonizing the activities of transcription factors such as AP-1, STAT3, and GATA4.

In conclusion, PPARγ ligands inhibited Ang II–induced cardiac hypertrophy in vitro and pressure overload–induced cardiac hypertrophy in vivo. Pressure overload induced more marked cardiac hypertrophy in PPARγ−/− mice than in WT mice. These results suggest that the PPARγ-dependent pathway inhibits the development of cardiac hypertrophy. Recently, many reports have suggested that insulin resistance and hyperinsulinemia are involved in cardiac hypertrophy. Our study suggests the potential clinical efficacy of the thiazolidinediones for prevention of cardiac hypertrophy. Further studies are necessary to elucidate whether inhibition of cardiac hypertrophy by PPARγ ligand improves prognosis or not.

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