Peroxisome Proliferator–Activated Receptor Activators Inhibit Lipopolysaccharide-Induced Tumor Necrosis Factor-α Expression in Neonatal Rat Cardiac Myocytes

Hiroyuki Takano, Toshio Nagai, Masayuki Asakawa, Tetsuya Toyozaki, Toru Oka, Issei Komuro, Toshihiro Saito, Yoshiaki Masuda

Abstract—Peroxisome proliferator–activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily. Recently, PPAR activators have been shown to inhibit the production of proinflammatory cytokines in macrophages or vascular smooth muscle cells. It has been reported that tumor necrosis factor-α (TNF-α) expression is elevated in the failing heart and that TNF-α has a negative inotropic effect on cardiac myocytes. Therefore, we examined the effects of PPARα and PPARγ activators on expression of TNF-α in neonatal rat cardiac myocytes. Northern blot analysis revealed expression of PPARα and PPARγ mRNA in cardiac myocytes. Immunofluorescent staining demonstrated that both PPARα and PPARγ were expressed in the nuclei of cells. When cardiac myocytes were transfected with PPAR responsive element (PPRE)-luciferase reporter plasmid, both PPARα and PPARγ activators increased the promoter activity. Cardiomyocytes were stimulated with lipopolysaccharide (LPS), and the levels of TNF-α in the medium were measured by ELISA. After exposure to LPS, the levels of TNF-α significantly increased. However, pretreatment of myocytes with PPARα or PPARγ activators decreased LPS-induced expression of TNF-α in the medium. Both PPARα and PPARγ activators also inhibited LPS-induced increase in TNF-α mRNA in myocytes. In addition, electrophoretic mobility shift assays demonstrated that PPAR activators reduced LPS-induced nuclear factor-κB activity. These results suggest that both PPARα and PPARγ activators inhibit cardiac expression of TNF-α in part by antagonizing nuclear factor-κB activity and that treatment with PPAR activators may lead to improvement in congestive heart failure. (Circ Res. 2000;87:596-602.)

Key Words: peroxisome proliferator–activated receptor ■ tumor necrosis factor-α ■ nuclear factor-κB ■ cardiac myocyte

Tumor necrosis factor-α (TNF-α) is a proinflammatory cytokine that has been implicated in the pathogenesis of cardiovascular diseases, including congestive heart failure, acute myocardial infarction, myocarditis, and dilated cardiomyopathy. Several lines of evidence indicate that the levels of circulating TNF-α are elevated in patients with congestive heart failure and that TNF-α expresses in the failing myocardium. Previous studies have demonstrated that TNF-α has direct negative inotropic effects on cardiac muscle cells. It has been suggested that TNF-α may contribute to the progression of congestive heart failure. Indeed, recent studies have shown that transgenic mice with myocardial expression of TNF-α develop a dilated cardiomyopathy and have documented a severe impairment of cardiac function. Although modulation of TNF-α in cardiovascular diseases is a critical therapeutic strategy, the molecular mechanisms that regulate cardiac TNF-α expression are unknown.

Peroxisome proliferator–activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily that heterodimerize with the retinoid X receptor and bind to specific response elements termed PPAR responsive elements (PPREs) in target gene promoters. PPARs have 3 isoforms, α, β (or δ), and γ. PPARα regulates genes involved in the β-oxidative degradation of fatty acids, whereas PPARγ promotes adipocyte differentiation and glucose homeostasis. Hypolipidemic drugs (clofibrate, gemfibrozil, and Wy14643) are known to be ligands for PPARα. Natural prostaglandin D2 metabolite, 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), and synthetic anti-diabetic thiazolidinedione (troglitazone and BRL49653) are identified as ligands for PPARγ. PPARα is present in liver, kidney, and muscle, whereas PPARγ is expressed predominantly in adipose tissue. It was recently reported that PPARα activators inhibit inflammatory responses in aortic smooth muscle cells, whereas PPARγ activators suppress production of inflammatory cytokines in activated macrophages. Therefore, we hypothesized that PPAR activators might regulate cardiac expression of TNF-α.
In the present study, we first examined the expressions of PPARα and PPARγ in neonatal rat cardiac myocytes. We demonstrate that both PPARα and PPARγ are expressed in the nuclei of cardiomyocytes. Furthermore, we investigated whether PPAR activators regulate lipopolysaccharide (LPS)–induced TNF-α expression. Our results show that PPAR activators inhibit TNF-α expression at the transcriptional level in part by preventing nuclear factor-κB (NF-κB) activity in cardiomyocytes.

Materials and Methods

Materials
Troglitazone, BRL49653, and gemfibrozil were generous gifts from Sankyo, SmithKline Beecham, and Parke-Davis, respectively. Wy14643 was purchased from Cayman Chemical. LPS was purchased from Sigma.

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. Cardiomyocytes were plated at a cell density of 10^3 cells/mm² on 35- or 100-mm culture dishes (Falcon Primaria) and cultured in MEM containing 5% bovine calf serum.

RNA Extraction and Northern Blot Analysis
Total RNA was isolated by the guanidinium thiocyanate–phenol chloroform method, and 20 μg of total RNA was used in Northern blot analysis. The following cDNA fragments were used as probes: rat PPARα cDNA, murine PPARγ1 cDNA, and murine TNF-α cDNA.

Immunocytochemistry
To prove that cardiomyocytes express PPARα and PPARγ, double staining was performed with goat polyclonal anti-PPARα or -PPARγ antibody (Santa Cruz Biotechnology) and mouse monoclonal anti-cardiac troponin I antibody (generous gift from Dr N. Toyota, Chiba University). Proteins were visualized using secondary Cy3-conjugated anti-goat IgG and FITC-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories). Substitution of the primary antibody with a normal goat IgG was used as control. Specificity of PPAR antibodies was checked by Western blot analysis.

Transient Transfection Assay
The reporter construct (PPRE)₃-TK-LUC was constructed by inserting 3 copies of the PPRE site from rat acyl-CoA oxidase promoter into the SalI site of the basal reporter construct TK-LUC, which contains a thymidine kinase promoter fused to a firefly luciferase gene. Cardiomyocytes were cotransfected with the reporter plasmid and pRL-SV40 control plasmid, which contains a Renilla luciferase gene, by the calcium phosphate method. At 6 hours after transfection, cells were washed with PBS, PPARα or PPARγ activator was added in the medium, and cells were incubated further for 24 hours.

Measurement of TNF-α
Cardiac myocytes were pretreated for 18 hours with various PPAR activators and stimulated with LPS from Escherichia coli 0127 (10 ng/mL) for 6 hours. At the end of the treatment period, the supernatants were collected, and the levels of TNF-α were measured with a rat TNF-α UltraSensitive ELISA kit (Biosource).

Electrophoretic Mobility Shift Assay (EMSA)
For EMSA, cardiac myocytes were pretreated with various PPAR activators for 24 hours before stimulation with LPS (10 ng/mL) for 1 hour. Nuclear extracts from cells were prepared as described previously. Nuclear extracts (5 μg) were incubated with ³²P-labeled NF-κB consensus oligonucleotide (AGTTGAGGG-GACCTTCCAGGC) (Promega). Specificity was determined by addition of an excess of unlabeled NF-κB oligonucleotide. Supershift analysis was performed by incubating nuclear extracts with anti-p50 or anti-p65 antibodies (catalogue Nos. sc-114X and sc-109X, respectively, Santa Cruz Biotechnology). EMSA was performed as described previously.

Statistical Analysis
Data are presented as mean±SEM. Statistical analysis was performed using 1-way ANOVA, and a Student t test was used to examine significance of difference in 2 groups. A value of P<0.05 was considered to be statistically significant.

Results

PPARα and PPARγ Are Expressed in Neonatal Rat Cardiac Myocytes
First, to investigate the expression of PPARs in rat cardiac myocytes, Northern blot analysis of total RNA isolated from neonatal rat cardiac myocytes was performed. Northern blot analysis demonstrated the presence of both PPARα and PPARγ mRNAs in cardiac myocytes (Figure 1A). To identify the subcellular localization of PPARα and PPARγ protein in cardiomyocytes, double staining with an antibody to cardiac troponin I, which specifically stains the cytoplasm of cardiac myocytes, and an antibody to PPARα or PPARγ was performed. Immunostaining with troponin I antibody demonstrated that a routine preparation contains 90% to 95% cardiomyocytes, as described previously. Staining with PPARα antibody revealed immunoreactivity in the nuclei of myocytes (Figure 1B, a). Similar results were seen when staining with PPARγ antibody was performed (Figure 1B, e). No immunoreactive PPARα or PPARγ was detectable when normal goat IgG was used (Figure 1B, b and f). Western blot analysis was performed using PPARα and PPARγ antibodies to determine the specificity of both antibodies (Figure 1C). Taken together, these data indicate that both PPARα and PPARγ are expressed in rat cardiac myocytes.

PPAR Activators Induce Transcriptional Activity of Endogenous PPARs
Because PPARs are ligand-dependent transcription factors, we sought to determine whether endogenous PPARs are transcriptionally active in cardiac myocytes. Myocytes were transiently cotransfected with the (PPRE)₃-TK-LUC reporter plasmid and pRL-SV40 control plasmid and subsequently treated with PPARα (Wy14643 and gemfibrozil) or PPARγ (BRL49653 and troglitazone) activator. After 24 hours, cells were harvested for assessment of luciferase activity. Both PPARα and PPARγ activators increased luciferase activity (Figure 2). These results suggest that endogenous PPARs function as ligand-dependent transcription factors, which bind to PPRE.

PPAR Activators Inhibit LPS-Induced TNF-α Production
Recently, it was reported that LPS (10 ng/mL, 6 hours) induces an increase in TNF-α secretion by cardiomyocytes. Therefore, we examined the effects of PPAR activators on LPS-induced TNF-α expression by ELISA. Cells were pre-
treated for 18 hours with various PPARα (Wy14643 and gemfibrozil) or PPARγ (BRL49653 and troglitazone) activators and subsequently stimulated with LPS for 6 hours. As previously described,21 LPS (10 ng/mL, 6 hours) significantly induced TNF-α production (116.6 ± 10.7 pg/mL) (Figure 3A). All PPAR activators significantly inhibited LPS-induced TNF-α expression (Figure 3A). However, when myocytes were exposed to PPAR activators at the time of or after LPS stimulation, TNF-α expression was not inhibited (data not shown). These changes were not due to cell death because cellular protein content was equal in each sample and myocyte beating appeared the same. Similarly, 15d-PGJ2, which is known as a ligand for PPARγ, inhibited LPS-induced TNF-α expression (data not shown). Although 15d-PGJ2 has been suggested to inhibit the production of inflammatory cytokines through the activation of PPARγ, recent study has shown that 15d-PGJ2 exerts a direct inhibitory effect on the activity of the inhibitor of nuclear factor-κB (IκB) kinase.22 As IκB kinase is responsible for NF-κB activation and TNF-α production, there is a possibility that 15d-PGJ2 may regulate TNF-α production, independent of PPARγ activation. Therefore, we used both BRL49653 and troglitazone as PPARγ activators to examine whether PPARγ activators inhibit TNF-α expression. These data indicate that both PPARα and PPARγ activators decrease LPS-induced TNF-α production in cardiomyocytes.

**PPAR Activators Repress LPS-Induced TNF-α mRNA Expression**

Northern blot analysis demonstrated increased TNF-α mRNA after stimulation of cardiac myocytes with LPS (10 ng/mL) for the indicated times (Figure 3B, a). TNF-α mRNA level started to increase 30 minutes after stimulation with LPS, peaked at 1 hour, and decreased gradually thereafter (Figure 3B, a). We next examined whether PPAR activators regulate LPS-induced TNF-α mRNA expression in myocytes. Cells were pretreated with PPARα (Wy14643) or PPARγ...
(BRL49653) (Figure 3B, b and c) activator for 23 hours and were subsequently stimulated with LPS (10 ng/mL) for 1 hour. Both PPAR activators inhibit LPS-induced TNF-α mRNA expression in cardiac myocytes. These results indicate that both PPARα and PPARγ activators inhibit LPS-induced TNF-α production at the mRNA level.

**PPAR Activators Reduce LPS-Induced NF-κB Activation**

TNF-α transcription is regulated by transcription factor binding sites present within the TNF-α promoter. It has been reported that LPS induction of TNF-α promoter activity is dependent on NF-κB binding sites and that LPS treatment of macrophages stimulates the nuclear translocation of NF-κB. Therefore, we performed EMSAs to investigate whether PPAR activators inhibit NF-κB activation in cardiac myocytes. Specificity of DNA-binding complexes was assessed in competition experiments by adding an excess of unlabeled NF-κB oligonucleotide to incubation mixtures. Characterization of NF-κB was performed by incubating nuclear extracts with antibodies directed against p50 and p65 subunits. Stimulation of cells with LPS (10 ng/mL) led to an increase in NF-κB binding activity reaching a plateau between 60 and 120 minutes (Figure 4A). Cells were pretreated with various PPAR activators for 23 hours before LPS (10 ng/mL) stimulation for 1 hour. Both PPARα and PPARγ activators reduced LPS-induced increase in NF-κB binding activity (Figures 4B and 4C). In the presence of excess consensus, the shifted band was abrogated. On the addition of anti-p50 or p65 antibody, the band was supershifted, thus showing that these incubation mixtures contained NF-κB. These results suggest that PPARs inhibit TNF-α gene expression in part by antagonizing the binding activity of NF-κB in cardiomyocytes.

**Discussion**

The present study demonstrates the expression of both PPARα and PPARγ in neonatal rat cardiac myocytes. Furthermore, we show that LPS induces TNF-α secretion by cardiac myocytes. This result is consistent with previous reports showing TNF-α secretion by neonatal mouse or neonatal rat cardiomyocytes after exposure to LPS. Both PPARα and PPARγ activators inhibit LPS-induced expression of TNF-α in myocytes at the mRNA level. Finally, we demonstrate that PPAR activators inhibit TNF-α gene expression, at least in part, by antagonizing the binding activity of NF-κB in cardiomyocytes.

It has been reported that PPARα is present in liver, kidney, and muscle, whereas PPARγ is expressed predominantly in adipose tissue. Previous studies have examined the expression of PPARs in heart by RNase protection assay, reverse transcription-polymerase chain reaction, or in situ hybridization. These results have shown that PPARα is expressed in variable amounts between individuals, whereas PPARγ is expressed at a low level, in adult rat or adult human heart. Our results demonstrate the expression of both PPARα and PPARγ by Northern blot analysis of total RNA from neonatal rat cardiac myocytes. Recently, it was reported that PPARα...
activators inhibit the inflammatory responses in aortic smooth muscle cells by negatively regulating NF-κB transcriptional activity\textsuperscript{15} and that PPARγ activators suppress production of inflammatory cytokines in macrophages by antagonizing the activities of activator protein-1 (AP-1), signal transducers and activators of transcription (STAT), and NF-κB.\textsuperscript{16} Our results suggest that both PPARα and PPARγ are involved in the regulation of proinflammatory cytokine TNF-α in cardiac myocytes.

Because LPS has been shown to induce TNF-α production in macrophages or cardiomyocytes,\textsuperscript{21,23–25} we stimulated neonatal rat myocytes in the same way. Regulation of TNF-α transcription is conferred by transcription factor binding sites present within TNF-α promoter. It has been reported that LPS-induced increase in TNF-α promoter activity occurs by activation of the NF-κB signaling pathway and is dependent on NF-κB binding sites.\textsuperscript{23,24} Therefore, we examined whether PPAR activators exert their effects on TNF-α gene transcription by preventing the binding activity of NF-κB. Our results from EMSAs showing that PPAR activators reduce LPS-induced increase in NF-κB binding activity suggest that both PPARα and PPARγ activators inhibit expression of TNF-α, at least in part, by antagonizing NF-κB binding activity.

However, the molecular mechanism by which PPARs interfere with NF-κB binding to the TNF-α promoter is unknown. Recently, several lines of evidence have implicated the functional significance of interaction between nuclear receptors and coactivators in transcriptional activation. cAMP response element binding protein (CREB)-binding protein (CBP)/p300 is a transcriptional coactivator of PPARα,

**Figure 4.** Effects of PPAR activators on LPS-induced NF-κB activation. A, EMSA of cardiac myocytes stimulated with LPS (10 ng/mL) for the indicated times (0, 15, 30, 45, 60, and 120 minutes). Nuclear extracts (5 μg) from cardiac myocytes were incubated with the \textsuperscript{32}P-labeled NF-κB consensus oligonucleotide. B, EMSA of cardiac myocytes stimulated with LPS (10 ng/mL) for 1 hour in the presence of Wy14643 (50 and 250 μmol/L). C, EMSA of cardiac myocytes stimulated with LPS (10 ng/mL) for 1 hour in the presence of BRL49653 (1 and 10 μmol/L). Nuclear extracts (5 μg) from cardiac myocytes were incubated with the \textsuperscript{32}P-labeled NF-κB consensus oligonucleotide. Specificity was assessed by addition of unlabeled NF-κB oligonucleotide (cold probe). Supershift analysis was performed by incubating nuclear extracts with antibodies directed against p50 or p65 subunit of NF-κB. Similar results were obtained from 3 independent experiments.
PPARγ, and NF-κB.26–28 Steroid receptor coactivator-1 (SRC-1) also functions as a coactivator for PPARγ and NF-κB.29,30 These observations raise the possibility that nuclear competition for limiting amounts of CBP/p300 or SRC-1 may occur between PPARs and NF-κB. Our data do not exclude the participation of coactivators in the inhibition of NF-κB activity by PPAR activators.

Previous studies have reported that the levels of circulating TNF-α are elevated in patients with congestive heart failure and that TNF-α expresses in the failing heart.2–4 In addition, overexpression of TNF-α in heart led to a phenotype characterized by dilated cardiomyopathy.7,8 It has been suggested that TNF-α may contribute to the progression of heart failure. Therefore, the results of the present study suggest that PPAR activators may prevent the development of congestive heart failure by inhibiting TNF-α expression. Although it is known that other cytokines such as interleukin-1β, interleukin-2, and interleukin-6 are also involved in the pathogenesis of congestive heart failure, the effects on expression of other cytokines by PPAR activators remain to be determined. Because our study uses only LPS to induce TNF-α production in cardiac myocytes, whether PPAR activators attenuate TNF-α expression in congestive heart failure in vivo is not yet understood.

More studies are necessary to characterize the effects of PPAR activators on the development of heart failure in vivo. PPARα activator gemfibrozil has been shown to reduce coronary events and improve plasma lipid levels. Gemfibrozil may prevent the critical inflammatory processes not only in atherosclerosis but also in heart failure. PPARγ activator troglitazone has been shown to reduce insulin resistance associated with obesity, hypertension, and impaired glucose tolerance in humans. Because it is known that diabetic cardiomyopathy, which is a major complication of diabetes, is characterized by systolic and diastolic dysfunctions, troglitazone appears to be beneficial to cardiac function impairment in patients with diabetes mellitus. It has been reported that high rates of fatty acid oxidation after ischemia decrease cardiac function during reperfusion.31 Excessive rates of fatty acid oxidation inhibit glucose oxidation and cause an uncoupling between glycolysis and glucose oxidation, resulting in an increase in the production of protons from glycolytically derived ATP.31 Therefore, the production of protons appears to be an important contributor to the impaired recovery of reperfused ischemic heart. Although PPARα activators stimulate the oxidation of fatty acid, it remains to be determined whether PPARα activators induce the same mechanism. Williams et al32 reported that T-174 (LY282449), a PPARγ activator, induces cardiac hypertrophy in rats. However, we recently found that pioglitazone, also a PPARγ activator, inhibits the pressure overload–induced cardiac hypertrophy and that pressure overload induces more prominent cardiac hypertrophy in heterozygous PPARγ-deficient (PPARγ+/-) mice than in wild-type mice (M. Asakawa et al, unpublished data, 2000). Therefore, PPARγ activators may also be useful in the treatment of cardiac hypertrophy. Recent studies have demonstrated that both PPARα and PPARγ are implicated in the pathogenesis of atherosclerosis.33–35

In conclusion, we demonstrate that PPAR activators inhibit LPS-induced TNF-α expression in cardiac myocytes. Given the involvement of TNF-α in the pathogenesis of heart failure, our findings suggest that PPARs may play a critical role in inflammatory response in heart failure. Further studies should continue to elucidate the role of PPARs in other TNF-α–related cardiovascular diseases, such as ischemia-reperfusion, myocardial infarction, myocarditis, and dilated cardiomyopathy.

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