Abstract—Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors belonging to the nuclear receptor superfamily. They regulate lipid metabolism, glucose homeostasis, cell proliferation, and differentiation and modulate inflammatory responses. We examined whether PPARγ is functional in cultured neonatal ventricular myocytes and studied its role in inflammation. Western blots revealed PPARγ in myocytes. When myocytes were transfected with a PPAR response element reporter plasmid (PPRE-TK-luciferase), the PPARγ activator 15-deoxy-Delta12,14-prostaglandin J2 (15dPGJ2) increased promoter activity, whereas cotransfection of a dominant negative PPARγ inhibited it. To determine the role of 15dPGJ2 in expression of proinflammatory genes, we tested its effect on interleukin-1β induction of cyclooxygenase-2 (COX-2). 15dPGJ2 decreased interleukin-1β stimulation of COX-2 by 40% and PGE2 production by 73%. We next questioned whether 15dPGJ2 was modulating the expression of inducible prostaglandin E2 synthase (PGES) and found that it completely blocked interleukin-1β induction of PGES. Use of a second PPARγ agonist, troglitazone, and the selective PPARγ antagonist GW9662 demonstrated that the effects seen were PPARγ-dependent. In addition, we found that 15dPGJ2 blocked interleukin-1β stimulation of inducible nitric oxide synthase (iNOS). We concluded that 15dPGJ2 may play an anti-inflammatory role in a PPARγ-dependent manner, decreasing COX-2, PGES, and PGE2 production, as well as iNOS expression. (Hypertension. 2003;42[part 2]:844-850.)

Key Words: myocytes ■ nitric oxide synthase ■ cyclooxygenase ■ prostaglandins

Peroxisome proliferator-activated receptors (PPARs) are a family of 3 nuclear hormone receptors, PPARα, PPARβ (also named PPARδ), and PPARγ, which are members of the steroid receptor superfamily. PPARs bind to cognate DNA elements called peroxisome proliferator response elements (PPRE) as obligate heterodimers with the retinoid X receptor (RXR). After ligand activation, they work as transcription factors.1,2 There are two PPARγ subtypes, PPARγ1 and PPARγ2, which are derived from alternative splicing and promoter usage.3,4 PPARγ1 is highly expressed in adipose tissue, whereas PPARγ2 has been found in the kidney, heart, liver, and activated monocytes.5,6 PPARγ can be activated by docosahexaenoic acid and certain prostaglandins.7 Other PPARγ ligands include the natural prostaglandin metabolite 15-deoxy-Delta12,14-prostaglandin J2 (15dPGJ2),8,9 polyunsaturated fatty acids,10 nonsteroidal anti-inflammatory drugs,11 and members of the thiazolidinedione family.12 PPARγ has been associated with control of inflammation by inhibiting cytokine stimulation of COX-2 and inducible nitric oxide synthase (iNOS) expression in different cell types,13-15 decreasing release of proinflammatory cytokines16 and inhibiting vascular smooth cell migration.17-19 PPARγ has also been shown to have antineoplastic and antigrowth properties.18 However, the precise mechanisms defining these effects are unknown.

We recently found that in neonatal ventricular myocytes (NVM), induction of cyclooxygenase-2 (COX-2), and prostaglandin E2 synthase (PGES) by the proinflammatory cytokine interleukin-1β (IL-1β) results in preferential production of PGE2.20 In addition, we found that PGE2 induces growth in NVM, suggesting that it plays a detrimental role in the heart. Also, it is known that iNOS is induced on stimulation with IL-1β21 and that it contributes to myocyte damage after infarction in the rat22 and mouse.23 Thus, we hypothesized that PPARγ would exert an anti-inflammatory effect in NVM, decreasing COX-2 and PGES expression and leading to decreased production of the hypertrophic prostanoid PGE2, as well as decreasing iNOS.

Methods

Cell Culture

Primary cultures of NVM were derived from digestion of 1- to 2-day-old neonatal Sprague-Dawley rat hearts (Charles River).24 This protocol was approved by the Henry Ford Hospital IACUC. Myocytes were plated at a density of 1×10⁶/cm² (1×10⁶ cells per well of a 6-well dish or 5.8×10⁵ cells/10-cm dish) in DMEM (Gibco-BRL) plus 10% FBS (Gibco) and 0.1 mmol/L bromodeoxyuridine for 40 hours. Serum-free medium supplemented with glutamine, insulin, selenium, and transferrin (SF-DMEM) was then added for 24 hours. Unless otherwise specified, all treatments lasted...
Isolation of Protein and Western Blot of COX-2, PGES, and iNOS

Protein was isolated from NVM through the use of lysis buffer and protease inhibitors. Lysate protein (50 µg per lane) was separated out by electrophoresis on an 8% SDS polyacrylamide gel (for COX-2 and iNOS detection) and transferred to an Immobilon-P PVDF membrane (Millipore); 72-kDa COX-2 and 130-kDa iNOS proteins were detected as described previously. Microsome-enriched membrane preparations were used for Western blot of PGES. For this, 5.8 × 10^6 cells per well of a 6-well plate were treated with vehicle, 1 µmol/L PGE2, PGF2α, and 15dPGJ2, GW9662 before treatment with 5 ng/mL IL-1β.

Enzyme Immunoassay for Measurement of PGE2, PGF2α, and the Stable PG12 Metabolite 6-Keto PGF1α

1 × 10^6 cells per well of a 6-well plate were treated with vehicle, IL-1β, 15dPGJ2, or 15dPGJ2 + IL-1β in 1 mL SF-DMEM. A 1-mL aliquot of medium from each well was dried down and resuspended in 0.15 mL ultrapure H2O. Aliquots were diluted if necessary and assayed for specific prostaglandins with the use of EIA kits from Cayman. Values from triplicate wells were averaged, and mean ± SEM values are expressed as nanograms per milligram of protein.

Isolation of RNA and RT-PCR of COX-2, PGES, and iNOS

Total RNA was isolated from control and IL-1β–treated NVM by means of Tri Reagent (MRC), according to the manufacturer’s instructions; 2 µg total RNA was reverse-transcribed in a total volume of 25 µL, using 1 µL random primer (Gibco/BRL) and 200 U MMLV reverse transcriptase (Promega). Five-microliter aliquots of the resulting cDNA were subjected to PCR in a 50-µL reaction volume for amplification of COX-2 (462 bp), PGES (473 bp), and GAPDH (554 bp). Oligonucleotide primers (5′-3′) for rat COX-2 were ATG ACG AAG ACC CTT ACG (sense) and TAA GTT GGT GGG CTT TCA AT (antisense). Primers for the complete PGES cDNA were ATG ACT TCC CTG GGT TTG GTG (sense) and TCA GCT GCT GGT CAC AGA TGG (antisense). Primers for rat GAPDH were AAT GCA TCC TGC ACC TCG (sense) and GGA GGC CAT GTA GGC CAT GAG GTC (antisense). A PTC-100 thermal controller (MJ Research) was used for amplification, and the program settings were denaturation for 30 seconds at 95°C, annealing for 60 seconds at 55°C for COX-2, and 60°C for PGES and GAPDH, and extension for 90 seconds at 72°C for 35 cycles (COX-2 and PGES) or 25 cycles (GAPDH). PCR products were separated out on a 2% agarose gel, stained with ethidium bromide, and photographed. Densitometry was used for quantification. COX-2 and PGES were normalized to GAPDH.

Transfection and Luciferase Assay

Transfection was performed by electroporation, and luciferase activity was assayed as described previously, containing 3 copies of PPRE, coupled to a minimal thymidine kinase (TK) promoter, kindly provided by Dr Ronald Evans, Salk Institute) was transfected per 3 × 10^6 NVM, and the cells were aliquoted into 3 wells of a 12-well plate. After 40 hours, the medium was changed to SF-DMEM, and 24 hours later the cells were treated with 15dPGJ2. After a 24-hour treatment period, NVM were lysed and assayed for luciferase activity using Promega reagents in an OptoComp 1 luminometer. Relative light units (RLU) from triplicate wells were averaged. RLU values in control (untreated) lysates were normalized, and the values of 15dPGJ2-treated lysates were compared with control to give the fold increase. Data are expressed as mean ± SEM.

Statistics

Values are represented as mean ± SEM. Differences in mean values were analyzed by Student t test or 1-way ANOVA, with pairwise multiple comparisons made by the Student-Newman-Keuls method. A value of P < 0.05 was considered significant.

Results

Detection and Activation of PPARγ in NVM

To determine whether PPARγ receptors are present in NVM, protein from cell lysates was analyzed by Western blot. PPARγ was detected as a 52-kDa protein (Figure 1A). We next tested whether endogenous PPARγ is transcriptionally active in cardiac myocytes. For this, NVM were transiently transfected with the PPRE-TK-luciferase reporter plasmid and treated with the PPARγ agonist 15dPGJ2. Figure 1B shows that 15dPGJ2 increased luciferase activity 12.0 ± 2.2-fold compared with untreated NVM. Finally, we cotransfected NVM with the PPRE-TK-Luc reporter gene and dominant negative PPARγ cDNA (dnPPARγ). As shown in Figure 1B, 15dPGJ2; activation of
the PPRE-TK-Luc reporter gene was significantly decreased.

**Effect of 15dPGJ$_2$ on IL-1$\beta$ Stimulation of COX-2, PGES, and Prostaglandin Production**

High PGE$_2$ resulting from induction of COX-2 participates in inflammation. We tested whether PPAR$\gamma$ activation is anti-inflammatory by examining the effect of the natural PPAR$\gamma$ agonist 15dPGJ$_2$ on PGE$_2$ production and COX-2 mRNA. 15dPGJ$_2$ reduced IL-1$\beta$–stimulated PGE$_2$ release by 73% (Figure 2A); however, IL-1$\beta$–induced PGF$_2\alpha$ and PGI$_2$ levels were not affected (data not shown). In addition, 15dPGJ$_2$ decreased IL-1$\beta$–induced COX-2 mRNA levels by 40% (Figure 2B). IL-1$\beta$ induction of COX-2 protein (72 kDa) was similarly inhibited (data not shown).

Since PGE$_2$ production was inhibited to a greater extent than COX-2 mRNA, we tested whether 15dPGJ$_2$ was affecting the inducible form of PGE$_2$ synthase (mPGES). Figure 3A shows that 15dPGJ$_2$ completely inhibited IL-1$\beta$ induction of PGES mRNA. Western blots of microsome-enriched preparations revealed that 15dPGJ$_2$ also blocked IL-1$\beta$ induction of PGES protein in NVM (Figure 3B).

**Effect of the PPAR$\gamma$ Agonist Troglitazone on COX-2 and PGE$_2$**

We used a second PPAR$\gamma$ agonist, troglitazone (TRO, 100 $\mu$mol/L) to verify our findings with 15dPGJ$_2$. TRO transactivated the PPRE-TK-LUC reporter plasmid (data not shown). TRO also blocked IL-1$\beta$–induced COX-2 (Figure 4A) and PGE$_2$ production (Figure 4B).

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**Figure 2.** Effect of PPAR$\gamma$ agonist 15dPGJ$_2$ on COX-2 expression and PGE$_2$ production. A, PGE$_2$ production; $y$-axis is PGE$_2$ production, expressed as percentage of IL-1$\beta$–induced PGE$_2$; $x$-axis is treatment. Basal PG production by control NVM (CONT) was in the range of 1 to 4 ng/mg protein. Bars represent mean±SEM (n=6). *$P<0.01$ vs IL-1$\beta$. B, RT-PCR of COX-2 mRNA; $y$-axis is COX-2 mRNA normalized to GAPDH mRNA, expressed as fold increase versus IL-1$\beta$; $x$-axis shows different treatments. Bars represent mean±SEM (n=7). *$P<0.01$ vs IL-1$\beta$. C, Representative RT-PCR results showing amplified cDNAs on 2% agarose gel.

**Figure 3.** Effect of the PPAR$\gamma$ agonist 15dPGJ$_2$ on PGES expression. A, PGES mRNA; $y$-axis is PGES mRNA normalized to GAPDH mRNA, expressed as fold increase versus IL-1$\beta$; $x$-axis is treatment. Bars represent mean±SEM (n=6). *$P<0.01$ vs IL-1$\beta$. Figure below is representative agarose gel of RT-PCR results. B, PGES protein; $y$-axis is PGES protein expressed as OD units; $x$-axis is treatment. Bars represent mean±SEM (n=3). *$P<0.01$ vs IL-1$\beta$. Below is a representative Western blot.
Effect of a PPARγ Antagonist, GW9662, on COX-2 and PGES

To further verify that the effects of 15dPGJ2 are PPARγ-dependent, we studied the effect of the PPARγ antagonist GW9662 (GW, 2 μmol/L). GW blocked the effects of 15dPGJ2 on IL-1β-induced COX-2 (Figure 5A) and PGES mRNA (Figure 5B). GW alone did not modify either COX-2 or PGES mRNA (data not shown).

Effect of the PPARγ Activator 15dPGJ2 on iNOS

We have previously shown that IL-1β induces iNOS expression and nitrite production in NVM.25 Since iNOS has been implicated in inflammation, we examined the effect of 15dPGJ2 on IL-1β induction of iNOS in NVM. Although treatment of NVM with IL-1β significantly increased iNOS protein, cotreatment with IL-1β and 15dPGJ2 inhibited iNOS expression (Figure 6A). Troglitazone mimicked the effect of 15dPGJ2 on IL-1β-induced iNOS expression (Figure 6B). GW was able to partially reverse the inhibitory effect of 15dPGJ2 on IL-1β-induced iNOS protein (Figure 6C).

Discussion

We found that PPARγ is present and functional in cardiac myocytes. We also demonstrated that its natural agonist 15dPGJ2 exerts anti-inflammatory effects by modulating IL-1β-stimulated COX-2, PGES, and iNOS in a PPAR-dependent manner.

The PGD2 dehydration product 15dPGJ2 is the most potent endogenous ligand for PPARγ yet discovered.8,9 Recently, 15dPGJ2 production was measured in tryptase-stimulated cells in culture as well as human breast tissue.30 Moreover, prostaglandin D synthase (PGDS) mRNA is expressed in the heart.31 Thus, locally produced PGD2 may result in 15dPGJ2 in myocytes or surrounding cells. Although it is not known whether 15dPGJ2 is produced in myocytes and has autocrine effects to activate PPARγ, it is known that the 15dPGJ2 precursor Δ12-15dPGJ2 is transported into cells and accumulates in the nucleus.32

We found that 15dPGJ2 decreased IL-1β induction of COX-2 mRNA. This inhibition probably occurred at the transcriptional level. The anti-inflammatory effects of PPARγ are thought to be mediated by antagonizing transcription factors such as NF-κB, AP-1, STAT, and NFAT, some of which are necessary for COX-2 transcription. In fact, Subbaramaiah et al13 showed that stimulation of a breast epithelial cell line with phorbol ester induced COX-2 and that two different PPARγ agonists interfered with AP-1-dependent activation of the COX-2 promoter. A PPARγ agonist also inhibited IL-β-induced COX-2 expression in mesangial cells, although the authors suggested that the effects seen are PPAR-independent.34

Previous studies have demonstrated that COX-2 and microsomal PGES are colocalted in the perinuclear envelope, suggesting coordinated biosynthetic activity between enzymes.35 In fact, induction of both COX-2 and PGES results in high level PGE2 production by cardiac myocytes.36 Our data show that 15dPGJ2 blocked IL-1β stimulation of PGES production but did not modify IL-1β stimulation of PGI2 or PGE2 production.
indicating that PPARγ ligand effects are specific for PGES. Naraba et al. reported that the mouse PGES gene contains several cis-acting elements including C/EBPα, AP-1, C/EBPβ, and CACCC-binding factor. In contrast to COX-2, no binding sites for NF-kB or CREB were found in the PGES promoter region. This may indicate that the mechanisms for PPAR regulation of COX-2 and PGES are distinct. In the same report, the authors showed that induction of PGES is exclusively regulated by the transcription factor early growth response-1 (Egr-1), which can be rapidly and transiently upregulated by various growth factors including IL-1β.

Interestingly, ligand-induced transactivation of PPARγ is achieved by receptor recruitment of the coactivator CBP/p300. Hence, we speculate that PPAR-mediated inhibition of PGES could result from competition for limited amounts of CBP/p300.

It has been shown that the PPAR ligand 15dPGJ2 exerts anti-inflammatory functions independent of PPAR activation. Some of the independent effects of 15dPGJ2 have been attributed to an electrophilic carbon that can react with nucleophiles. To validate our results, we tested a different PPAR agonist. Troglitazone, an antidiabetic drug belonging to the thiazolinedione family, mimicked the effect of 15dPGJ2 on IL-1β-induced COX-2, iNOS, and PGE2 production. We found that troglitazone was more effective than 15dPGJ2 in inhibiting IL-1β induction of COX-2. This may result from the fact that these two selective PPARγ agonists bind PPAR and alter its structure in specific ways, resulting in distinct PPARγ-coactivator interactions. It is also possible that 15dPGJ2 and troglitazone may differ in their stability and affinity binding constant for PPARγ in NVM.

Recently, a PPAR-selective antagonist, GW9662, was identified. GW9662 has been demonstrated to covalently modify PPAR in a selective and irreversible manner and was found to block the effects of 15dPGJ2 in bone marrow, hepatocytes, and human smooth muscle cells. By using this PPAR-specific antagonist, we demonstrated that the effects of 15dPGJ2 on IL-1β-induced COX-2 and PGES are essentially mediated by PPAR.

As expected, activation of PPARγ inhibited IL-1β stimulation of the proinflammatory gene iNOS. Previous reports demonstrated that 15dPGJ2 inhibited IL-1β regulation of iNOS by blocking NF-kB activation. A similar mechanism is probably responsible for regulation of iNOS in cardiac myocytes in our studies, since we know that IL-1β activates NF-kB in cardiac myocytes, and 15dPGJ2 can partially abrogate this effect (Mendez and LaPointe, unpublished data). However, PPAR-independent effects have been reported. Our results show that GW only partly reverses the effect of 15dPGJ2 on IL-induced iNOS protein. Thus, we cannot rule out the possibility of some PPAR-independent effect of the natural ligand 15dPGJ2 on iNOS regulation. At this time, we know of no studies that definitively address the PPAR-independent mechanisms that regulate iNOS expression in cardiac myocytes.

iNOS and inducible COX-2 have been implicated in pathological events in the heart. We reported that PGE2 is produced by COX-2 in cardiac myocytes and...
fibroblasts and that PGE₂ analogues stimulate myocyte hypertrophy²⁰ and fibroblast proliferation in vitro (Mendez and LaPointe, unpublished observations). In addition, COX-2 inhibition reduces hypertrophy and fibrosis in vivo in a mouse model of myocardial infarction (LaPointe, unpublished data). PPAR inhibitors have been shown to reduce protein synthesis in myocytes⁵,¹⁵ and vascular smooth muscle cell proliferation.¹⁸ It is interesting to speculate that 15dPGJ₂ exerts its antigrowth effects in NVM by blocking the release of the hypertrophic prostaglandin PGE₂.

**Perspectives**

PPARγ has been shown to participate in cell growth, differentiation, and inhibition of inflammatory responses in multiple cell types important to cardiovascular function, such as vascular smooth muscle cells and cardiac myocytes. In this study, we provide compelling evidence that PPARγ activation blunts the release of the hypertrophic and proinflammatory prostaglandin PGE₂, along with blockade of the inflammatory genes COX-2 and PGE₂, in a PPAR-dependent manner. In addition, blockade of iNOS is both PPAR-dependent and PPAR-independent. Knowledge of key components of the transcriptional regulation of inflammatory genes by PPARγ may provide a viable therapeutic target in the control of cardiac diseases resulting from inflammatory stimuli.

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**References**


