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Conditional deletion of cardiomyocyte peroxisome proliferator-activated receptor- γ enhances myocardial ischemia-reperfusion injury in mice

Michael J. Hobson^{*}, Paul W. Hake^{*}, Michael O'Connor^{*}, Christine Schulte[‡], Victoria Moore[‡], Jeanne M. James[‡], Giovanna Piraino^{*}, and Basilia Zingarelli^{*}

^{*}Division of Critical Care Medicine, Cincinnati Children's Hospital Medical Center, and The University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, Ohio 45229, USA

[‡]Cardiovascular Imaging Core of the Heart Institute, Cincinnati Children's Hospital Medical Center, and The University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, Ohio 45229, USA

Abstract

The nuclear transcription factor peroxisome proliferator-activated receptor- γ (PPAR γ) is a key regulator of the inflammatory response to an array of biologic insults. We have previously demonstrated that PPAR γ ligands reduce myocardial ischemia-reperfusion injury in rodents. In the current study, we directly determined the role of cardiomyocyte PPAR γ in ischemia-reperfusion injury, employing a model of conditional cardiomyocyte-specific deletion of PPAR γ *in vivo*. In mice, α -myosin heavy chain-restricted Cre-mediated PPAR γ deficiency was induced by tamoxifen treatment (30 mg/kg intraperitoneally) for 4 days (PPAR γ ^{-/-} mice); whereas controls included mice treated with the oil diluent vehicle (PPAR γ ^{+/+} mice). Western blot and histochemical analyses confirmed that expression of PPAR γ protein was abolished in cardiomyocytes of mice treated with tamoxifen, but not with vehicle. After tamoxifen or vehicle treatment, animals were subjected to 30 min ligation of the left anterior descending coronary artery followed by 2 hrs reperfusion. In PPAR γ ^{-/-} mice, myocardial ischemia and reperfusion induced extensive myocardial damage, which was associated with elevated tissue activity of myeloperoxidase, indicating infiltration of neutrophils, and elevated plasma levels of troponin-I when compared to PPAR γ ^{+/+} mice. PPAR γ ^{-/-} mice also demonstrated ventricular dilatation and systolic dysfunction upon echocardiographic analysis. Plasma levels of the pro-inflammatory cytokines interleukin-1 β and interleukin-6 were higher in PPAR γ ^{-/-} mice when compared to PPAR γ ^{+/+} mice. These pathological events in PPAR γ ^{-/-} mice were associated with enhanced nuclear factor- κ B DNA binding in the infarcted hearts. Thus, our data suggests that cardiomyocyte PPAR γ is a crucial protective receptor and may prevent reperfusion injury by modulating mechanisms of inflammation.

Keywords

Cre-loxP system; PPAR γ ; NF- κ B; cardiomyocytes; neutrophils; IL-6; IL-1 β

Author for correspondence: Basilia Zingarelli, M.D., Ph.D. Division of Critical Care Medicine Cincinnati Children's Hospital Medical Center 3333 Burnet Avenue, Cincinnati, Ohio 45229, USA Phone: (513) 636-8704; fax: (513) 636-4892 basilia.zingarelli@cchmc.org.

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INTRODUCTION

Coronary artery disease and myocardial infarction remain a leading cause of morbidity and mortality in the United States (1). Modern therapies such as thrombolytic medications and coronary angioplasty reverse myocardial ischemia and restore coronary blood flow. However, concurrent with the restoration of blood flow and re-oxygenation, ischemic myocardial tissue may paradoxically suffer reperfusion injury. Myocardial ischemia and reperfusion injury results in further myocyte death and worsening myocardial dysfunction beyond that expected from the ischemic insult alone. Although the mechanisms underlying the phenomenon of ischemia and reperfusion injury have not been precisely defined, toxicity by reactive oxygen free radicals and oxidants, leukocyte-endothelial cell adhesion and a marked inflammatory reaction have been implicated in the process of injury (2, 3).

Peroxisome proliferator-activated receptor- γ (PPAR γ) is a member of the superfamily of ligand-activated nuclear receptors. Upon activation, PPAR γ binds to the retinoid X receptor, and this heterodimer subsequently serves as a transcription factor for the promoter regions of various target genes (4). PPAR γ is expressed most prominently in adipose tissue, but is also seen in endothelium, vascular smooth muscle, monocytes, macrophages and cardiac myocytes (5). Functionally, PPAR γ has a prominent role in several biologic processes, including lipid and glucose homeostasis, modulation of cellular proliferation, and regulation of several inflammatory disorders (4-6).

With the use of specific ligands for the receptor, we have previously indicated a role for PPAR γ in regulating myocardial damage by governing the inflammatory process, which incites reperfusion injury at the nuclear level (7). In particular, in a rodent model of myocardial ischemia and reperfusion injury, treatment with the endogenous PPAR γ ligand 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ and the thiazolidinedione ciglitazone reduced myocardial damage, neutrophil infiltration and systemic cytokine production via enhanced PPAR γ DNA binding and reduced activation of nuclear factor- κ B (NF- κ B) (7), an important transcription factor for pro-inflammatory genes (8). Other experimental studies have supported that treatment with thiazolidinedione PPAR γ ligands reduce myocardial infarct size (9) and improve cardiac function following an acute myocardial ischemic insult (10-12).

Given the potential benefit for PPAR γ ligands to attenuate myocardial ischemia and reperfusion injury, further investigation is merited to determine the exact biologic site of action of PPAR γ within the heart. As PPAR γ is expressed within cardiac myocytes as well as coronary endothelium and vascular smooth muscle (4-6), we sought to investigate the role of the cardiomyocyte PPAR γ on myocardial ischemia and reperfusion injury by employing a conditional cardiomyocyte-specific PPAR γ knockout animal model. Here, we provide evidence that cardiomyocyte PPAR γ plays a prominent role in protecting the myocardium against the ischemia and reperfusion injury. This endogenous cardio-protective effect of PPAR γ appears a result of nuclear regulation of NF- κ B activation.

MATERIALS AND METHODS

Generation of cardiomyocyte-specific PPAR γ knockout mice

The investigation conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and was approved by the Institutional Animal Care and Use Committee. Mice expressing a tamoxifen-inducible Cre recombinase fused to mutant estrogen-receptor ligand-binding domains (MerCreMer) under the control of the α -myosin heavy chain promoter (B6.Cg-Tg(Myh6-cre/Esr1)1Jmk/J) (13) and PPAR γ^{loxP} mice (B6.129-Ppargtm2Rev/J), both on C57BL6 genetic background, were obtained from Jackson Laboratories (Bar Harbor, Maine). MerCreMer mice were crossed with PPAR γ^{loxP} mice

thus generating cardiac-specific inducible PPAR $\gamma^{Cre/loxP}$ animals. At 8-12 weeks of age, male PPAR $\gamma^{Cre/loxP}$ animals were given tamoxifen (30 mg/kg daily) intraperitoneally (ip) for four days in order to generate cardiomyocyte PPAR γ knockout (PPAR $\gamma^{-/-}$) mice. Tamoxifen was dissolved in peanut oil at a concentration of 2.5 mg/ml. PPAR $\gamma^{Cre/loxP}$ mice treated with oil alone served as controls (PPAR $\gamma^{+/+}$ mice).

Myocardial ischemia and reperfusion

Myocardial ischemia and reperfusion was conducted as previously described (7). Twenty-four hrs after the last treatment with tamoxifen or oil PPAR $\gamma^{+/+}$ and PPAR $\gamma^{-/-}$ mice were anesthetized with thiopentone sodium (4 mg/ml, 10 μ l/g body weight ip). A tracheostomy was performed to provide mechanical ventilation. The chest was opened via a left thoracotomy incision in order to expose the left ventricle. The left anterior descending (LAD) coronary artery was occluded for 30 minutes by ligation with a 6.0 silk suture passed underneath the LAD and subsequently anchored over a 3-mm air balloon, which was placed on top of the vessel. Reperfusion was allowed for 2 hrs following deflation of the balloon. Animals were euthanized at the end of the reperfusion period by a lethal dose of thiopentone sodium. Plasma samples and the left ventricles were collected for subsequent histological and biochemical studies. A separate group of mice underwent the above procedure without LAD ligation, thus serving as the sham control group.

Echocardiographic assessment of left ventricle structure and function

Cardiac function was assessed by echocardiography as previously described using a VisualSonics 2100 system equipped with a 30 MHz transducer (14). Left ventricle (LV) internal dimensions, including end-diastolic and end-systolic dimensions (LVIDd and LVIDs, respectively), interventricular septal thickness in diastole and systole (IVSd and IVSs, respectively) and LV posterior wall thickness in diastole and systole (LVPWd and LVPWs, respectively) were measured directly. Echocardiographic measurements were obtained before LAD ligation (baseline measurements, n=11-16 mice for each group) and at the end of the reperfusion period (n=3 mice for each group).

Histopathological analysis and immunohistochemical staining for PPAR γ

Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. For histological evaluation, sections were stained with hematoxylin and eosin. For immunohistochemistry of PPAR γ expression, binding sites of PPAR γ primary antibody were visualized with an avidin-biotin peroxidase complex immunoperoxidase technique using diaminobenzidine as recommended by the protocol provided by the manufacturer (Vector Laboratories, Burlingame, CA).

Plasma cardiac troponin activity

Plasma levels of troponin-I were evaluated as an index of cardiac cellular damage using a high sensitivity mouse cardiac troponin-I ELISA kit (Life Diagnostics, Inc., West Chester, PA).

Myeloperoxidase activity

Myeloperoxidase (MPO) activity was determined as a marker of neutrophil migration into myocardial tissue following ischemia-reperfusion. Cardiac tissues were homogenized in a solution containing 0.5% hexa-decyl-trimethyl-ammonium bromide in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 4,000 g at 4 $^{\circ}$ C. An aliquot of the supernatant was allowed to react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM hydrogen peroxide. The rate of change in absorbance was measured by

spectrophotometry at 650 nm. A unit of MPO activity was defined as the quantity of enzyme that degraded 1 μ mol of hydrogen peroxide per min at 37° C per 100 mg weight of tissue.

Plasma levels of cytokines

Plasma levels of interleukin (IL)-1 β , IL-6, IL-10, keratinocyte-derived chemokine (KC), macrophage inflammatory protein-1 α (MIP-1 α) and tumor necrosis factor- α (TNF α) were evaluated by a commercially available Milliplex mouse cytokine magnetic bead panel kit (Millipore Corporation, Billerica, MA) using the protocols recommended by the manufacturer.

Subcellular fractionation and nuclear protein extraction

Heart samples were homogenized in a buffer containing 0.32 M sucrose, 10 mM tris-HCl pH 7.4, 1 mM EGTA, 2 mM EDTA, 5 mM sodium azide, 10 mM β -mercaptoethanol, 20 μ M leupeptin, 0.15 μ M pepstatin A, 0.2 mM phenylmethanesulfonyl fluoride, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 0.4 nM microcystin. The homogenates were centrifuged (1,000 g, 10 minutes). The pellets were solubilized in Triton buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM sodium orthovanadate, 20 μ M leupeptin A, 0.2 mM phenylmethanesulfonyl fluoride). The lysates were centrifuged (15,000 g, 30 minutes, 4° C), and the supernatant (nuclear extract) was collected for evaluation of content of PPAR γ and DNA binding of NF- κ B.

Western blot analysis

Nuclear expression of PPAR γ was determined by immunoblot analysis. Nuclear extracts were boiled in loading buffer (125 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, and 10% 2-mercaptoethanol) and 35 μ g of protein were loaded per lane on a 10% Tris-glycine gradient gel. Proteins were separated electrophoretically and transferred to nitrocellulose membranes. For immunoblotting, membranes were blocked with 5% nonfat dried milk in Tris-buffered saline for 1 h and then incubated with primary antibodies against PPAR γ for 1 h. The membranes were washed in Tris-buffered saline with 0.1% Tween 20 and incubated with secondary peroxidase-conjugated antibody. Immunoreaction was visualized by chemiluminescence. Densitometric analysis was performed using ImageQuant (Molecular Dynamics).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed using an oligonucleotide probe corresponding to NF- κ B consensus sequence (5'-AGT TGA GGG GAC TTT CCC AGG C-3') as previously described (9). The oligonucleotide probe was labeled with γ -(³²P)ATP using T4 polynucleotide kinase and purified in Bio-Spin chromatography columns (BioRad, Hercules, CA). Twenty-five micrograms of nuclear protein was incubated with EMSA buffer (12 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid pH 7.9, 4 mM Tris-HCl pH 7.9, 25 mM potassium chloride, 5 mM magnesium chloride, 1 mM EDTA, 1 mM dithiothreitol, 50 ng/ml poly (d[I-C]), 12% glycerol vol/vol, and 0.2 mM phenylmethanesulfonyl fluoride) and radiolabeled oligonucleotide. The specificity of the binding reactions was determined by co-incubating duplicate nuclear extract samples with unlabeled oligonucleotide (competitor assay). Protein-nucleic acid complexes were then resolved using a non-denaturing polyacrylamide gel and run in 0.5X Tris-HCl (45 mM), boric acid (45 mM), and EDTA (1mM) for 1 h at constant current (30 mA). Gels were then transferred to Whatman 3M paper, dried under a vacuum at 80° C for 1 h, and exposed to photographic film at -70° C with an intensifying screen. Densitometric analysis was performed using ImageQuant (Molecular Dynamics).

Materials

The primary antibody directed to PPAR γ was obtained from Thermo Scientific (Rockford, IL). The oligonucleotide for NF- κ B was obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). All other chemicals were obtained from Sigma/Aldrich (St. Louis, MO).

Statistical analysis

All values in the figures and text are expressed as mean \pm SEM of n observations ($n = 3-16$ animals for each group). The results were examined by one-way analysis of variance followed by the Bonferroni's correction post hoc t test. Data were analyzed using Systat software (Sigma Plot 12.3). A P value of < 0.05 was considered significant.

RESULTS

Conditional deletion of cardiomyocyte PPAR γ by tamoxifen treatment

PPAR γ conditional knockout mice were generated using the Cre-loxP system. PPAR $\gamma^{\text{Cre/loxP}}$ animals were given tamoxifen (30 mg/kg, ip) or vehicle for four days in order to delete cardiomyocyte PPAR γ . Western blot analysis revealed that PPAR γ expression was markedly decreased in the heart samples of mice treated with tamoxifen (PPAR $\gamma^{-/-}$ mice) when compared to control animals receiving vehicle alone (PPAR $\gamma^{+/+}$ mice) (Figure 1). To confirm cell location of PPAR γ deletion, we further performed immunohistochemical staining specific for PPAR γ . At immunohistochemical evaluation cardiac sections of PPAR $\gamma^{+/+}$ mice treated with oil only for four days showed a dark staining in the nuclear compartment of cardiac cells and in vessels under sham conditions and after ischemia and reperfusion (Figure 2). Staining for PPAR γ was absent in the myocytes of PPAR $\gamma^{-/-}$ animals who received four days of tamoxifen therapy. Of note, PPAR γ expression was maintained in the vasculature of these animals, thus confirming the cardiomyocyte specificity of the gene deletion (Figure 2).

Conditional deletion of cardiomyocyte PPAR γ enhances cardiac dysfunction and tissue injury following myocardial ischemia and reperfusion

PPAR $\gamma^{-/-}$ animals undergoing ischemia of the LAD coronary artery followed by reperfusion showed significant myocardial tissue injury, which was characterized by areas of interstitial edema, development of contracture bands, hemorrhage, margination of neutrophils along vessel walls and tissue infiltration of inflammatory cells, when compared to milder pathological signs of hemorrhage and edema of PPAR $\gamma^{+/+}$ mice (Figure 3). The severe histologic injury in knockout animals was paralleled by a rise in plasma cardiac-specific troponin-I (Figure 4A). Two hrs following reperfusion, plasma levels of troponin-I in PPAR $\gamma^{-/-}$ animals (52.5 ± 5.1 ng/ml) were significantly elevated in comparison to those of PPAR $\gamma^{+/+}$ mice (40.1 ± 2.8 ng/ml; $P < 0.05$). By echocardiography analysis, mice of both genotypes exhibited a significant decrease in systolic function as evidenced by lower fractional shortening and ejection fraction after ischemia and reperfusion. However, PPAR $\gamma^{-/-}$ mice had a more severe change when compared to baseline values. Furthermore, PPAR $\gamma^{-/-}$ mice, but not wild-type mice, exhibited a significant increase in the left ventricle internal dimension both at diastole and systole when compared to baseline measurements, suggesting the occurrence of left ventricle dilatation most probably due to impairment of cardiac contractility (Table 1). Interestingly, the PPAR $\gamma^{-/-}$ mice demonstrated decreased systolic thickening of the interventricular septum after ischemia and reperfusion. However, thickening of the LV posterior wall was not affected in these animals, thus suggesting that PPAR γ deficiency renders the area at risk particularly vulnerable to reperfusion injury (Table 1).

Conditional deletion of cardiomyocyte PPAR γ enhances neutrophil infiltration following ischemia and reperfusion

The activity of MPO, an enzyme specific to granulocytes, was used to quantify the degree of neutrophil infiltration into the myocardium following ischemia and reperfusion injury. MPO activity in the myocardial tissue of *PPAR γ ^{-/-}* animals (77.95 ± 10.50 U/100 mg tissue) was significantly elevated compared to the *PPAR γ ^{+/+}* counterparts (50.60 ± 8.44 U/100 mg tissue, $P < 0.05$; Figure 4B).

Conditional deletion of cardiomyocyte PPAR γ enhances levels of plasma pro-inflammatory cytokines

Plasma IL-1 β levels of *PPAR γ ^{-/-}* mice were significantly elevated at the end of reperfusion (93.1 ± 11.8 pg/ml) in comparison to *PPAR γ ^{+/+}* wild-type animals (63.5 ± 7.8 pg/ml, $P < 0.05$; Figure 5A). Similarly, *PPAR γ ^{-/-}* mice had significantly higher plasma levels of IL-6 (72.9 ± 4.9 ng/ml) in comparison to *PPAR γ ^{+/+}* counterparts (63.3 ± 1.7 ng/ml, $P < 0.05$; Figure 5B). Interestingly, plasma levels of IL-10, MIP-1 α , KC and TNF- α were not different between the two genotypes (Figure 5).

Conditional deletion of cardiomyocyte PPAR γ enhances DNA binding of NF- κ B

To investigate the mechanisms underlying the enhanced inflammatory response in the *PPAR γ ^{-/-}* hearts we evaluated NF- κ B activity. Cardiac DNA binding of NF- κ B was significantly increased in *PPAR γ ^{-/-}* animals undergoing sham procedure in comparison to *PPAR γ ^{+/+}* sham mice. After ischemia and reperfusion both genotypes experienced an elevation in NF- κ B activity. However, the degree of NF- κ B DNA binding was significantly higher in *PPAR γ ^{-/-}* animals in comparison to *PPAR γ ^{+/+}* mice (Figure 6).

DISCUSSION

The cardioprotective role of PPAR γ ligands in myocardial ischemia-reperfusion injury has been well established (7, 9-12). While PPAR γ is biologically active in many sites within the heart, the precise role of cardiomyocyte PPAR γ during myocardial ischemia and reperfusion has not yet been elucidated. In the present study we demonstrated that cardiomyocyte-restricted PPAR γ knockout enhanced myocardial ischemia and reperfusion injury. The underlying cellular mechanisms of this excessive vulnerability to injury related to increased neutrophil infiltration and increased production of IL-6 and IL-1 β , most probably mediated by enhanced NF- κ B activation.

Gene inactivation approaches have been used to investigate the functional role of PPAR γ in the heart. These studies have demonstrated that PPAR γ is clearly necessary for cellular development and metabolism. Studies using global homozygous PPAR γ null mice have shown embryonic lethality with documented severe myocardial thinning (15). Conventional Cre-lox cardiac-specific PPAR γ models have also demonstrated that mice with chronic cardiac PPAR γ deficiency since early stages of embryonic development may survive to adulthood, but they develop age-dependent pathological changes, including cardiac hypertrophy and oxidative damage (16, 17). Thus, to avoid these potential pathological developmental confounds and other phenotypic differences, in our study we used a loss-of-function strategy of short-term deletion of PPAR γ . Conditional knockout mice were generated using the Cre/LoxP system to allow for regional control of the deletion of the *PPAR γ* gene through the Cre recombinase driven by the α -myosin heavy chain promoter. This Cre recombinase was fused to mutant estrogen-receptor ligand-binding domains (MerCreMer), which drives expression in cardiomyocytes even in adulthood after a short treatment with tamoxifen (13). Our data demonstrated that even an acute deficiency of cardiomyocyte-PPAR γ rendered the heart more susceptible to reperfusion stress and caused

severe damage, as demonstrated by histology assessment and high levels of plasma troponin I, the biomarker of infarct extension in humans and experimental animals (18-20). Importantly, the severe myocardial damage in *PPAR γ ^{-/-}* mice translated into severe LV dysfunction and LV dilatation when compared to *PPAR γ ^{+/+}* mice as measured by echocardiography. It should be noted that other non-myocyte cell types, such as macrophages, endothelial cells, neutrophils, monocytes, and platelets contribute to the pathological processes of myocardial damage during ischemia and reperfusion (3). Our laboratory and others have previously demonstrated that PPAR γ is also an important anti-inflammatory modulator in those cells (21-25). Our current study, therefore, suggest that PPAR γ in myocytes constitutes an important requisite for regulating both the inflammatory response as well as the cardiac performance during early reperfusion injury. However, given the importance of PPAR γ in myocardial metabolic homeostasis (4-6), it remains to be determined whether cardiomyocyte PPAR γ deficiency may also impact the progressive process of cardiac remodeling and recovery.

Ischemia and reperfusion results in significant cardiac dysfunction and injury owing in part to the release of potent pro-inflammatory cytokines (2). The myocardium is critical for the production of several inflammatory cytokines during reperfusion injury as a mechanism to start tissue repair, but it also becomes a target of their cytotoxic effects (26). For example, in patients with myocardial infarction IL-6 appears to be synthesized in the myocardium during the ischemic period and its secretion is further triggered by the reperfusion process (27, 28). Increased IL-6 is associated with poor prognosis in patients with myocardial infarction or heart failure (29) and with life-threatening events, such as shock, decreased myocardial contractility and arrhythmias (30, 31). As with IL-6, IL-1 β is another marker of severe cardiac injury after ischemia and reperfusion (26, 32). IL-1 β is toxic for the cardiac myocyte and inhibition of its activity affords cardioprotective effects as demonstrated by studies in patients with myocardial infarction and animal models of ischemia and reperfusion (33-35). Additional production of inflammatory cytokines and chemokines takes place through endothelial dysfunction and direct recruitment of inflammatory cells to the site of injury, which amplifies the local inflammatory response (26). In experimental models of ischemia and reperfusion cardiac mast cells, macrophages and endothelial cells are main sources of TNF α (36), while IL-10 appears to be mainly secreted by T-lymphocytes (37). The chemokine KC (or the human homologue IL-8) is produced by activated neutrophils to mediate their intra-parenchymal migration (38-40). MIP-1 α levels are elevated during acute coronary syndromes (41) and are produced by endothelial cells (42) as well as macrophages, dendritic cells, and lymphocytes (43). In our study, *PPAR γ ^{-/-}* mice displayed a significant increase in plasma levels of IL-6 and IL-1 β after myocardial ischemia and reperfusion when compared to *PPAR γ ^{+/+}* mice. Considering the important role of both IL-6 and IL-1 β in myocardial ischemia and reperfusion, our study therefore suggests that the combined elevation of these cytokines can significantly contribute to tissue damage. Our hypothesis is supported by previous studies demonstrating that pro-inflammatory cytokines, including IL-6 and IL-1 β , are secreted by cardiomyocytes during inflammation and act synergistically to cause cardiac contractile dysfunction (44). Interestingly, the expression of other established inflammatory markers, such as TNF α , KC, MIP-1 α and IL-10 were not significantly different in *PPAR γ ^{+/+}* mice and cardiomyocyte-specific *PPAR γ ^{-/-}* mice, in which the expression of PPAR γ remains intact in the endothelium and inflammatory cells. Therefore, the differential effect on plasma cytokine levels between the two genotypes clearly reflects the different cell source in response to the ischemia and reperfusion challenge. Our findings, in fact, indicate that PPAR γ activity in the myocardium is required for the regulation of IL-6 and IL-1 β synthesis. Our data also indicate that endothelial cells, infiltrated neutrophils, macrophages and other immunocompetent cells can maintain a sustained production of other important inflammatory cytokines and chemokines including TNF α , KC, MIP-1 α and IL-10. This differential cytokine response may also explain the

enhanced leukosequestration in damaged tissue in cardiomyocyte-specific *PPAR* γ ^{-/-} mice when compared to *PPAR* γ ^{+/+} mice. Neutrophils infiltrate into the myocardium following ischemia and reperfusion and are important players in the development of irreversible tissue injury. Among chemokine and cytokines, IL-6 and IL-1 β appear important factors to drive migration of neutrophils into myocardium (45, 46). For example, it has been shown that IL-6 receptor inhibition suppressed infiltration of neutrophils, thereby reducing MPO activity in the infarct area and the border zone (45). Similarly, IL-1 receptor deficient mice exhibited markedly reduced neutrophil infiltration of the infarcted myocardium (46). Thus, our study suggests that cardiomyocyte *PPAR* γ is an important contributor to the modulation of myocardial neutrophil infiltration through specific regulation of inflammatory cytokines.

As a nuclear transcription factor, *PPAR* γ binds to recognition sites on *PPAR* response elements in the promoter region of target genes, many of which serve to maintain metabolic homeostasis (5). Similar to other nuclear receptors, *PPAR* γ is also able to bind to co-repressors or other transcription factors and cause transrepression (6). Previously we have shown that the cardioprotective effects of *PPAR* γ ligands are in part due to their ability to downregulate the pro-inflammatory transcription factor NF- κ B (7), thus suggesting a mechanism of transrepression of the inflammatory response induced by reperfusion injury. In the present study, we further confirmed that *PPAR* γ in cardiomyocytes is crucial for the negative regulation of NF- κ B, as cardiac DNA binding of NF- κ B was markedly enhanced in cardiomyocyte-specific *PPAR* γ ^{-/-} mice when compared to *PPAR* γ ^{+/+} mice. The ability of the cardiomyocyte *PPAR* γ to negatively regulate NF- κ B activation may extend beyond the setting of myocardial ischemia-reperfusion. For example, rat cardiomyocytes pretreated with *PPAR* γ ligands showed reduced NF- κ B DNA binding when stimulated with lipopolysaccharide (47). In our study, there was also a modest but significant increase in DNA binding of NF- κ B, which was associated with an increase in neutrophil infiltration under basal sham conditions in cardiomyocyte-specific *PPAR* γ ^{-/-} mice when compared to *PPAR* γ ^{+/+} mice. Thus, our data also support that *PPAR* γ plays a critical role in maintaining the physiological homeostasis of cell phenotype and suggest that even acute deficiency of *PPAR* γ function may induce a pathological imbalance rendering cardiac tissue susceptible to sham surgical procedures, such as thoracotomy and balloon positioning.

In conclusion, by means of cell type-specific gene knockout technology, the current study provides conclusive data that *PPAR* γ expression in cardiomyocytes functions as a protective factor against reperfusion injury. As the thiazolidinedione class of *PPAR* γ ligands has remained under close scrutiny in the clinical arena due to a potential risk of adverse cardiovascular events (48-50), further insight into the exact biological mechanism and site of action of *PPAR* γ within the cardiovascular system may help to provide better clarity as to the local function of *PPAR* γ and the proper clinical use of these medications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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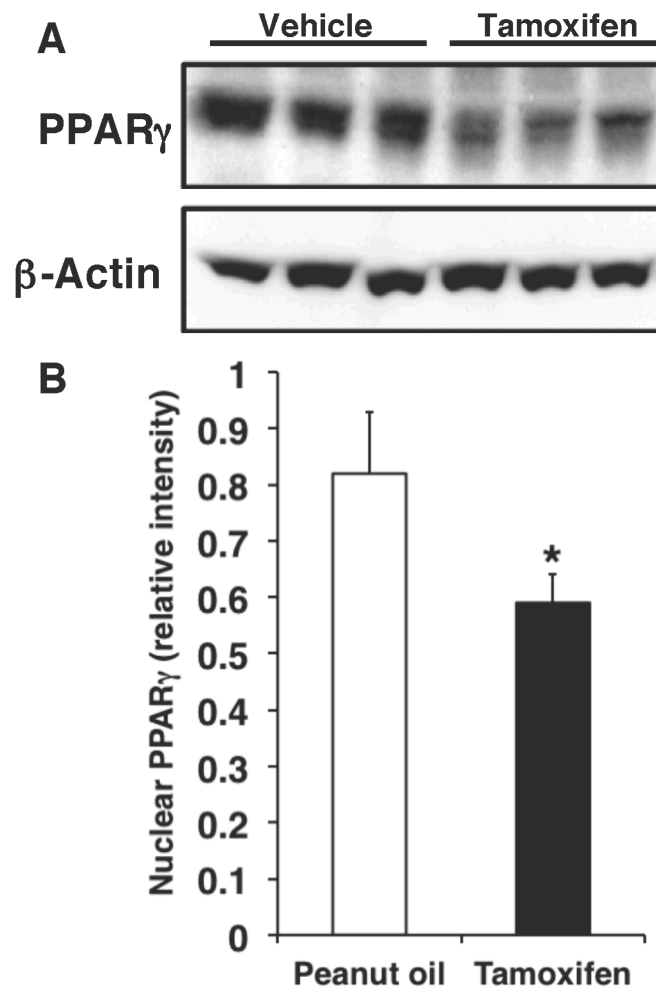


FIG. 1. Nuclear PPAR γ expression in cardiac samples

(A) Representative Western blot of PPAR γ in nuclear extracts from hearts of PPAR $\gamma^{Cre/loxP}$ mice receiving tamoxifen (30 mg/kg daily, ip) or vehicle (peanut oil) for four days. Expression of β -actin was used as loading control protein. (B) Quantitative analysis of PPAR γ expression determined by densitometry. Relative intensity was normalized to β -actin expression. *Represents $P < 0.05$ versus vehicle treatment.

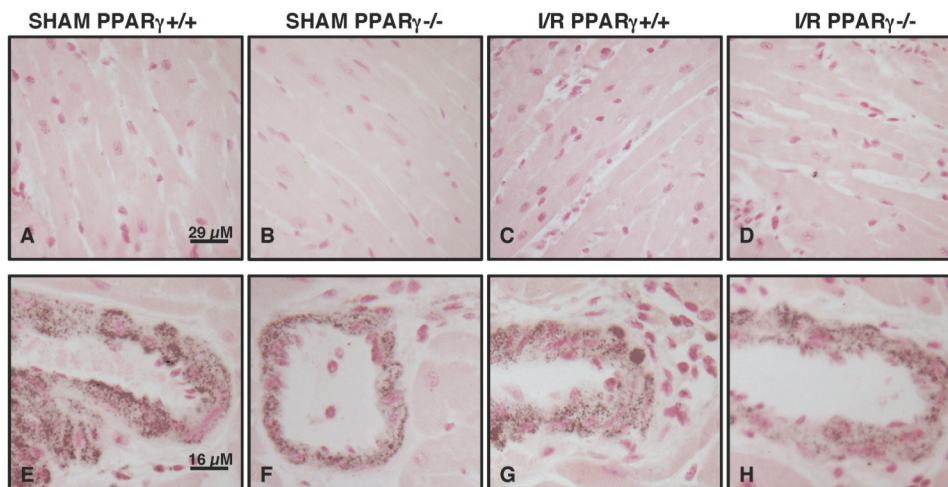


FIG. 2. Immunohistochemistry of PPAR γ in cardiac sections of $PPAR\gamma^{+/+}$ and $PPAR\gamma^{-/-}$ mice subjected to sham procedure or myocardial ischemia and reperfusion (I/R)

Immunohistochemistry for PPAR γ showed positive staining in $PPAR\gamma^{+/+}$ mice subjected to sham procedure or myocardial I/R both in cardiomyocytes (A and C) and endothelial cells (E and G). There was no staining for PPAR γ in cardiomyocytes of $PPAR\gamma^{-/-}$ mice subjected to sham procedure or myocardial I/R (B and D). Positive staining for PPAR γ was observed in endothelial cells of $PPAR\gamma^{-/-}$ mice subjected to sham procedure or myocardial I/R (F and H). Figure is representative of at least four different experiments.

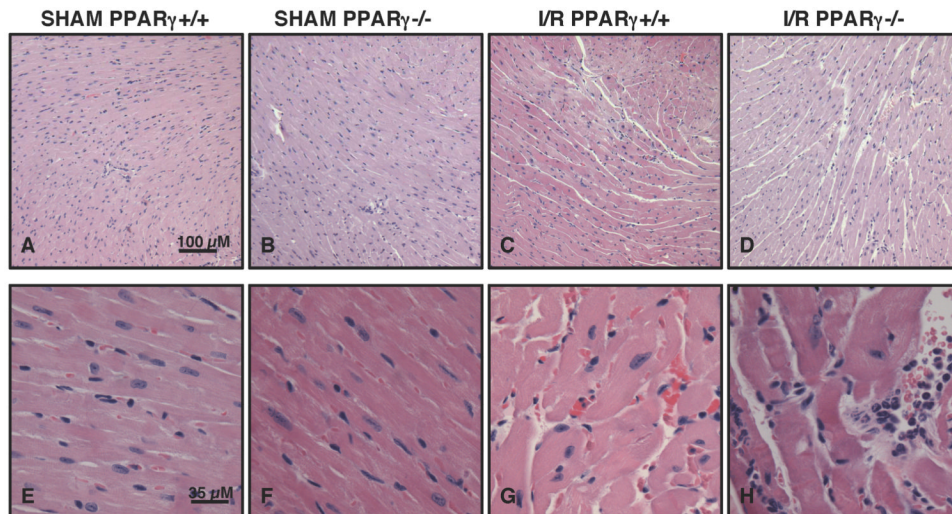


FIG. 3. Histology of cardiac sections from $PPAR\gamma^{+/+}$ and $PPAR\gamma^{-/-}$ mice subjected to sham procedure or myocardial ischemia and reperfusion (I/R)
 Representative sections from sham $PPAR\gamma^{+/+}$ (A and E) and $PPAR\gamma^{-/-}$ (B and F) mice revealed normal myocardial structure. After myocardial I/R areas of hemorrhage and extracellular edema were observed in cardiac sections of $PPAR\gamma^{+/+}$ mice (C and G). $PPAR\gamma^{-/-}$ mice undergoing I/R exhibited a more severe damage characterized by disruption of myofibrils, larger vacuole of edema formation, neutrophil margination and infiltration (D and H). Figure is representative of at least four different experiments.

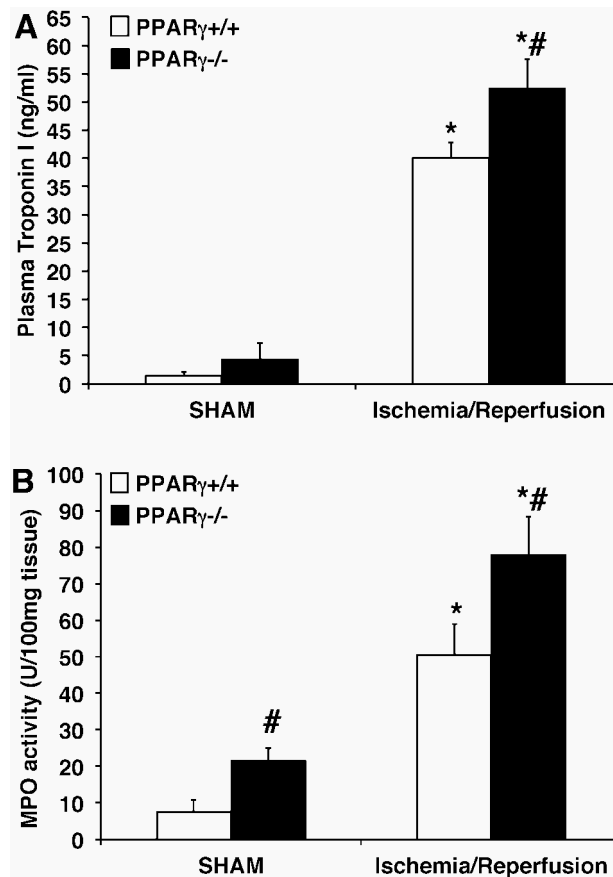


FIG. 4. Plasma levels of Troponin I (A) and cardiac myeloperoxidase activity (B) of $PPAR\gamma^{+/+}$ and $PPAR\gamma^{-/-}$ mice subjected to sham procedure or myocardial ischemia and reperfusion Each data point represents the mean \pm SEM of 4-8 animals for each group. * $P < 0.05$ versus sham mice of the same genotype; # $P < 0.05$ versus $PPAR\gamma^{+/+}$ mice.

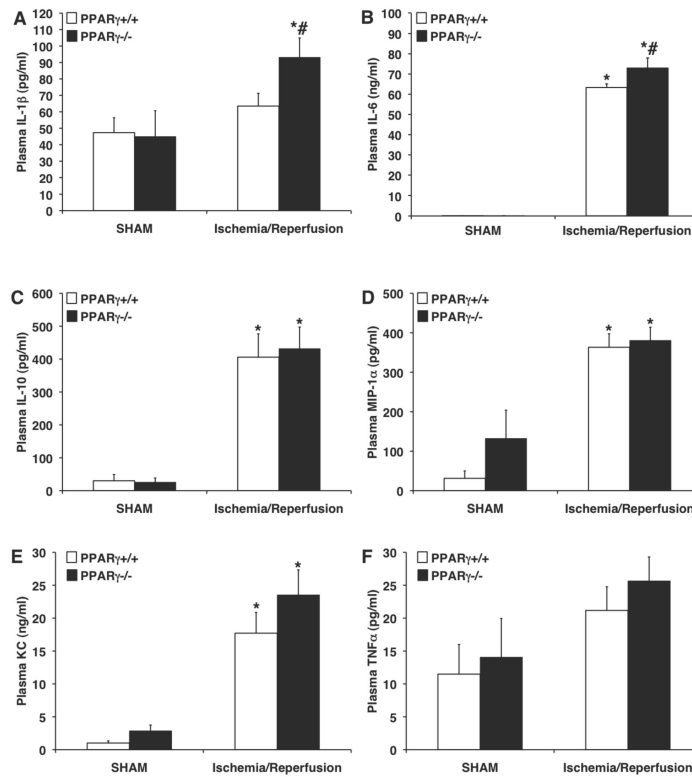


FIG. 5. Plasma levels of IL1- β (A), IL-6 (B), IL-10 (C), MIP-1 α (D), KC (E) and TNF- α (F) of $PPAR\gamma^{+/+}$ and $PPAR\gamma^{-/-}$ mice subjected to sham procedure or myocardial ischemia and reperfusion

Each data point represents the mean \pm SEM of 4-8 animals for each group. * $P < 0.05$ versus sham mice of the same genotype; # $P < 0.05$ versus $PPAR\gamma^{+/+}$ mice.

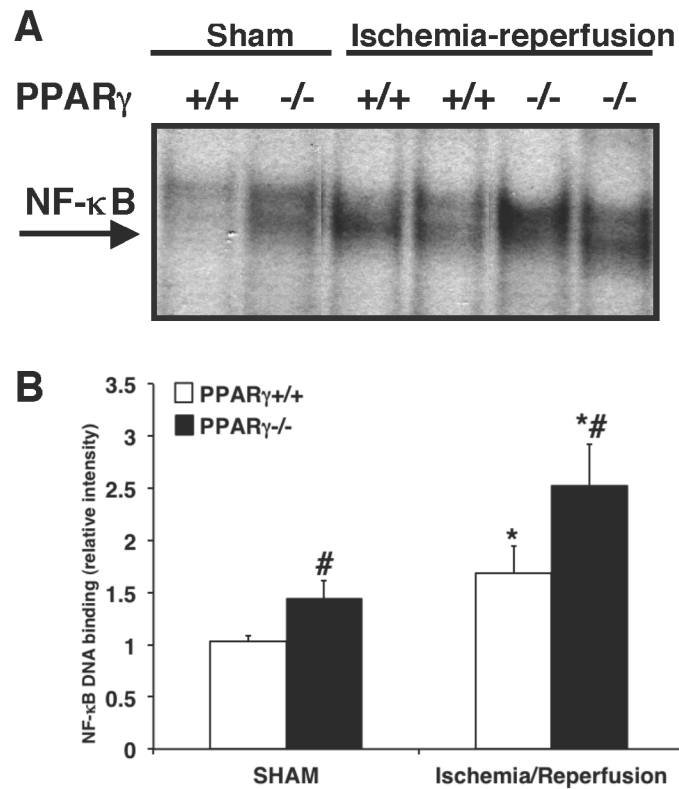


FIG. 6. DNA binding activity of NF- κ B in cardiac samples of PPAR $\gamma^{+/+}$ and PPAR $\gamma^{-/-}$ mice subjected to sham procedure or myocardial ischemia and reperfusion

(A) Representative autoradiographs of electrophoretic mobility shift assay for NF- κ B DNA binding in nuclear extracts from hearts of PPAR $\gamma^{+/+}$ and PPAR $\gamma^{-/-}$ mice subjected to sham procedure or myocardial ischemia and reperfusion. (B) Quantitative analysis of NF- κ B DNA binding determined by densitometry. Each data point represents the mean \pm SEM of 4-7 animals for each group. * $P < 0.05$ versus sham mice of the same genotype; # $P < 0.05$ versus PPAR $\gamma^{+/+}$ mice.