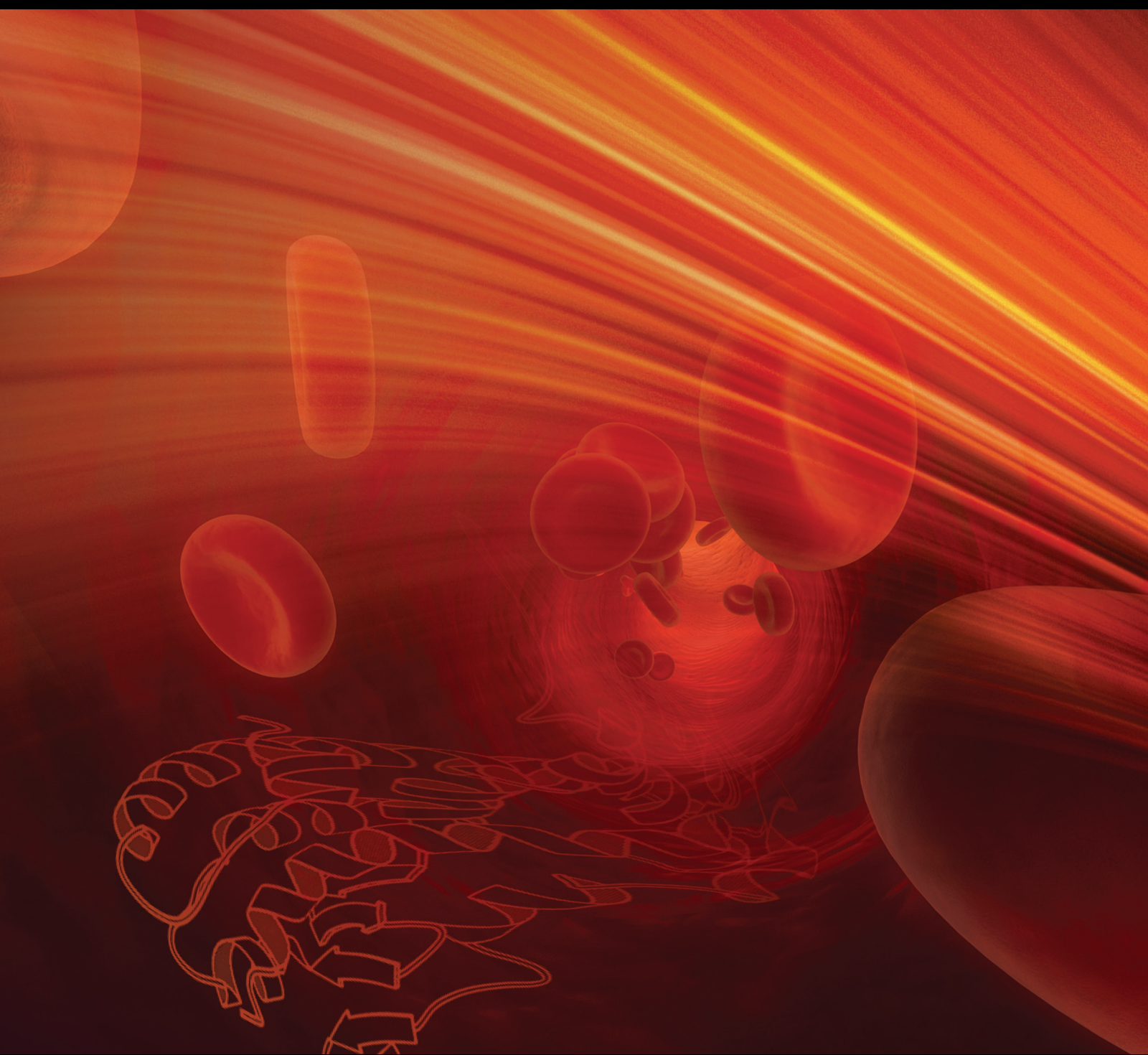
PPAR Research

PPAR in Cardiovascular Disorders

Guest Editors: Alexander N. Orekhov, Nigora Mukhamedova,
Ekaterina A. Ivanova, and Manfredi Rizzo



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Editorial

PPAR in Cardiovascular Disorders

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Peroxisome proliferation-activated receptors (PPARs) are ligand-inducible transcription factors that, upon binding their ligands, translocate into the nucleus, where they regulate transcription of numerous genes that have the peroxisome proliferator response element (PPRE) in the promoter region [1].

In humans, there are 3 PPAR isoforms: PPAR-α, PPAR-β/δ, and PPAR-γ. The isoforms have partially overlapping spectra of activity and are differently expressed in organs and tissues [2]. PPAR-α is expressed mostly in tissues characterized by high catabolic activity, including skeletal muscle, liver, proximal tubular cells in kidneys, and brown fat. This PPAR isoform regulates components of β-oxidation pathway, enzymes, and transporters involved in fatty acid metabolism and promotes lipolysis and fatty acid oxidation. PPAR-α can be activated by fatty acids, prostaglandins, fibric acid derivatives (fibrates), and a number of recently developed specific agonists. Activation of PPAR-α has a beneficial effect on processes involved in the development of atherosclerosis, as it decreases plasma triglyceride levels, increases high density lipoprotein cholesterol, and reduces inflammatory response. Therefore, PPAR-α agonists gain attention as potential components of antiatherosclerotic therapy [3, 4].

PPAR-β/δ is expressed in many organs and tissues, with relatively high levels present in skeletal muscle, liver, kidney, and macrophages. It is activated by fatty acids and carbaprostacyclin, stimulates fatty acid oxidation, and improves insulin sensitivity in insulin-resistant animal models. This isoform is also known to have potential antiatherosclerotic properties and is considered for treatment of cardiovascular disorders [5, 6].

PPAR-γ is mainly expressed in white and brown fat and can also be found in other organs and tissues, including liver, kidney, and immune cells. It is activated by fatty acids implicated in regulation of glucose homeostasis, lipid metabolism, and adipogenesis. Synthetic PPAR-γ agonists, thiazolidinediones, such as pioglitazone and rosiglitazone, are currently used as insulin sensitizers but can have a broader therapeutic potential for treatment of conditions associated with increased cardiovascular risk [7].

Therefore, PPARs have a wide spectrum of biological activities relevant to prevention and treatment of cardiovascular diseases. Moreover, the availability of natural and synthetic small molecule agonists, many of them being relatively well studied by now, makes PPARs attractive therapeutic targets. To date, PubMed literature database delivers more than 3300 articles found by key words "PPAR" + "cardiovascular". In this special issue, we are happy to present several important works revealing various aspects of PPAR involvement in cardiovascular conditions. The importance of PPAR-α signalling for regulation of cardiomyocyte metabolism is highlighted by the research articles of E. Czarnowska with coauthors, who studied the correlation of PPAR-α activity and cardiomyocyte function during heart failure, and J. Yang with coauthors, who

demonstrated that PPAR-α upregulation mediated the effect of testosterone replacement on cardiac metabolic remodelling after myocardial infarction. G. Barreto-Torres with S. Javadov and W.-Y. Wei with coauthors presented the links between PPAR activation and the key cellular signalling network, which includes the AMPK and AKT pathways and regulates cellular metabolism, growth, and response to stress. Another evidence of anti-inflammatory properties of pioglitazone in patients with drug eluting stents is presented by Z. Wang and coauthors. H.-J. Liu and coauthors provide evidence for the critical role of PPAR-γ in cardiac fibrosis, and A. Pleskovič with coauthors reported that PPAR-γ polymorphisms have a minor effect on atherosclerosis markers in diabetic patients. K.-D. Wagner with coauthors demonstrates that inducible vascular-specific overexpression of PPAR-β/δ causes cardiac hypertrophy. Finally, a review article of W.-S. Lee and J. Kim provides an overview of the roles of PPARs in the heart. Together, the works collected in this special issue add to our growing knowledge on the PPARs and their activators in the context of cardiovascular disorders.

Alexander N. Orekhov
Nigora Mukhamedova
Ekaterina A. Ivanova
Manfredi Rizzo

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Research Article

Testosterone Replacement Modulates Cardiac Metabolic Remodeling after Myocardial Infarction by Upregulating PPARα

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Despite the importance of testosterone as a metabolic hormone, its effects on myocardial metabolism in the ischemic heart remain unclear. Myocardial ischemia leads to metabolic remodeling, ultimately resulting in ATP deficiency and cardiac dysfunction. In the present study, the effects of testosterone replacement on the ischemic heart were assessed in a castrated rat myocardial infarction model established by ligating the left anterior descending coronary artery 2 weeks after castration. The results of real-time PCR and Western blot analyses showed that peroxisome proliferator-activated receptor α (PPARα) decreased in the ischemic myocardium of castrated rats, compared with the sham-castration group, and the mRNA expression of genes involved in fatty acid metabolism (the fatty acid translocase CD36, carnitine palmitoyltransferase I, and medium-chain acyl-CoA dehydrogenase) and glucose transporter-4 also decreased. A decline in ATP levels in the castrated rats was accompanied by increased cardiomyocyte apoptosis and fibrosis and impaired cardiac function, compared with the sham-castration group, and these detrimental effects were reversed by testosterone replacement. Taken together, our findings suggest that testosterone can modulate myocardial metabolic remodeling by upregulating PPARα after myocardial infarction, exerting a protective effect on cardiac function.

1. Introduction

Cardiac metabolic remodeling is characterized by impairments in substrate utilization and mitochondrial biogenesis and function, leading to adenosine triphosphate (ATP) deficiency [1]. Regional myocardial infarction, which induces cardiac remodeling, decreases the capacity of the heart to generate sufficient ATP to maintain cardiac function. As these metabolic changes can lead to heart failure [2], the modulation of cardiac metabolism may be an alternative approach to protect against cardiac dysfunction in myocardial infarction.

Myocardial infarction leads to partial insulin resistance accompanied by reduced fatty acid oxidation and impaired mitochondrial biogenesis in addition to the downregulation of metabolic genes [3–5]. Peroxisome proliferator-activated receptor α (PPARα) is a nuclear receptor that functions as the primary transcriptional regulator of fatty acid metabolism in the heart. PPARα target genes include fatty acid translocase (CD36) and carnitine palmitoyltransferase I (mCPT-1), which are involved in the import of fatty acids into the cell and mitochondria, and medium-chain acyl-CoA dehydrogenase (MCAD), which catalyzes the rate-limiting step in medium-chain fatty acid β oxidation [6]. Besides, PPARα also modulates glucose metabolism. Heart-specific PPARα overexpression induces the transcription of fatty acid metabolism genes and downregulates genes associated with glucose transport and PPARα null mice show increased glucose transporter-4 (GLUT-4) expression and downregulation of PPARα targeted genes of fatty acid metabolism [7]. PPARα has emerged as an attractive target to improve metabolic remodeling.

The role of androgens in myocardial infarction is controversial. Studies showed that high levels of testosterone had adverse effects on cardiac remodeling and function after myocardial infarction [8, 9]; however, in a different study,

chronic testosterone treatment had no detrimental effects after myocardial infarction and was suggested to improve long-term outcomes, reducing left ventricular end-diastolic pressure and wall stress [10]. Testosterone has also been shown to reduce the infarct size in ischemia-reperfusion of orchidectomized rats [11, 12]. In patients with coronary artery disease, testosterone deficiency is associated with poor outcomes associated with heart failure and has a significant negative impact on survival [13]. Testosterone is an important hormone that is involved in the regulation of carbohydrate, fat, and protein metabolism [14]. Low testosterone levels are associated with impaired insulin sensitivity, increased body fat percentage, truncal obesity, and dyslipidemia [15], and testosterone deficiency is a risk factor for cardiovascular morbidity and mortality among men [16]. Although testosterone has effects on cardiac metabolism [17], little is known about the role of testosterone in the regulation of cardiac metabolic remodeling in the ischemic heart. This experiment was designed to assess the effects of testosterone replacement on the cardiac metabolic remodeling via regulating the expression of PPARα and its downstream genes in a castrated rat myocardial infarction model.

2. Materials and Methods

2.1. Animals. Male Wistar rats weighing 220–250 g were obtained from the Laboratory Animal Center of the First Affiliated Hospital of Harbin Medical University. The rats were maintained under temperature-controlled (22–24°C) and circadian conditions with free access to rodent chow and tap water. All experiments were performed in accordance with the protocols for the care and use of laboratory animals of the National Research Council and were approved by the ethics committee of our hospital.

2.2. Castration and Hormone Replacement. Rats were anesthetized with intraperitoneal injection of 10% chloral hydrate (3 mL/kg), and castration (Cas) or sham-castration (S-Cas) was randomly performed following a previously described method [18]. The animals were then randomly assigned into the four groups: (1) sham-castration+placebo (S-Cas), (2) castration+placebo (Cas), (3) castration+testosterone (Cas+T), and (4) castration+testosterone and flutamide (Cas+T+F). The different interventions were carried out according to the groupings on the same day of surgery to avoid disruption of hormonal effects [19]. Testosterone propionate (Amino Acids, P.F, Tianjin, China) dissolved in peanut oil was injected subcutaneously at a physiological dose of 2 mg/kg/d, and flutamide (Sigma Chemical Co., St. Louis, MO, USA), an antagonist of the androgen receptor (AR), dissolved in propylene glycol was injected at a dose of 30 mg/kg/d [20]. Peanut oil (2 mg/kg/d), serving as placebo, was injected into rats in groups 1 and 2.

2.3. Myocardial Infarction Model. Two weeks after castration, the rats received left coronary artery ligation to establish the myocardial infarction model [21]. Briefly, the rats were anesthetized with intraperitoneal injection of chloral hydrate (3 mL/kg), and then they were given mechanical positive pressure ventilation with a frequency of 65–70/min by a ventilator. The left coronary artery was ligated with 3/8 needle and 6-0 sutures. The success of establishment was confirmed by blanching of the anterior wall of the left ventricle and typical ST-segment elevation. A total of 27 rats (8 S-Cas, 6 Cas, 7 Cas+T, and 6 Cas+T+F) were included in the analysis performed 14 days after the ligation and subcutaneous injection of testosterone with/without flutamide. Additional normal rats ($n = 8$) that underwent the same procedure without occlusion were used as the control group.

2.4. Echocardiographic Studies. Echocardiography was performed under anesthesia at 14 days after coronary ligation. Two-dimensional and M-mode images were used to record the left ventricular end-diastolic diameter and left ventricular end-systolic diameter (LVDd and LVSd, resp.) from the parasternal long-axis views using an ultrasound machine (SONOS 7500, Philips) equipped with a 12 MHz transducer. Left ventricular ejection fraction (EF) and fractional shortening (FS) were calculated in real time. All measurements were averaged on three consecutive cardiac cycles.

Rats were euthanized, and blood samples were collected from the heart and centrifuged at 1000 ×g for 20 min to obtain serum. The hearts were excised and irrigated with saline solution. After removal of the atria, right ventricle, great vessels, and valves, the left ventricle was rapidly frozen in nitrogen and stored at −80°C or fixed in 4% paraformaldehyde and embedded in paraffin for further histological analysis.

2.5. Measurements of ATP. ATP concentration was measured using a kit from Jiancheng Biological Technical Institute (Nanjing, China). All procedures were performed according to the manufacturer's instructions. The peri-infarct cardiac tissues were homogenized in saline and centrifuged at 10000 ×g for 5 min. Tissue ATP was measured by spectrophotometer colorimetry at 636 nm [22].

2.6. Real-Time PCR. Total RNA was extracted with RNAiso Plus and reverse-transcribed to first-strand cDNA using a PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Otsu, Japan) according to the manufacturer's protocol. The mRNA levels of PPARα, CD36, mCPT-1, MCAD, and GLUT-4 in the peri-infarct cardiac tissues were measured by real-time PCR with SYBR Green (Roche, Germany) incorporation on an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster, CA, USA). The relative quantification of gene expression was determined by comparing the target-amplified product to GAPDH, which was used as an internal standard. The primer sequences are described in Table 1.

2.7. Western Blotting. Proteins were extracted from the left ventricular peri-infarct tissues, separated by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes as described previously [23]. Then, the membranes were exposed to primary antibodies against PPARα (1 : 200, Santa Cruz, Dallas, Texas, USA), GLUT-4 (1 : 800, Cell Signaling Technology, Danvers, MA, USA), and GAPDH (1 : 5000, KangChen, Shanghai, China) followed by the corresponding horseradish peroxidase-conjugated secondary antibodies

Table 1: Primers for real-time PCR.

	Primer
PPARα	F: 5′-TTTGTGGGGCTGGAGGGTTCGTG-3′
	R: 5′-GCCACAGAGCACCAATCTGTGA-3′
CD36	F: 5′-CCTATTGGGAAAGTTATTGCG-3′
	R: 5′-GTTGTCTGGGTTCTGGAGTG-3′
mCPT-1	F: 5′-CGAGTTCAGAAACGAACGCCC-3′
	R: 5′-GTGCATGCCACCCCTTATGC-3′
MCAD	F: 5′-TGTGTGCCTACTGCGTGACA-3′
	R: 5′-TCGGCCTCCACGATGAATCC-3′
GLUT-4	F: 5′-AGGCCGGGACACTATACCCTA-3′
	R: 5′-TCTGTGGGGCGTTGATGACT-3′
GAPDH	F: 5′-GGAAAGCTGTGGCGTGAT-3′
	R: 5′-AAGGTGGAAGAATGGGAGTT-3′

(1 : 2000, ZhongShan, Beijing, China). Protein bands were visualized using enhanced chemiluminescence detection reagents (Thermo Scientific™, Waltham, MA, USA) and exposure to X-ray film. Developed films were digitized with a scanner (Canon LiDE 110, Japan). Band intensities (area × OD) were analyzed using NIH ImageJ software (Wayne Rasband, Bethesda, MD, USA), and protein levels were normalized to GAPDH.

2.8. Histopathology. Myocardial samples were cut into 5 μm thick cross sections along the centre of the fibrotic scar and stained with Masson-Trichrome to estimate myocardial fibrosis. The fibrotic area percent was calculated and used to quantify the degree of cardiac fibrosis in the peri-infarct region. Randomly selected digital photographs of each slice were analyzed using image analysis software (Image-Pro Plus 6.0, Media Cybernetics, Rockville, MD, USA). The percentage of fibrotic area was calculated as the ratio of positively blue-stained fibrotic area to total myocardium area.

Apoptosis was determined using the TUNEL assay as described previously [21]. The procedure was conducted by the instructions of the In Situ Cell Death Detection Kit, POD (Roche, Mannheim, Germany). The percentage of apoptotic cells was calculated from the overall number of counted cells in at least six randomly selected fields at ×400 magnification under a microscope using Image-Pro Plus 6.0 software.

2.9. Measurement of Serum Testosterone and Estradiol. Serum testosterone and 17β-estradiol levels were measured with commercially available enzyme-linked immunoassay (ELISA) kits (Uscn Life Science, Inc., Houston, TX, USA). All procedures were performed according to the manual as previously described [24]. The limit of detection was 0.0437 ng/mL for testosterone and 4.75 pg/mL for 17β-estradiol.

2.10. Statistical Analysis. Results were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was carried out to determine differences among groups by using SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

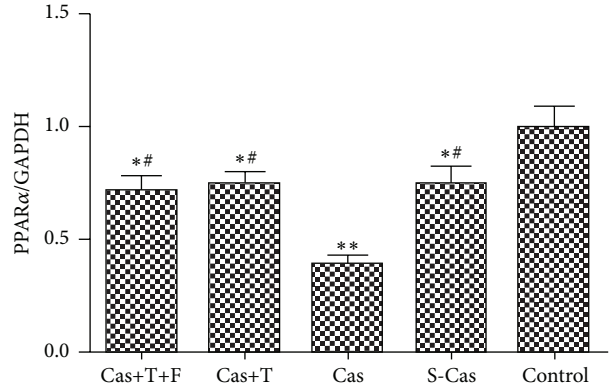

Figure 1: Effects of castration and testosterone replacement on the mRNA expression of PPARα in heart. Values are means ± SD; $n = 3$. S-Cas: sham-castration; Cas: castration; T: testosterone; F: flutamide. $^{*}P < 0.01$ and $^{**}P < 0.001$ versus control group; $^{\#}P < 0.01$ versus Cas.

3. Results

3.1. Alterations in the mRNA and Protein Expression of PPARα. PPARα plays a key role in modulating cardiac energy metabolism. In the present study, real-time PCR (Figure 1) and Western blotting (Figures 2(a) and 2(b)) showed that PPARα was downregulated at the mRNA and protein levels in the S-Cas group compared with the control group ($P < 0.01$), indicating that myocardial infarction-induced metabolic remodeling involved the suppression of PPARα signaling. Castration further decreased PPARα expression of mRNA and protein (Cas versus S-Cas, $P < 0.01$), which was rescued by exposure to testosterone with an increase of mRNA and protein expression (Cas+T versus Cas, $P < 0.01$); additional flutamide treatment did not downregulate PPARα levels compared with the group treated with testosterone alone ($P > 0.05$). Taken together, these results suggested that endogenous testosterone deprivation impaired PPARα signaling in a rat model of myocardial infarction, and this effect was reversed by testosterone replacement.

3.2. mRNA Expression of Fatty Acid Metabolism Related Genes. The mRNA levels of key regulators of fatty acid metabolism (CD36, mCPT-1, and MCAD) were assessed in the different groups (Figure 3). The expression of CD36, mCPT-1, and MCAD ($P < 0.05$) was downregulated in the S-Cas group compared with the control group. Castration further decreased the mRNA levels of CD36, mCPT-1, and MCAD ($P < 0.01$) compared with the S-Cas group, whereas testosterone replacement increased their expression ($P < 0.01$) compared with the castrated rats. Flutamide did not antagonize the effects of testosterone replacement on the expression of CD36, mCPT-1, and MCAD ($P > 0.05$) (Cas+T+F versus Cas+T).

3.3. Effects of Testosterone on the Expression of GLUT-4. The mRNA and protein levels of GLUT-4 (Figures 4 and 5), which was in charge of glucose transport, were higher in the S-Cas group compared with the control group but without

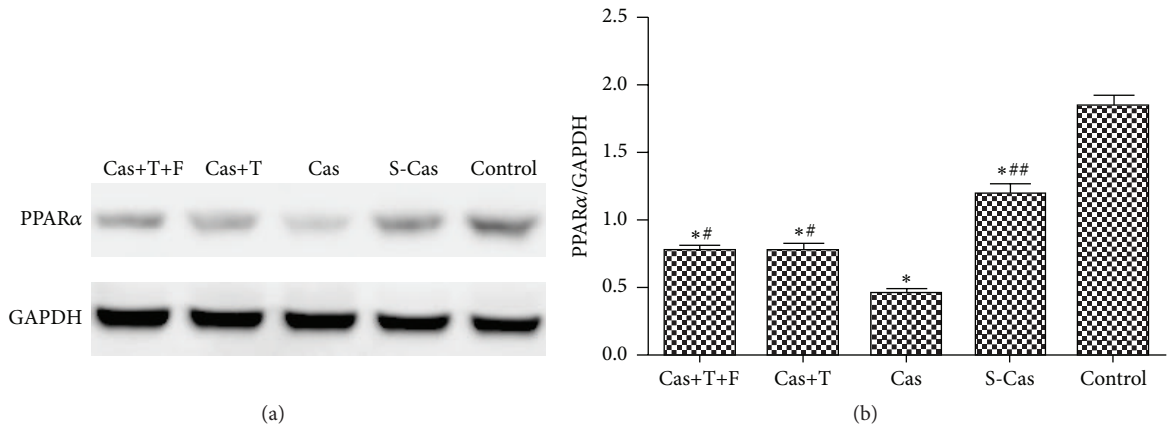

Figure 2: Effects of castration and testosterone replacement on the protein expression of PPARα in heart. (a) Western blotting result for PPARα protein level. (b) Quantitation of PPARα protein level. Values are means ± SD; $n = 3$. S-Cas: sham-castration; Cas: castration; T: testosterone; F: flutamide. $^{*}P < 0.001$ versus control group; $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ versus Cas.

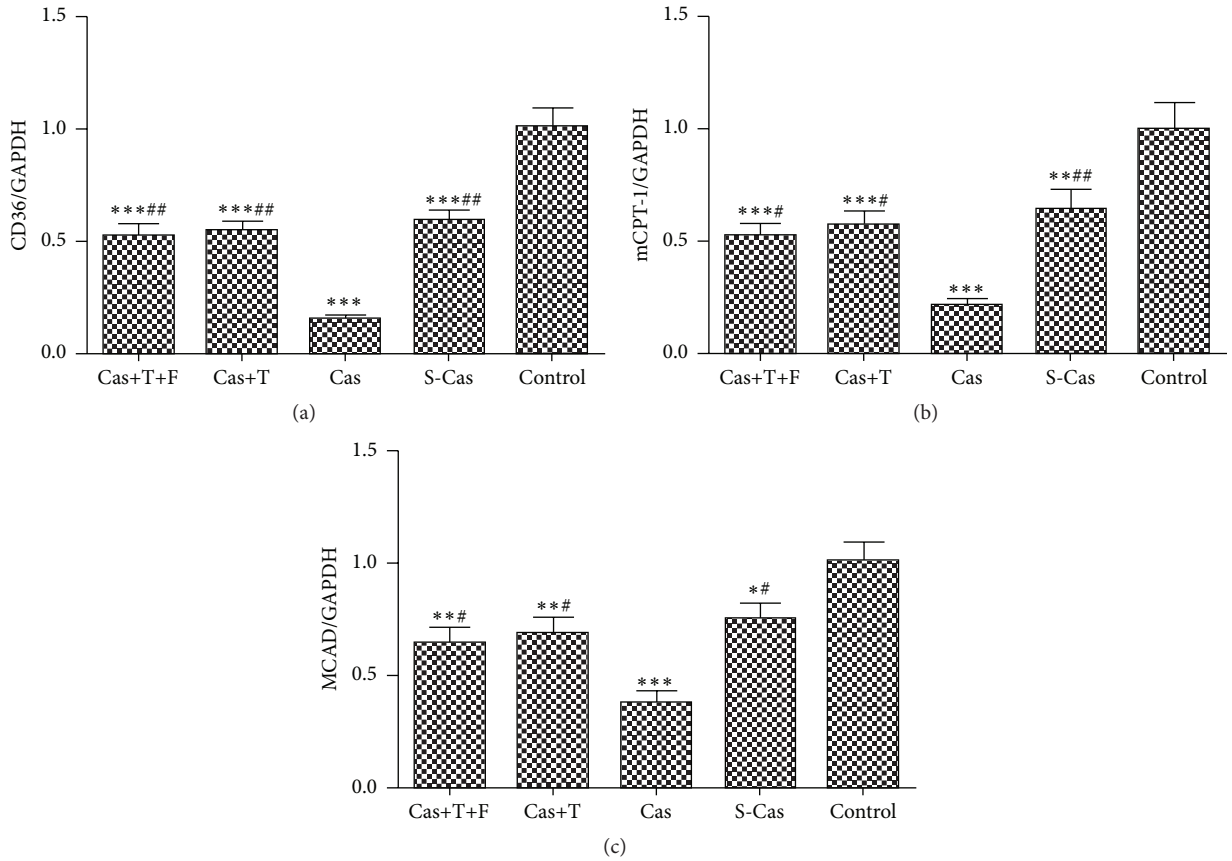

Figure 3: Effects of castration and testosterone replacement on the mRNA expression of fatty acid metabolism. (a–c) mRNA expression levels of CD36, mCPT-1, and MCAD, respectively. Values are means ± SD; $n = 3$. S-Cas: sham-castration; Cas: castration; T: testosterone; F: flutamide. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control group; $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ versus Cas.

significant differences ($P > 0.05$). Castration decreased the mRNA and protein levels of GLUT-4 (Cas versus S-Cas, $P < 0.01$), while testosterone could attenuate the decrease in GLUT-4 compared with the castrated rats ($P < 0.05$). Flutamide did not block the effect of testosterone (Cas+T+F versus Cas+T, $P > 0.05$).

3.4. Changes in ATP Concentration. The concentration of ATP in rat left ventricular tissues was compared among groups (Figure 6). ATP levels were lower in the S-Cas group than in the control group (794.80 ± 82.97 versus 1109.67 ± 140.17 μmol/gprot, $P < 0.001$), and castration further decreased the levels of ATP compared with the S-Cas group

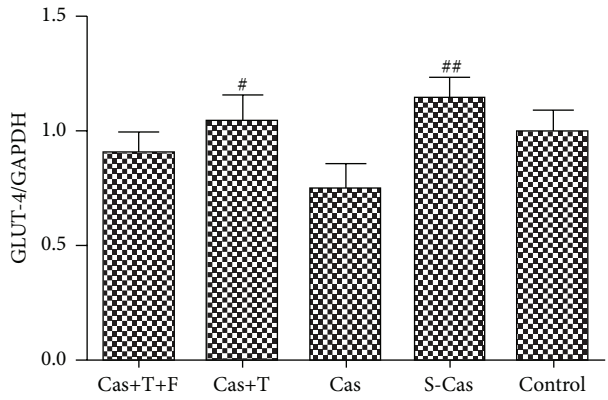

Figure 4: Effects of castration and testosterone on the mRNA expression of GLUT-4. Values are means ± SD; $n = 3$. S-Cas: sham-castration; Cas: castration; T: testosterone; F: flutamide. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus Cas.

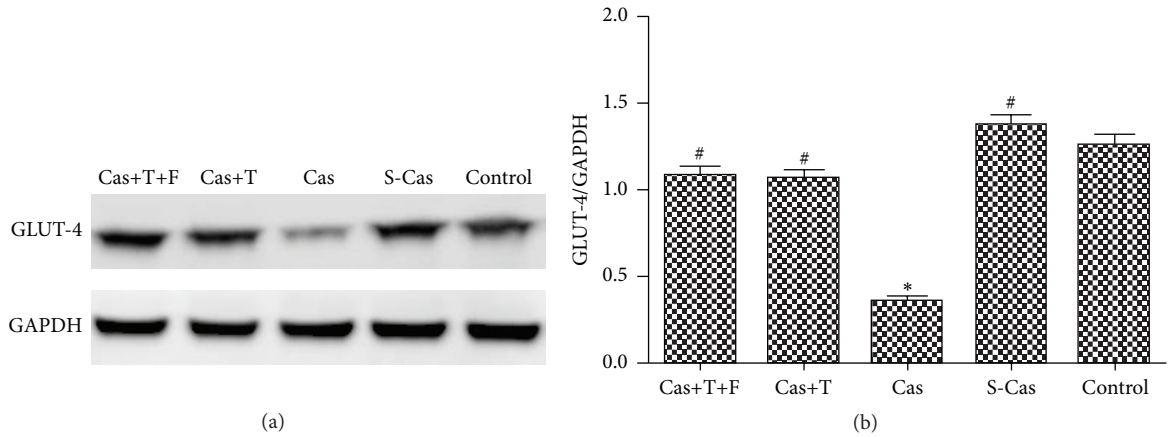

Figure 5: Effects of castration and testosterone on the protein expression of GLUT-4. (a) Western blotting result for GLUT-4 protein level. (b) Quantitation of GLUT-4 protein level. Values are means ± SD; $n = 3$. S-Cas: sham-castration; Cas: castration; T: testosterone; F: flutamide. $^{*}P < 0.001$ versus control group; $^{\#}P < 0.001$ versus Cas.

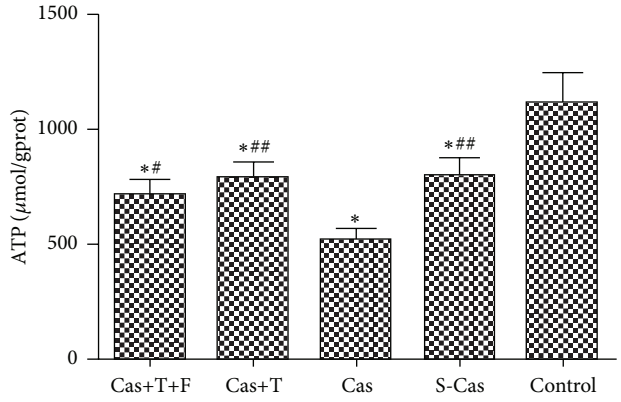

Figure 6: Comparison of ATP concentration in different groups. Values are means ± SD; $n = 6$. S-Cas: sham-castration; Cas: castration; T: testosterone; F: flutamide. $^{*}P < 0.001$ versus control group; $^{\#}P < 0.01$ and $^{\#\#}P < 0.001$ versus Cas.

(514.96 ± 56.96 versus 794.80 ± 82.97 μmol/gprot, $P < 0.001$). With the treatment of testosterone, the levels of ATP were restored, compared with the castrated rats (783.81 ± 76.22 versus 514.96 ± 56.96 μmol/gprot, $P < 0.001$). Additional flutamide treatment decreased ATP levels compared with the testosterone group but without statistical significance (715.04 ± 67.57 versus 783.81 ± 76.22 μmol/gprot, $P > 0.05$).

3.5. Effects of Testosterone on Cardiac Function. Cardiac function was evaluated by echocardiography at 14 days after

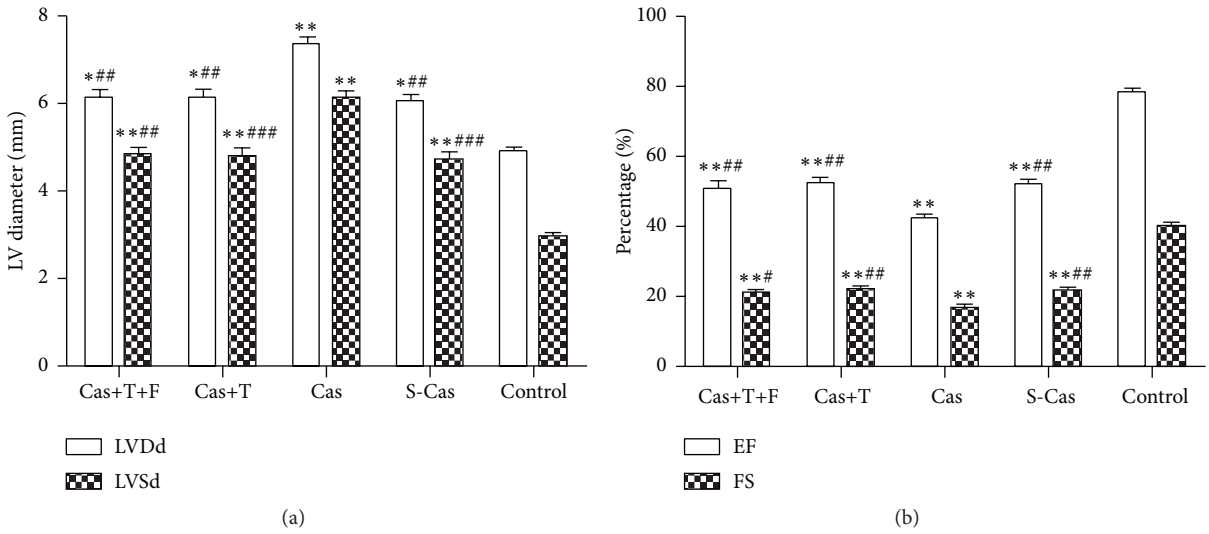

Figure 7: Echocardiography results 14 days after ligation. (a) Left ventricular end-diastolic diameter (LVDd) and left ventricular end-systolic diameter (LVSd) measurements for all groups. (b) Ejection fraction (EF) and fractional shortening (FS) results for all groups. Values are means ± SD; $n = 4$. S-Cas: sham-castration; Cas: castration; T: testosterone; F: flutamide. $^{*}P < 0.01$ and $^{**}P < 0.001$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus Cas.

ligation (Figure 7). The left ventricular end-diastolic and end-systolic diameters of myocardial infarction hearts were higher than those of the control group ($P < 0.01$), indicating cardiac dilation, whereas the EF and FS were decreased in myocardial infarction hearts ($P < 0.001$), suggesting impaired cardiac function. Castration aggravated the impairment in cardiac function, further reducing EF (42.22 ± 2.29% versus 51.98 ± 2.95%, $P < 0.01$) and FS (16.72 ± 1.09% versus 21.71 ± 1.61%, $P < 0.01$) and increasing LVDd (7.32 ± 0.35 versus 6.02 ± 0.32 mm, $P < 0.01$) and LVSd (6.10 ± 0.34 versus 4.72 ± 0.33 mm, $P < 0.001$), compared with the S-Cas group. Testosterone replacement decreased the LVDd to 6.09 ± 0.43 mm ($P < 0.01$) and LVSd to 4.76 ± 0.42 mm ($P < 0.001$) and improved myocardial performance, as indicated by the increase in EF (52.41 ± 3.00% versus 42.22 ± 2.29%, $P < 0.01$) and FS (21.95 ± 1.65% versus 16.72 ± 1.09%, $P < 0.01$) values, compared with the castrated rats. The differences in these parameters between the Cas+T+F group and the Cas+T group did not reach statistical significance (all $P > 0.05$).

3.6. Effects of Testosterone on Myocardial Apoptosis and Fibrosis. The results of TUNEL staining in the different groups were shown in Figure 8(g). The number of TUNEL-positive nuclei was higher in the S-Cas group than in the control group (31.63 ± 2.29% versus 10.68 ± 0.93%, $P < 0.001$), and castration exacerbated myocardial apoptosis compared to the S-Cas group ($P < 0.001$). Testosterone treatment significantly inhibited apoptosis with a lower percent of apoptotic cells than that of the castrated group (35.10 ± 3.52% versus 51.59 ± 5.45%, $P < 0.01$).

Masson-Trichrome staining was used to estimate the degree of myocardial fibrosis of all groups in Figures 8(a)–8(f). The area of fibrosis was significantly higher in S-Cas rats than in control animals (10.29 ± 1.47% versus 1.25 ± 0.14%, $P < 0.001$), and castration aggravated myocardial fibrosis ($P < 0.01$). Testosterone replacement attenuated the degree of fibrosis to 10.72 ± 1.51%, as compared to the castrated group of 15.63 ± 1.63% ($P < 0.01$). Flutamide had no effect on myocardial apoptosis and fibrosis ($P > 0.05$). The results indicated that testosterone plays a protective effect against myocardial apoptosis and fibrosis.

3.7. Serum Testosterone and Estrogen Concentration. As shown in Figure 9(a), castration significantly decreased the testosterone levels (Cas versus S-Cas, $P < 0.001$), whereas testosterone replacement restored the serum levels of testosterone compared with the castrated rats (5.36 ± 0.43 versus 3.48 ± 0.25 ng/mL, $P < 0.001$). There were no statistically significant differences in serum testosterone levels between the control group, the Cas+T group, and the Cas+T+F group.

The average serum 17β-estradiol concentrations were comparable between the groups (Figure 9(b)). These results suggested that estrogen would exert a similar effect on each experimental group.

4. Discussion

In the present study, the effects of testosterone on cardiac metabolism in the ischemic heart were investigated using a rat model of myocardial infarction. Our results showed that castration decreased the levels of PPARα and inhibited downstream signaling, downregulating the expression of fatty acid and glucose metabolism related genes. Castration reduced the concentration of ATP and increased cardiomyocyte apoptosis and cardiac fibrosis, aggravating cardiac dysfunction associated with myocardial infarction. Testosterone therapy reversed these unfavorable outcomes.

Alterations in cardiac metabolism play a key role in the pathogenesis and progression of myocardial ischemia and heart failure [25]. These metabolic alterations, which

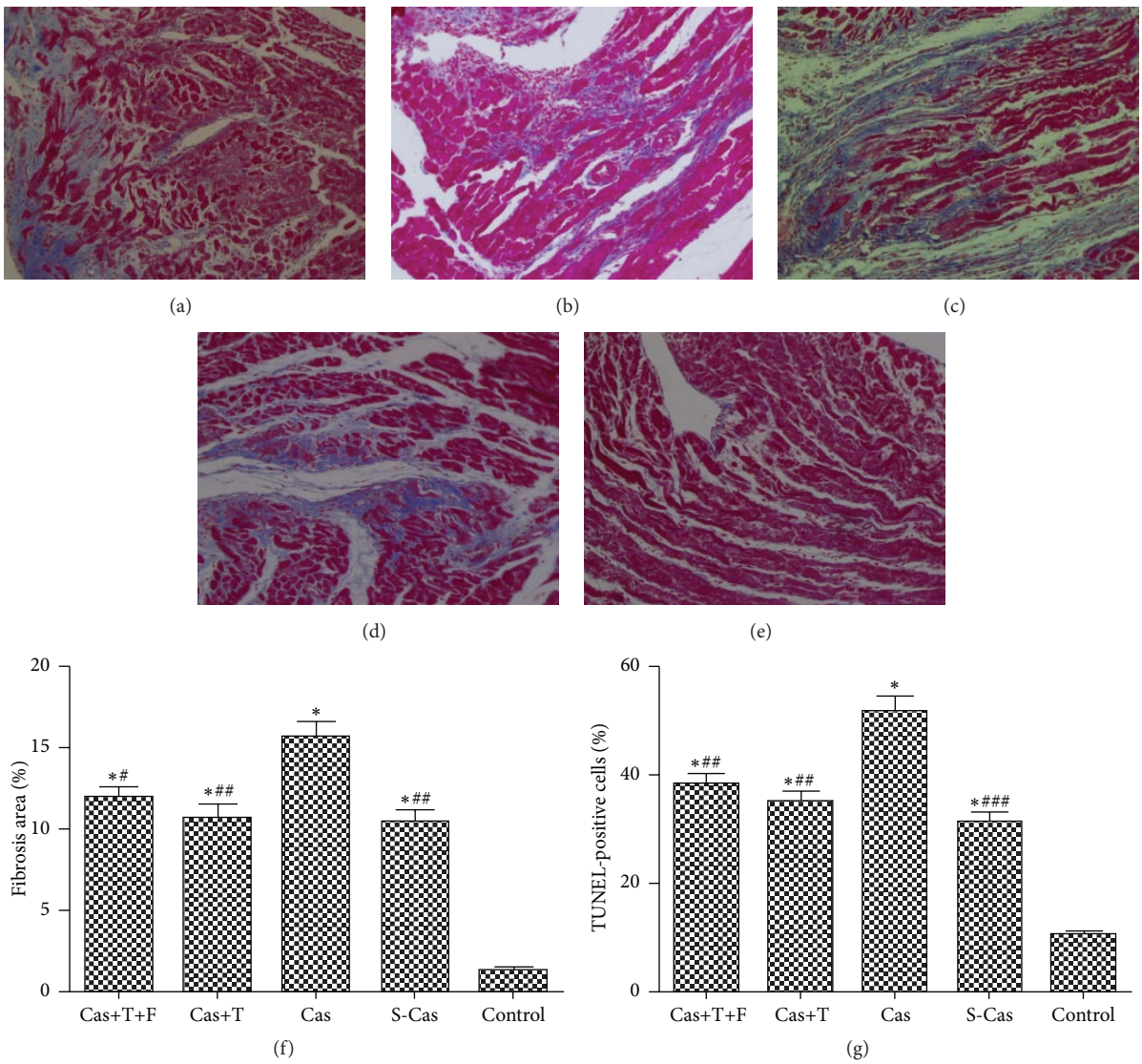

Figure 8: Effects of castration and testosterone replacement on myocardial fibrosis and apoptosis. Representative magnification (200x) of Masson-Trichrome sections of the heart. (a) Cas+T+F group, (b) Cas+T group, (c) Cas group, (d) S-Cas group, (e) control group, and (f) quantitative analysis of fibrotic area. (g) The TUNEL-positive cells percent expressed as a percent of normal nuclei. Values are means ± SD; $n = 3$. S-Cas: sham-castration; Cas: castration; T: testosterone; F: flutamide. $^{*}P < 0.001$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus Cas.

are termed metabolic remodeling, include a shift from fatty acids to glucose as the preferred energy substrate, decreased oxidative phosphorylation, and impaired energy transfer, leading to ATP deficiency and subsequent contractile dysfunction. Because of the close association of myocardial function with energy metabolism, metabolic pathways are potential therapeutic targets for the treatment of cardiac dysfunction [26]. During the early stages of cardiac remodeling, the myocardial energy source switches from fatty acids to glucose. A reduction in cardiac fatty acid metabolism, including the downregulation of fatty acid transporters and oxidative enzymes, has been reported in rat models of myocardial infarction-induced systolic dysfunction; however, myocardial infarction-induced alterations in cardiac glucose metabolism remain increased [3–5]. Despite the higher efficiency of glucose metabolism compared with that of fatty acids, the increase in ATP yield could not be sufficient to make up for the ATP deficiency, aggravating the progression of heart failure [27]. Lou et al. demonstrated that boosting fatty acid oxidation not glucose could enhance the energy production of infarct-remodeled rat hearts after conditioning against ischemia/reperfusion injury, which may promote postischemic contractile recovery [28]. Thus, reversal of metabolic shift may be beneficial for improving postischemic contractile dysfunction [29].

The transcription factor PPARα plays an important role in the modulation of cardiac metabolism by optimizing substrate selection. Reduced activity of PPARα results in

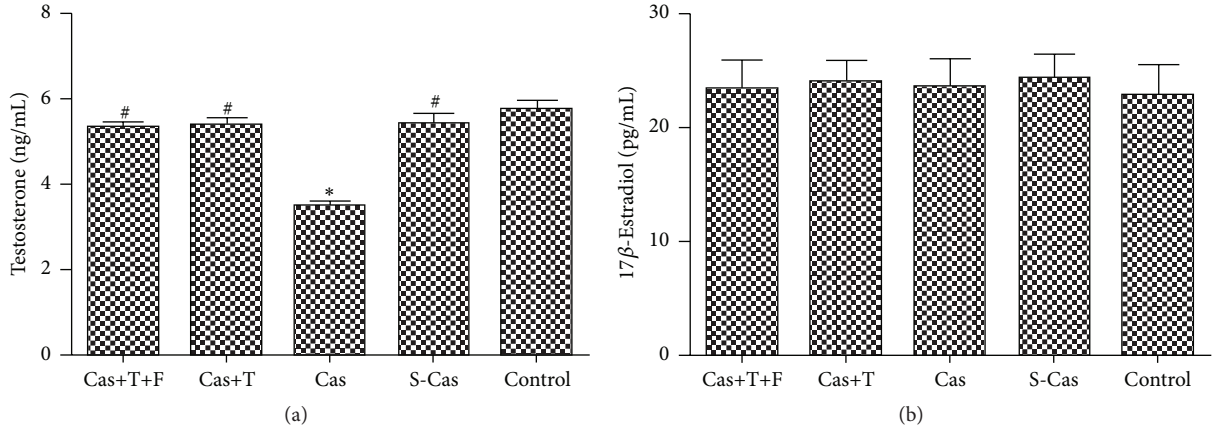

FIGURE 9: Serum testosterone levels (a) and 17β-estradiol levels (b). Values are means ± SD; $n = 6$. S-Cas: sham-castration; Cas: castration; T: testosterone; F: flutamide. $^{*}P < 0.001$ versus control group; $^{\#}P < 0.001$ versus Cas.

downregulation of the expression of genes involved in fatty acid transport and metabolism [30]. Meanwhile, PPARα KO mice exhibited reliance on glucose for cardiac ATP production with increased glucose uptake and GLUT4 expression [31, 32]. Therefore, modulation of PPARα activation has been proposed as a therapeutic approach to improve myocardial function [33]. PPARα is downregulated in response to cardiac hypertrophy [34], myocardial infarction [35], and heart failure [36] associated with the decrease in fatty acid utilization. The results of the present study showed that castration decreased the mRNA and protein expression of PPARα in the ischemic myocardium and this effect was reversed by testosterone replacement therapy. Our results also showed that genes related to fatty acid uptake and oxidation were downregulated in myocardial infarction rats compared with control rats. The expression of fatty acid metabolism genes, including CD36, mCPT-1, and MCAD, was further downregulated by castration, whereas expression levels were restored by testosterone. Besides, testosterone replacement restored ATP levels in the castrated rat after myocardial infarction. These results suggested that testosterone could enhance fatty acid metabolism to increase ATP generation for the ischemic heart by upregulating PPARα. We have demonstrated that testosterone could protect mitochondria in the postinfarct myocardium and attenuates a decrease in ATP levels and cardiomyocyte apoptosis [24]. In line with our findings, chronic activation of PPARα upregulates the fatty acid metabolic pathway despite the accumulation of myocardial triglycerides without worsening left ventricular dysfunction in a rat infarct model of heart failure [37].

Although Collett et al. suggested that PPARα is an androgen-negative gene in the human prostate [38], another study showed that the adrenal androgen dehydroepiandrosterone could induce peroxisome proliferative response in rats, probably by the androgen-mediated increase in PPARα [39]. In the present study, we also demonstrated that testosterone could upregulate PPARα expression. However, the effects of testosterone on PPARα and the expression of downstream fatty acid metabolism genes could not be antagonized by flutamide. Flutamide, an antagonist of the AR, could block the effects induced by testosterone. The above-mentioned studies indicated that testosterone could modulate PPARα expression through AR-independent mechanism. We have proved that testosterone can partly via the AMP-activated protein kinase- (AMPK-) peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) pathway protect against mitochondrial dysfunction and cardiomyocyte apoptosis in the postinfarct myocardium [24]. PGC-1α can bind to the heterodimers formed by PPARα and retinoic acid-activated receptor (RXR) and then coactivate PPARα to enhance fatty acid utilization in myocardium [6]. Moreover, AMPK activator can upregulate PPARα signaling pathway to inhibit cardiac hypertrophy [40]. However, Tennakoon et al. demonstrate that androgens regulate prostate cancer cell growth via AR-AMPK-PGC-1α signaling to promote mitochondrial biogenesis and induce metabolic switch [41]. Therefore, we should further investigate the interaction among AR, PPARα, and RXR and elucidate the exact mechanisms of testosterone modulating metabolic remodeling in postinfarcted heart.

The effects of testosterone can be mediated by the conversion of testosterone to estrogen by the enzyme aromatase. Therefore, we measured serum estradiol concentrations to exclude its influence. The lack of differences in serum 17β-estradiol levels among the groups implied that the effects of estrogen would be similar in each group.

MHC-PPARα mice, which are characterized by cardiac-specific PPARα overexpression, show decreased glucose transport [42, 43]. However, in the present study, testosterone increased the expression of PPARα as well as GLUT-4. The effect of testosterone on GLUT-4 mRNA levels may be mediated by AMPK. This was supported by a previous study showing that testosterone increased GLUT4-dependent glucose uptake, which was mediated by Ca^{2+}/calmodulin protein kinase and AMPK in cultured cardiomyocytes [44]. To elucidate the mechanism by which testosterone modulates glucose metabolism in the ischemic heart, future studies will be aimed at investigating the effect of testosterone on insulin signaling and glucose oxidation, as well as other glucose metabolism pathways, such as glycolysis and the pentose phosphate pathway.

In conclusion, the present study showed that testosterone insufficiency downregulated PPARα and altered the mRNA expression of fatty acid metabolism and glucose transport related genes, impairing ATP production in the ischemic myocardium. Testosterone replacement therapy reversed these unfavorable changes and improved cardiac metabolic remodeling and cardiac dysfunction. These data provide new application of testosterone in cardiovascular diseases.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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Clinical Study

Pioglitazone Attenuates Drug-Eluting Stent-Induced Proinflammatory State in Patients by Blocking Ubiquitination of PPAR

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The inflammatory response after polymer-based drug-eluting stent (DES) placement has recently emerged as a major concern. The biologic roles of peroxisome proliferator-activated receptor-γ (PPAR-γ) activators thiazolidinedione (TZD) remain controversial in cardiovascular disease. Herein, we investigated the antiinflammatory effects of pioglitazone (PIO) on circulating peripheral blood mononuclear cells (MNCs) in patients after coronary DES implantation. *Methods and Results*. Twenty-eight patients with coronary artery disease and who underwent DES implantations were randomly assigned to pioglitazone (30 mg/d; PIO) or placebo (control; Con) treatment in addition to optimal standard therapy. After 12 weeks of treatment, plasma concentrations of high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and matrix metalloproteinase-9 (MMP-9) were significantly decreased in PIO group compared to the Con group ($P = 0.035$, 0.011, 0.008, and 0.012, resp.). DES-induced mRNA expressions of IL-6, TNF-α, and MMP-9 in circulating MNC were significantly blocked by PIO ($P = 0.031$, 0.012, and 0.007, resp.). In addition, PIO markedly inhibited DES-enhanced NF-κB function and DES-blocked PPAR-γ activity. Mechanically, DES induced PPAR-γ ubiquitination and degradation in protein level, which can be totally reversed by PIO. *Conclusion*. PIO treatment attenuated DES-induced PPAR loss, NF-κB activation, and proinflammation, indicating that PIO may have a novel direct protective role in modulating proinflammation in DES era.

1. Introduction

Polymer-based drug-eluting (everolimus, zotarolimus, sirolimus, paclitaxel, etc.) stents (DES) have been known as a standard treatment for coronary artery diseases (CAD) undergoing percutaneous coronary angioplasties. Although DES has demonstrated efficacy and safety in clinical studies, human pathological data have raised concerns about the long-term healing and the potential for local inflammatory reactions [1, 2]. Also, it has been reported that DES induced hypersensitivity reactions with interacting lymphocytes, macrophages, multinucleated giant cells, and eosinophils and pervasive inflammation throughout the stented arterial segment by autopsy [3]. DES-induced inflammatory reaction has been found as early as 30 days after implantation but progressed in frequency and severity through 90 to 180 days in porcine model [1]. Furthermore, our previous studies found that DES implantation induced specific systematic inflammatory state, as evidenced by the enhanced NF-κB activity, suppressed PPAR-γ activity, and elevated plasma inflammatory factors, compared with no-stent implantation or bare metal stent (BMS) implantation [4, 5]. However, how to refine DES-induced proinflammation remains unknown.

In vascular wall and atherosclerosis, PPAR-γ is expressed in macrophages, T cells, endothelial cells, and vascular smooth muscle cells [6, 7]. Recent data have shown that synthetic antidiabetic thiazolidinediones (TZDs), which are known as PPAR-γ activators, inhibit inflammatory cytokine

Table 1: Baseline characteristics of the study population.

Parameters	Placebo ($n = 14$)	Pioglitazone ($n = 14$)	P value
Sex, M/F, n/n	11/3	12/2	1.00
Age, yr	55.5 ± 10.9	56.2 ± 10.4	0.74
Body mass index, kg/m^2	23.7 ± 4.9	24.5 ± 2.9	0.58
Systolic BP, mm Hg	124 ± 19	121 ± 16	0.72
Diastolic BP, mm Hg	78 ± 10	76 ± 10	0.55
Smoking	9	12	0.39
Fasting glucose, mmol/L	5.28 ± 0.57	5.32 ± 0.37	0.86
HbA1c, %	5.42 ± 0.59	5.18 ± 0.49	0.25
Total cholesterol, mmol/L	3.78 ± 0.76	3.61 ± 0.45	0.48
Triglycerides, mmol/L	1.75 ± 1.27	1.81 ± 1.23	0.89
HDL cholesterol, mmol/L	0.90 ± 0.17	0.88 ± 0.20	0.74
LDL cholesterol, mmol/L	2.35 ± 0.67	2.15 ± 0.49	0.34
Gensini score	12.6 ± 8.8	10.9 ± 5.1	0.54
hsCRP, mg/L	4.0 (0.8, 11.8)	2.9 (1.3, 12.3)	0.58
Treatment after stenting			
Aspirin	14	14	1.00
Clopidogrel	14	14	1.00
Blocker	12	14	0.48
ACE inhibitors/ARBs	14	14	1.00
Statins	14	14	1.00

Data are reported as mean ± SD, median (interquartile range), or *n*. BP, blood pressure; hs-CRP, high sensitivity C-reactive protein; ACE, angiotensin-converting enzyme; ARB, angiotensin II type 1 receptor blocker.

production by cells of monocyte/macrophage lineage [8]. These activators inhibit gene expression in part by antagonizing the activities of transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) [9, 10]. Several animal studies have demonstrated the antiatherogenic effects of TZDs [11, 12]. Moreover, pioglitazone (PIO) significantly decreased the occurrence of all-cause mortality, nonfatal myocardial infarction, and stroke in diabetic populations in the Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROactive) [13] and in a meta-analysis study [14]. However, a meta-analysis of rosiglitazone trials in diabetic patients showed that rosiglitazone was associated with increased myocardial infarction and cardiovascular death [15]. In an animal study, PIO was found to increase macrophage apoptosis and plaque necrosis in advanced lesions in LDLr-deficient mice [16]. Another animal study found that pioglitazone could induce excessive hepatic triglyceride accumulation and increase the plasma cholesterol [17].

These conflicting and controversial findings highlight the uncertainty regarding the effectiveness of using TZDs to treat atherothrombotic disease. Whether PIO have atheroprotective effects in DES-treated CAD patients remains unknown. Here, we performed a random single-blind placebo controlled clinical study to investigate whether PIO have anti-inflammatory effects in DES-implanted coronary artery disease patients.

2. Methods

2.1. Patients and Study Design. All subjects were consecutively recruited from the First Affiliated Hospital of Xi'an Jiaotong University. 28 nondiabetic patients with coronary artery disease (CAD) and who underwent DES implantations were randomized into two groups: placebo group (Con, $n = 14$) and pioglitazone group (PIO, $n = 14$). The nondiabetic state was determined by a negative history of diabetes mellitus, no treatment with antidiabetic drugs, and assessment of fasting blood glucose and oral glucose tolerance test (OGTT). The diagnosis of CAD was in accorded with the WHO definition. The exclusion criteria included clinical evidence of acute inflammation, tumor and rheumatic condition checked by the elevated CRP and ESR, liver and renal diseases, severe heart failure (NYHA class $\geq$ II), ejection fraction (EF) $< 50\%$, contraindications to treatment with pioglitazone, and patients who were given immunosuppressants. Table 1 summarized all subjects' demographic data. Study medication (30 mg/d) for 12 weeks was given in addition to optimal standard treatment, including aspirin, clopidogrel, β receptor blockers, angiotensin converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs), and statins. We followed up the patients twice after 4 and 12 weeks. Pioglitazone and placebo were provided by Zhongmei Huadong Pharmaceutical Co., Ltd. (Hangzhou, China).

Table 2: Metabolic and other parameters at baseline and after 12 weeks.

Parameters	Placebo controls		Pioglitazone	
	0 wk	12 wk	0 wk	12 wk
Body mass index, kg/m^2	23.7 ± 4.9	23.3 ± 3.3	24.5 ± 2.9	24.7 ± 2.5
Weight, kg	65.4 ± 12.7	66.7 ± 12.7	68.8 ± 9.4	69.3 ± 8.3
Waist to hip ratio, %	89.1 ± 5.8	89.3 ± 5.6	89.9 ± 5.9	90.2 ± 5.5
Systolic BP, mm Hg	124 ± 19	126 ± 13	121 ± 16	121 ± 15
Diastolic BP, mm Hg	78 ± 10	77 ± 8	76 ± 10	73 ± 9
Fasting glucose, mmol/L	5.28 ± 0.57	5.30 ± 0.49	5.32 ± 0.37	5.31 ± 0.45
Fasting insulin, U/L	6.73 ± 3.98	6.10 ± 4.02	6.79 ± 4.44	5.21 ± 3.62
HbA1c, %	5.42 ± 0.59	5.72 ± 0.46	5.18 ± 0.49	5.79 ± 0.35
Total cholesterol, mmol/L	3.78 ± 0.76	3.12 ± 0.74**	3.61 ± 0.45	3.01 ± 0.51**
Triglycerides, mmol/L	1.75 ± 1.27	1.38 ± 0.91*	1.81 ± 1.23	1.14 ± 0.39*
HDL cholesterol, mmol/L	0.90 ± 0.17	1.16 ± 0.41*	0.88 ± 0.20	1.27 ± 0.41**
LDL cholesterol, mmol/L	2.35 ± 0.67	1.67 ± 0.40**	2.15 ± 0.49	1.69 ± 0.40*
hsCRP, mg/L	4.0 (0.9, 11.8)	2.2 (0.8, 6.8)**	2.9 (1.3, 12.3)	1.0 (0.5, 3.8)$^{**\#}$
NT-pro BNP, pg/mL	220 (79, 839)	231 (140, 664)	259 (73, 706)	260 (102, 703)
EF, %	61.3 ± 9.9	62.7 ± 9.5	60.4 ± 8.1	60.6 ± 7.4

$n = 14$ for each group. Values are mean ± SD or median (interquartile range).
$^{*}P < 0.05$ and $^{**}P < 0.01$ compared with baseline, and $^{\#}P < 0.01$, compared with the placebo group.

We obtained each patient's medical and family history and general information such as smoking and drinking by medical history interview. This study complies with the Declaration of Helsinki, and the research protocol has been approved by the Ethics Committee of Xi'an Jiaotong University. The informed consents were obtained from the subjects.

2.2. Mononuclear Cell (MNC) Isolation. Peripheral blood MNC samples before medication and at the end of the 12th week after DES stent implantation were collected. Peripheral blood MNC samples were isolated by Ficoll standard density gradient centrifugation. The upper layer containing MNC was harvested and washed with Hanks' balanced salt solution and then with PBS.

2.3. Plasma Concentrations of Proinflammatory Cytokines. The concentrations of plasma interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and matrix metalloproteinase-9 (MMP-9) were assayed by ELISA. ELISA was performed by adding 100 μL or 200 μL of each sample to wells in a 96-well plate of a commercially available ELISA Kit (ExCell, Shanghai) according to the manufacturer's instructions. High-sensitivity C-reactive protein (hs-CRP) assays were performed by our hospital's clinical centre laboratory.

2.4. Total RNA Isolation and mRNA Detection. Total RNA was extracted from MNC with the RNAfast kit (Fastgen) according to manufacturer's protocol, and real-time reverse transcription-polymerase chain reaction was performed as previous report [4].

2.5. NF-κB and PPAR-γ DNA Binding Activity. Nuclear proteins were extracted according to the manufacturer's instructions (Pierce). The NF-κB and PPAR-γ DNA binding activity were measured with NF-κB p65/p50 and PPAR-γ transcription factor assay kit (Abcam) according to the manufacturers' instructions.

2.6. Western Blotting. Polyclonal or monoclonal antibodies (Santa Cruz Biotechnology) were used. Densitometry was performed with the Bio-Rad molecular analyst software, and all values were corrected by loading with Gapdh.

2.7. Statistical Analysis. Discrete variables were expressed as numbers and percentages and compared by the χ^2 test. Summary values are expressed as mean ± SE. Skewed data were reported as median (interquartile range). Analysis of the changes from baseline was performed by paired t-test. Holm-Sidak two-way repeated-measures ANOVA (TWR-MANOVA) method was used for all multiple comparisons between the Con and PIO groups as previous reports [4, 5]. Statistical significance was assumed at the 5% α-error level ($P < 0.05$).

3. Results

3.1. General Clinical Data. All 28 patients fulfilled the 12 weeks' follow-up without any drug related side effects. The characteristics of patients were shown in Table 1. There were no differences between the PIO and placebo groups with respect to baseline characteristics. After 12 weeks of treatment, there was no significant change in body mass index (BMI), weight, waist to hip ratio, blood pressure (SBP or DBP), fasting glucose and insulin, NT-pro BNP, and EF of the two groups compared to baseline (Table 2). Considering the unchanged BMI, weight, waist to hip ratio, NT-pro BNP, and EF between two groups, there was no evidence of PIO

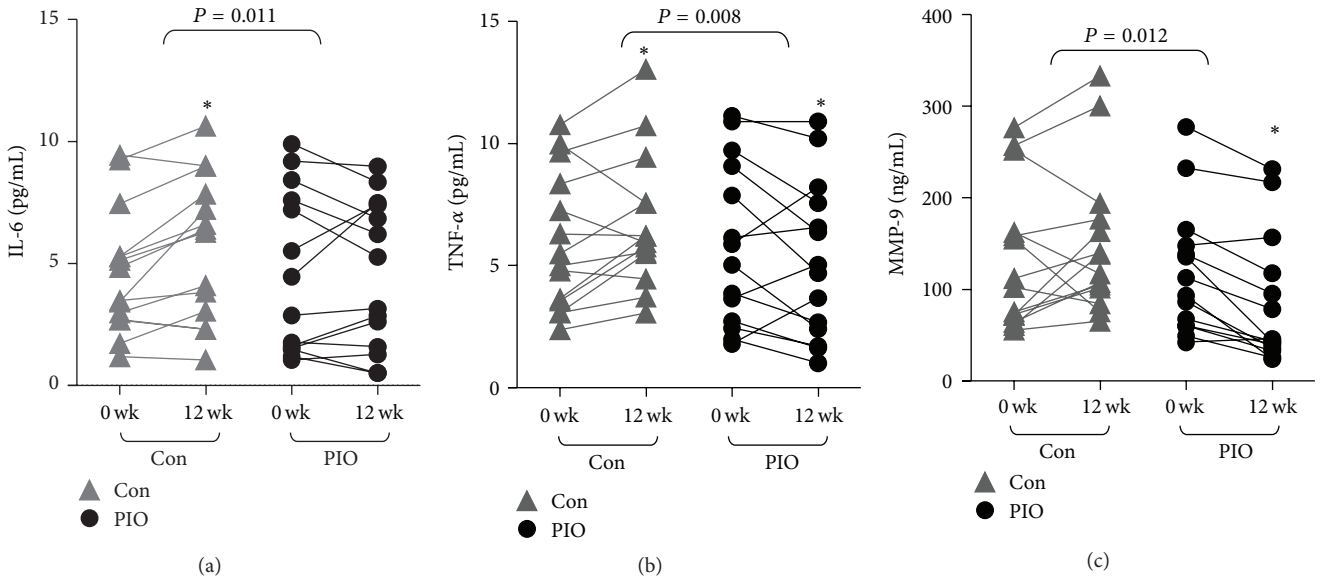

Figure 1: Pioglitazone decreased plasma proinflammatory factors concentrations in patients after coronary DES implantation. The results are presented as raw data. Compared to the control group, PIO significantly reduced plasma IL-6, TNF-α, and MMP-9 concentrations by TWRMANOVA ($P = 0.011$, 0.008, 0.002, and 0.012, resp.). $^{*}P < 0.05$ compared with baseline.

increasing the occurrence of heart failure in the present study. Total cholesterol, triglycerides, and LDL were reduced significantly after medication; however, no disparities of the reduction between two groups were observed.

3.2. Pioglitazone Reduced the Plasma Concentrations of Proinflammatory Cytokines. In the control group, plasma concentrations of IL-6, TNF-α, and MMP-9 were increased by 138 ± 16%, 136 ± 15%, and 110 ± 7%, respectively, compared with the baseline level at 12 wk (paired t-test, $P = 0.033$, 0.041, and 0.142, resp., Figures 1(a)–1(c)), indicating DES-related proinflammatory status. In the PIO treatment group, plasma concentrations of IL-6, TNF-α, and MMP-9 were decreased by 89 ± 8%, 64 ± 16%, and 86 ± 6% compared with the baseline level at 12 wk (paired t-test, $P = 0.204$, 0.041, and 0.037, resp., Figures 1(a)–1(c)). Compared to the control group, PIO treatment significantly reduced the plasma IL-6, TNF-α, and MMP-9 concentrations (TWRMANOVA, $P = 0.011$, 0.008, and 0.012, resp.). Moreover, hs-CRP in both Con and PIO treatment groups were decreased significantly and more drastically in PIO treatment group (TWRMANOVA, $P = 0.035$, Table 2).

3.3. Pioglitazone Downregulated the Expressions of Proinflammatory Factors in MNC. To examine whether the reduction of plasma proinflammatory cytokines by PIO treatment might be associated with downregulation of the mRNA expressions of proinflammatory cytokines in MNC, quantitative real-time PCR was used to calculate the mRNA expression. In the Con group, the mRNA expressions of IL-6, TNF-α, and MMP-9 at 12 weeks' follow-up were increased compared with the baseline (paired t-test, $P = 0.009$, 0.025, and 0.105, resp., Figures 2(a)–2(c)). Compared with the Con group, PIO treatment significantly reduced the mRNA expressions of IL-6, TNF-α, and MMP-9 (TWRMANOVA, $P = 0.031$, 0.012, and 0.007 resp., Figures 2(a)–2(c)), indicating that PIO attenuate DES-induced proinflammatory factors expression in MNC.

3.4. Pioglitazone Regulated the DES-Induced NF-κB/PPAR-γ Imbalance In Vivo. To evaluate whether pioglitazone interacts with the proinflammatory transcription factor NF-κB in MNC, we measured the nuclear NF-κB DNA binding activity in MNCs (Figure 2(a)). As depicted in Figure 2(d), DES enhanced the DNA binding activity of NF-κB ($P = 0.023$), which were attenuated by PIO (TWRMANOVA, $P = 0.017$).

Next, we detected the expressions of the p50 subunit and p65 subunit of NF-κB in MNCs. As shown in Figure 2(e), PIO treatment significantly reduced DES-induced p50 expression (TWRMANOVA, $P = 0.017$) but did not alter p65 expression.

Moreover, we found that PPAR-γ were dramatically decreased in the DES group ($P = 0.038$, Figure 3(a)), which were reversed by PIO treatment (TWRMANOVA, $P = 0.027$, Figure 3(a)), suggesting that pioglitazone regulated the DES-induced NF-κB/PPAR-γ imbalance in vivo.

3.5. Pioglitazone Regulated the DES-Induced PPAR-γ Ubiquitination and Degradation. To refine the changes of protein and mRNA expressions of PPAR-γ, Western blot and quantitative real-time PCR were performed. As shown in Figure 3(b), DES markedly decreased PPAR-γ protein level, which were blocked by PIO treatment (TWRMANOVA, $P = 0.031$, Figure 3(b)). We also found that PIO did not alter PPAR-γ mRNA expression, suggesting that PIO may regulate the protein stability in vivo. Furthermore, we detected the ubiquitination of PPAR-γ by Western blot. As depicted in

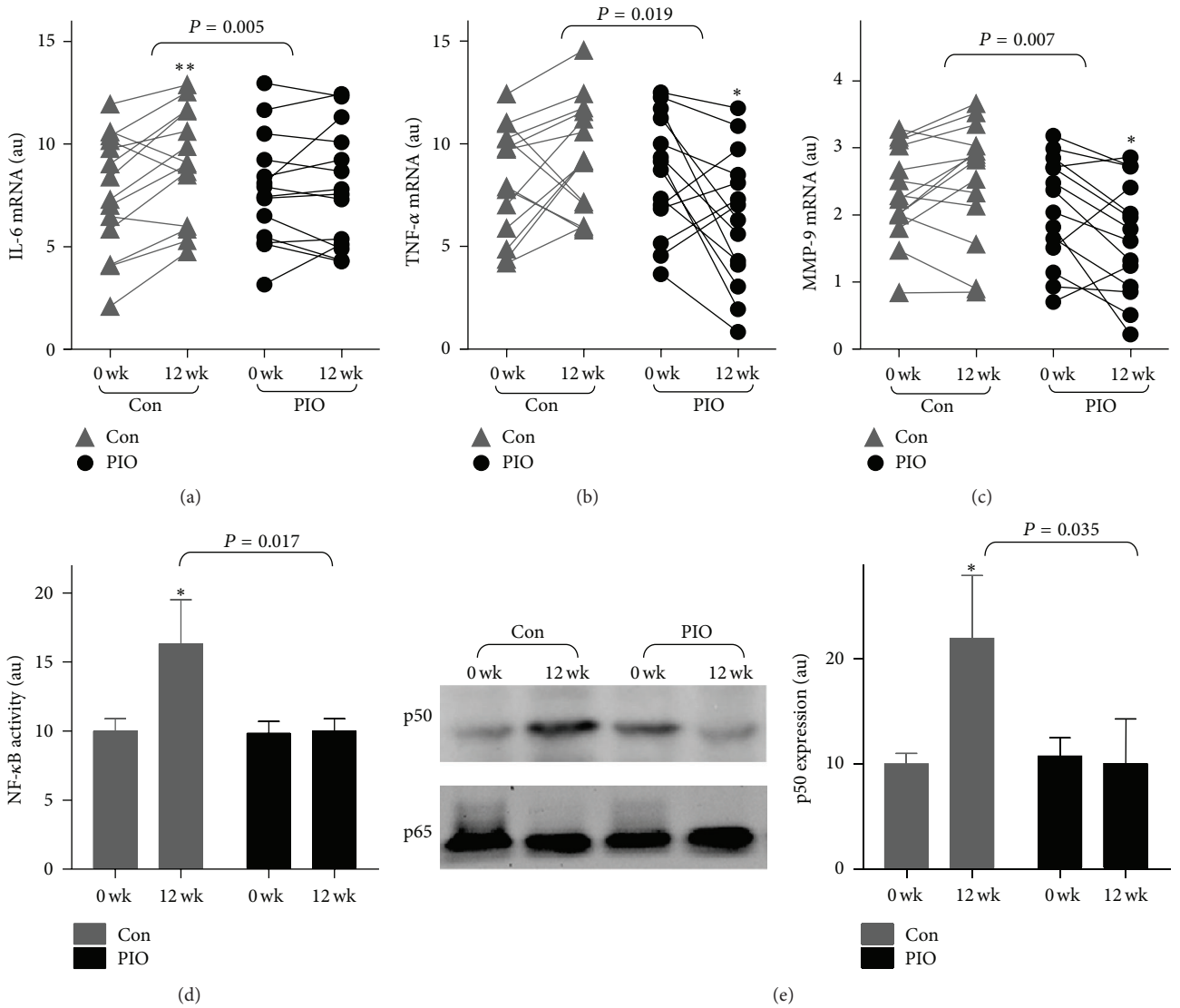

Figure 2: Pioglitazone downregulated the proinflammation in MNC. (a)–(c) Change in mRNA expression of peripheral blood mononuclear cells (MNC). The mRNA levels of IL-6, TNF-α, and MMP-9 were detected by quantitative real-time PCR. (d) NF-κB DNA binding activity was detected. (e) The expression of p50 subunit and p65 subunit in MNC. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with baseline.

Figure 3(d), DES significantly induced PPAR-γ ubiquitination ($P = 0.043$), which was blocked by PIO, implying that pioglitazone regulated the DES-induced PPAR-γ ubiquitination and degradation in vivo.

4. Discussion

The present study clearly showed that the circulating inflammatory responses were increased after the implantation of DES in the CAD patients treated with optimal drug combinations, which were blocked by addition of oral pioglitazone. PIO sequentially acts through PPAR-γ activation, NF-κB blockade, and inhibition of inflammatory cytokine expressions. These findings suggested that PIO may have a novel direct protective role in modulating the proinflammatory responses after coronary DES implantation in CAD patients, thus providing further optimizations of the drug therapy in those patients.

Vascular inflammation is recognized as the foundation mechanism of atherosclerosis, and proinflammatory mediators including IL-6, TNF-α, and MMP-9 play a pivotal role in atherosclerosis [18]. IL-6 and TNF-α are classic proinflammatory cytokines, which play key roles in vascular disease [19]. Excessive degradation and remodeling of the extracellular matrix, a promoter of the instability of plaques, are the major effect of matrix metalloproteinases (such as MMP-9) [20]. DESs left target vascular intima partially unendothelialized for a sustaining period [21], resulting in intensely inflammation-arousing vessel segments, which may facilitate the release of multiple proinflammatory factors into serum and then proinflammation.

It has been reported that PIO may exhibit several antiatherosclerotic effects through multiple mechanisms, including modulation of blood pressure, lipid concentrations, matrix remodeling activation of matrix proteases, and finally

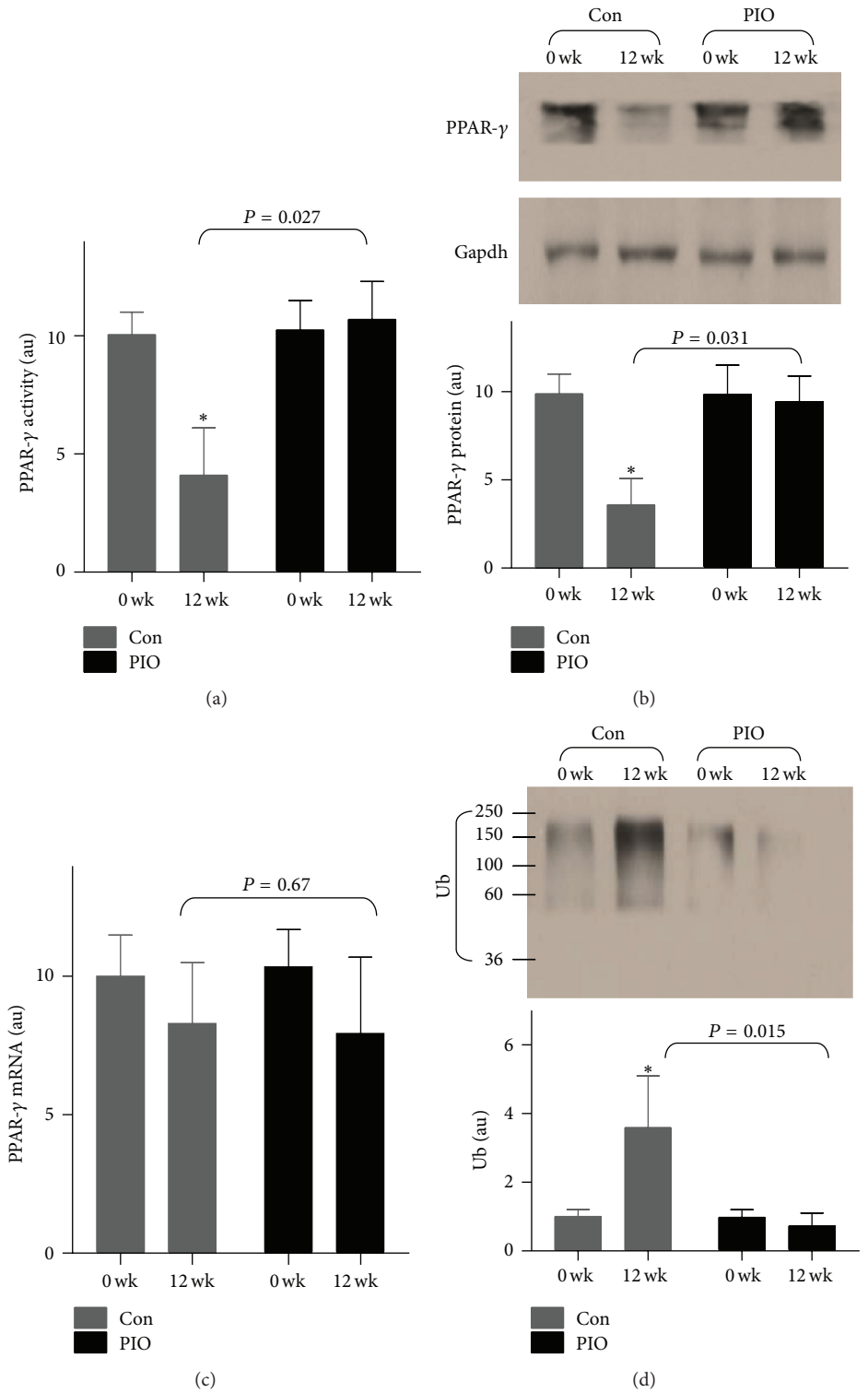

Figure 3: Pioglitazone blocked the DES-induced PPAR-γ ubiquitination and degradation in vivo. (a) PPAR-γ DNA binding activity was shown. (b) PPAR-γ protein was tested by Western blot. (c) The mRNA expression of PPAR-γ. (d) The ubiquitination of PPAR-γ was detected by coimmunoprecipitation assay. $^{*}P < 0.05$, compared with baseline.

induction of inflammation [22]. In the present study, we failed to observe a reduction of blood pressure, blood glucose, insulin, or HbA1c levels in both groups. Firstly, the unchangeable blood pressure and other metabolic parameters may be explained as follows: first, the blood pressure of all subjects was already under control by optimal treatment and the patients included in this study were all nondiabetics. Moreover, it has been reported that PIO treatment had no significant effect on the level of total cholesterol, LDL, and HDL. The improved lipid profile and hs-CRP are mainly attributed to the standard drug therapy.

Herein, we found a novel mechanism that PIO enhance PPAR-γ binding activity though inhibiting its ubiquitination and degradation, which may play a key role in PPAR function in vivo [23].

Taken together, our study demonstrated that PIO treatment attenuated the proinflammatory state in circulating MNCs; and our results suggest that PIO treatment may sequentially act through PPAR-γ activation, blocking of NF-κB activation, and inhibition of inflammatory cytokine expressions. These findings suggest that PIO may have a novel direct atheroprotective role by modulating the local and circulating proinflammatory responses in patients with coronary polymer-based drug-eluting stent implantation.

Competing Interests

The authors declare no competing interests.

Acknowledgments

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Review Article

Peroxisome Proliferator-Activated Receptor-γ Is Critical to Cardiac Fibrosis

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Peroxisome proliferator-activated receptor-γ (PPARγ) is a ligand-activated transcription factor belonging to the nuclear receptor superfamily, which plays a central role in regulating lipid and glucose metabolism. However, accumulating evidence demonstrates that PPARγ agonists have potential to reduce inflammation, influence the balance of immune cells, suppress oxidative stress, and improve endothelial function, which are all involved in the cellular and molecular mechanisms of cardiac fibrosis. Thus, in this review we discuss the role of PPARγ in various cardiovascular conditions associated with cardiac fibrosis, including diabetes mellitus, hypertension, myocardial infarction, heart failure, ischemia/reperfusion injury, atrial fibrillation, and several other cardiovascular disease (CVD) conditions, and summarize the developmental status of PPARγ agonists for the clinical management of CVD.

1. Introduction

Cardiac fibrosis is an inevitable process of varieties of cardiovascular diseases (CVDs) and is characterized by abnormal accumulation of extracellular matrix (ECM) in the myocardial interstitium. The ECM, composed of collagens, elastic fibers, glycosaminoglycan, and glycoproteins [1], are derived mainly from fibroblasts. Under physiological conditions, ECM is necessary to maintain the normal structure and function of the heart, the formation and degradation of ECM retain in dynamic balance, while in pathological conditions, because of excessive activation of renin-angiotensin-aldosterone system (RAAS), maladjustment of matrix metalloproteinases (MMP), and excessive secretion of some regulation cytokines such as transforming growth factor beta (TGFβ), the dynamic balance would be broken which resulted in ECM deposition and eventually cardiac fibrosis [2]. This pathological process is the beginning of cardiac remodeling and directly leads to arrhythmia [3], impaired cardiac function [4, 5] heart failure (HF), and even sudden cardiac death [6].

Although there are no effective strategies for treatment of cardiac fibrosis right now, it is firmly convinced that inhibition or reversion of myocardial fibrosis will be a promising way for prevention and treatment of HF in the nearby future [7]. Currently, the strategies for treatments of cardiac fibrosis mainly target RAAS system and inflammatory response; however, more and more other molecular mechanisms have been recognized to involve the regulation of cardiac fibrosis [8].

Interestingly, peroxisome proliferator-activated receptor-γ (PPARγ) has been identified to have the function of antimyocardial fibrosis [9–11]. According to published investigations, PPARγ has a wide spectrum of functions in regulating metabolism, attenuating inflammation, maintaining the balance of immune cells, inhibiting apoptosis and oxidative stress, and improving endothelial function [12]. All of these biological functions will be benefit for preventing the cardiac function from deterioration. However, the underling mechanisms of PPARγ in the regulation of cardiac fibrosis are not fully illustrated yet. This review will mainly summarize

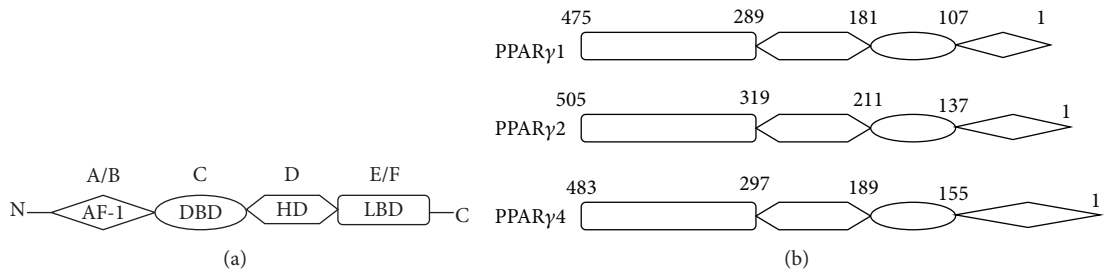

Figure 1: Schematic structure of peroxisome proliferator-activated receptor-γ and its protein isoforms. A/B, C, D, and E/F indicate the N-terminal A/B domain containing a ligand-independent AF-1, the DNA-binding domain, the hinge region, and the C-terminal LBD containing AF-2, respectively. AF-1 is responsible for phosphorylation, while AF-2 promotes the recruitment of coactivators for gene transcription. PPAR: peroxisome proliferator-activated receptor; AF: activation function; DBD: DNA-binding domain; HD: hinge domain; LBD: ligand-binding domain. Figure adapted from [13].

the reports about PPARγ and its agonist in the regulation of cardiac fibrosis.

2. Structure and Function of PPARγ

PPARs, belonging to the nuclear hormone receptor superfamily and consisting of three isoforms, PPARα, PPARβ/δ, and PPARγ, are ligand-inducible transcription factors. They are encoded by three separate genes and are distributed in different organs and tissues [14]. Because of the different expression and distribution profile, each of them presents unique biological function [14–16]. Activated by their specific ligands, PPARs can transfer into nucleus and form heterodimers with the retinoid X receptor. The heterodimeric complexes then band to the promoter region of target genes carrying peroxisome proliferator response elements (PPREs) and regulate transcription of target genes [17, 18]. Being similar to other nuclear receptors, PPAR isoforms possess five or six structural regions within four functional domains [14, 17, 19]. Activation function-1 motif (AF-1) locates at the N-terminal and is the target of phosphorylation kinase. The DNA-binding domain (DBD) consists of two highly conserved zinc finger motifs and is responsible for binding to PPRE. The hinge domain (BD) serves as a docking site for cofactors. The ligand bind domain (LBD) located at the C-terminal (E/F domain) is in charge of ligand specificity and activation of PPARs that bind to the PPRE, which increases target gene expression (Figure 1) [14, 17, 19].

The PPARγ gene is located on human chromosome 3p25 [20]. Seven transcripts have been identified, termed PPARγ1, PPARγ2, PPARγ3, PPARγ4, PPARγ5, PPARγ6, and PPARγ7 [17]. The PPARγ1, PPARγ3, PPARγ5, and PPARγ7 mRNA transcripts translate PPARγ1 protein and PPARγ2 mRNA yields PPARγ2 protein, while PPARγ4 and γ6 mRNA transcripts translate PPARγ4 protein [21–23]. Because of different transcript, translation, and tissue distribution, each protein has different biological functions in a variety of organs and cells (Table 1) [13]. So it is not a surprise that PPARγ plays important roles in CVDs including hypertension [17, 24, 25], atherosclerosis [26], HF [27], diabetic cardiomyopathy [11, 28], angiogenesis [29], valvular calcification [30], aortic aneurysm [31], restenosis following cardiovascular interventions [32], and ischemia/reperfusion (I/R) injury [33, 34].

Table 1: Tissue and cell distribution of PPARγ mRNA transcripts. Modified from [13].

PPARγ mRNA transcripts	Tissue and cell distribution
PPARγ1	Cardiac muscle, skeletal muscle, kidney, adrenal, spleen, intestine, pancreatic β-cells, and vascular smooth muscle cells
PPARγ2	Adipose tissue
PPARγ3	Adipose tissue, colon, and macrophages
PPARγ4	Macrophages
PPARγ5	Macrophages
PPARγ6	Macrophages and adipose tissue
PPARγ7	Macrophages and adipose tissue

3. PPARγ and Cardiac Fibrosis

The primary of activation of PPARγ is to lower serum glucose and improve the insulin sensitivity. In the clinical practice, the specific ligands of PPARγ have been accepted for treatment of diabetes mellitus. However, more and more researches had indicated that activation of PPARγ presents pleiotropic biological effects involving regulation of inflammation and energy metabolism. Because of its pleiotropic effects, PPARγ has been recognized as a target for the treatment of cardiac fibrosis. The characteristics of PPARγ regulate myocardial fibrosis in different CVDs as described below.

3.1. Diabetic Cardiomyopathy. The diabetic cardiomyopathy is accompanied by myocardial hypertrophy, dilated ventricular chamber, and fibrosis [49]. The specific PPARγ ligands, thiazolidinediones (TZDs), are used in clinical practice to improve insulin sensitivity in type 2 diabetes mellitus (T2DM). As shown in Table 2, evidences have demonstrated that TZDs could decrease myocardial fibrosis and improve cardiac dysfunction. In the animal experiment, Ihm and his colleagues found that the PPARγ ligand, rosiglitazone, significantly decreased myocardial fibrosis in the Otsuka Long-Evans Tokushima Fatty (OLETF) rats [35]. The underlying mechanism may be involved in the inhibiting nuclear factor-κB (NF-κB) activation in the myocardium. This biological function directly resulted in downregulation of receptor

Table 2: Effects of PPARγ ligands on diabetic related cardiac fibrosis.

Study model	Dose/duration/route	Major cardiac findings and conclusions	Ref.
Male OLETF rats, LETO rats, 20 weeks old	Rosiglitazone 20 mg/kg/d for 20 weeks, gavage	Suppression of RAGE and CTGF expression in the diabetic myocardium appears to contribute to the antifibrotic effect of rosiglitazone	[35]
Male STZ-induced diabetic Sprague-Dawley rats (200 ± 20 g)	Pioglitazone 10 mg/kg/d for 14 weeks, gavage	Activation of the PPARγ signal pathway could repress cardiac fibrosis in diabetic rats and partly improve cardiac remodeling and function by downregulating activity of RAS level	[36]
Male offspring of Wistar rats fed NP diet or LP diet, 3 months old	Rosiglitazone 5 mg/kg/d for three months, gavage	Rosiglitazone showed beneficial effects on rat offspring programmed by low protein diet during gestation decreasing cardiac fibrosis and enhancing myocardial vascularization	[37]
Alloxan-induced diabetic rabbits 1.8–2.5 Kg	Rosiglitazone 2 mg/kg/d for 4 weeks, unclear	Rosiglitazone attenuates arrhythmogenic atrial structural remodeling and atrial fibrillation promotion	[38]
Male OLETF rats, LETO rats, 20 weeks old	Pioglitazone 10 mg/kg/d for 20 weeks, per orem	Activation of PPARγ may decrease collagen concentration and reduce cardiac fibrosis by exerting regulatory effects on cardiac telomere biology	[39]
Male WT, $CBS^{+/+}$, $CBS^{+/-}$, and $Ins2^{+/-}/CBS^{+/-}$ rats, 20 weeks old	Ciglitazone 3 mg/kg/d for 4 weeks, orally	Treatment with ciglitazone alleviated MMP-9 activity and fibrosis and improved end diastolic diameter	[40]
Male OLETF rats, LETO rats, 28 weeks old	Rosiglitazone 3 mg/kg/d and losartan 5 mg/kg/d for 12 weeks, gavage	A combination of rosiglitazone and losartan attenuates myocardial fibrosis and dysfunction	[41]
Male diabetic hypertensive rats 179–219 g	Rosiglitazone 3 mg/kg/d or combination of felodipine 5 mg/kg/d for one month, orally	The combined treatment can improve dyslipidemia and decrease TNFα, TGFβ, collagen I, and collagen III, and increased MMP-2 but within a greater effect than treatment with rosiglitazone alone	[28]

OLETF: Otsuka Long-Evans Tokushima Fatty, LETO: Long-Evans Tokushima Otsuka, RAGE: receptor for advanced glycation end products, CTGF: connective tissue growth factor, WT: wild type, $CBS^{+/-}$: cystathionine beta synthase mutant, $Ins2^{+/-}$: insulin 2 mutant, MMP: matrix metalloproteinases, TNF: tumor necrosis factor, TGF: transforming growth factor, NP: normal protein (19% protein), LP: low protein (5% protein), STZ: streptozotocin, and RAS: renin-angiotensin system.

for advanced glycation end products and connective tissue growth factor (CTGF) expression [35], which have been convinced to play a key role in cardiac fibrosis [50, 51]. As we know, activation of RAAS may also lead to collagen deposition and result in cardiac fibrosis [2, 52]. Research has shown that pioglitazone activation of PPARγ can attenuate cardiac fibrosis in diabetic rats and partly ameliorates cardiac remodeling and function by suppressing activity of RAS [36]. The interesting finding is that rosiglitazone is able to decrease cardiac fibrosis and enhance myocardial vascularization in rat offspring programmed by low protein diet during gestation, which may be implicated in rosiglitazone administration which can decrease angiotensin (Ang) II and endothelin-(ET-) 1 and increase endothelial nitric oxide synthase (eNOS) [37]. Moreover, rosiglitazone reduces atrial interstitial fibrosis and AF promotion in the diabetic rabbits via modulating oxidative stress and inflammation [38]. The selective PPARγ, pioglitazone, could attenuate cardiac fibrosis and collagen concentration by upregulating insulin-like growth factor 1 (IGF-1), phosphorylated Akt, and eNOS in OLETF rats [39]. Furthermore, the PPARγ agonist ciglitazone may alleviate MMP-9 and fibrosis and improve end diastolic diameter in diabetic mice hearts [40]. Unfortunately, a recent study in the same animal model gave a negative conclusion that treatment with rosiglitazone had little cardioprotection and there is no indication for the regulation of NF-κB signaling pathway [41]. But the combination of rosiglitazone and losartan obviously attenuated the interstitial fibrosis and collagen deposition of the heart by inhibiting TGFβ and tumor necrosis factor-α (TNFα), along with the proinflammatory cytokines, interleukin- (IL-) 1β, and IL-6 [41]. Therefore, the authors declared that the benefit may be not derived from the activation of PPARγ. In addition, combination treatment with rosiglitazone and felodipine could improve the metabolic abnormalities and decrease TNFα, TGFβ, collagen I, and collagen III and increased MMP-2, while treatment with rosiglitazone alone had no effect on attenuating the hypertension and only exerted a minimal effect

on reducing cardiac fibrosis and improving dyslipidemia and hyperglycemia in diabetic hypertensive rats [28]. Thus, on one hand, whether the activation of PPARγ which could attenuate myocardial fibrosis remains unclear, the improving of cardiac function may not be related to the attenuation of cardiac fibrosis. On the other hand, the discrepancy results may partly be due to the dosage and length of observation time. Thirdly, the selective ligand, rosiglitazone, presents more discrepancy in the published data, so different structure of selective ligand may show different biological function. More investigations are needed to clarify these perplex.

It has been reported recently that the muscle specific ubiquitin ligase muscle ring finger-2 (MuRF2) and MuRF3 regulate PPARγ1 activity to protect against diabetic cardiomyopathy [53, 54]. Although MuRF2$^{-/-}$ hearts have significant increases in fibrosis and PPARγ1-regulated cardiac genes, the expression of PPARγ1 mRNA has no differences in MuRF2$^{-/-}$ hearts and wild-type mice. Unfortunately, only minimal amount of fibrosis was detected in MuRF3$^{-/-}$ hearts and has no differences compared to wild-type controls. Furthermore, PPARγ1 target genes showed increases in both MuRF3$^{-/-}$ and wild-type hearts, but the mRNA expression levels have no differences between the two groups. Thus it can be seen that MuRF2 and MuRF3 inhibit cardiac PPAR isoforms expression to protect against high fat diet-induced diabetic cardiomyopathy, which mainly improve systolic dysfunction and attenuate left ventricular mass and heart weight but do not include cardiac fibrosis. Therefore, more research needs to prove the role of different PPARγ subtypes in myocardial fibrosis.

3.2. Hypertension. There is considerable evidence regarding arterial hypertension which leads to cardiac hypertrophy and myocardial fibrosis [10, 55]. For this reason, it is significant to explore novel strategies to protect the hypertension related cardiac remodeling [56]. Fortunately, despite low expression in the heart, PPARγ acts as a functional antifibrogenic factor in hypertensive heart disease [42]. Recent studies have indicated that treatment with the PPARγ activators resulted in the reduction of ECM deposition and cardiac fibrosis, while PPARγ antagonist GW9662 or T0070907 reversed these changes [10, 42, 43]. In addition, a significant negative correlation was observed between myocardial interstitial fibrosis and mRNA expression of PPARγ [56]. Furthermore, mice with a dominant-negative point mutation in PPARγ (P465L) developed significantly more severe cardiac fibrosis to Ang II-induced hypertension [57].

Despite the fact that the role of PPARγ in chronic pressure overload-induced cardiac fibrosis has been hypothesised previously (details are shown in Table 3), the molecular mechanisms are not fully understood. It has been suggested that activation of PPARγ inhibited both the expressions of TGFβ1 [10, 42–44, 56] and phosphorylation of Smad2/3 [10] in vivo and cultured neonatal rat cardiomyocytes and cardiac fibroblasts. In addition, the PPARγ agonist pioglitazone significantly decreased cardiac inflammatory response by inhibiting NF-κB and activator protein-1 (AP-1) binding activities, the expression of TNFα, and the adhesion of platelet endothelial cell adhesion molecule in stroke-prone spontaneously hypertensive rats (SHRSP) [45]. On the other hand, the downregulation of reactive oxygen species (ROS) mediated by an upregulation PPARγ may play a role in pressure overload-induced cardiac fibrosis [46, 47, 56]. However, Shinzato et al. [44] found that ROS production was not improved in SHRSP treated with pioglitazone. Furthermore, long-term administration of pioglitazone attenuates the development of left ventricular (LV) hypertrophy and fibrosis and inhibited phosphorylation of mTOR and p70S6 kinase in the heart, which are likely attributable to both the activation of AMPK signaling through stimulation of adiponectin secretion and the inhibition of Akt signaling [48].

3.3. Myocardial Infarction (MI). Adverse LV remodeling after MI is characterized by myocyte hypertrophy and interstitial fibrosis of the noninfarcted myocardium [58]. Accumulating evidence suggests that angiotensin II receptor blockers (ARBs) induce the activity of PPARγ which inhibit unfavorable LV remodeling [41, 58–60]. PPARγ protein expression is mainly in cardiac myocytes and fibroblasts in the infarcted area three weeks after MI, suggesting the critical role of PPARγ in cardiac fibrosis [59]. A study conducted by Maejima et al. [58] verified that telmisartan effectively inhibits infaust LV remodeling through a reduction of infiltration of macrophages, activation of MMP2 and MMP9, and expression of TGFβ1, CTGF, and osteopontin, while expression of PPARγ and activation of tissue inhibitor of metalloproteinase-1 (TIMP-1) were enhanced in the noninfarcted myocardium of rats. And in in vitro experiments, they got the similar results. Pioglitazone, a PPARγ activator, has been proved to reduce TNFα, TGFβ, and monocyte chemoattractant protein-1 and attenuate myocyte hypertrophy and interstitial fibrosis in MI mice [61]. This indicated that an anti-inflammatory effect mediated by PPARγ activation plays a critical role in post-MI LV remodeling in rats. More recently, a multicenter randomized double-blind study demonstrated that Qiliqiangxin, a traditional Chinese medicine, ameliorates unfavorable myocardial remodeling after acute MI including improved cardiac function, decreased apoptosis, and reduced fibrosis by increasing PPARγ levels. However, the expression of well-known signaling pathways including Akt, SAPK/Jun NH_2-terminal kinase phosphorylation (JNK), and ERK was not altered by Qiliqiangxin treatment [62]. Interestingly, Birnbaum et al. showed that pioglitazone is able to limit myocardial infarct size by activating Akt and upregulating cytosolic phospholipase A2 and cyclooxygenase-2 [63]. These suggest that the underlying mechanism may be varied from different drugs, but PPARγ play a critical role in myocardial fibrosis after MI is indisputable. Besides, TZDs also have neutral [64] or detrimental [65] effects on cardiac remodeling or mortality after MI. Therefore, the exact role of TZDs in myocardial remodeling after MI remains controversial and further studies should be done to elucidate the precise effects and mechanisms.

3.4. HF. Although the initial indications for PPAR agonist treatment mainly focus on hyperlipidemia and diabetes, there

Table 3: Effects of PPARγ ligands on hypertension related cardiac fibrosis.

Study model	Dose/duration/route	Major cardiac findings and conclusions	Ref.
Male SHR and WKY rats, 8 weeks old Cell culture: CFs form SD rats, 1-2 days old	Curcumin 100 mg/kg/d or curcumin 100 mg/kg/d plus GW9662 10 mg/kg/d for 12 weeks, gavage	Curcumin attenuates cardiac fibrosis in SHRs and inhibits Ang II-induced production of CTGF, PAI-1, ECM, TGFβ1, and phosphorylation of Smad2/3 in CFs in vitro	[10]
Male DnTGFβRII and WT C57BL/6 mice, 8–10 weeks old subjected to TAC	Rosiglitazone 10 mg/kg/d or T0070907 1.5 mg/kg/d for 3 weeks, gavage	Downregulation of endogenous PPARγ expression by TGFβ may be involved in pressure overload-induced cardiac fibrosis	[42]
Male Wistar rats, weights 250–300 g subjected to abdominal aortic banding at 4 weeks after ligation Cell culture: CFs form Wistar rats, 1–3 days old	Rosiglitazone 6 g/kg/d or GW9662 0.2 g/kg/d 2 h prior to rosiglitazone 6 g/kg/d for 1 week, intraperitoneal injection	Activation of PPARγ significantly inhibited cardiac remodeling by suppression the expressions of Brq1 and TGFβ1 through the NF-κB pathway	[43]
Male SHRSP and WKY rats, 24 weeks old	Pioglitazone 10 mg/kg/d for 8 weeks, mixed with food	Pioglitazone decreased interstitial fibrosis and number of myofibroblasts; mRNA levels of collagen I and BNP; MMP2 activity and protein level of CTGF. However, the mRNA level of collagen III and TGFβ1, MMP9 activity, and ROS production were not improved	[44]
Male SHRSP, 6 weeks old	Pioglitazone 10 mg/kg/d for 20 weeks, mixed with food	Subepicardial interstitial fibrosis, left ventricular NF-κB and AP-1 binding activities, the TNFα expression, and the adhesion of PECAM were decreased by pioglitazone treatment	[45]
Male SHRSP and WKY rats, 11 weeks old	Pioglitazone 1 mg/kg/d or 2 mg/kg/d, candesartan 0.3 mg/kg/d for 4 weeks, gavage	Pioglitazone suppressed cardiac inflammation and fibrosis and reduced vascular endothelial dysfunction by inhibition of cardiovascular NADPH oxidase, and the combination of pioglitazone and candesartan exerted more beneficial effects	[46]
Male C57BL/6J rats, 8 weeks old subjected to abdominal aortic banding	Ciglitazone 2 mg/kg/d for 4 weeks, administered in drinking water	Ciglitazone decreased interstitial and perivascular fibrosis and inhibition of an induction of NOX4, iNOS, MMP-2/MMP-13 expression, and collagen synthesis/degradation	[47]
Male inbred Dahl salt- sensitive rats, 7 weeks old	Pioglitazone 2.5 mg/kg/d for 4 weeks, gavage	Pioglitazone treatment ameliorated LV hypertrophy and fibrosis and improved diastolic function by activating AMPK signaling and inhibiting Akt signaling.	[48]

DnTGFβRII: dominant-negative mutation of the human TGFβ type II receptor, WT: wild type, TGF: transforming growth factor, TAC: transverse aortic constriction, CFs: cardiac fibroblasts, NF-κB: nuclear factor-κB, SHR: spontaneously hypertensive rats, WKY: Wistar Kyoto rats, SD: Sprague-Dawley, CTGF: connective tissue growth factor, PAI-1: Plasminogen activator inhibitor-1, ECM: extracellular matrix, SHRSP: stroke-prone spontaneously hypertensive rats, BNP: brain natriuretic peptide, MMP: matrix metalloproteinases, ROS: reactive oxygen species, NADPH: nicotinamide adenine dinucleotide phosphate, NOX4: nicotinamide adenine dinucleotide phosphate oxidase 4, iNOS: inductive nitric oxide synthase, AP-1: activator protein-1, TNF: tumor necrosis factor, PECAM: platelet endothelial cell adhesion molecule, and AMPK: adenosine monophosphate-activated protein kinas.

is a growing body of data which suggest that they maybe improve cardiac function with decreased fibrosis, improved contractility, and endothelial function in animal models of systolic HF [66]. In a rabbit model with nonischemic HF induced by combined aortic regurgitation and aortic stenosis, decreased ejection fraction and unfavorable myocardial remodeling including increased collagen volume fraction were observated. Moreover, the activity and expression of NF-κB subunits p65, RhoA, and Rho GTPase significantly increased. Interestingly, all these changes were reversed and the mRNA and protein expression of PPARγ were significantly increased with simvastatin treatment. Based on these results, the authors declared that simvastatin inhibited RhoA and Rho GTPase signaling to restrain NF-κB activation by the PPARγ-dependent pathway, thus attenuating LV hypertrophy and fibrosis [67]. In addition, pioglitazone treatment reduced the duration of atrial fibrillation (AF) and attenuated atrial structural remodeling including atrial fibrosis via attenuating the expression of TNFα, TGFβ1, and ERK but left unaffected p38 and JNK activation in the rabbit model with congestive heart failure [68]. Therefore, it is conceivable that PPARγ activation suppresses cardiac fibrosis by antagonizing inflammatory and hypertrophic signaling pathways. Likewise, PPARγ acts as a modulator of cardiac fibrosis in human as well. Cardiac remodeling occurring in patients with end-stage heart failure due to ischemic cardiomyopathy is related to PPAR activity, whereby inactivation of PPARα and PPARγ would lead to an increase in the production of ET-1 and the presence of cardiac fibrosis [69]. Nevertheless, rosiglitazone treatment had no significant effects on myocardial fibrosis compared with the vehicle group in MI-induced HF rats [70]. This result should raise questions with regard to these models or the particular species at large. Further studies are needed to test the variety and potential mechanisms.

3.5. I/R Injury. Early reperfusion of ischemic myocardium is necessary to salvage myocardial tissue from ultimate death. Nevertheless, reperfusion always results in cardiomyocyte death, microvasculature injury, and cardiac fibrosis, which ultimately cause myocardial remodeling and dysfunction [71, 72]. Recently, research has shown that rosiglitazone alleviated I/R injury by inhibiting inflammatory, improving endothelial function, reducing oxidative stress, and calcium overload [33]. Likewise, rosiglitazone treatment can effectively suppress the inflammatory induced by I/R injury and promote myocardial functional recovery [73] with an inhibition of JNK, AP-1 DNA-binding activity, and NF-κB signaling pathway [33, 73]. These data demonstrated that rosiglitazone limits postischemic injury, suggesting an important function for PPARγ in the heart.

Snail, a zinc finger transcription factor, activation induces lung, liver, and kidneys fibrosis [74–76]. Recently, its role in cardiac fibrosis after I/R injury and the probable underlying mechanisms had been identified. Lee and her colleagues [77] found that I/R injury to mouse hearts significantly increases the expression of Snail. In addition, the author showed that the cell source of Snail induction is endothelial cells. Moreover, Snail overexpression-mediated endothelial-to-mesenchymal transition-like cells markedly stimulated fibroblasts to myofibroblasts transdifferentiation via secretion of CTGF. What is more, they found that PPARγ agonist rosiglitazone, a selective Snail suppressor, remarkably suppressed cardiac fibrosis, improved cardiac function, and reduced Snail and CTGF expression in vivo. Based on this, the authors suggested that Snail might be a potential target molecule in the treatment of cardiac fibrosis.

3.6. AF. The relevance of atrial fibrosis and AF is well established and the causal relationship between them is interdependent. Atrial fibrosis expedites the development of AF by causing alterations of electrical properties [78]; on the other hand, AF itself promotes atrial fibrosis [79]. Although the underlying mechanisms are not fully understood, inflammation may promote the persistence of AF and atrial remodeling. A study conducted by Chen et al. [80] suggested that the PPARγ mRNA was significantly decreased in the hypertensive AF patients and PPARγ had a negative correlation with inflammatory cytokines TNFα, IL-6, and IL-1. The similar results were observed in elderly patients with AF [81]. In addition, pioglitazone was able to attenuate Ang II-induced electrical and structural remodeling by inhibiting both the TGFβ1/Smad2/3 and the non-Smad TGFβ1/tumor necrosis factor receptor associated factor 6/TGFβ-associated kinase 1 signaling pathways in vitro cellular models [82], which adds further evidence to the benefits of PPARγ agonist for the prevention of AF. Thus, PPARγ is at least partly involved in the pathogenesis of AF by regulation of inflammation through the NF-κB pathway; PPARγ agonist is potential useful in suppressing cardiac fibrosis and preventing AF occurrence.

3.7. Other CVD Conditions. It has been demonstrated that myocardial fibrosis is a common pathological change in radiation-induced heart diseases [83]. In Sprague-Dawley rats receiving chest radiation, the protein expression of TIMP-1 and TGFβ1 was higher than that in rats without radiation in the heart; the PPARγ mRNA and protein expression levels are upregulated in heart injured by radiation as well. However, upregulation of PPARγ failed to inhibit the expression of TIMP-1 and TGFβ1 [84]. Therefore, it is a possible mechanism that PPARγ itself has protective effect in response to radiation-induced heart injury. Regrettably, the authors did not use PPARγ agonists or inhibitors to further discuss its function in radiation-induced heart diseases. Besides, study on experimental animals demonstrated that tenascin-x, an ECM glycoprotein exclusively expressed in fibroblasts, can inhibit myocardial fibrosis via upregulation of TGFβ1 and downregulation of PPARγ in alcoholic cardiomyopathy [85]. These data suggested that PPARγ plays a crucial role in inhibiting cardiac fibrosis; further understanding of cardioprotection properties of PPARγ activator came from the study of pioglitazone influence on experimental autoimmune myocarditis. The authors suggested that pioglitazone could alleviate cardiac inflammation and fibrosis by inhibiting macrophage inflammatory protein-1α expression and modulating the Th1/Th2 balance [86].

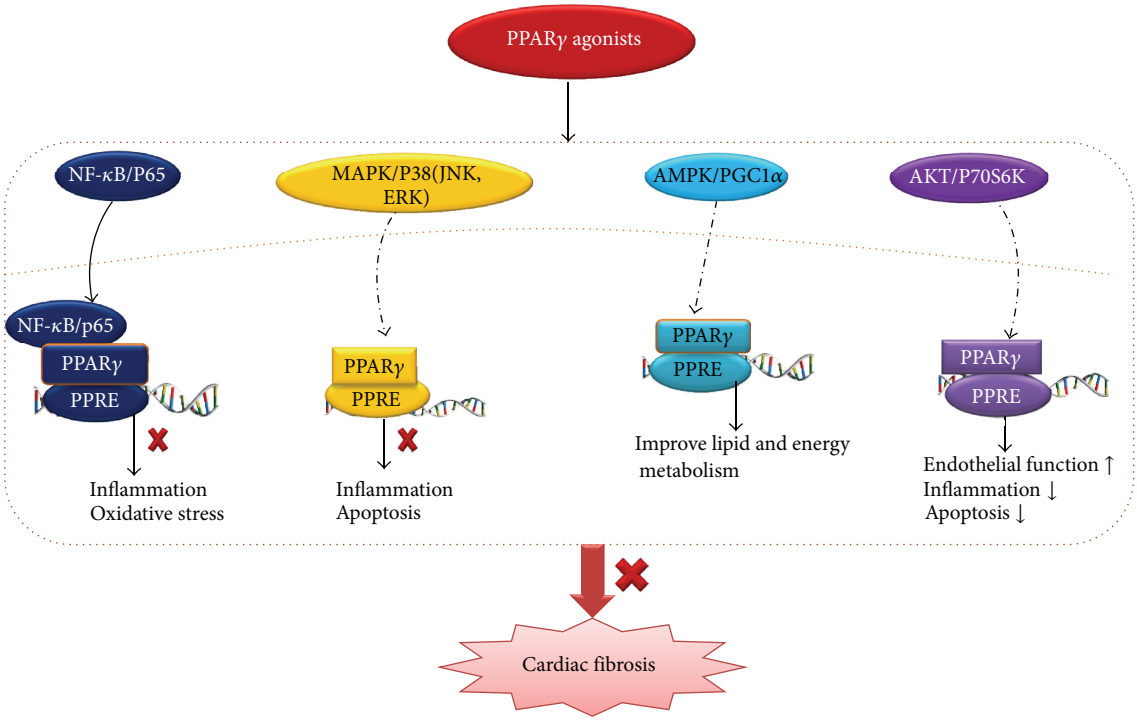

Figure 2: The possible underlying mechanisms involved in PPARγ agonists alleviate cardiac fibrosis. PPARγ agonists show pleiotropy functions associated with inhibiting cardiac fibrosis via variety of signaling pathways. PPARγ: peroxisome proliferator-activated receptor-γ; PPREs: peroxisome proliferator response elements; NF-κB: nuclear factor-κB; MAPK: mitogen-activated protein kinase; JNK: Jun NH_2-terminal kinase phosphorylation; ERK: extracellular signal-regulated kinase; AMPK: adenosine monophosphate-activated protein kinas; PGC1α: peroxisome proliferator-activated receptor gamma coactivator-1α; AKT: also known as protein kinase B.

Coincidentally, PPARγ shows a pivotal role in multiple other cardiovascular disease states. Singh et al. [87] demonstrated that rosiglitazone relieves cardiac hypertrophy and myocardial fibrosis in a dose-dependent manner possibly through its antioxidant activity in hyperhomocysteinemia rats. Moreover, simvastatin treatment has beneficial effects on augmentation of the PPARγ, PPARα expression, and reducing cardiac interstitial fibrosis biochemical makers including MMP-9 and cathepsin S in apolipoprotein E-deficient mice fed with a high fat diet [88]. More importantly, irbesartan prevents myocardial hypertrophy and fibrosis via activation of the PPARγ and suppression of the TGFβ-CTGF-ERK signaling in angiotensin-converting enzyme 2 knockout mice [9]. Finally, activation of PPARγ inhibits isoprenaline-induced myocardial fibrosis and remodeling via the NF-κB and MAPKs-dependent mechanism in rats [89–92].

3.8. Cardiac Fibroblasts (CFs) Culture In Vitro. Apart from in vivo experiments, PPARγ have been reported to have a number of cardioprotective properties in vivo. Due to a large number of stresses including growth and vasoactive factors, cytokines, and mechanical stimuli [93], fibroblasts proliferate and differentiate into myofibroblasts, a cell type with an increased secretion capacity of ECM [94]. There is convincing evidence that PPARγ ligands, rosiglitazone, pioglitazone, and 15-deoxy-$\Delta^{12,14}$-prostaglandin J2, all inhibit Ang II-induced CFs proliferation and differentiation, collagen synthesis, and ECM production [95–97], which are the critical steps in the pathogenesis of cardiac fibrosis. In addition, rosiglitazone can prevent myocardial fibrosis induced by advanced glycation end products in cultured neonatal rat CFs via inhibiting CFs proliferation, decreasing nitric oxide production, and CTGF expression [98]. Collectively, these data suggest that PPARγ activation has an antifibrotic effect. Despite these findings, the underlying mechanisms for the regulatory effects of PPARγ ligands on cardiac fibrosis are ambiguity and the specific role of PPARγ in this process has not yet been fully elucidated. The molecular mechanisms probably involved NF-κB/TGFβ/Smad2/3 and JNK signaling pathways [95, 99–101].

4. Conclusions and Future Prospects

Cardiac fibrosis is associated with varied cardiovascular disease and thus is a pivotal determinant of clinical outcome in heart diseases. Although the last decade has seen enormous progress insight into cardiac fibrosis, there is no precise and effective therapy. At the same time, accumulating evidences demonstrate that PPARγ exerts a broad spectrum of biological functions, including the beneficial effects of alleviating myocardial fibrosis. However, the cardioprotection mechanisms are currently not fully established, and the potential mechanisms were shown in Figure 2. Therefore, in-depth understanding of the potential molecular mechanisms

of PPARγ and its ligands in preventing cardiac fibrosis may provide valuable information in the design of novel treatment strategies in HF.

Unfortunately, despite many beneficial features of PPARγ agonists, they also exhibit adverse effects associated with long-term use. It has been proposed that PPARγ agonists are not free from side effects including edema, headache, hypoglycemia, myalgia, HF, weight gain, bone fractures, increased risk of MI and mortality, and possibly bladder cancer [13, 14, 17, 102–104]. Rosiglitazone, pioglitazone, and troglitazone have been approved for treatment of type 2 diabetes in clinical practice. Contrary to pioglitazone, rosiglitazone and troglitazone were associated with significant tissue toxicities after a relatively short-term exposure [15, 102]. In addition, the dual PPAR agonist ragaglitazar, MK-0767, naveglitazar, tesaglitazar, and muraglitazar for diabetes have failed due to various safety concerns. Aleglitazar, the most recent dual PPARα/γ agonist, has shown a significant dose-dependent reduction in HbA1c and beneficial effects on lipid subfractions [14]. Unfortunately, aleglitazar increased the risks of HF, renal dysfunction, bone fractures, gastrointestinal hemorrhage, and hypoglycemia [105]. Thus, new PPARγ-directed therapeutic modalities should be considered as possible approaches to reducing the adverse events seen with current TZDs. The pan-PRAR agonists bezafibrate, selective PPARγ modulators S26948 and INT131, partial PPARγ agonists balaglitazone, MBX-102, MK-0533, PAR-1622, PAM-1616, KR-62776, and SPPARγ M5, new dual PPARα/γ agonists saroglitazar, have a reduced tendency to cause the adverse effects and might be available in clinical management in the near future [14].

PPARγ agonists convey beneficial effects as therapeutic agents for cardiac fibrosis; however, their functions are not fully established yet. As such, PPARγ agonists possess different properties for different species, and the mechanisms by which they attenuate cardiac fibrosis are required in both experimental animal models and humans [106]. Moreover, the adverse side effects of PPARγ agonists and the potential mechanisms responsible for these effects should be clarified in detail, particularly in humans [106]. Last but not the least, it is necessary to focus on interactions between PPARγ-activating agents and other cardiovascular drugs [106]. Intensive research on these targets should be of great assistance to the development of safety and efficacy PPARγ agonists in the near future.

Abbreviations

PPARγ: Peroxisome proliferator-activated receptor-γ
CVD: Cardiovascular disease
ECM: Extracellular matrix
RAAS: Renin-angiotensin-aldosterone system
MMP: Matrix metalloproteinases
TGFβ: Transforming growth factor beta
HF: Heart failure
PPREs: Peroxisome proliferator response elements
AF-1: Activation function-1 motif
DBD: DNA-binding domain
LBD: Ligand bind domain
I/R: Ischemia/reperfusion
TZDs: Thiazolidinediones
T2DM: Type 2 diabetes mellitus
OLETF: Otsuka Long-Evans Tokushima Fatty
NF-κB: Nuclear factor-κB
CTGF: Connective tissue growth factor
Ang II: Angiotensin II
ET-1: Endothelin-1
IGF-1: Insulin-like growth factor 1
eNOS: Endothelial nitric oxide synthase
TNFα: Tumor necrosis factor-α
IL-1β: Interleukin-1β
MuRF2: Muscle specific ubiquitin ligase muscle ring finger-2
AP-1: Activator protein-1
SHRSP: Stroke-prone spontaneously hypertensive rats
ROS: Reactive oxygen species
LV: Left ventricular
MI: Myocardial infarction
ARB: Angiotensin II receptor blocker
TIMP-1: Tissue inhibitor of metalloproteinase-1
JNK: Jun NH_2-terminal kinase phosphorylation
AF: Atrial fibrillation
CFs: Cardiac fibroblasts.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Huang-Jun Liu and Hai-Han Liao contributed equally to this study.

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Research Article

Pioglitazone Protected against Cardiac Hypertrophy via Inhibiting AKT/GSK3β and MAPK Signaling Pathways

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Peroxisome proliferator activated receptor γ (PPARγ) has been closely involved in the process of cardiovascular diseases. This study was to investigate whether pioglitazone (PIO), a PPARγ agonist, could protect against pressure overload-induced cardiac hypertrophy. Mice were orally given PIO (2.5 mg/kg) from 1 week after aortic banding and continuing for 7 weeks. The morphological examination and biochemical analysis were used to evaluate the effects of PIO. Neonatal rat ventricular cardiomyocytes were also used to verify the protection of PIO against hypertrophy in vitro. The results in our study demonstrated that PIO remarkably inhibited hypertrophic response induced by aortic banding in vivo. Besides, PIO also suppressed cardiac fibrosis in vivo. PIO treatment also inhibited the activation of protein kinase B (AKT)/glycogen synthase kinase-3β (GSK3β) and mitogen-activated protein kinase (MAPK) in the heart. In addition, PIO alleviated angiotensin II-induced hypertrophic response in vitro. In conclusion, PIO could inhibit cardiac hypertrophy via attenuation of AKT/GSK3β and MAPK pathways.

1. Introduction

Cardiac hypertrophy is characterized by the dilation of heart, the enlargement of cardiac myocytes, and the accumulation of collagen. Cardiac hypertrophy could result in ventricular arrhythmia, heart failure, and sudden cardiac death [1, 2]. Although the causes and effects of cardiac hypertrophy have been extensively investigated, the underlying molecular mechanisms of cardiac hypertrophy remain unclear. Numerous studies have implicated that activation of protein kinase B (AKT)/glycogen synthase kinase-3β (GSK3β) and mitogen-activated protein kinase (MAPK) was closely involved in the process of cardiac hypertrophy [3]. Therefore, discovering new drugs which could suppress these signaling pathways would be of great importance for the treatment of cardiac hypertrophy.

Peroxisome proliferator activated receptors (PPARs), which were named for the ability to induce hepatic peroxide proliferation in response to xenobiotic stimuli, are members of nuclear receptor superfamily [4]. There are three isotypes of PPARs: PPAR-α, PPAR-β, and PPAR-γ [5]. PPARγ expressed predominantly in adipose tissue. PPARγ was found to regulate adipogenesis and insulin sensitivity [6, 7]. Studies later showed that PPARγ also expressed in rat cardiac myocytes [8] and was closely involved in the process of cardiac hypertrophy. Cardiomyocyte-specific PPARγ knockout mice could induce cardiac hypertrophy [9] and activation of PPARγ by the specific agonist, rosiglitazone, can inhibit cardiac hypertrophy in vivo and in vitro [10]. Pioglitazone (PIO), another PPARγ agonist, also displayed protective effects in the cardiovascular diseases. Pioglitazone could inhibit atherosclerosis [11] and improved left ventricular remodeling in mice with postmyocardial infarction [12]. However, the role of PIO in cardiac hypertrophy is still unclear. Previous study indicated that the PPARγ agonist suppressed GSK3β in colon cancer cell [13]. The activation of PPARγ could result in the inhibition of extracellular signal-regulated kinase (ERK) [14]. Thus, whether PIO has antagonistic actions on these signaling pathways also still needs to be investigated.

In this study, we used an animal model of cardiac hypertrophy induced by pressure overload to determine

whether PIO could protect against cardiac hypertrophy, and we also uncovered the molecular mechanisms underlying the protective effects.

2. Materials and Methods

All animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication, revised 2011) and approved by the Animal Care and Use Committee of Renmin Hospital of Wuhan University, China.

2.1. Reagents. PIO was purchased from Sigma-Aldrich (CDS021593, purity > 98% determined by HPLC). Angiotensin II (Ang II, A9525) was also purchased from Sigma-Aldrich. Anti-PPARγ (sc-7196) was purchased from Santa Cruz Biotechnology. The first antibodies followed were purchased from Cell Signaling Technology: anti-AKT (#4691), anti-phospho-AKT (#4060), anti-GSK3β (#9315), anti-phospho-GSK3β (#9323P), anti-ERK (#4695), anti-phospho-ERK (#4370P), anti-P38 (#9212P), and anti-phospho-P38 (#4511P). Anti-GAPDH (#ab8245) was obtained from ABCAM. Anti-α-actin was obtained from Millipore. The secondary antibodies were obtained from LI-COR Biosciences. All other chemicals were of analytical grade.

2.2. Animals and Treatments. All 60 male C57BL/6 mice (8–10-week-old; male body weight: 23.5–27.5 g) were purchased from the Institute of Laboratory Animal Science, CAMS & PUMC (Beijing, China), and housed with controlled temperature and humidity. All the mice were allowed free access to food and water under a 12 h light-dark cycle in the Cardiovascular Research Institute of Wuhan University (Wuhan, China). The animals were randomly divided to either a sham surgery or AB group, which were orally treated with or without PIO (2.5 mg/kg body weight/day) for 7 weeks, and beginning 1 week after aortic banding. The AB was performed as described previously [15, 16]. The dose of PIO was selected according to our preliminary experiment (see Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/9174190). PIO was dissolved in normal saline for in vivo experiments. Eight weeks after surgery, the animals were anesthetized with 1.5% isoflurane and subjected to echocardiographic measurements, whereafter the mice were euthanized with excessive sodium pentobarbital (200 mg/kg). Hearts were dissected and weighed to calculate the heart weight/body weight (HW/BW) and heart weight/tibia length (HW/TL) ratios in the PIO-treated and vehicle-treated mice. Collected left ventricle of heart tissues was snap-frozen in liquid nitrogen and stored in minus 80°C until further experiments.

2.3. Echocardiography Analysis. Echocardiography was performed on euthanized mice by using the Mylab 30CV (Esaote S.P.A., Genoa, Italy) equipped with a 10 MHz linear array ultrasound transducer, as previously described [17]. Parasternal long-axis views, short-axis views, and 2D guided M-mode images of short axis at the papillary muscle level were recorded. Left ventricular end-systolic diameter (LVSD) and end-diastolic diameter (LVDD) were measured tracing with a sweep speed of 50 mm/s.

2.4. Histological Analysis. The removed heart tissues were arrested with 10% KCl and fixed with 10% formalin. Then we embedded the heart in paraffin and sectioned transversely. After rehydration, the sections (4-5 μm) of heart were obtained and mounted onto slides and stained with haematoxylin-eosin (HE) or picrosirius red (PSR). After staining, the cross-sectional areas of the myocytes and the average collagen volume were determined by a quantitative digital analysis system (Image-Pro Plus, version 6.0; Media Cybernetics, Bethesda, MD, USA). The sections were examined blind.

2.5. Western Blot Analysis. The frozen heart tissues were lysed by a RIPA buffer, which is 720 μL of RIPA, 100 μL of cocktail, 100 μL of Phos-stop, 50 μL of NaF, 20 μL of PMSF, and 10 μL of Na_3VO_4 compounded in every 1 mL of lysis buffer. The protein concentrations were subsequently measured using the BCA Protein Assay Kit (cat. number 23227; Thermo Fisher Scientific, Waltham, MA, USA). Then the protein was resolved to the 10% SDS PAGE and transferred to a PVDF membrane (cat. number IPFL00010; EMD Millipore, Billerica, MA, USA). After that, the membranes were blocked and incubated with primary antibodies and secondary antibodies. Finally, the blots were observed and analyzed using Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA).

2.6. Real-Time Polymerase Chain Reaction Analysis. The total RNA was harvested from frozen left ventricle tissues or cell lysates using TRIzol (cat. number 15596026; Invitrogen Life Technologies, Carlsbad, CA, USA). 1 μg RNA of each sample was used to reverse-transcribe into cDNA using the PrimeScript RT reagent Kit (cat. number RR047Q; Takara Biotechnology (Dalian) Co., Ltd.). PCR was performed using a LightCycler 480 SYBR Green Master Mix (cat. number 04896866001; Roche Diagnostics GmbH). All primer details were provided in Table 1. The mRNA levels were normalized to GAPDH.

2.7. Cell Culture. The isolation of primary neonatal rat ventricular cardiomyocytes (NRVCMs) was performed according to previous study [18]. Bromodeoxyuridine (0.1 mM) was used to inhibit the growth of cardiac fibroblast. Isolated primary neonatal rat ventricular cardiomyocytes were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F12) 1 : 1 mixture (GIBCO, C11995). NRVCMs were provided with 10% fetal bovine serum (FBS) (GIBCO, 10099), streptomycin (100 mg/mL; GIBCO, 15140), and penicillin (100 U/mL). Then the cells were cultured in an atmosphere of 5% CO_2 with a humidified incubator (SANYO 18M) at 37°C. The cells were seeded onto six-well culture plates or 24-well plates for 24 h with DMEM and 10% FBS, later cultured with 0.5% DMEM for another 12 h. After that, Ang II (1 μmol) was added to the medium in the presence or absence of PIO (20 μmol/L). The viability of neonatal rat ventricular

Table 1: Primers used in the study.

Gene	Species		Sequence (5′-3′)
GAPDH	Mouse	Forward	ACTCCACTCACGGCAAATTC
		Reverse	TCTCCATGGTGGTGAAGACA
GAPDH	Rat	Forward	GACATGCCGCCTGGAGAAAC
		Reverse	AGCCCAGGATGCCCTTTAGT
ANP	Mouse	Forward	ACCTGCTAGACCACCTGGAG
		Reverse	CCTTGGCTGTTATCTTCGGTACCGG
ANP	Rat	Forward	AAAGCAAACTGAGGGCTCTGCTCG
		Reverse	TTCGGTACCGGAAGCTGTTGCA
β-MHC	Mouse	Forward	CCGAGTCCCAGGTCAACAA
		Reverse	CTTCACGGGCACCCTTGGA
BNP	Mouse	Forward	GAGGTCACTCCTATCCTCTGG
		Reverse	GCCATTTCCTCCGACTTTTCTC
Collagen I	Mouse	Forward	TGGTACATCAGCCCGAAC
		Reverse	GTCAGCTGGATAGCGACA
Collagen III	Mouse	Forward	GTCAGCTGGATAGCGACA
		Reverse	GAAGCACAGGAGCAGGTGTAGA
CTGF	Mouse	Forward	TGTGTGATGAGCCCAAGGAC
		Reverse	AGTTGGCTCGCATCATAGTTG
Fibronectin	Mouse	Forward	CCGGTGGCTGTCAGTCA GA
		Reverse	CCGTTCCCACTGCTGATTTATC

cardiomyocytes was determined by CCK-8 in 5 independent experiments.

2.8. Immunofluorescence Staining. NRVCMs cultured on cover slips were pretreated with or without PIO (20 μmol/L) and stimulated with 1 μmol Ang II for 24 h. For staining the cells, the NRVCMs were fixed with 4% formaldehyde and permeabilized in 0.1% Triton X-100. Subsequently, the cells were stained with anti-α-actin (1 : 100 dilution) and incubated using Alexa Fluor 568-goat anti-mouse (Invitrogen, A11017). The cross-sectional areas were measured using Image-Pro Plus 6.0. More than 100 myocytes were outlined in each group.

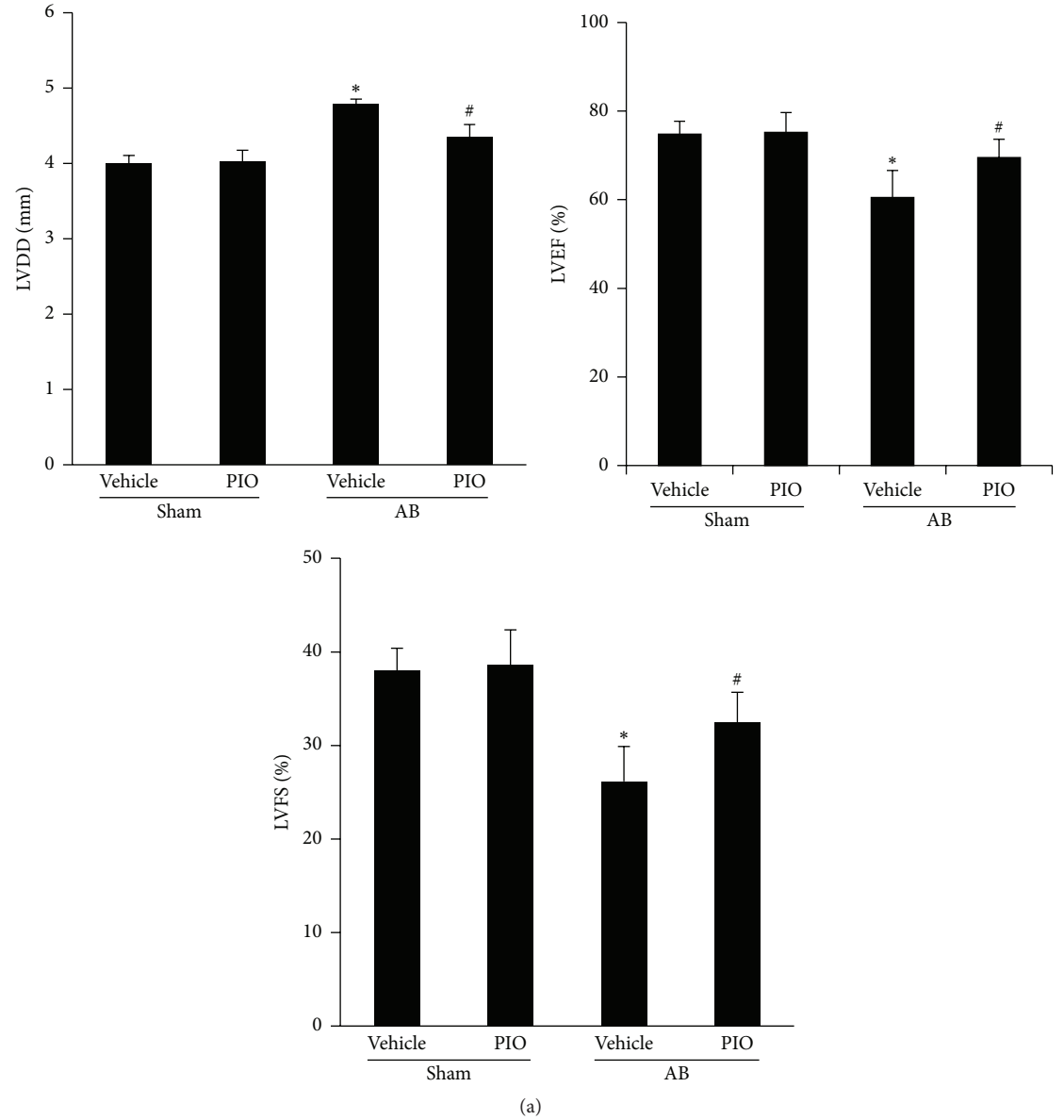
2.9. Statistical Analysis. Data are presented as mean ± SD. Comparisons were undertaken by one-way ANOVA followed by a post hoc Tukey's test. A value of $P < 0.05$ was considered significant.

3. Results

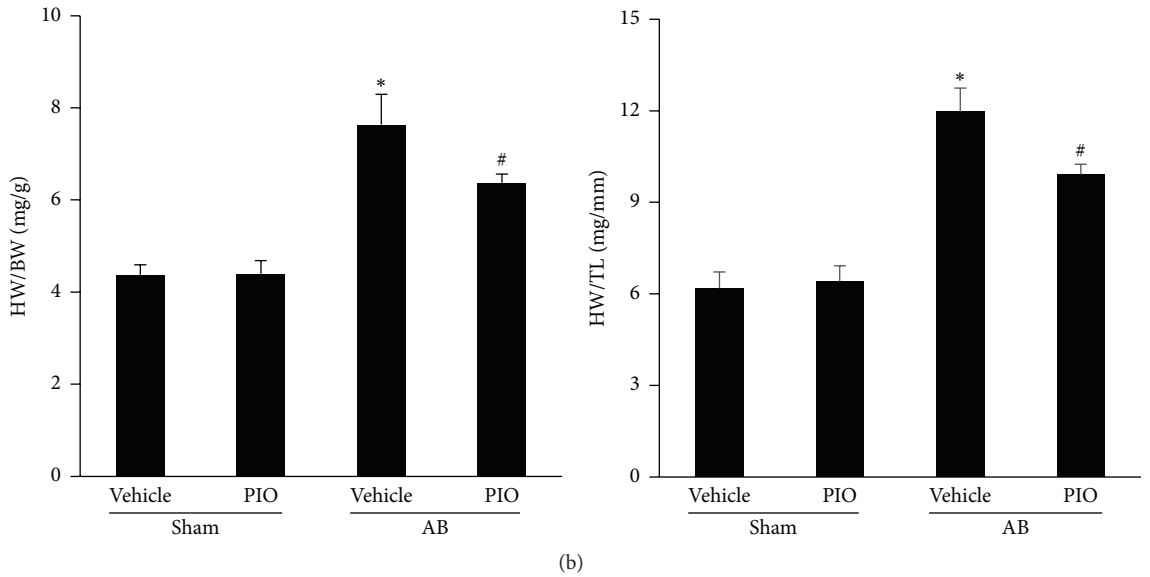
3.1. PIO Suppressed Pressure Overload-Induced Cardiac Hypertrophy In Vivo. Compared with mice in sham-operated group, mice subjected to aortic banding developed a marked increase of LVDD and a decrease of heart function (Figure 1(a)). AB mice displayed a hypertrophic response as measured by the ratios of HW/BW, HW/TL, the gross heart size, and cross-sectional areas (Figures 1(b) and 1(c)). Conversely, compared with mice subjected to AB, the hypertrophic response induced by pressure overload was attenuated in mice with PIO treatment. In addition, the markers of cardiac hypertrophy, including atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and β-myosin heavy chain (β-MHC), were also checked (Figure 1(d)). Our data demonstrated the hypertrophic markers were attenuated in AB mice treated with PIO. And no significant difference was observed in the sham-operated mice with or without PIO.

3.2. PIO Attenuated Cardiac Fibrosis Induced by Aortic Banding In Vivo. Fibrosis, which is one of major features of cardiac hypertrophy, is characterized by the disproportionate accumulation of collagen and perivascular fibrosis [19, 20]. Thus, heart sections were stained with PSR and analyzed quantitatively to evaluate the extent of fibrosis. As shown in Figure 2(a), marked interstitial and perivascular fibrosis and increased collagen volume were observed in the mice with AB surgery, and PIO treatment could attenuate the fibrotic response. The mRNA expression levels of collagen I, collagen III, connective tissue growth factor (CTGF), and fibronectin were increased in AB group (Figure 2(b)). Though PIO cannot affect the fibrotic genes at baseline, PIO treatment decreased the increased levels of fibrotic markers induced by pressure overload.

3.3. PIO Protected against Cardiac Hypertrophy by Inhibiting MAPK and AKT/GSK3β Pathways. Our data indicated that the levels of PPARγ were decreased after long-term pressure overload. And PIO, as a PPARγ agonist, apparently upregulated the expression levels of PPARγ in mice given PIO with or without aortic banding (Figure 3). The MAPK and AKT/GSK3β signaling pathways were also checked in our study. As illustrated by Figure 3, PIO treatment alone inhibited the phosphorylated AKT and GSK3β in the mice without surgery. Moreover, PIO could also decrease the



(a)



(b)

Figure 1: Continued.

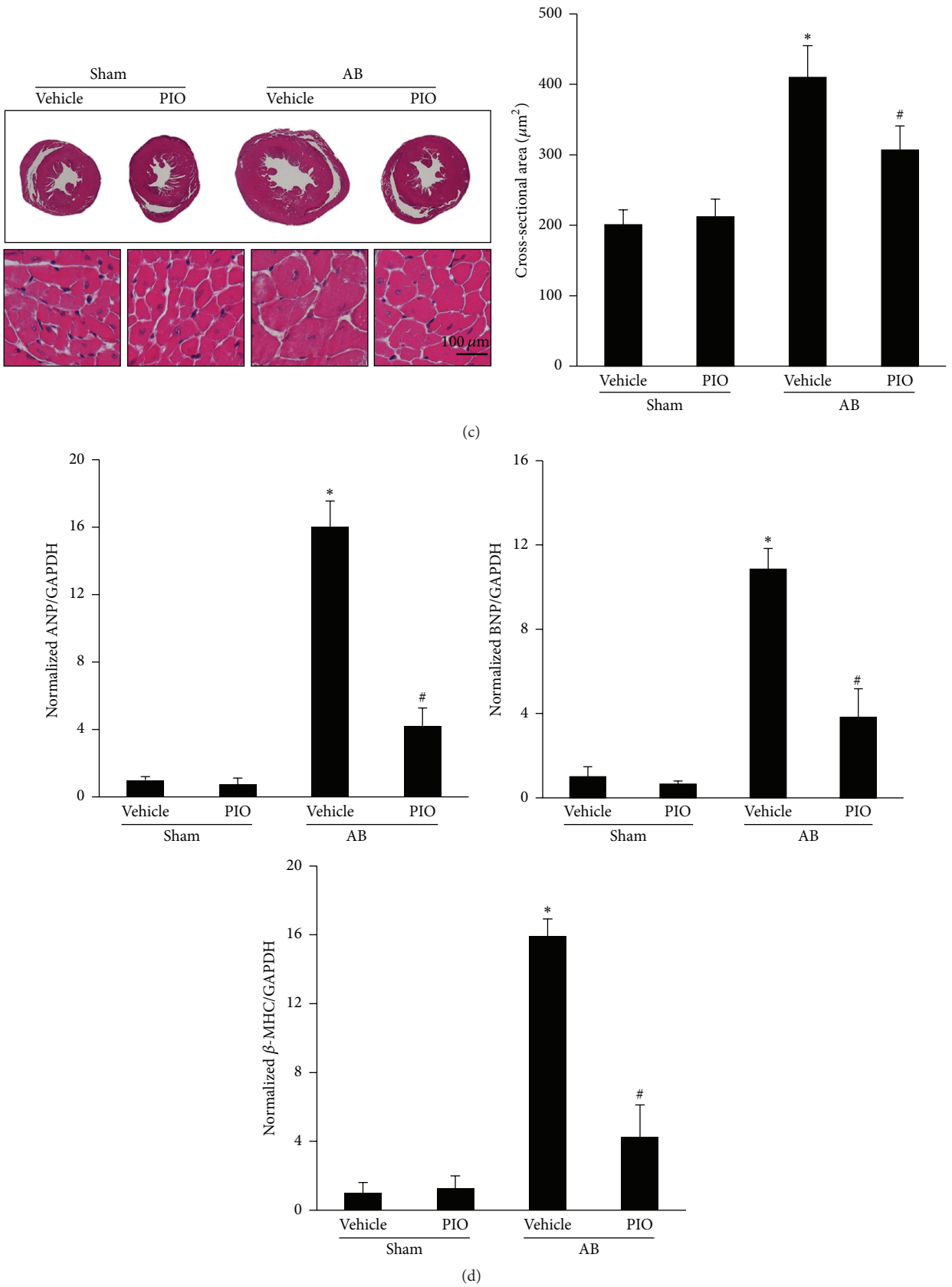
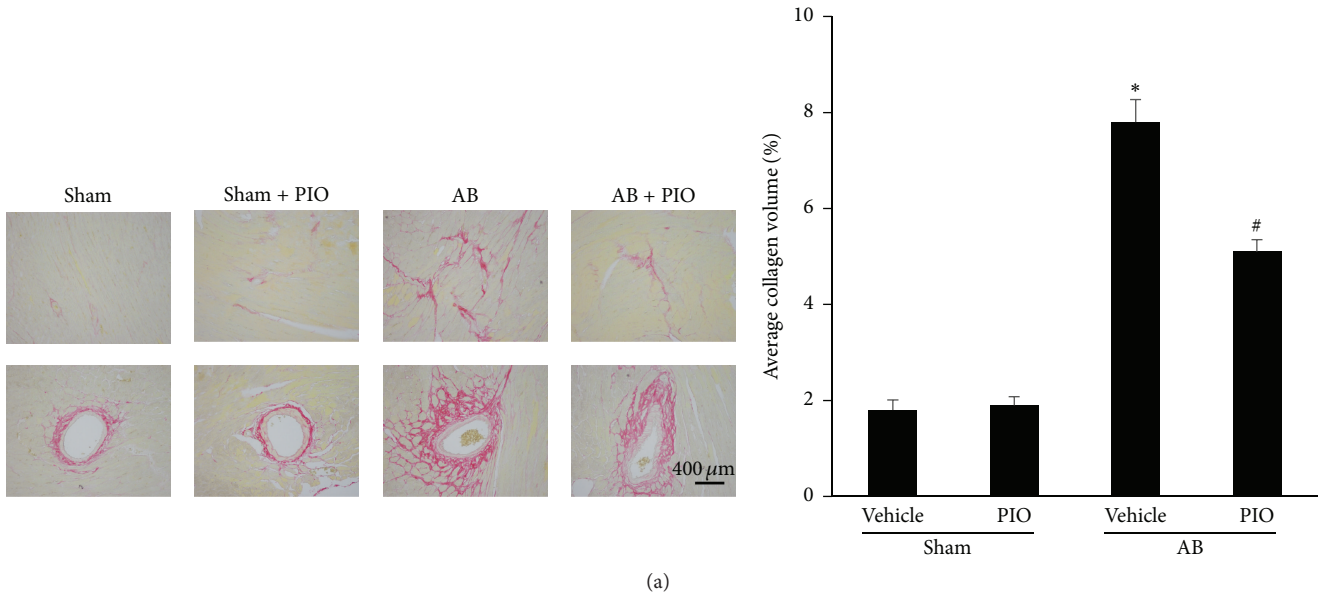
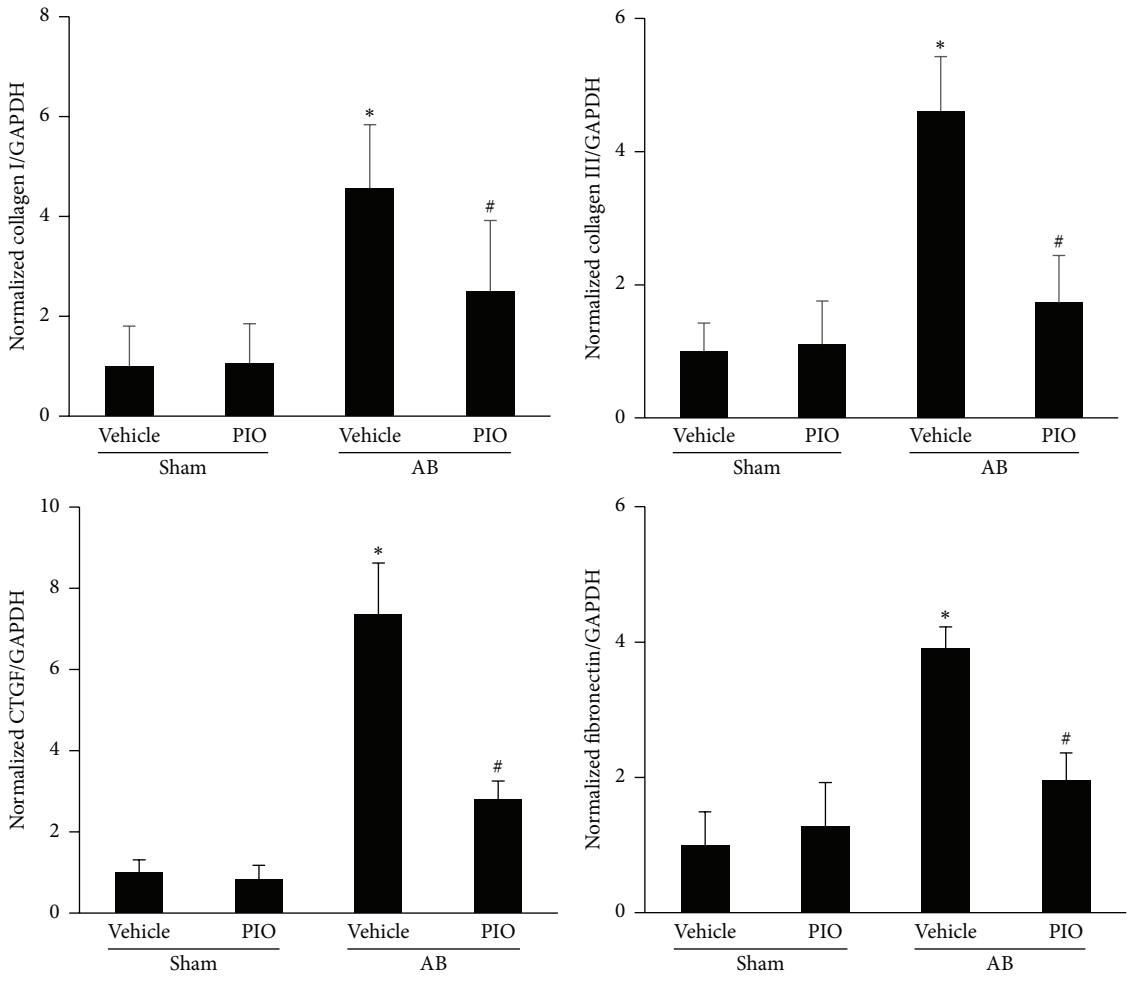


FIGURE 1: PIO inhibited cardiac hypertrophy in vivo. (a) Echocardiography results of LVDD, LVEF, and LVFS of the indicated groups (n = 10–12). (b) Results of the HW/BW, HW/TL ratios of the indicated groups (n = 13–15). (c) Gross hearts, HE staining, and cross-sectional area after 8 weeks of AB or sham with or without PIO (n = 4). (d) Expression levels of the transcripts for ANP, BNP, and β-MHC normalized by GAPDH of the indicated groups (n = 4). $^{*}P < 0.05$ as compared with the corresponding sham group. $^{\#}P < 0.05$ versus the AB group.



(a)



(b)

Figure 2: PIO attenuated cardiac fibrosis in vivo. (a) Histological sections of the left ventricle were stained for PSR and average collagen volume in group of AB or sham with or without PIO ($n = 4$). (b) Expression levels of the transcripts for collagen I, collagen III, connective tissue growth factor (CTGF), and fibronectin normalized by GAPDH of the indicated groups by real-time PCR ($n = 4$). $^{*}P < 0.05$ as compared with the corresponding sham group. $^{\#}P < 0.05$ versus the AB group.

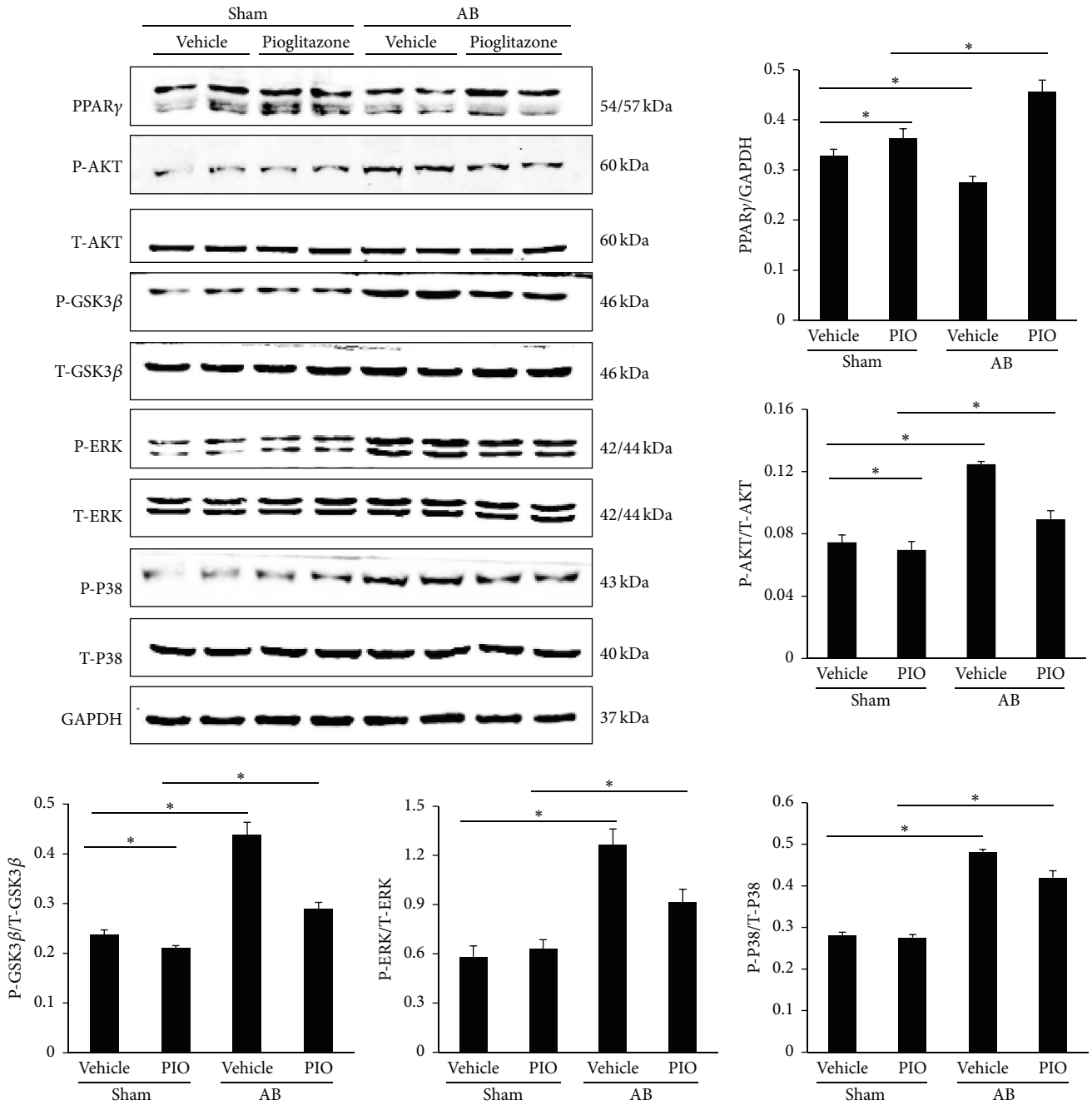


Figure 3: PIO protects against cardiac hypertrophy by inhibition of MAPK and AKT/GSK3β pathways. Representative and quantitative expression of PPARγ, phosphorylated AKT, GSK3β, ERK, P38, and the effects of PIO in group of AB or sham with or without PIO ($n = 6$). $^{*}P < 0.05$.

increased phosphorylation of AKT and GSK3β caused by AB. The protein levels of phosphorylated ERK and P38 were also increased after 8 weeks of aortic banding surgery, and treatment of PIO could reduce these pathways. However, there was no significant change of P-ERK and P-P38 between sham and sham + PIO group.

3.4. PIO Alleviated Hypertrophy of Cardiac Myocytes In Vitro. Taking the cardiovascular protective role of PIO into consideration, neonatal rat ventricular cardiomyocytes were used to further examine the protection of PIO against hypertrophy of myocytes in vitro. Ang II was used to induce hypertrophy in that hypertrophy induced by aortic banding was mediated partly by Ang II [21]. AKT/GSK3β and MAPK signaling could also be induced by Ang II [22–24]. And the treatment of PIO could not cause significant cytotoxicity in the cultured cells (Figure 4(a)). As expected, Ang II induced hypertrophy of myocytes, characterized by the increase of ANP (Figure 4(b)) and the enlargement of cross-sectional areas (Figure 4(c)). PIO treatment dose-dependently attenuated

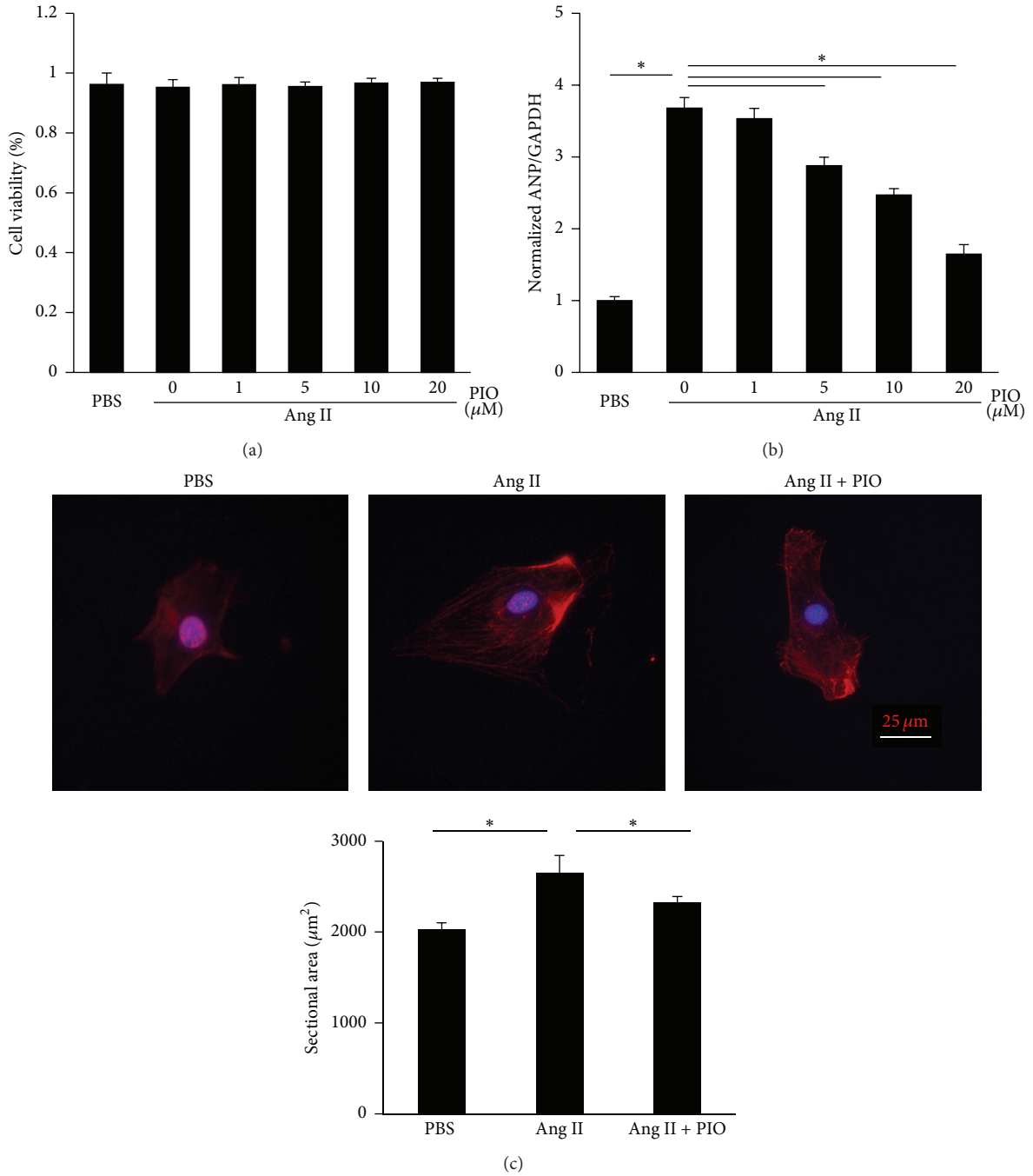


FIGURE 4: PIO alleviated hypertrophy of cardiac myocytes in vitro. (a) The effects of PIO on viability of neonatal rat ventricular cardiomyocytes were determined by CCK-8 in 5 independent experiments. (b) The levels of ANP of cardiomyocytes in indicated groups ($n = 5$). (c) The immunofluorescence of cardiomyocytes and the sectional areas of myocytes ($n = 5$). $^{*}P < 0.05$.

the hypertrophic response, while no significant difference was observed in baseline.

4. Discussion

Our study demonstrated that PIO protected against cardiac hypertrophy induced by aortic banding and inhibited hypertrophy of myocytes stimulated by Ang II. Moreover, it was observed that PIO attenuated the increases of P-AKT, P-GSK3β, P-ERK, and P-P38, induced by AB. Besides, PIO could attenuate the fibrosis caused by long-time pressure overload. These findings indicated that PIO played a prominent role in the protection of cardiac hypertrophy by inhibiting AKT/GSK3β and MAPK signaling pathways.

A number of published studies implicated that a wide array of intracellular signaling pathways were involved in the

hypertrophic response. AKT is a crucial regulator of cardiac hypertrophy [25]. Activated AKT phosphorylates and inactivates the downstream GSK3β signaling, further promoting the development of pathological cardiac hypertrophy [26]. Previous study indicated that long-term activation of AKT could accelerate the process of heart failure [27]. Cardiac-specific overexpression of constitutive activated AKT mutant could lead to decompensation of hypertrophy; conversely, AKT1 knockout mice were resistant to cardiac hypertrophy [28]. The data from our previous studies demonstrated that inhibition of AKT/GSK3β alleviated AB-induced cardiac hypertrophy [29, 30]. Consistent with these results, we also found that PIO treatment reduced the activation of AKT/GSK3β pathways, accompanied with the increase of PPARγ. The potential mechanism underlying downregulation of AKT signaling caused by PIO may be correlated with PPARγ-ligand-mediated upregulation of PTEN. And PTEN could restrain activation of AKT by dephosphorylating inositol phospholipid intermediates of the PI3K pathway [31]. In addition, PPARγ attenuated AKT in a PPAR-γ-independent manner [32]. Future study using the specific inhibitor of PPARγ will be of interest.

ERK and P38, as the members of MAPK signaling pathways, participate in gene expression associated with cardiac hypertrophy. Previous studies indicated that ERK could be activated in cardiac myocytes in response to hypertrophic stimulus, and the blockade or deletion of cardiac ERK aggravated the development of cardiac hypertrophy [33]. P38 was also activated during the pressure overload-induced-hypertrophy in vivo. And overexpression of P38 in cultured cardiomyocytes by recombinant adenoviruses caused characteristic hypertrophic responses [34]. Ji et al. found that pioglitazone could inhibit microglia inflammation by blocking p38 signaling pathways [35]. Moon et al. demonstrated that alpha-eleostearic acid, as an agonist of PPARγ, could play a role of anticancer by activating PPARγ and downregulating the phosphorylation of ERK [36]. Li et al. verified that pioglitazone attenuated ERK phosphorylation through stimulation of adiponectin levels [37]. Consistent with these findings, our study also found that PIO reduced the phosphorylation levels of ERK and P38 in mice subjected to aortic banding. Inconsistent with our data, previous studies demonstrated that PIO could protect against ischemia-reperfusion injury via upregulation of ERK [38]. Therefore, substantial work is needed for the precise clarification of the cardiovascular protection of PIO.

Inconsistent with our study, agonist of PPARγ induced cardiac hypertrophy in both the wild type mice and cardiomyocyte-specific PPARγ knockout mice, implying that the in vivo effects of the agonist on the heart are mediated by non-PPAR effects [9]. Indeed, this prohypertrophic effect does not occur in humans for the limited doses [39, 40]. In view of that high level of PPARγ in the heart increased uptake of fatty acid and glucose [41], a relatively small dose of PIO was used in our study, which may explain the inconsistent results.

Fibrosis was a pathophysiologic process of cardiac hypertrophy, which is characterized by the accumulation of collagen and the increase of the extracellular matrix [42]. Koitabashi et al. found that cardiac hypertrophy was accompanied by increased level of CTGF [43]. Morais et al. indicated that fibronectin contributed to maladaptive cardiac hypertrophy [44]. It was reported that rosiglitazone could inhibit Ang II-induced CTGF expression in vascular smooth muscle cells. In this study, we also observed that the increased collagen volume and the molecular markers, including collagen I, collagen III, CTGF, and fibronectin, were alleviated after PIO treatment. Previous studies demonstrated that myocardial interstitial fibrosis induced by pressure overload may be mediated through MAPK and AKT/GSK3β pathways [28, 29]. The suppression of AKT/GSK3β and MAPK pathways caused by PIO may be the potential mechanism that mediated the antifibrotic effects.

In conclusion, the results of the present study demonstrated that PIO protected against cardiac hypertrophy in vivo and inhibited myocyte hypertrophy in vitro. And the cardioprotective effects were mediated by the inhibition of MAPK and AKT/GSK3β pathways. Our study provides theoretical evidence for treating cardiac hypertrophy with PIO in the clinical application.

Disclosure

Wen-Ying Wei and Zhen-Guo Ma are the co-first authors.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Wen-Ying Wei and Zhen-Guo Ma contributed equally to this work.

Acknowledgments

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Research Article

Possible Role of Interaction between PPARα and Cyclophilin D in Cardioprotection of AMPK against *In Vivo* Ischemia-Reperfusion in Rats

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Activated AMPK protects the heart from cardiac ischemia-reperfusion (IR) injury and is associated with inhibition of mitochondrial permeability transition pore (PTP) opening. On the other hand, pharmacological inhibition of the PTP reduces infarct size and improves cardiac function. However, it is unclear whether beneficial effects of AMPK are mediated through the PTP and, if they are not, whether simultaneous activation of AMPK and inhibition of the PTP exert synergistic protective effects against cardiac IR injury. Here, we examined the effects of the AMPK activator, A-769662 in combination with the PTP inhibitor, sanglifehrin A (SfA) on *in vivo* cardiac IR. Cardiac dysfunction following IR injury was associated with decreased activity of the mitochondrial electron transport chain (ETC) and increased mitochondrial ROS and PTP opening. Administration of A-769662 or SfA individually upon reperfusion improved cardiac function, reduced infarction size, and inhibited ROS production and PTP opening. However, simultaneous administration of SfA and A-769662 did not provide synergistic improvement of postischemic recovery of cardiac and mitochondrial function, though both compounds disrupted IR-induced interaction between PPARα and CyP-D. In conclusion, A-769662 or SfA prevents PPARα interaction with CyP-D, improving cardiac outcomes and increasing mitochondrial function, and simultaneous administration of the drugs does not provide synergistic effects.

1. Introduction

AMPK is a serine/threonine kinase that is activated by increased intracellular AMP levels. In the heart, AMPK has been shown to regulate cellular uptake and subsequent entry of fatty acids into the mitochondria to increase fatty acid oxidation [1]. Also, AMPK stimulates translocation of GLUT-4 to the sarcolemma to increase glucose uptake [2] and activates glycolysis [3]. AMPK is activated in response to oxidative and energy stress induced by ischemia-reperfusion (IR) in the heart [4, 5]. Notably, increased AMP/ATP ratio in cardiac IR activates AMPK through stimulation of threonine (Thr^{172}) phosphorylation [4] or inhibition of AMPK dephosphorylation [5]. Active AMPK switches off energy-consuming processes like protein and lipid synthesis and stimulates ATP-generating mechanisms thereby maintaining ATP production despite the lack of oxygen in the heart [6–8]. Previous studies using genetic mouse models lacking the cardiac-specific α2-AMPK isoform have collectively demonstrated that downregulation of AMPK induces greater cardiac injury, activates apoptosis, and worsens cardiac recovery after IR [9, 10], whereas the activation of AMPK protects the heart and improves mitochondrial function [11].

Based on the potential therapeutic benefits observed in animal models, extensive studies have been performed to develop pharmacological activators of AMPK. Early studies determined that biguanides, including metformin, increased the phosphorylation and activation of AMPK, thereby protecting the heart against IR injury [12]. Yet, the mechanism of metformin in AMPK activation is still poorly understood. Metformin has been shown to exert pleiotropic effects, activating AMPK-independent pathways of cell survival [13]. Also, numerous studies suggest that metformin acts through the inhibition of the electron transport chain

(ETC) complex I, increasing AMP levels due to inhibition of oxidative phosphorylation [14, 15]. Notably, the inhibition of AMPK activity prevented the cardioprotective effects of metformin, suggesting a central role of AMPK in mediating beneficial effects of metformin. Furthermore, the beneficial effects of metformin are abrogated by inhibition of the p38 mitogen-activated protein kinase and PKC [13]. Interest in the development of a more specific AMPK activator led to the identification of a potent thienopyridone called A-769662. Instead of activating AMPK indirectly by increasing the AMP : ATP ratio, A-769662 directly binds to the regulatory β-subunit of AMPK and, thereby, allosterically stimulates AMPK [16]. A-769662 has also been shown to inhibit AMPK dephosphorylation and increase AMPK resistance to protein phosphatases [17].

The cardioprotective effects of AMPK activation are associated with inhibition of the mitochondrial permeability transition pores (PTP) [18–20]. The PTP is a nonselective channel that renders mitochondria permeable to any solute up to approximately 1.5 kDa, inducing mitochondrial swelling. In turn, the outer membrane ruptures, releasing proapoptotic factors that eventually stimulate cell death via apoptosis or necrosis [21, 22]. Although the molecular identity of the PTP complex is controversial, a key regulator of the pores is cyclophilin D (CyP-D), a cis-trans isomerase found exclusively in the mitochondrial matrix that is essential for proper protein folding [23].

In this study, we examined whether simultaneous PTP inhibition and AMPK activation provided synergistic cardioprotection against *in vivo* cardiac IR. To avoid the undesired AMPK-independent effects of metformin, we used A-769662 in this study to activate AMPK. Since AMPK mediates cardioprotection by inhibiting PTP opening, we hypothesized that combined treatment with SfA and A-769662 would not provide synergistic effects. Our experiments revealed that treatment with SfA and/or A-769662 protects cardiac function and mitochondria without affecting AMPK phosphorylation. Both individual treatments reduced mitochondrial ROS (mitROS) levels, improved the activities of ETC complexes, and prevented IR-induced PPARα-CyP-D interaction and PTP opening. The combination of SfA and A-769662 did not provide synergistic cardioprotective effects on cardiac IR.

2. Materials and Methods

2.1. Animals. Male Sprague-Dawley rats (250–275 g, Charles River, Wilmington, MA) were housed in individual cages in a temperature controlled room under a regular light-dark cycle. Water and food were provided *ad libitum*. All experiments were performed according to protocols approved by the University Animal Care and Use Committee and conformed to the National Research Council Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (2011, eighth edition).

2.2. In Vivo Model of Cardiac IR. Animals were anesthetized with an anesthetic cocktail containing (mg per kg body weight, IP) xylazine 4.2, ketamine 87.5, and acepromazine 0.88 and artificially ventilated with room air using a small-animal ventilator (Kent Scientific Corp., Torrington, CT). The respiration rate was maintained at 65 to 70 breaths per minute, and body temperature was maintained at 37°C by placing the animals on a homeothermic surgery table [24]. Lateral thoracotomies were performed to gently expose the hearts. The left anterior descending coronary arteries were ligated ~3 mm from their origins with firmly tied silk sutures (7-0), inducing myocardial infarction. Electrocardiograms (ECG) were recorded to monitor the hearts' electrical activity throughout the surgeries by using the MouseMonitor™ (Indus Instruments, Houston, TX) heating pad with needle ECG leads. For the Sham procedures the ligatures were placed in an identical fashion but not tied. The resulting coronary artery ligations (CAL) were maintained for 30 min, after which the sutures were removed and followed by reperfusion for 24 h. The animals were randomly divided into the following five groups: Sham (no CAL, $n = 7$), IR ($n = 8$), IR + SfA ($n = 4$), IR + A-769662 (IR + A, $n = 6$), and IR + SfA + A-769662 (IR + SfA + A, $n = 4$). Animals having no ST-segment elevation on the ECG following CAL were excluded from IR groups with or without treatment due to a lack of myocardial infarction. SfA (25 mg/kg), A-769662 (10 mg/kg), or their combination was administered by intravenous bolus immediately before reperfusion. The doses for SfA and A-769662 were based on previous studies that demonstrated that SfA treatment [25] or A-769662 treatment [26] had cardioprotective effects in murine models of *in vivo* IR.

2.3. Echocardiography. Echocardiographic measurements were performed as described previously [24]. Briefly, rats were anesthetized and placed in a supine position. M-mode and 2D echocardiography images were obtained with a high-frequency 8–4 MHz 10-mm broadband phased P10 probe attached to a digital portable ultrasound system Micromaxx (Sonosite Inc., Bothell, WA). Diastolic and systolic measurements of LV dimensions (LVIDd, LVIDs), LV end-systolic and end-diastolic volumes (LVESV, LVEDV), and HR were recorded. Then, stroke volume (SV), cardiac output (CO), and ejection fraction (EF) were calculated as SV = LVEDV − LVESV, CO = (SV ∗ HR)/1000, and EF = ((LVEDV − LVESV)/LVEDV) ∗ 100, respectively.

2.4. Infarction Size. Rats were sacrificed, the hearts were excised, and blood was washed for 10 min by retrograde perfusion with Krebs-Henseleit solution [27]. Then, the hearts were placed in −20°C for 1-2 h, manually sliced into 5-6 uniform slices, and incubated for 20 min in the phosphate buffer (0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4, pH 7.4 at 37°C) containing 10 mg/mL tetrazolium chloride (TTC). The heart sections were fixed in 10% formaldehyde overnight and then photographed. Digital images of heart sections were analyzed for infarct size (TTC negative) using the NIH ImageJ software. The infarction size was calculated as a percentage of the entire left ventricle.

2.5. Measurement of PTP Opening in Isolated Mitochondria. Opening of the mitochondrial pore was determined by Ca^{2+} induced swelling of isolated mitochondria, measured

as a reduction in light scattering at 520 nm, as previously described [28]. Mitochondria containing 50 μg of protein were incubated at 25°C in 200 μL buffer containing 150 mM KSCN, 20 mM MOPS, 10 mM Tris, and 2 mM nitrilotriacetic acid, supplemented with 0.5 μM rotenone, 0.5 μM antimycin, and 2 μM A23187. Swelling of mitochondria was initiated by progressive additions of $CaCl_2$ (100 μM every 5 min for a total of 5 times), and rates of swelling were determined by monitoring the decrease in light scattering at 525 nm, quantified using a Spectramax M3 plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.6. Mitochondrial ETC Complexes Enzyme Activity. Enzymatic activities of ETC complexes were determined in isolated cardiac mitochondria, as previously described [29]. Briefly, mitochondria were diluted with a hypertonic media buffer (25 mM KH_2PO_4, 5 mM $MgCl_2$, and 0.5 mg/mL BSA), supplemented with saponin (0.55 mg/mL). To access the ETC complexes embedded in the inner mitochondrial membrane, the mitochondria were disrupted three times by freeze-thawing and, then, incubated for 30 min at 4°C. All measurements were recorded at the Thermo Scientific GENESYS™ 10S UV-Vis spectrophotometer at 30°C. Activities of all ETC complexes were normalized to citrate synthase activity.

Citrate synthase activity was determined by measuring coenzyme A formation at the Thermo Scientific GENESYS™ 10S UV-Vis spectrophotometer and was expressed as nmol oxaloacetate/min per mg protein [28].

2.7. Mitochondrial ROS Levels. Amplex Red (Life Technologies, Carlsbad, CA), a dye that reacts with H_2O_2 to produce the highly fluorescent molecule, resorufin, was used for measurement of ROS levels in isolated mitochondria. Mitochondria were incubated with 100 μM Amplex Red for 15 min, and the fluorescence intensity was quantified using a Spectramax M3 plate reader (Molecular Devices, Sunnyvale, CA, USA) at an excitation of 460 nm and emission of 490 nm.

2.8. SDS-PAGE and Western Blotting. Protein concentration in homogenate and mitochondria was determined by the Bradford assay (Bio-Rad, Hercules, CA). Equal amounts (50 μg/well) of protein were loaded onto 10% SDS-PAGE gels, run, and transferred onto Amersham Hybond ECL nitrocellulose membranes (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The membranes were immunoblotted with AMPK, P-AMPKα_1^{Thr172} (Cell Signaling, Boston, MA), PPARα, and P-PPARα^{Ser21} (Santa Cruz Biotechnology, Santa Cruz, CA). The signals were visualized using Thermo Scientific Pierce ECL Western Blotting Detection reagents (Thermo Scientific, Rockford, IL) at the VersaDoc 3000 Gel Imaging System (Bio-Rad, Hercules, CA).

2.9. Co-Immunoprecipitation. Protein samples were incubated with anti-PPARα or anti-CyP-D antibodies overnight at 4°C, and the immunoprecipitates were harvested with Dynabeads® Protein G (Life Technologies, Carlsbad, CA). The immunoprecipitated complexes were washed and, then, subjected to SDS-PAGE, followed by immunoblotting using antibodies for CyP-D (Abcam, Cambridge, MA) or PPARα.

2.10. Statistical Analysis. Data are presented as means ± SE. Differences among groups were compared by two-tailed Student's t-tests. Differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Cardiac Function and Infarct Size. The heart-to-body weight ratio (HW/BW) was increased by 24% ($P < 0.05$) in the IR group compared to Sham-operated rats. Treatment with A-769662 and/or SfA significantly prevented the IR-induced increase of the HW/BW ratio (Figure 1(a)).

Next, we determined the effects of A-769662 and/or SfA on infarct size with the TTC method. As shown in Figures 1(b) and 1(c), the infarct size was 71% ($P < 0.01$), 33% ($P < 0.05$), and 49% ($P < 0.05$) less in IR + A-769662, IR + SfA, and IR + SfA + A groups, respectively, compared to the IR group. Interestingly, SfA alone did not reduce infarct size as much as the other two treatments (A-769662 and SfA + A-769662). Analysis of cardiac function demonstrated that rats subjected to IR had a 51% and 55% ($P < 0.01$ for both) lower cardiac output and ejection fraction, respectively, than the Sham-operated counterparts (Figures 2(a) and 2(b)). In all treatment groups, cardiac output and ejection fraction were remarkably preserved compared to the IR (untreated) group. No significant differences were observed between the treated groups with regard to changes of cardiac output and ejection fraction.

Overall, these results suggest that activation of AMPK and inhibition of PTP protect the heart during cardiac IR by reducing infarct size and improving cardiac function.

3.2. Phosphorylation of AMPK and PPARα. We examined the effects of A-769662 and/or SfA on phosphorylation of AMPK and PPARα during cardiac IR. Results demonstrated a 2-fold ($P < 0.05$ versus Sham) increase in P-AMPK$^{\text{Thr172}}$ levels in IR hearts. Neither treatment with A-769662 nor treatment with SfA had any effects on IR-induced AMPK phosphorylation. However, simultaneous treatment with SfA and A-769662 returned the expression of P-AMPK$^{\text{Thr172}}$ to the level shown in Sham-operated hearts (Figures 3(a) and 3(b)). Also, IR, with or without treatment, increased the level of P-PPARα by 100%, 88%, 68%, and 68% ($P < 0.05$ for all) in IR, IR + A-769662, IR + SfA, and IR + SfA + A, respectively, compared to the Sham group (Figures 3(d) and 3(e)). Total levels of AMPK and PPARα were not affected by IR, with or without treatment (Figures 3(c) and 3(f)).

Overall, these results indicate that, unlike metformin, A-769662 does not phosphorylate AMPK at Thr^{172}. Furthermore, cardiac IR-stimulated PPARα phosphorylation is not prevented by A-769662 and/or SfA.

3.3. Mitochondrial PTP Opening and mitROS Production. In the next set of experiments, we evaluated the effects of A-769662, SfA, or the combination on mitROS production in cardiac IR. We measured H_2O_2 levels in isolated mitochondria using the Amplex Red assay. IR induced a 54% ($P < 0.01$) increase of mitROS levels compared to the Sham group, and treatment with A-769662 and/or SfA at reperfusion blocked

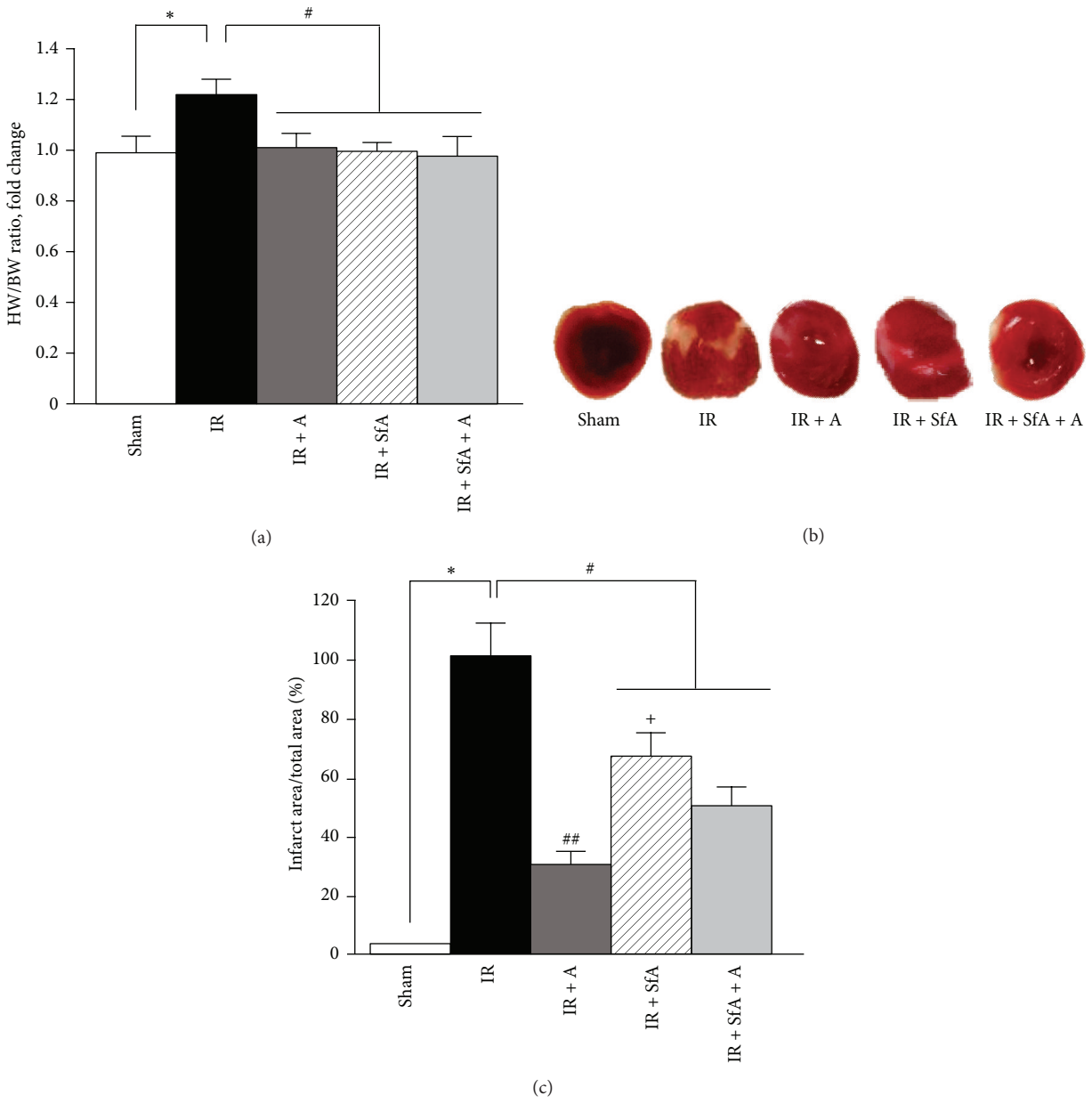

Figure 1: Effects of A-769662 (A) and/or SfA on the heart-to-body weight ratio (HW/BW) and infarct size during cardiac IR. (a) HW/BW expressed as a fold change of the Sham group; (b) representative images of heart cross sections, showing viable (*red*) and infarcted tissue (*white*) for all experimental groups; (c) quantitative results of TTC staining, expressed as percentages of the IR group. The number of animals in each group for analysis of HW/BW (a): Sham ($n = 7$), IR ($n = 8$), IR + SfA ($n = 4$), IR + A-769662 ($n = 6$), and IR + SfA + A ($n = 4$). Additional 3 hearts from each group were analyzed for quantification of infarction size (b and c). $^{*}P < 0.01$ versus Sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus IR; $^{+}P < 0.01$ versus IR + A and IR + SfA + A.

the IR-induced increase of mitROS levels (Figure 4(a)). Since elevated mitROS levels play a causal role in cell damage by inducing PTP opening, we assessed whether pore opening is affected by cardiac IR, with or without treatment. As shown in Figures 4(b) and 4(c), hearts subjected to IR had an 80% ($P < 0.01$ versus Sham) increase in Ca^{2+}-induced mitochondrial swelling (a marker of PTP opening), which was significantly attenuated in all three treatment groups (IR + A-769662, IR + SfA, and IR + SfA + A-769662). Altogether, these data demonstrate that treatment with A-769662 and/or SfA decreases mitROS levels and inhibits PTP opening.

3.4. Enzymatic Activity of Mitochondrial ETC Complexes. Analysis of enzymatic activity of ETC complexes demonstrated that cardiac IR reduced the activity of complexes I, III, and IV by 28%, 44%, and 50% ($P < 0.05$ for all), respectively, compared to Sham-operated animals (Figure 5). Treatment with A-769662 improved the activities of complexes I and

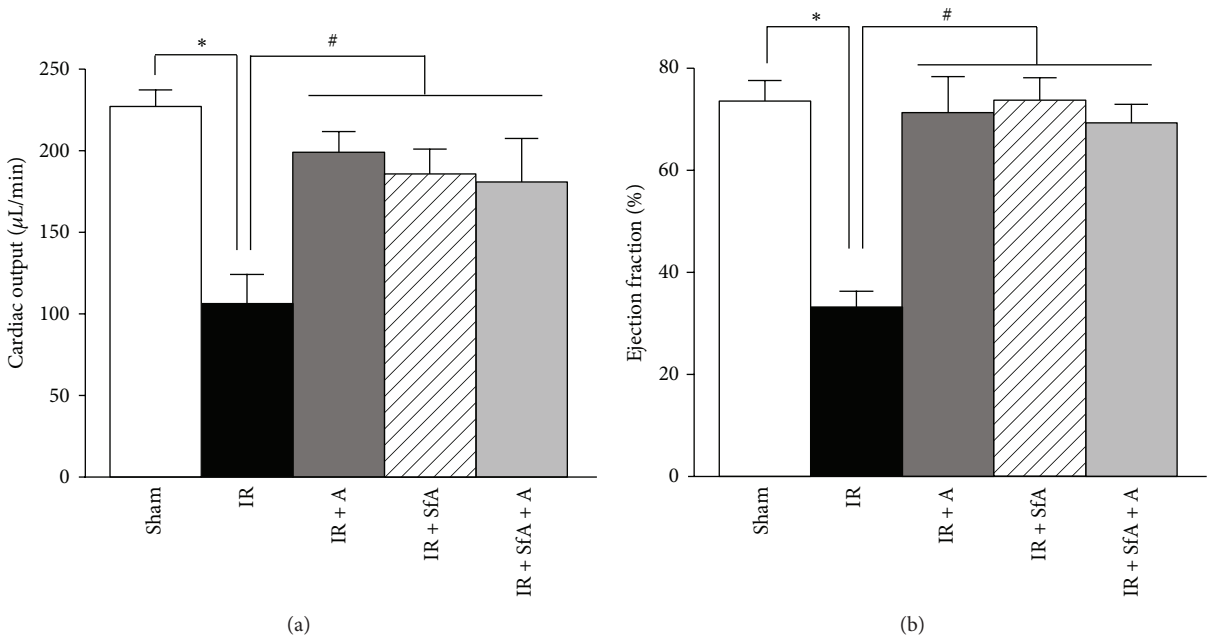

Figure 2: Effects of A-769662 (A) and/or SfA on cardiac output (a) and ejection fraction (b) of hearts after IR or Sham procedure. Calculations of cardiac output and ejection fraction are given in Section 2. The number of animals: Sham ($n = 7$), IR ($n = 8$), IR + SfA ($n = 4$), IR + A-769662 ($n = 6$), and IR + SfA + A ($n = 4$). $^{*}P < 0.01$ versus Sham; $^{\#}P < 0.01$ versus IR.

IV, which were 23% and 40% ($P < 0.05$) higher, respectively, compared to the IR group. Likewise, the activities of complexes I and IV in SfA-treated hearts were 38% and 47% ($P < 0.05$ for both) higher, respectively, than that in untreated hearts. The combination of SfA with A-769662 did not exert additional effects on the activity of the ETC complexes. Likewise, neither treatment with SfA, A-769662 nor the combination improved IR-induced suppression of complex III activity (Figure 5(b)).

Next, we measured citrate synthase activity as a marker of mitochondrial mass. Our results showed that IR reduced citrate synthase activity in mitochondria. Treatment with SfA, A-769662, or their combination did not prevent the effect of IR, suggesting that improvements observed with ETC complexes are not due to increased mitochondrial mass (Figure 5(d)).

These results demonstrate that cardiac IR decreased the activity of ETC complexes I, II, III, and IV. Furthermore, A-769662 and/or SfA significantly prevented inactivation of all the complexes, except for complex III.

3.5. Physical Interaction between CyP-D and PPARα. In our *in vitro* studies, oxidative stress stimulated protein-protein interactions between PPARα and CyP-D in H9c2 cardioblasts, and those interactions were prevented by metformin [20]. Therefore, we sought to examine whether PPARα and CyP-D interaction occurred in an *in vivo* model of cardiac IR. We applied two contrasting technical approaches to verify the interaction when mitochondrial proteins were immunoprecipitated with PPARα or CyP-D antibodies followed by immunoblotting with CyP-D or PPARα antibodies (Figures 6(a) and 6(b)). In both cases, cardiac IR significantly increased interactions between PPARα and the PTP regulator, CyP-D ($P < 0.05$), when compared with the Sham group. However, treatment with A-769662 and/or SfA ameliorated this interaction (Figures 6(a) and 6(b)). In conclusion, these data demonstrate the existence of physical interactions between PPARα and CyP-D in response to IR, and A-769662 and/or SfA are able to abrogate these interactions.

4. Discussion

This study demonstrated that (i) SfA, A-769662, or their combination attenuated cardiac dysfunction and infarct size induced by *in vivo* IR; (ii) *in vivo* cardiac IR increased phosphorylation of AMPK, which was not affected by treatments with A-769662; (iii) SfA, A-769662, or their combination reduced IR-induced mitROS levels and PTP opening in cardiac mitochondria; (iv) *in vivo* cardiac IR induced an interaction between PPARα and CyP-D which was attenuated by SfA, A-769662, or their combination; and (v) IR-induced decrease in ETC activity was abrogated by SfA, A-769662, or their combination. These data, for the first time, provide evidence that although both AMPK activation and PTP opening exert cardioprotective effects against *in vivo* IR, the simultaneous application of both therapeutic approaches (SfA + A-769662) has no synergistic effects.

Activation of AMPK by A-769662 has been previously shown to protect endothelial [30], neuronal [31], and liver [32] cells, as well as the whole heart [26, 33], from oxidative damage. A-769662 protected hearts against IR injury, and this protection was associated with decreased infarct size and PTP opening in Goto-Kakizaki diabetic rats [18]. However,

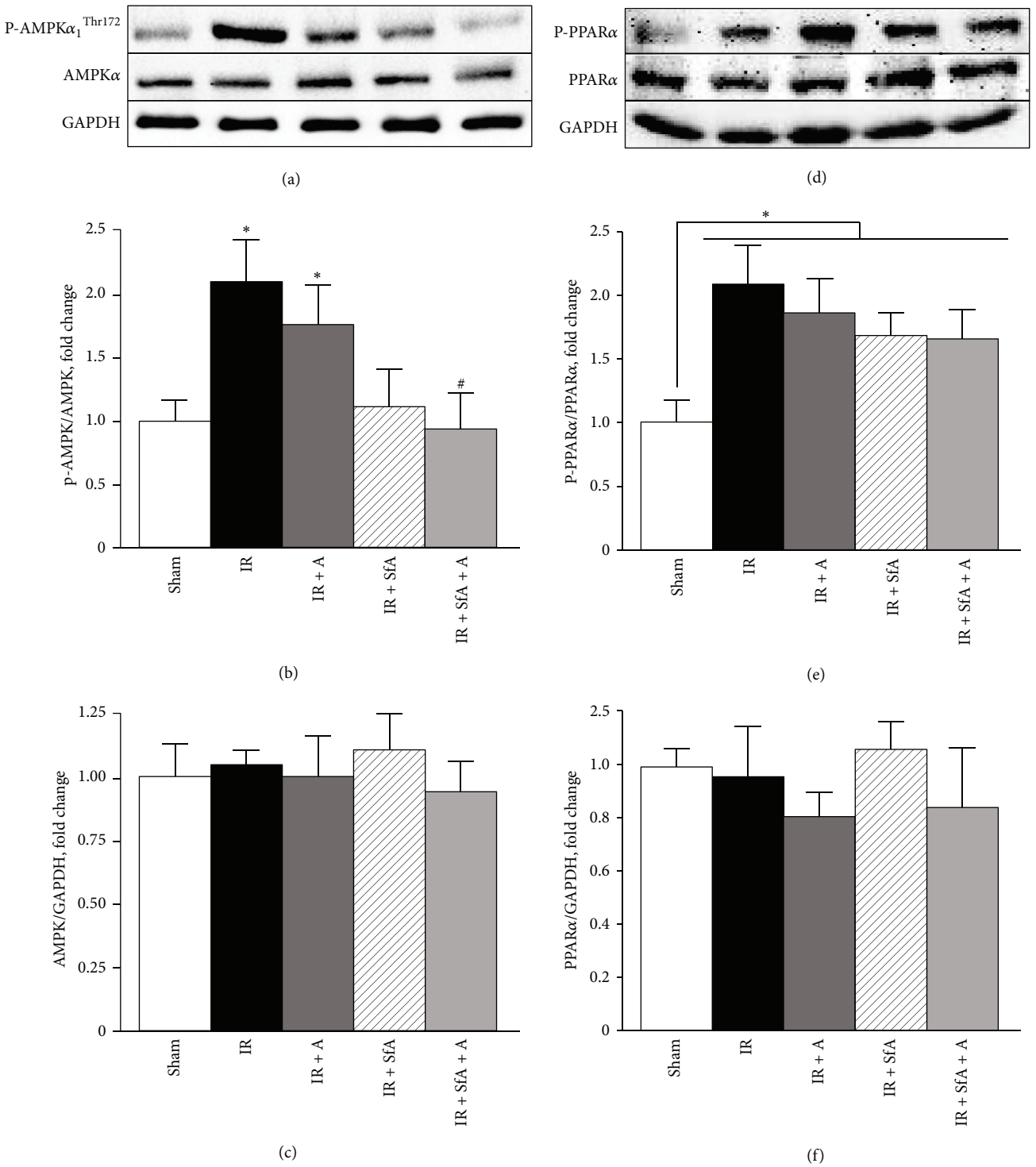

FIGURE 3: Protein levels of phosphorylated and total AMPKα (a–c) and PPARα (d–f) in heart homogenates. Protein levels of P-AMPKα_1 ^{Thr172} and P-PPARα ^{Ser21} were normalized to total AMPKα_1 and PPARα, respectively, whereas the levels of AMPK and PPARα were normalized to GAPDH. Top panels (a, d) are representative western blots in each group. Bottom panels represent quantitative data of protein expression for P-AMPKα_1 ^{Thr172} (b), AMPKα_1 (c), P-PPARα ^{Ser21} (e), and PPARα (f). Results are expressed as a fold change of the Sham group. The number of animals: Sham ($n = 7$), IR ($n = 8$), IR + SfA ($n = 4$), IR + A-769662 ($n = 6$), and IR + SfA + A ($n = 4$). $^{*}P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus IR.

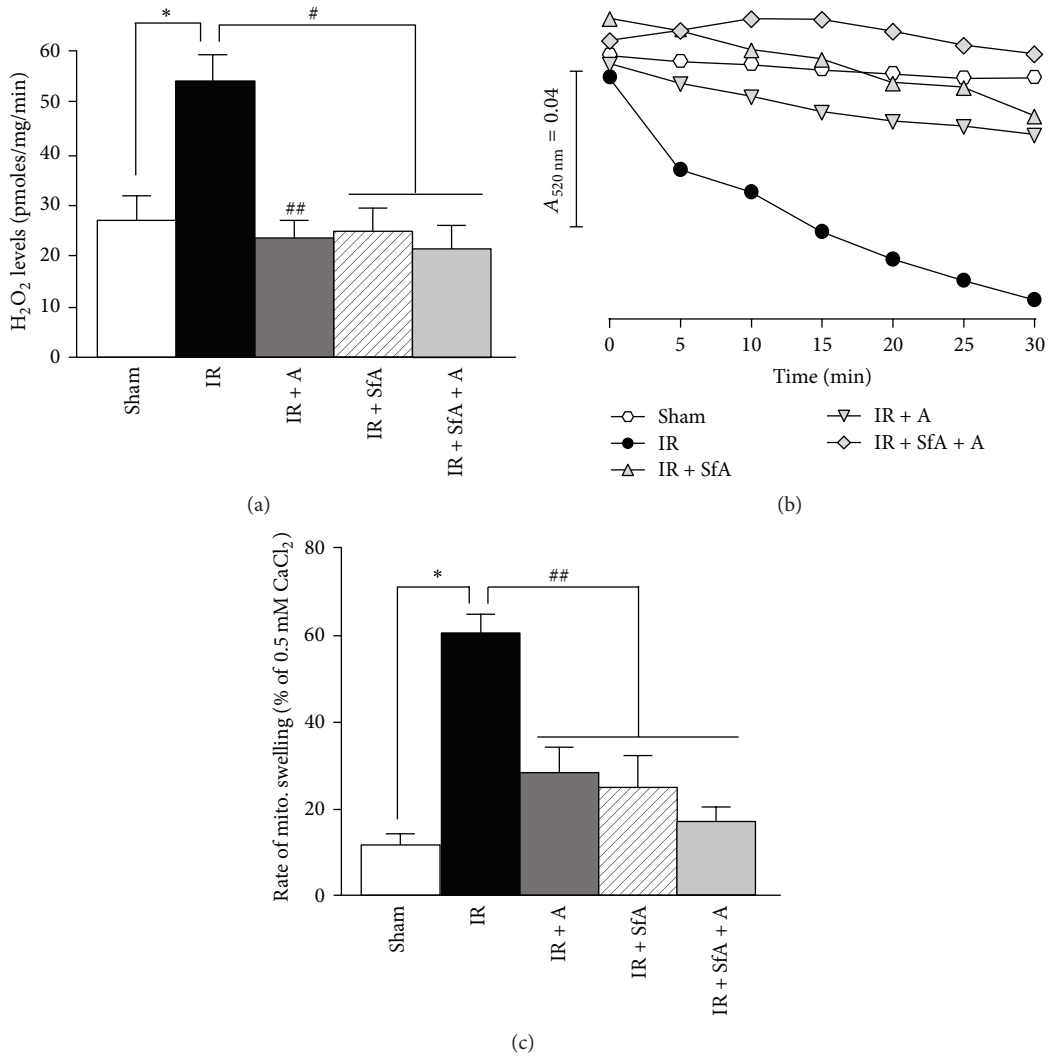

Figure 4: ROS levels and Ca^{2+}-induced mitochondrial swelling as an indicator of PTP opening in cardiac mitochondria isolated from rats treated with A-769662 (A) and/or SfA. (a) ROS levels in mitochondria, expressed as pmoles of H_2O_2 per mg of protein per minute. (b) Representative traces of mitochondrial swelling, shown as calcium-induced decrease in light scattering at 520 nm. (c) Quantitative data of PTP opening, shown as the rate of swelling and expressed in percentage of maximum swelling induced by 0.5 mM $CaCl_2$. The number of animals: Sham ($n = 7$), IR ($n = 8$), IR + SfA ($n = 4$), IR + A-769662 ($n = 6$), and IR + SfA + A ($n = 4$). $^{*}P < 0.01$ versus Sham; $^{\#}P < 0.01$ versus IR.

there are no studies elucidating simultaneous effects of AMPK activation and PTP inhibition on cardiac IR. Our results show that A-769662 and SfA independently protected hearts against cardiac IR, as evidenced by reduced infarct size and improved cardiac output and ejection fraction. Interestingly, the protective effects provided by combined therapy of A-769662 and SfA on infarct size and cardiac function were similar to those provided by A-769662 alone. Based only on these data, it is impossible to conclude whether these compounds had synergistic or nonsynergistic effects. Interestingly, SfA treatment alone did not reduce infarct size as much as A-769662, although it protected cardiac function as much as A-769662 or A-769662 + SfA. These results suggest that the cardioprotective mechanism of PTP inhibition is not dependent solely on infarct size reduction, but rather due to additional beneficial effects such as coronary vasodilation. Another possible explanation for these findings is that A-769662 could also activate other PTP-independent mechanisms to decrease infarct size.

Previous studies found that pretreatment with A-769662 did not stimulate phosphorylation of AMPK at Thr^{172} in the Langendorff-perfused mouse heart subjected to *ex vivo* IR [26]. However, treatment with A-769662 in combination with other classical AMPK activators (metformin, AICAR, phenformin, and oligomycin) induced a dramatic increase in AMPK phosphorylation and stimulated glucose uptake [34]. It has been suggested that A-769662 activates AMPK by not increasing its phosphorylation at the Thr^{172} site of the alpha subunit; it acts through the regulatory β-subunit to allosterically activate AMPK and its downstream targets [26]. Consistent with this, we observed no further increase in P-$AMPK^{Thr172}$ levels in the hearts pretreated with A-769662.

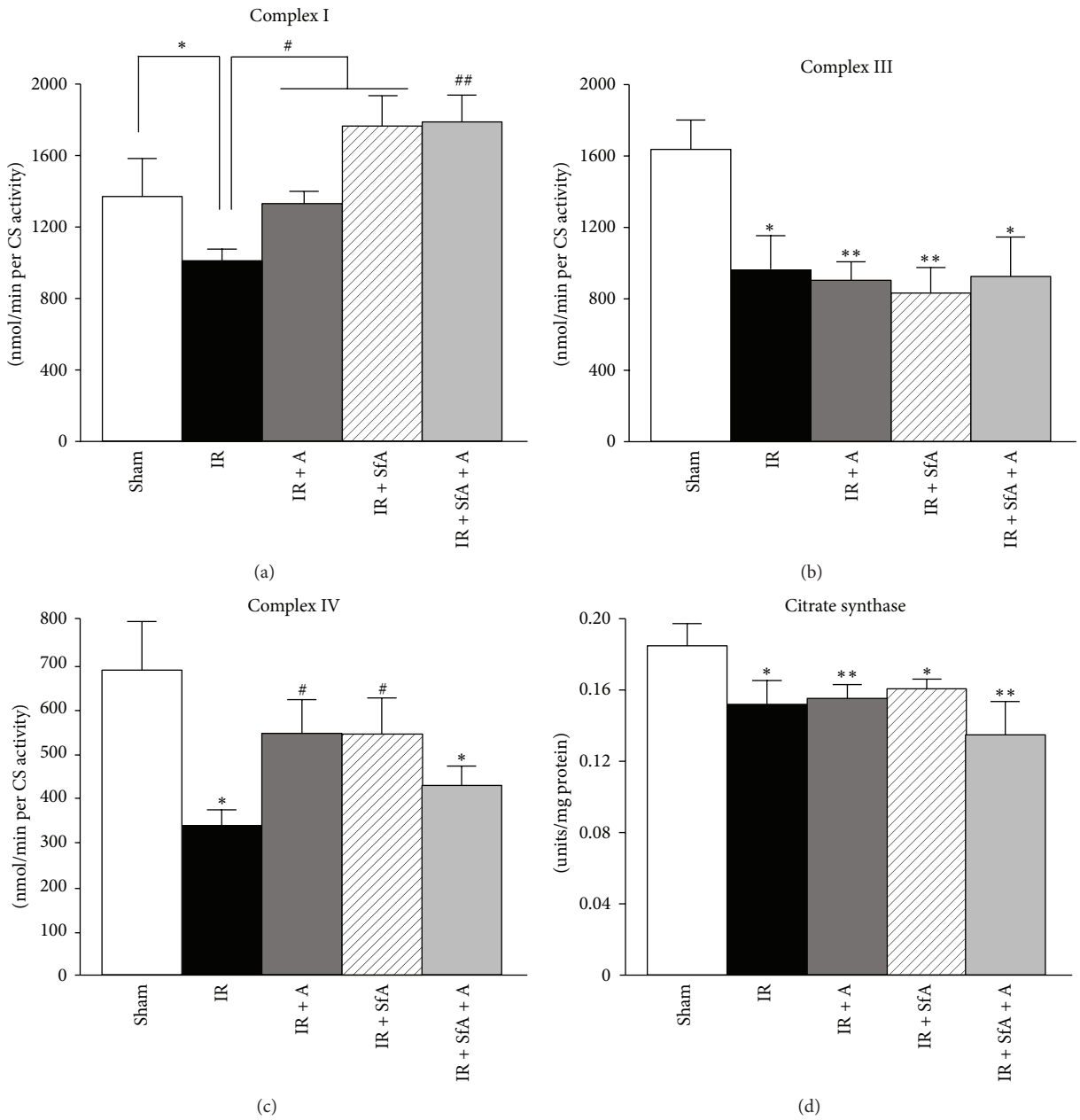

Figure 5: Effects of SfA, A-769662 or their combination (A) on the activity of the ETC complexes I (a), III (b), and IV (c), and citrate synthase (d) in cardiac mitochondria. Activities of ETC complexes and citrate synthase (CS) were normalized to citrate synthase or mg mitochondrial protein, respectively. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus IR, $^{*}P < 0.05$, $^{**}P < 0.01$ versus Sham.

It has been suggested that the cardioprotective effects of AMPK activation are mediated mainly through the mitochondria. Mitochondria isolated from mouse hearts expressing kinase-dead (KD) AMPK demonstrated increased hydrogen peroxide production and decreased resistance to PTP opening compared to WT counterparts [35]. Conversely, pretreatment with A-769662 inhibited PTP formation, induced by *ex vivo* IR, in Langendorff-perfused hearts [18] and oxidative stress in H9c2 cardioblasts [20]. Consistent with these studies, we found that A-769662 causes inhibition of the PTP immediately upon reperfusion in *in vivo* cardiac IR. CyP-D is a key regulator of the PTP and has emerged as an important target for PTP inhibition [21, 36]. The pharmacological inhibitors of CyP-D, CsA, and SfA, have been shown to attenuate PTP opening and exert cardioprotective effects against IR [25, 37–39]. SfA is a CsA analogue that, unlike CsA, does not inhibit the activity of the Ca^{2+}-activated phosphatase, calcineurin [37]. Furthermore, SfA and CsA inhibit PTP opening through different mechanisms; CsA prevents interaction of CyP-D with the adenine nucleotide translocase, another key PTP regulator, whereas SfA inhibits the enzymatic activity of CyP-D [36].

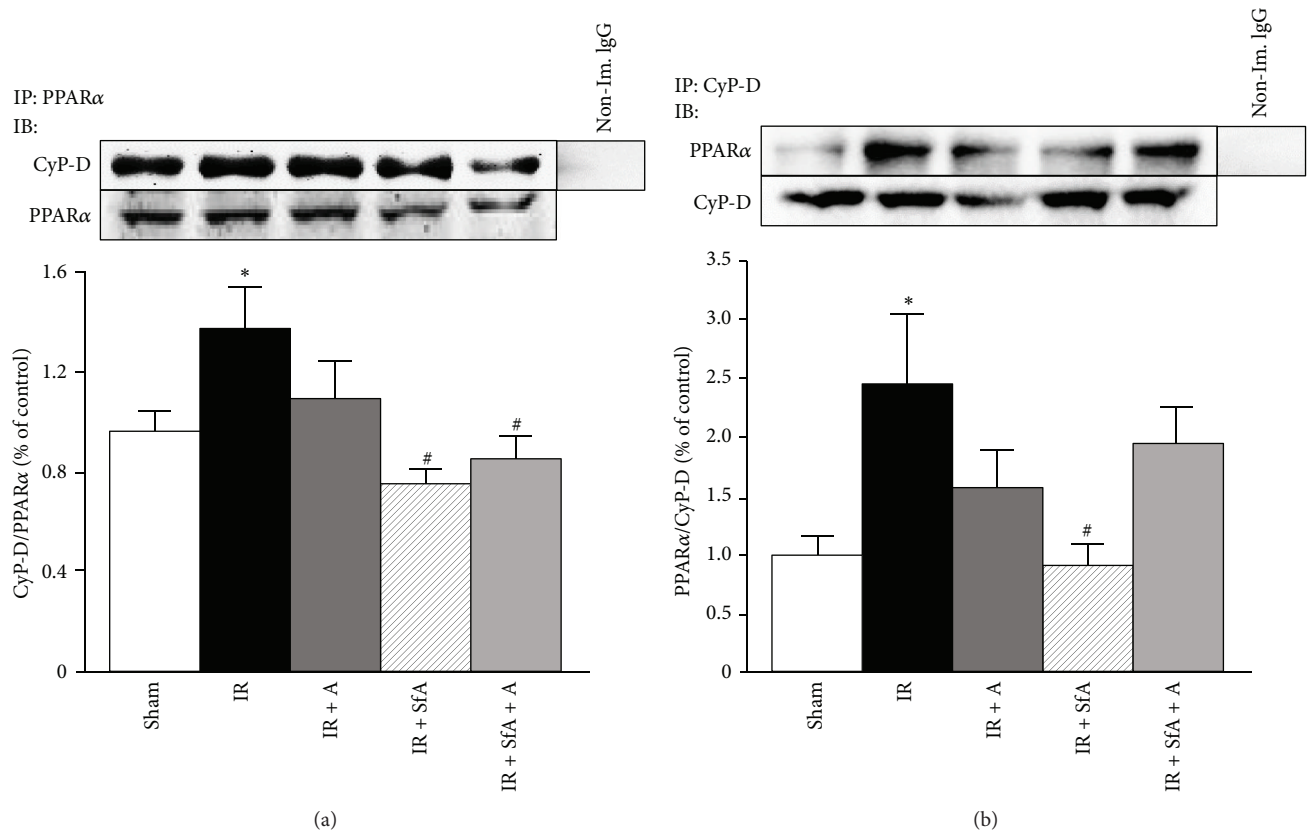

Figure 6: Effects of SfA, A-769662, or their combination on IR-induced CyP-D-PPARα interaction. Isolated mitochondria were immunoprecipitated (IP) with anti-PPARα (a) or anti-CyP-D (b) antibodies. The complexes were subjected to SDS-PAGE, followed by immunoblotting (IB) with CyP-D (a) or PPARα (b) antibodies. Representative immunoblots (a and b, top panels) show the effects of A-769662 and/or SfA on the interaction between PPARα and CyP-D. Quantitative results (a and b, bottom panels) were expressed as a fold change, compared with Sham. n = 3-4 per each group. $^{*}P < 0.05$ versus Sham; $^{\#}P < 0.05$ versus IR.

Notably, PTP opening occurs at reperfusion, but not during ischemia, and reaches a maximum within 10–15 min of reperfusion [36, 40]. Cardioprotective effects were observed only when SfA was administered during the first 15 min of reperfusion. Administration of SfA after 15 min of reperfusion had no cardioprotection against *ex vivo* IR injury [39]. Likewise, SfA administered 5 min prior to reperfusion showed a marked decrease in infarct size [25]. In line with these studies, in our experiments, SfA was administered immediately upon reperfusion, which significantly abrogated cardiac dysfunction and reduced infarct size. Choosing the right time to administer PTP inhibitors is apparently important for maximum protective effects in treatment of cardiac IR. This factor might be one of the main reasons that the recent CIRCUS clinical trials with CsA failed to protect hearts against reperfusion injury in STEMI patients [41].

Although we and others have established that the beneficial effects of AMPK activation against cardiac IR are mediated through PTP formation, the specific mechanisms associated with AMPK-induced inhibition of the PTP are still unclear. One potential mechanism may involve the indirect modulation of CyP-D. We have previously shown that the beneficial effects of metformin on mitochondria are mediated through PPARα, since the PPARα inhibitor GW6471 prevented cardioprotective effects of metformin against IR in rat hearts [19]. PPARα is one of central mediators involved in the mitochondrial transcriptional network that regulates cardiac mitochondrial metabolism and biogenesis under both physiological and pathological conditions [42, 43]. PPARα seems to be a downstream target for AMPK that could modulate PTP formation. Indeed, our studies with cultured H9c2 cells demonstrated that H_2O_2-induced oxidative stress promoted protein-protein interactions between PPARα and CyP-D that were associated with PTP opening. Conversely, activation of AMPK prevented this interaction, suggesting that AMPK was indirectly involved in regulating pore formation by diminishing the interaction of PPARα with CyP-D [20]. Likewise, *in vivo* cardiac IR induces protein-protein interactions between PPARα and CyP-D (Figure 6), and pretreatment with A-769662 abrogated this interaction, confirming the results previously observed with our *in vitro* model of oxidative stress.

Our data also indicate that the mechanism underlying the protective action of A-769662 is not associated with phosphorylation of PPARα. Inhibition of the PTP might be due to phosphorylation of GSK-3β, a downstream target of AMPK activation. Recent studies demonstrate that A-769662 increased the phosphorylation of GSK-3β, inhibiting

PTP formation [18] and reducing the levels of mitROS [44] in cardiac IR. AMPK could also inhibit the PTP through posttranslational modifications of CyP-D. Previous studies have demonstrated that acetylation of CyP-D [24, 45] can also initiate its interaction with other proteins and promote PTP formation. We have showed that the beneficial effects of metformin against oxidative stress-induced injury were not associated with acetylation or phosphorylation of CyP-D, although other types of posttranslational protein modifications may be involved [23]. Recent studies demonstrated a possible role of AMPK-induced JNK inhibition to increase the resistance of cardiac mitochondria to PTP opening in the kinase-dead AMPK mouse [35]. However, in our previous studies, the specific JNK inhibitor SU3327 had noninhibitory effects on the mitochondrial PTP in rat hearts subjected to global IR [28].

In summary, combination of A-769662 and SfA exerted no additional protective effects on cardiac function, mitochondri-al ETC activity, PTP opening, or mitROS levels compared to individual treatments. On the other hand, it is difficult to make conclusion on the synergistic effect of A-769662 and SfA as treatment with each compound returned the changes observed in IR to the Sham level for most parameters. Further studies are needed to elucidate the synergistic effect of AMPK activation and PTP inhibition and understand the molecular mechanisms of cardioprotection induced by the AMPK/PTP pathway.

Abbreviations

AMPK: AMP-activated protein kinase
CsA: Cyclosporin A
CyP-D: Cyclophilin D
ECG: Electrocardiography
ETC: Electron transport chain
IR: Ischemia-reperfusion
HW/BW: Heart weight to body weight ratio
CAL: Coronary artery ligation
mitROS: Mitochondrial ROS
PGC-1α: PPAR-gamma co-activator 1-alpha
PPAR: Peroxisome proliferator-activated receptor
PTP: Permeability transition pore
ROS: Reactive oxygen species
SfA: Sanglifehrin A
TTC: 2,3,5-Triphenyltetrazolium chloride.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Giselle Barreto-Torres performed the research, analyzed the data, and prepared the draft of the paper. Sabzali Javadov designed the study, performed interpretation of the data, contributed essential reagents, techniques, and tools, and revised and approved the final version of the paper.

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Research Article

Inducible Conditional Vascular-Specific Overexpression of Peroxisome Proliferator-Activated Receptor Beta/Delta Leads to Rapid Cardiac Hypertrophy

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Peroxisome proliferator-activated receptors are nuclear receptors which function as ligand-activated transcription factors. Among them, peroxisome proliferator-activated receptor beta/delta (PPARβ/δ) is highly expressed in the heart and thought to have cardioprotective functions due to its beneficial effects in metabolic syndrome. As we already showed that PPARβ/δ activation resulted in an enhanced cardiac angiogenesis and growth without impairment of heart function, we were interested to determine the effects of a specific activation of PPARβ/δ in the vasculature on cardiac performance under normal and in chronic ischemic heart disease conditions. We analyzed the effects of a specific PPARβ/δ overexpression in endothelial cells on the heart using an inducible conditional vascular-specific mouse model. We demonstrate that vessel-specific overexpression of PPARβ/δ induces rapid cardiac angiogenesis and growth with an increase in cardiomyocyte size. Upon myocardial infarction, vascular overexpression of PPARβ/δ, despite the enhanced cardiac vessel formation, does not protect against chronic ischemic injury. Our results suggest that the proper balance of PPARβ/δ activation in the different cardiac cell types is required to obtain beneficial effects on the outcome in chronic ischemic heart disease.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear receptor superfamily. There are three members of the PPAR family (α, β/δ, and γ) with distinct, but overlapping spatial, temporal, and regulated expression patterns. For all PPARs, lipids are endogenous ligands and PPARs are considered as important transcriptional regulators of genes involved in lipid metabolism and cardiac energy production [1].

PPARβ/δ is the predominant subtype in the heart, and several lines of evidence suggest a cardioprotective function of PPARβ/δ. Cardiac PPARβ/δ deletion in mice resulted in cardiac dysfunction, hypertrophy, and congestive heart failure [2]. Furthermore, it has been shown that the PPARβ/δ agonist L-165041 inhibits pharmacologically induced hypertrophy of cardiomyocytes through the interaction of PPARβ/δ to NF-κB and a subsequent downregulation of NF-κB target genes [3, 4]. An *in vivo* study demonstrated that cardiac specific overexpression of PPARβ/δ led to increased myocardial glucose utilisation and did not alter cardiac function but tended to exert a protective effect to ischemia/reperfusion-induced myocardial injury. This was attributed to an activation of the Glut-4 promoter by PPARβ/δ and the subsequently increased cardiac glucose utilisation [5]. Finally, we recently showed that pharmacological activation of PPARβ/δ with GW0742

or GW501516 in mice led to rapid cardiomyocyte growth with a preserved myocardial function. We demonstrated that PPARβ/δ directly activates the Calcineurin gene [6], which is known to induce cardiac growth [7, 8]. Most interestingly, we observed in our study a rapid induction of cardiac angiogenesis upon pharmacological PPARβ/δ activation, a matter which surprisingly had not been investigated before, although the correlation between cardiac growth and angiogenesis seems quite evident. PPARβ/δ expression in endothelial cells has already been reported in 1999 by Bishop-Bailey and Hla [9]. Pharmacological activation of endothelial and endothelial progenitor cells with PPARβ/δ agonists had been shown to increase the migration, proliferation, and tube formation of these cells [10, 11].

Furthermore, PPARβ/δ knockout mice exhibited a diminished blood flow and immature microvascular structures in subcutaneously induced tumors, which could be rescued by reexpression of PPARβ/δ [12]. In human pancreatic tumors, PPARβ/δ expression strongly correlated with the advanced tumor stage and increased risk of tumor recurrence and distant metastasis. PPARβ/δ has therefore been suggested to be involved in the regulation of the angiogenic switch in tumor progression [13].

PPARβ/δ is also involved in physiological angiogenesis. As we and others showed, treatment with the PPARβ/δ agonists GW0742 and GW501516 induced an exercise-like phenotype in the heart. Both agonists induced a surprisingly rapid (after 24 h) remodelling of mouse hearts [6] and skeletal muscle [14] by increasing microvessel densities.

However, until now it was not clear if either the increase of the cardiac vasculature drives the myocardial hypertrophy or the enhanced cardiac angiogenesis might be a potential indirect effect of cardiomyocyte-specific PPARβ/δ overexpression.

In our present work, we address this question through the generation of transgenic mice with an inducible conditional vascular-specific overexpression of PPARβ/δ and analyze the normal cardiac phenotype and function as well as function and histology after experimental myocardial infarction.

We show that inducible vessel-specific overexpression of PPARβ/δ results in a rapid induction of angiogenesis, cardiac hypertrophy, and impairment of cardiac function as reflected by enhanced end-diastolic and end-systolic volumes, reduced fractional shortening, and decreased ejection fractions. Additionally, we demonstrate that, after myocardial infarction, despite the higher collateral vessel formation, the animals with vascular-specific PPARβ/δ overexpression display bigger infarct lesions, higher cardiac fibrosis, and further reduced cardiac function. This points to a more careful view about the potential benefits of PPARβ/δ agonists in the treatment of cardiovascular diseases, as the proper balance between cardiomyocytic and vascular PPARβ/δ seems to be crucial for cardiac health, especially under ischemic conditions.

2. Materials and Methods

2.1. Animals. All animals were used in accord with local Home Office regulations. *PPARβ/δ-flox*$^{+/-}$ [15] and *Tie2-CreERT2* [16] animals were crossed to generate *Tie2-CreERT2;PPARβ/δ-flox*$^{+/-}$ mice, further referred to as *Tie2-CreERT2;PPARβ/δ*. The *Tie2*-Cre-line was backcrossed four times onto C57BL6. Age- and sex-matched *Tie2-CreERT2;PPARβ/δ* animals were injected for one week intraperitoneally either with sunflower oil (vehicle) or Tamoxifen dissolved in sunflower oil in a dose of 33 mg/kg per day [17]. *Tie2-CreERT2* animals injected with Tamoxifen served as an additional control. Anaesthetized mice were examined by echocardiography using the iE33 xMATRIX system with a 12 MHz transducer (Philips Healthcare, DA Best, Netherlands). Myocardial infarctions were induced by ligation of the left coronary artery (LAD) as described [18]. Briefly, anaesthetized mice were endotracheally intubated, the skin was incised on the left thorax side, the pectoralis muscles were mobilized, a thoracotomy between the third and fourth rib was performed, and the LAD permanently was closed with a 7-0 suture distal to the left auricle. This resulted in large myocardial infarctions. The thoracotomy and the skin wound were closed with 4-0 sutures and the mice remained intubated until spontaneous respiration was reestablished. Lethality of the procedure was approximately 50% independent of the genotype of the mice.

2.2. Genotyping. The genotype of animals was identified by PCR. PCR conditions and primer sequences are available on request.

2.3. Tissue Samples, Histology, and Immunohistology. Histology and measurement of cardiomyocyte diameters were performed according to established protocols [19]. Samples from at least five different animals per group (*Tie2-CreERT2;PPARβ/δ* + vehicle, *Tie2-CreERT2* + Tamoxifen, and *Tie2-CreERT2;PPARβ/δ* + Tamoxifen) were analyzed. Investigators were blinded for the genotype of the mice. Three μm paraffin sections were used for histological and immunohistological procedures.

Haematoxylin-Eosin staining was routinely performed on all tissue samples; additionally, sections were stained with Trichrome Masson and Picrosirius red. For PPARβ/δ and Pecam-1 immunohistology, after heat-mediated antigen retrieval and quenching of endogenous peroxidase activity, the antigen was detected after antibody application Pecam-1 (CD31) (1 : 100, rabbit polyclonal, ab28364, Abcam) or PPARβ/δ (1 : 100, rabbit polyclonal, ab154395, Abcam) using EnVision™ Peroxidase/DAB Detection System from Dako (Trappes, France). Sections were counterstained with Hematoxylin (Sigma). Omission of the first antibody served as a negative control. Additionally, some slides were incubated with IgG Isotype Controls (1 : 100, rabbit monoclonal, clone SP137, Abcam). Slides were viewed under an epifluorescence microscope (DMLB, Leica, Germany) connected to a digital camera (Spot RT Slider, Diagnostic Instruments, Scotland).

Area densities for all immunohistological stainings were determined using the ImageJ software. Vessel area density was analyzed on at least five different sections of hearts per mouse.

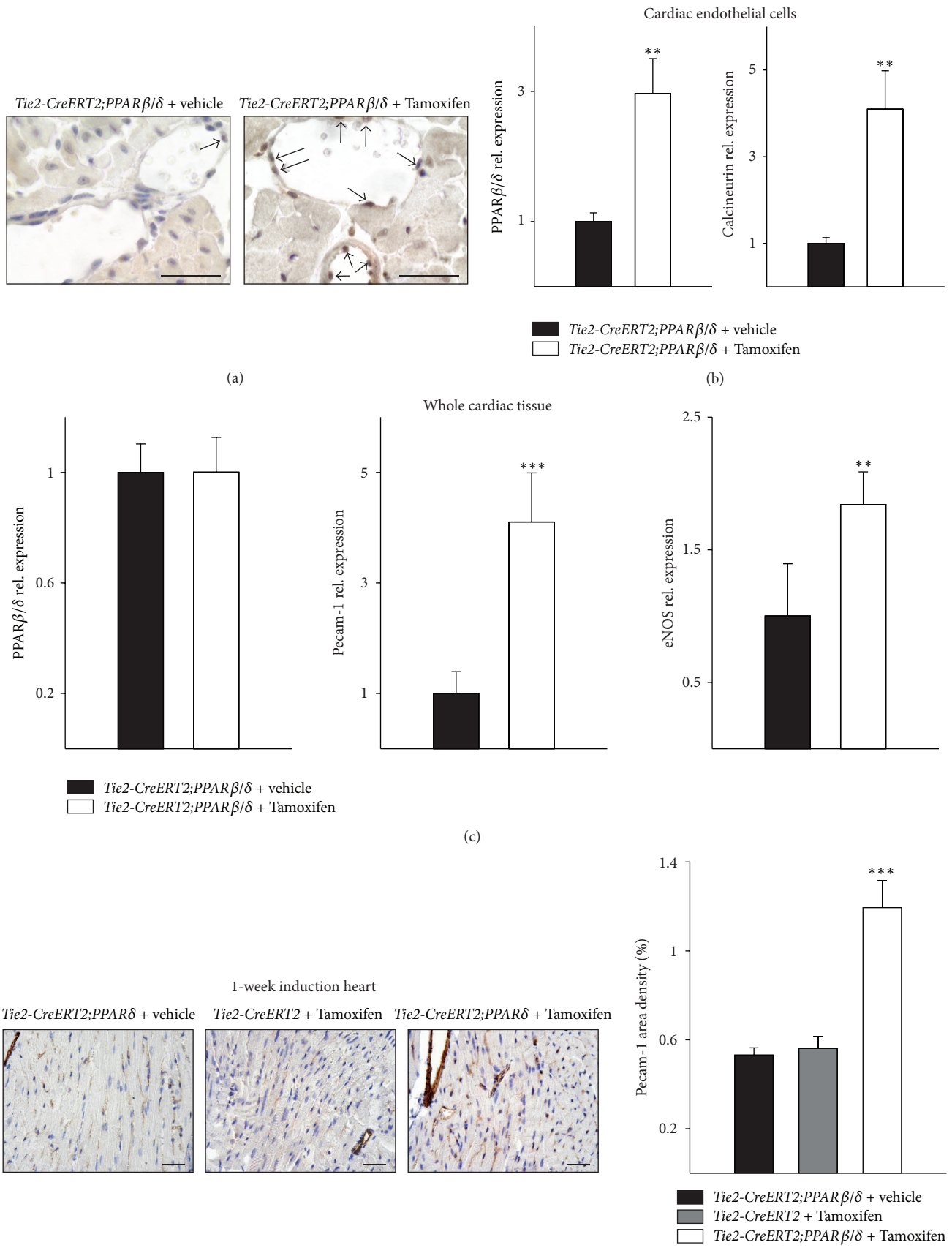


Figure 1: Continued.

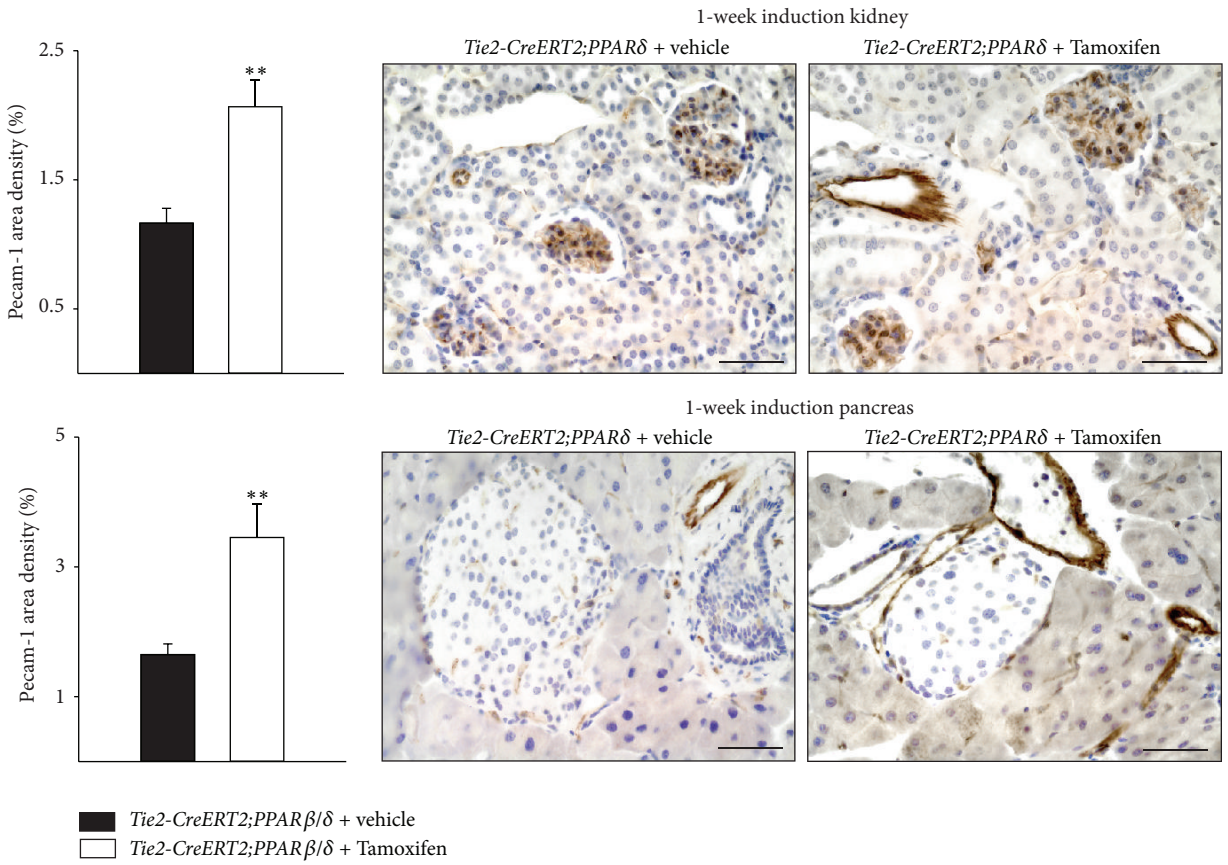


Figure 1: Increased cardiac vessel density upon Tie2-Cre-mediated conditional PPARβ/δ overexpression. (a) PPARβ/δ immunostaining from heart sections of *Tie2-CreERT2; PPARβ/δ* + vehicle and *Tie2-CreERT2;PPARβ/δ* + Tamoxifen animals indicates higher expression levels in the endothelium of *Tie2-CreERT2;PPARβ/δ* + Tamoxifen animals. Arrows mark PPARβ/δ positive endothelial cells. (b) Quantitative real-time PCRs for PPARβ/δ and Calcineurin in cardiac endothelial cells from *Tie2-CreERT2;PPARβ/δ* + vehicle and *Tie2-CreERT2;PPARβ/δ* + Tamoxifen animals ($n = 5$ for each group). (c) Expression levels for PPARβ/δ, Pecam-1, and eNOS determined by quantitative real-time PCRs from whole mouse heart RNA preparations for both groups ($n = 5$ for each group). (d) Pecam-1-immunostaining in mouse heart sections and quantification of Pecam-1 signal area density (*Tie2-CreERT2;PPARβ/δ* + Tamoxifen, $n = 5$, *Tie2-CreERT2;PPARβ/δ* + vehicle, $n = 5$, and *Tie2-CreERT2* + Tamoxifen, $n = 5$). (e) Quantification of Pecam-1 signal area densities and Pecam-1-immunostainings in mouse kidney (upper panel) and pancreas (lower panel) sections (*Tie2-CreERT2;PPARβ/δ* + Tamoxifen, $n = 3$, and *Tie2-CreERT2;PPARβ/δ* + vehicle, $n = 3$). Scale bars indicate 50 μm. Data are means ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$.

2.4. Real-Time RT-PCR. Total RNA was isolated from hearts and cardiac endothelial cells, sorted with CD31 MicroBeads (Miltenyi Biotec) from the mouse hearts using the Trizol reagent (Invitrogen). The RNA pellet was dissolved in diethyl pyrocarbonate-treated H_2O. First-strand cDNA synthesis was performed with 0.5 μg of total RNA using oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen). One μL of the reaction product was taken for real-time RT-PCR amplification (ABI Prism 7000, Applied Biosystems) using a commercial SYBR® Green kit (Eurogentec, Angers, France). Primer sequences are available on request. Expression of each gene was normalized to the respective *Gapdh, Actb, and Rplp0* expression.

2.5. Statistical Analysis. Data are expressed as means ± SEM. ANOVA with Bonferroni test as *post hoc* test or Mann-Whitney tests was performed as indicated. A p value of less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. PPARβ/δ Vascular-Specific Overexpression Rapidly Increases Cardiac Vessel Density. Immunohistochemistry for PPARβ/δ of heart sections proved the upregulation of PPARβ/δ protein expression in the endothelium of *Tie2-CreERT2;PPARβ/δ* mice induced with Tamoxifen as compared to vehicle-treated *Tie2-CreERT2;PPARβ/δ* animals (Figure 1(a)). Quantitative RT-PCRs from cardiac endothelial cells enriched with Pecam-1/CD31 MicroBeads were performed to confirm the vascular overexpression of PPARβ/δ upon Cre-mediated recombination. Endothelial cells isolated from hearts of Tamoxifen induced *Tie2-CreERT2;PPARδ* animals

showed a modest upregulation of PPARβ/δ and Calcineurin expression compared to cardiac vascular cells of vehicle-treated animals (Figure 1(b)). In contrast, no significant changes in PPARβ/δ expression levels in whole heart RNA preparations could be detected (Figure 1(c)), additionally confirming specificity of vascular PPARβ/δ overexpression, as endothelial cells contribute only to around seven percent of the total cell numbers in the mouse heart [20]. An increase in cardiac vessel density became evident on the RNA (Figure 1(c)) as well as on the protein level (Figure 1(d)) already one week after Cre-mediated vascular PPARβ/δ overexpression. Additionally, increased cardiac eNOS expression confirmed the enhanced cardiac angiogenesis (Figure 1(c)). The detection of Pecam-1 protein expression by immunohistochemistry allowed determining that this upregulation of Pecam-1 was due to the formation of new microvessels (for comparison, see Figure 1(d) right photomicrograph, which depicts higher microvessel formation in the hearts of *Tie2-CreERT2;PPARβ/δ* animals induced with Tamoxifen as compared to vehicle-treated *Tie2-CreERT2;PPARβ/δ* animals on the left or Tamoxifen treated *Tie2-CreERT2* animals in the middle). Determination of Pecam-1 area density indicated a doubling of Pecam-1 positive vascular structures (Figure 1(d)). This angiogenic response to transgenic overexpression of PPARβ/δ in the endothelium was also observed in the kidney and the pancreas (Figure 1(e)), indicating a general proangiogenic action of PPARβ/δ in endothelial cells. These findings are in line with previous studies, which reported a rapid enhancement of vessel density upon pharmacological PPARβ/δ activation [6, 14] and the general view of PPARβ/δ as a proangiogenic factor [21].

3.2. Specific Vascular Overexpression of PPARβ/δ Induces Cardiac Hypertrophy. Already one week after induction of PPARβ/δ expression in vessels, it became evident that cardiac growth was enhanced in the animals with Cre-mediated recombination as compared to both controls, vehicle-treated Tie2-CreERT2;PPARβ/δ and Tie2-CreERT2 mice treated with Tamoxifen. Heart/body weight measurements confirmed the macroscopic observation. This growth induction became more enhanced after three weeks and remained then stable for up to two months, the latest time point studied (Figure 2). The cause of this cardiac growth was an increase in cardiomyocyte size, as determined by cardiomyocyte diameter measurements at the different time points. On average, the cardiomyocyte diameter increased about 30% compared to the respective controls (Figure 3). Vascular formation during embryonic development is crucial for organ growth; for example, the inhibition of coronary vessel formation abolishes cardiac growth [22]; however, the factors determining organ size in an adult organism are not completely understood, but some lines of evidence suggest that, during tissue repair or in response to physiological stimuli vessel formation is required for organ enlargement [23]. Some evidence that at least for the heart vascular growth indeed led to an increase in the cardiac mass under normal conditions came from a study using transgenic mice with a cardiomyocyte-specific on/off regulatable system for the secretion of the proangiogenic factor PR39. The authors concluded that myocardial hypertrophy observed after three weeks was due to the induction of angiogenesis. They suggested that increased NO production due to increased endothelial cell mass mediated the observed hypertrophy [24]. This is in accordance with our finding of enhanced eNOS expression in the hearts of mice with vascular-specific overexpression of PPARβ/δ (Figure 1(b)). However, PR39 is a macrophage derived peptide, which inhibits degradation of hypoxia inducible factor 1α protein, thus activating angiogenesis through the induction of VEGF and fibroblast growth factor signalling and acting on all cardiac and other cell types. It can therefore not be excluded that part of the observed effects in this study was due to actions of PR39 on other cell types of the heart compared to only endothelial cells. The fact that we could observe cardiac hypertrophy already one week after vascular-specific overexpression of PPARβ/δ is mostly due to the overexpression of PPARβ/δ in endothelial cells, which induced angiogenesis leading to hypertrophy of the cardiomyocytes. Our approach was more direct as targeting the secretion of a proangiogenic factor like PR39 by cardiomyocytes, which affects secondarily the endothelium and in the end the increase in cardiomyocyte size is solely attributed to the increased angiogenesis. However, in the mentioned study, it cannot be excluded that the forced secretion of a proangiogenic molecule by cardiomyocytes also acts on other cell types compared to only endothelial cells, including cardiomyocytes themselves. The endothelial-specific conditional induction of PPARβ/δ in our model excludes a potential interference with possibly in parallel ongoing actions in other cardiac cell types.

3.3. PPARβ/δ Vascular-Specific Overexpression Also Increases Capillary Density in the Setting of Myocardial Infarction but Fails to Ameliorate the Outcome after Chronic Ischemic Heart Disease. To investigate the effect of PPARβ/δ driven angiogenesis on myocardial function in pathological settings, the left anterior descending (LAD) coronary artery in *Tie2-CreERT2;PPARβ/δ* animals induced with Tamoxifen or treated with vehicle was ligated. Immunohistochemical investigation of Pecam-1 expression demonstrated a significant increase in capillary density not only in the infarct zone but also in the border zone of the infarcted area and in the remote myocardial area of the right ventricle of *Tie2-CreERT2; PPARβ/δ* animals induced with Tamoxifen compared to those treated with vehicle only. This was additionally confirmed by quantification of Pecam-1 area densities (Figure 4). Heart/body weight determination demonstrated a hypertrophic effect of vascular-specific overexpression of PPARβ/δ also in the setting of chronic ischemic heart disease, due to an increase in cardiomyocyte size (Figure 5(a)). Interestingly, histological analyses revealed much bigger infarct sizes in animals with vascular-specific overexpression of PPARβ/δ as compared to controls (Figure 5(b)) and an enhanced cardiac fibrosis, as determined by Picrosirius red staining for collagen (Figure 5(c)). This is in contrast to the study using a cardiomyocyte-specific on/off regulatable system for

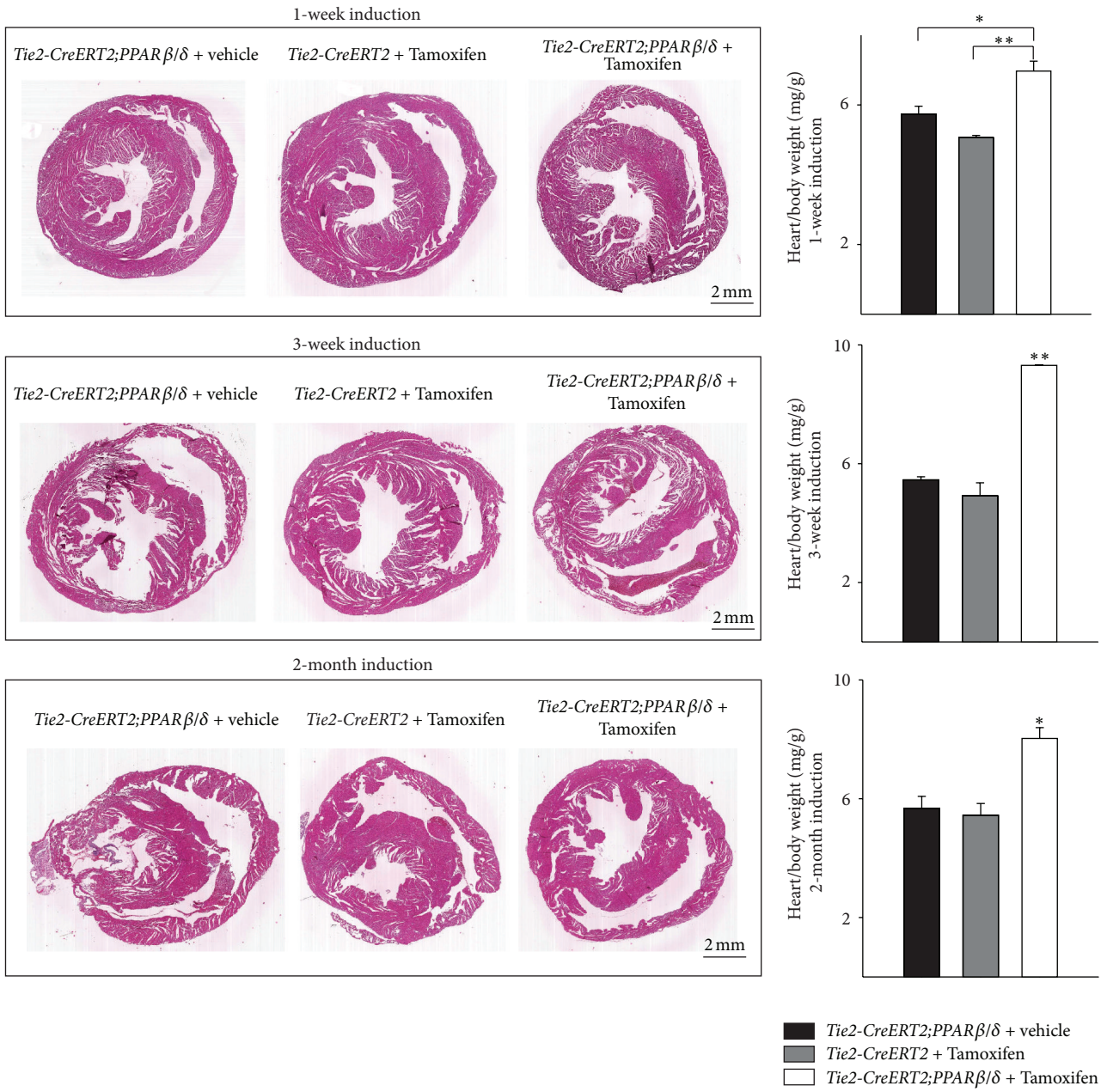


FIGURE 2: Rapid induction of cardiac growth by Tie2-Cre-mediated conditional PPARβ/δ overexpression. Photomicrographs of Hematoxylin-Eosin- (HE-) stained cross sections of the hearts and respective heart-to-body weight ratios (*Tie2-CreERT2;PPARβ/δ* + Tamoxifen, $n = 7$, *Tie2-CreERT2;PPARβ/δ* + vehicle, $n = 6$, and *Tie2-CreERT2* + Tamoxifen, $n = 6$). Scale bars indicate 2 mm. Data are means ± SEM. $^{*}p < 0.05$ and $^{**}p < 0.01$.

the secretion of the proangiogenic factor PR39 from cardiomyocytes; the secretion of PR39 reduced infarct sizes after myocardial infarction [24]. However, as stated before, this study was based on the effects of PR39, a macrophage derived proangiogenic molecule, which might act on all cardiac cell types rather than solely on endothelial cells. Our results are in agreement with clinical studies suggesting cardiac hypertrophy as a risk factor for arteriosclerosis, myocardial infarction, and heart failure [25]. This is probably due to the increased energy consumption of hypertrophic myocardium.

To test whether the angiogenesis induced cardiac hypertrophy affects cardiac function, we performed premyocardial and three-week postmyocardial infarction echocardiographic examinations in *Tie2-CreERT2;PPARβ/δ* animals induced with Tamoxifen and the respective controls treated with vehicle. Consistent with the observed cardiac

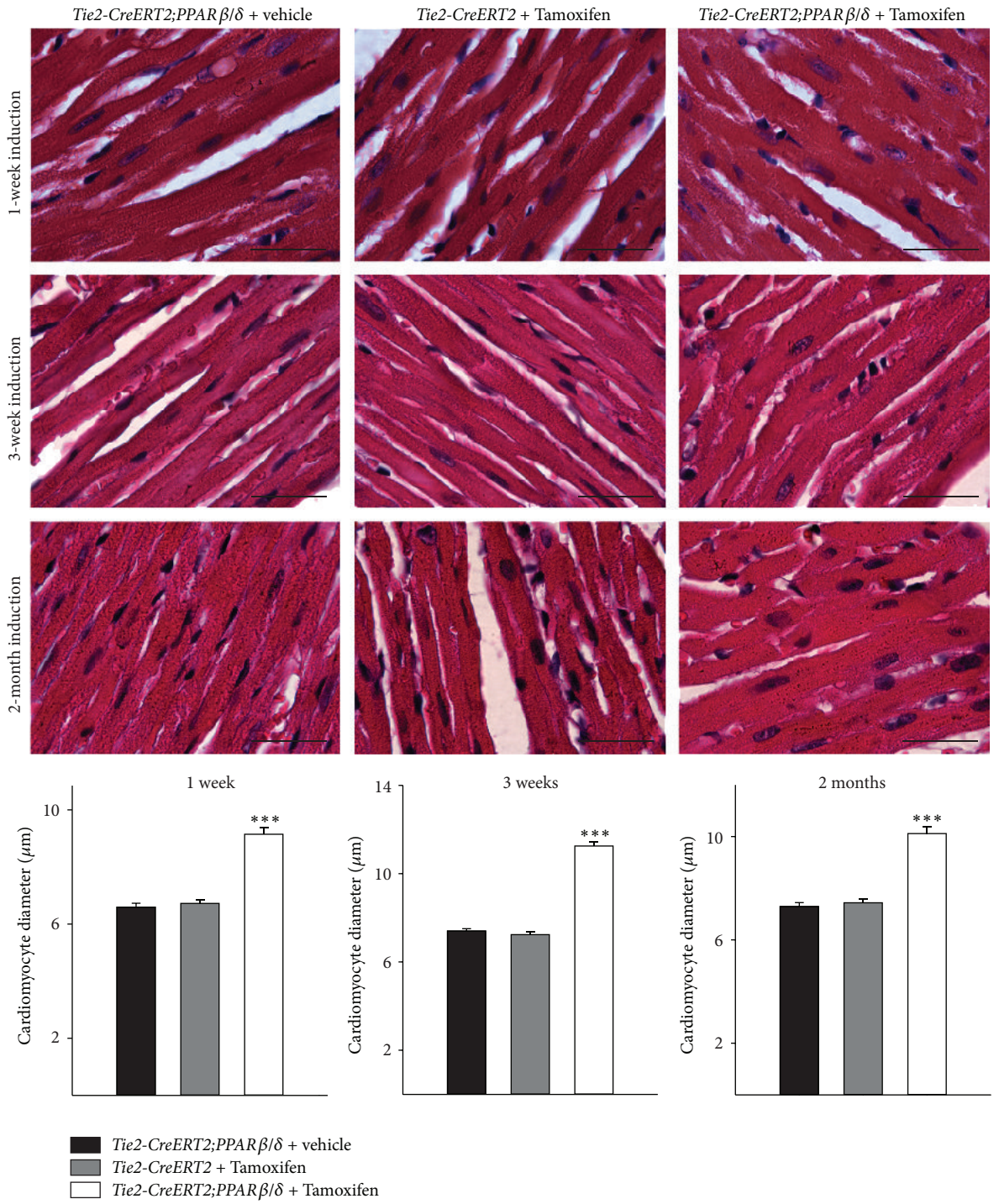


FIGURE 3: Enhanced cardiomyocyte diameter upon vascular-specific PPARβ/δ overexpression. High power photomicrographs of HE-stained sections showing individual cardiomyocytes and quantification of cardiomyocyte diameters. Scale bars indicate 50 μm. Data are means ± SEM. $^{***}p < 0.001$.

hypertrophy, mice with vascular-specific overexpression of PPARβ/δ showed an increase in left ventricular end-diastolic (LVED) and -systolic (LVES) volume. Fractional shortening and the ejection fraction were slightly reduced as compared to the respective controls (Figure 6(a)).

Three weeks after myocardial infarction, control *Tie2-CreERT2;PPARβ/δ* animals treated with vehicle also showed an increase in the left ventricular end-diastolic and -systolic volume as well as a reduction in the fractional shortening and ejection fraction when compared to their healthy status

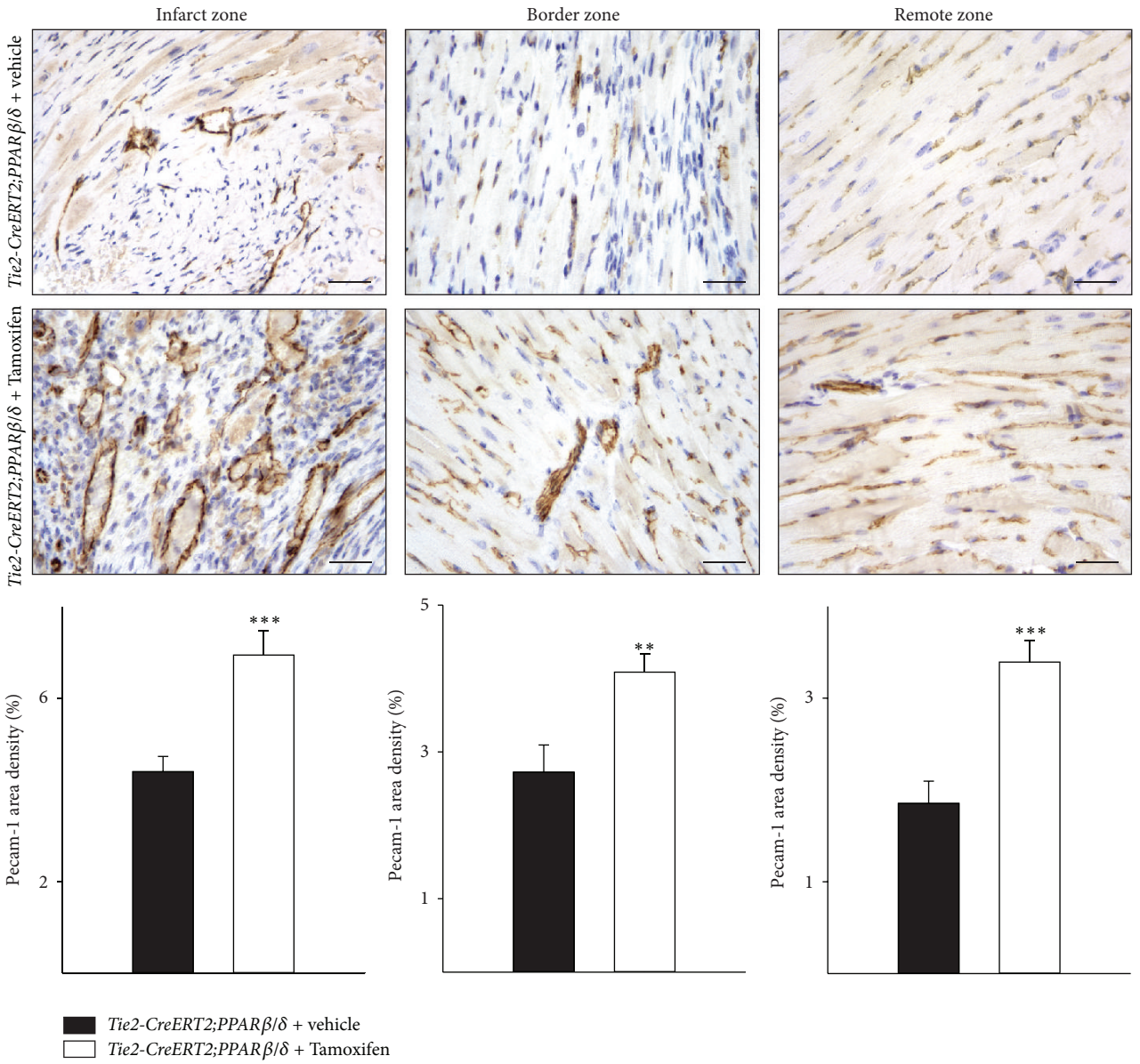


FIGURE 4: Increased vessel formation after myocardial infarction in the hearts of mice with vascular-specific PPARβ/δ overexpression. Pecam-1-immunostaining in mouse heart sections and quantification of Pecam-1 signal area density (*Tie2-CreERT2;PPAR*β/δ + Tamoxifen, $n = 5$, and *Tie2-CreERT2;PPAR*β/δ + vehicle, $n = 5$). Scale bars indicate 50 μm. Data are means ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$.

before chronic ischemic heart disease. However, the situation was far worse in the mice with vascular-specific overexpression of PPARβ/δ; both LVED and LVES volume were highly increased and the fractional shortening and ejection fraction severely diminished (Figure 6(b)). Most studies attributed to PPARβ/δ a cardioprotective role, as *in vitro* and *in vivo* data suggested that PPARβ/δ inhibits cardiomyocyte apoptosis [26], protects against lipotoxicity [2], reduces cardiomyocyte hypertrophy [27], and, if overexpressed in cardiomyocytes, reduces myocardial injury due to ischemia/reperfusion [5]. Animals treated with PPARβ/δ agonists showed a rapid increase of the cardiac vasculature and an enhanced cardiac growth without functional impairment [6]. It seems as if the proper balance between PPARβ/δ activation in endothelial cells and cardiomyocytes (and maybe other cardiac cell types as fibroblasts) is required to confine the attribute "cardioprotective" to PPARβ/δ. Our results indicate that the specific, unbalanced activation of PPARβ/δ only in the vasculature, despite its effects on vessel and cardiac growth, is not sufficient to protect against chronic ischemic heart disease. Nevertheless, it is possible that activation of PPARβ/δ in the vasculature might have beneficial effects in the settings of smaller infarct sizes or in slowly developing arteriosclerosis, which will be subject of future studies.

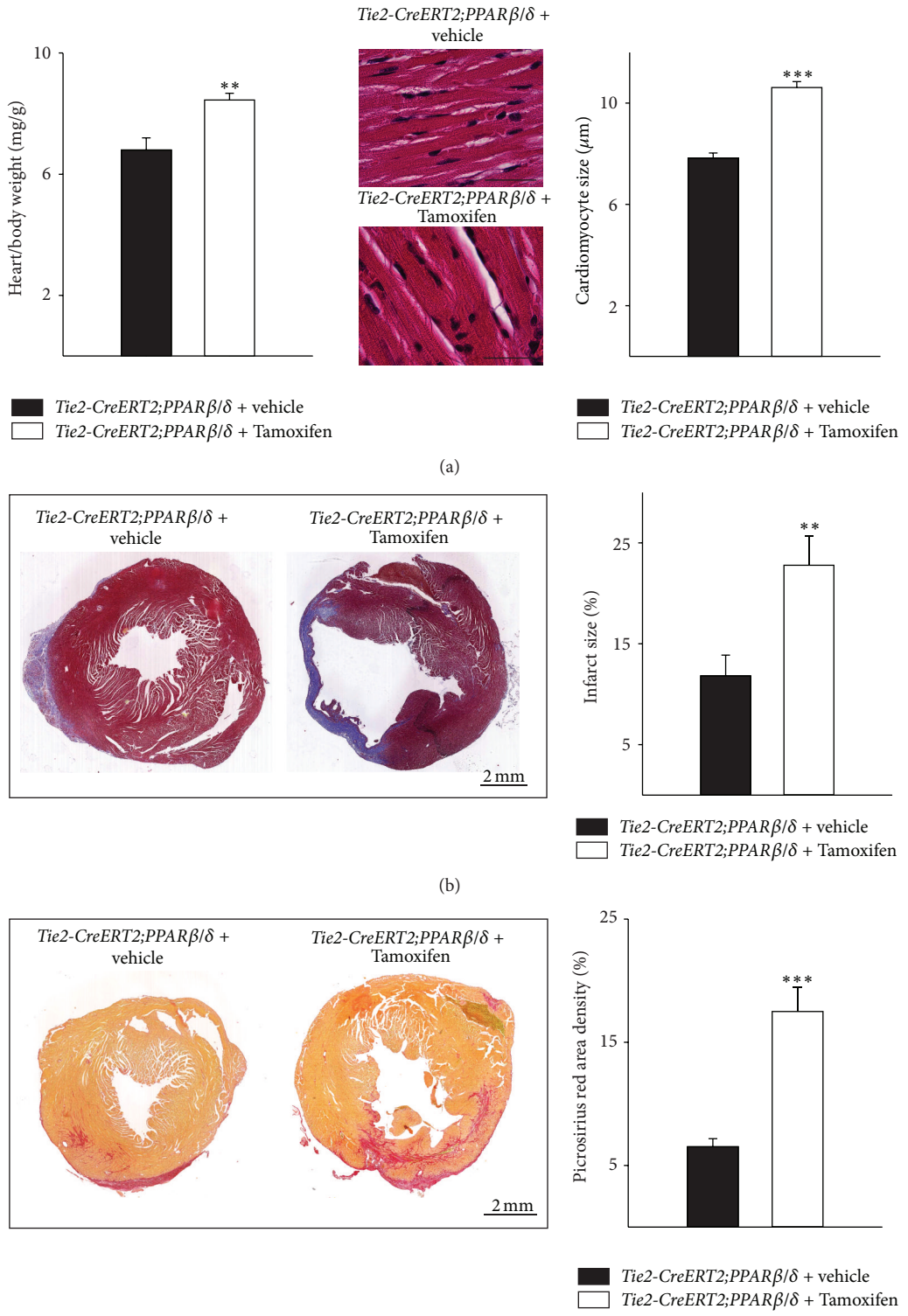
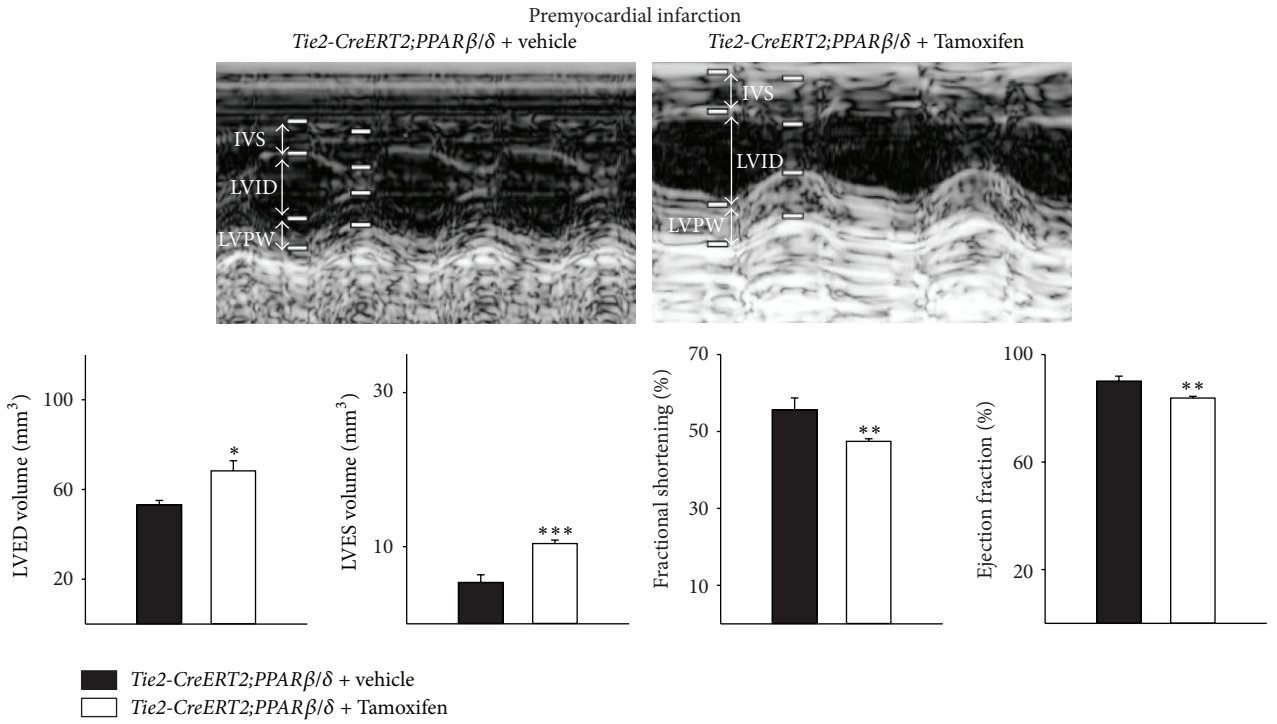
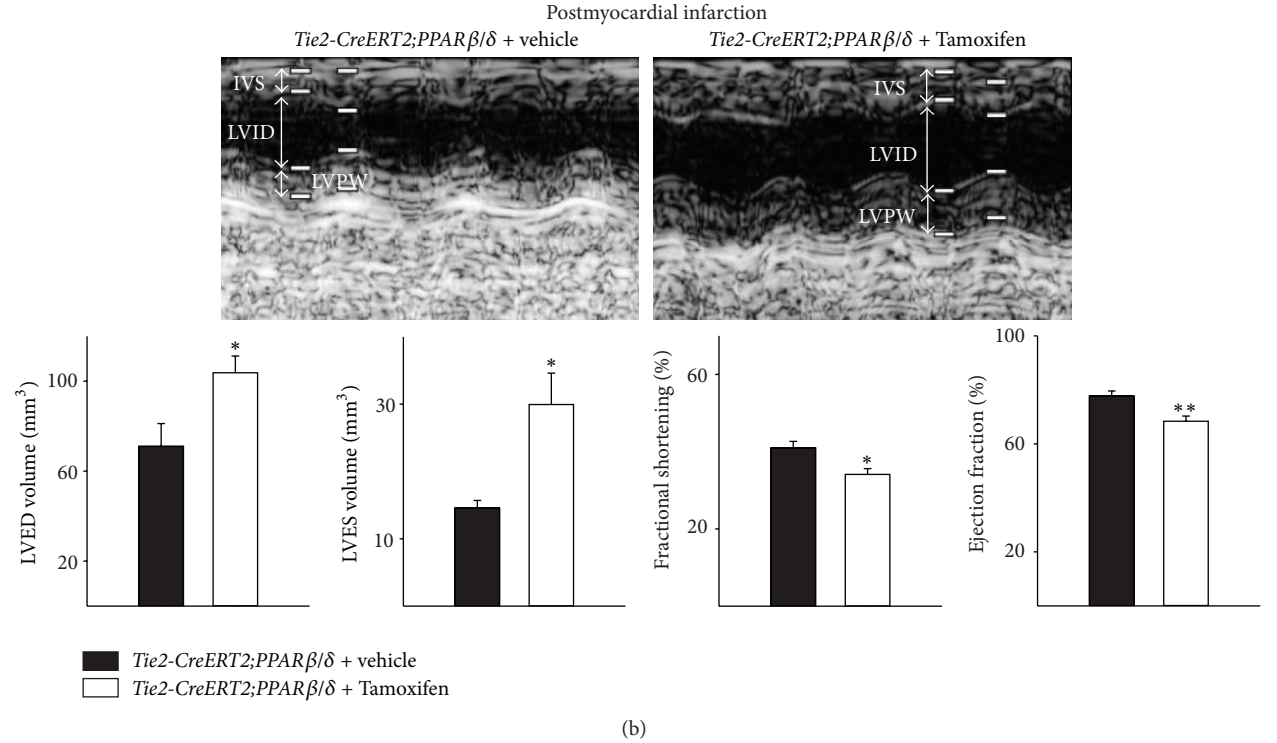


Figure 5: Increased infarct sizes and higher cardiac fibrosis in animals with vascular-specific PPARβ/δ overexpression. (a) Respective heart-to-body weight ratios and high power photomicrographs of HE-stained heart sections showing individual cardiomyocytes and quantification of cardiomyocyte diameters. Scale bars indicate 50 μm. (b) Photomicrographs of Trichrome Masson stained cross sections and quantification of the infarct sizes (*Tie2-CreERT2;PPARβ/δ* + Tamoxifen, $n = 8$, and *Tie2-CreERT2;PPARβ/δ* + vehicle, $n = 5$). Scale bars indicate 2 mm. (c) Photomicrographs of Picrosirius red stained cross sections and quantification of cardiac fibrosis. Scale bars indicate 2 mm. Data are means ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$.



(a)



(b)

Figure 6: Impaired cardiac function upon vascular-specific PPARβ/δ overexpression, which worsens after myocardial infarction. (a) Echocardiographic examination indicates increased systolic and diastolic volumes, a reduced fractional shortening, and a decreased ejection fraction in animals with vessel-specific overexpression of PPARβ/δ, which becomes more evident after myocardial infarction (b) (*Tie2-CreERT2;PPARβ/δ* + Tamoxifen, $n = 8$, and *Tie2-CreERT2;PPARβ/δ* + vehicle, $n = 5$). IVS: interventricular septum; LVID: left ventricular internal diameter; LVPW: left ventricular posterior wall. Data are means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

4. Conclusions

In this study, we investigated the effects of a vascular-specific overexpression of PPARβ/δ on cardiac phenotype and function. The rapid induction of cardiac vessel formation was accompanied by an induction of cardiac growth, characterized by an increase in cardiomyocyte diameter. Upon myocardial infarction, the increased cardiac angiogenesis neither reduced infarct sizes nor improved the cardiac function. The proper balance of PPARβ/δ activation in the different cardiac cell types may be important for potential cardioprotective effects of PPARβ/δ.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Research Article

The Correlation of PPARα Activity and Cardiomyocyte Metabolism and Structure in Idiopathic Dilated Cardiomyopathy during Heart Failure Progression

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This study aimed to define relationship between PPARα expression and metabolic-structural characteristics during HF progression in hearts with DCM phenotype. Tissue endomyocardial biopsy samples divided into three groups according to LVEF ((I) 45–50%, $n = 10$; (II) 30–40%, $n = 15$; (III) <30%, $n = 15$; and control (donor hearts, >60%, $n = 6$)) were investigated. The PPARα mRNA expression in the failing hearts was low in Group (I), high in Group (II), and comparable to that of the control in Group (III). There were analogous changes in the expression of FAT/CD36 and CPT-1 mRNA in contrast to continuous overexpression of GLUT-4 mRNA and significant increase of PDK-4 mRNA in Group (II). In addition, significant structural changes of cardiomyocytes with glycogen accumulation were accompanied by increased expression of PPARα. For the entire study population with HF levels of FAT/CD36 mRNA showed a strong tendency of negative correlation with LVEF. In conclusion, PPARα elevated levels may be a direct cause of adverse remodeling, both metabolic and structural. Thus, there is limited time window for therapy modulating cardiac metabolism and protecting cardiomyocyte structure in failing heart.

1. Introduction

Heart failure (HF) can be of various etiology [1]. Despite substantial improvement in HF prevention and management, this clinical syndrome continues to be a major health concern, since more than 50% of HF patients die within 5 years of being diagnosed [2]. Therefore, an enhanced understanding of differences in cardiac status during the disease's progress is required for more precise therapy.

Routine histopathological examinations of human cardiac muscle collected *via* endomyocardial biopsy demonstrate the diversity of anomalies in myocardial tissue but have not been used to establish their peculiarities in association with metabolic changes and the degree of cardiac dysfunction. Cardiac remodeling underlying HF progression is known to be associated with cardiomyocyte hypertrophy, structural impairment and death, and fibrosis irrespective of disease etiology [3] on one hand and with changes in substrate uptake and metabolism [4] on the other. Most studies reveal reduced fatty acid (FA) use and impaired glucose oxidation in the failing heart [5]. The uptake and use of both substrates are closely linked and coregulated by the peroxisome proliferator-activated receptors alpha (PPARα) that is highly expressed in the myocardium [6, 7]. Findings

in PPARα-null mice [8] and MHC-PPARα mice [9] suggest that the lack of PPARα or increase in its level leads to cardiac hypertrophy and dilated cardiomyopathy (DCM). Furthermore, experimental data show that overactivation of PPARα is associated with impaired cardiac function [10–13]. The research on PPARα in the failing human heart had concerned only end-stage HF [14–18].

Therefore, to enhance our understanding of the phenomena associated with HF progression and DCM phenotype we analyzed sections of endomyocardial tissue taken during biopsy sampling. The aim of this study was to define the temporal relationship between PPARα expression and cardiomyocyte metabolic and structural remodeling in idiopathic DCM during HF progression.

Analyses revealed phase changes of PPARα during HF, among which significant structural damage and cardiomyocyte death were associated with PPARα increase.

2. Material and Methods

2.1. Patients. Forty consecutive patients with idiopathic dilated cardiomyopathy (iDCM) and six donor hearts (which had not been transplanted for technical reasons), all from the database of Silesian Medical University (Poland), were included in this study. All tissue samples were obtained with a signed consent from patients or patients' families. Exclusion criteria included diabetes, secondary heart failure (due to hypertension, ischemic heart disease, primary valvular disease, and alcohol abuse), and familial disease.

DCM groups were defined according to left ventricle ejection fraction (LVEF): Group (I) with mildly reduced LVEF of 45–50% ($n = 10$), Group (II) with moderately reduced LVEF of 30–40% ($n = 15$), and Group (III) with severely reduced LVEF of <30% ($n = 15$). Donor hearts (LVEF > 60%, $n = 6$) were served as the control group. The age of patients in groups with HF was between 30 and 40 years, and only single subjects had about 50 years, while in the control group the age was between 22 and 40 years. All patients with HF were on typical therapeutic regimens including digitalis, diuretics (furosemide, 40–80 mg/d, and spironolactone, 100 mg/d), an ACE inhibitor (captopril 50–75 mg/d), β-blockers (metoprolol, 50 to 100 mg/d), and an antiarrhythmic drug (amiodarone, 200 to 400 mg/d). Detailed characteristics of entire study population are presented in Table 1.

2.2. Histopathology with Morphometric Analyses. Deparaffinized 5 μm thick sections of biopsy samples taken for diagnostic reasons were routinely stained with hematoxylin and eosin (HE), Masson's Trichrome (MT), and periodic acid Schiff (PAS) and PAS with diastase digestion and described for general histopathology. Images from these sections taken under identical lightning conditions were analyzed by two independent investigators blinded to the NYHA class and LVEF value. Measurements were done with the Cell^{sense} program (Olympus). Finally, obtained data were presented as mean ± SEM for each group. Cardiomyocyte cross-sectional area and longitudinal area were measured systematically throughout the entire tissue section. Fibrosis quantification was performed on TM stained sections with the software by defining blue stained fibrotic areas automatically as the percentage of the total analyzed tissue. PAS-positive area was calculated as the percentage of the whole analyzed tissue section. The percentage of PAS-positive scored as 0 for area 5%, as 1 for area 6–30%, as 2 for area 31–60%, and as 3 for area >60%. Additionally, PAS staining intensity was scored as 0 for lack of staining, 1 for weak positive staining, 2 for moderate positive staining, and 3 for strong staining. The final score of glycogen accumulation resulted from both PAS staining intensity and the percentage of stained area as follows: normal glycogen abundance for a score of <1, low glycogen storage for a score of 1–4, and high glycogen storage for a score of ≥5.

2.3. Cardiomyocyte Immunohistochemistry and Numerical Cell Density. Deparaffinized 5 μm thick sections mounted on poly-L-lysine coated slides were blocked against endogenous peroxidase and nonspecific binding of the respective primary antibodies. Anti-desmin antibodies (1 : 50, DakoCytomation) and anti-ubiquitin antibodies (1 : 50, Abcam) were used for visualization of the desmin cytoskeleton and the intensity of proteasome/lysosomal degradation to estimate cardiomyocyte impairment and degeneration. Next, the secondary IgG antibody conjugated with horseradish peroxidase (EnVision System HRP ready to use, DakoCytomation) was applied. Nuclei were stained with hematoxylin. Numerical density of cardiomyocytes with pathology of the desmin cytoskeleton (according to the features described in detail previously [19]) was identified in each section and calculated as the percentage of the total cardiomyocyte number. Four categories of desmin patterns in cardiomyocytes were identified: normal, with regular distribution of fine desmin fibrils (type I), compensatory pattern, with increased desmin expression and properly arranged desmin (type IIA), and pathological pattern, with desmin visible as intercellular aggregates (type IIB) or with lack of desmin staining (type III).

Apoptotic and autophagic cardiomyocyte death was visualized with anti-active caspase-3 (1 : 200, Millipore) and anti-Beclin-1 (1 : 500, Novus Biotechnologies) primary antibodies, respectively. The secondary detection system was Alexa Fluor 488 goat anti-rabbit (Invitrogen). Nuclei were stained with DAPI (1 μL/1 mL, Millipore). Slices were investigated with a confocal microscope (FV-1000, Olympus). The autophagic and apoptotic cardiomyocytes were presented as the percentage of the total cardiomyocyte number.

The specificity of all staining was verified by omission of primary antibodies. All analyses were done in a blinded manner by two independent investigators.

2.4. Ultrastructure and Analysis. Biopsy sections routinely stained for ultrastructural evaluation and obtained from tissue samples processed into Epon blocks were examined under an electron microscope (Jem 1011, Jeol) and morphometrically evaluated using iTEM software. The intensity of anomalies in cardiomyocytes was graded as follows: contractile apparatus with thickened or blurred Z-bands (grade 1), myofibril loss (grade 2); mitochondria of normal shape, increased in number, and forming clusters (grade 1), polymorphic mitochondria with locally lucent matrix and lost cristae and decreased in number (grade 2); T-tubule loss

Table 1: Clinical data.

	Control ($n = 6$) LVEF > 60%	Group (I) ($n = 10$) LVEF 50–45%	Group (II) ($n = 15$) LVEF 44–30%	Group (III) ($n = 15$) LVEF < 30%
Age (y)	32.6 ± 10.8	34 ± 9.39	38.8 ± 7.87	41.13 ± 11.83
Men/women (n)	5/1	8/2	12/3	11/4
NYHA class	—	1.22 ± 0.44	1.6 ± 0.57	2.10 ± 0.47
BMI	22.05 ± 0.92	21.7 ± 1.43	26.58 ± 2.89	25.35 ± 3.88
LV EF [%]	63 ± 4	47.77 ± 2.81	35.80 ± 3.42	22.33 ± 4.06
LV EDD [mm]	51.34 ± 3.74	53.44 ± 1.74	65.13 ± 7.38	71.53 ± 6.65
LV ESD [mm]	30 ± 1.4	34.55 ± 4.74	47.73 ± 8.75	60.13 ± 5.46
ESV [mL]	45 ± 8	52 ± 4.2	124.2 ± 44.76	188.93 ± 49.25
EDV [mL]	102 ± 13.2	105 ± 24.8	182.86 ± 57.9	241.8 ± 79.15
NT-proBNP [pg/mL]	64.4 ± 1.4	78.61 ± 1.3	999.8 ± 1625.1	1709.6 ± 2314.3
Creatinine [μmol/L]	68.9 ± 10.2	72.01 ± 10.5	72.22 ± 13.09	76.46 ± 12.5
Glucose [mmol/L]	5 ± 0.40	5.03 ± 0.45	5.02 ± 0.40	5.51 ± 0.91
Cholesterol [mmol/L]	3.3 ± 1.42	3.33 ± 1.97	5.13 ± 0.58	4.99 ± 1.55
TG [mmol/L]	1.45 ± 0.21	0.85 ± 0.74	1.62 ± 0.77	1.53 ± 0.86
HDL [mmol/L]	1.2 ± 0.81	1.05 ± 0.84	1.39 ± 3.14	1.40 ± 0.56
LDL [mmol/L]	2.12 ± 0.50	2.01 ± 1.39	3.14 ± 0.60	2.45 ± 1.34
Diuretics	—	1	8	14
Digitalis	—	1	4	9
ACE inhibitors	—	2	3	3
β-blockers	—	3	6	5

at the Z-line (grade 1), dilated T-tubules with absent sarcoplasmic reticulum (SR) junction (grade 2); isolated lipid droplets in the cytoplasm (grade 1), numerous lipid droplets (grade 2); dispersed glycogen with slight accumulation in the perinuclear area and between myofibrils (grade 1), abundant glycogen throughout the cytoplasm (grade 2). Cumulative intensity of ultrastructural pathologies was calculated for each patient as a mean value. Additionally, the numerical density of cardiomyocytes with ultrastructural anomalies was determined and scored as 0 for 5%, 1 for 6–30%, 2 for 31–60%, and 3 for >60%. The final ultrastructural pathology score was calculated from total points obtained in both analyses and described as no pathology for score ≤2, mild pathology for score 3, moderate pathology for score 4, and severe pathology for score 5.

2.5. Quantitative Real-Time PCR. Total myocardial RNA was extracted from frozen myocardial tissue sections via a standard method. The quality and quantity of RNA were determined using gel electrophoresis and the spectrophotometric method (GeneQuant II RNA/DNA Calculator (Pharmacia Biotech)). Reverse transcription, followed by quantitative polymerase chain reaction (RT-PCR) for PPARα, FAT/CD36, CPT-1, GLUT4, PDK-4, Beclin-1, and caspase-3 mRNAs, was done according to a previously reported technique [20]. The mRNA copy numbers of examined genes were determined on the basis of the commercially available standard of β-actin (TaqMan DNA Template Reagent Kit, Applied Biosystems) and expressed per μg of total RNA. The quantity of PCR amplicons was determined after each round of amplification using the fluorescent dye SYBR-Green I (Qiagen). Oligonucleotide primers are shown in Table 2.

2.6. Statistical Analyses. All data are presented as mean ± standard deviation. A nonparametric approach was used, since the patient population did not show a normal distribution of clinical variables (Shapiro-Wilk normality test). The Mann-Whitney, Kruskal-Wallis, and *post hoc* tests were used for the statistical significance of differences yielded by mRNA evaluations. Then, Spearman coefficients were calculated for PPARα expression and metabolic and structural parameter in each investigated group and in the whole population with HF. Differences between groups were considered significant when the p value was <0.05.

3. Results

3.1. Expression of PPARα and the Related Genes during HF Progression. We detected a decrease in PPARα expression in hearts with mildly reduced EF (Group I), a marked increase in patients with moderate cardiac dysfunction (Group II), and a renewed decline in hearts with severely reduced EF (Group III) in comparison with the control group (Figure 1). Similar, heart failure stage-related changes were observed for FAT/CD36 and CPT-1 mRNA levels in Groups (I) and (III). However, there was only a tendency of CPT-1 mRNA level increase in Group (II) compared with the control. Subsequently, there was high expression of GLUT-4 mRNA

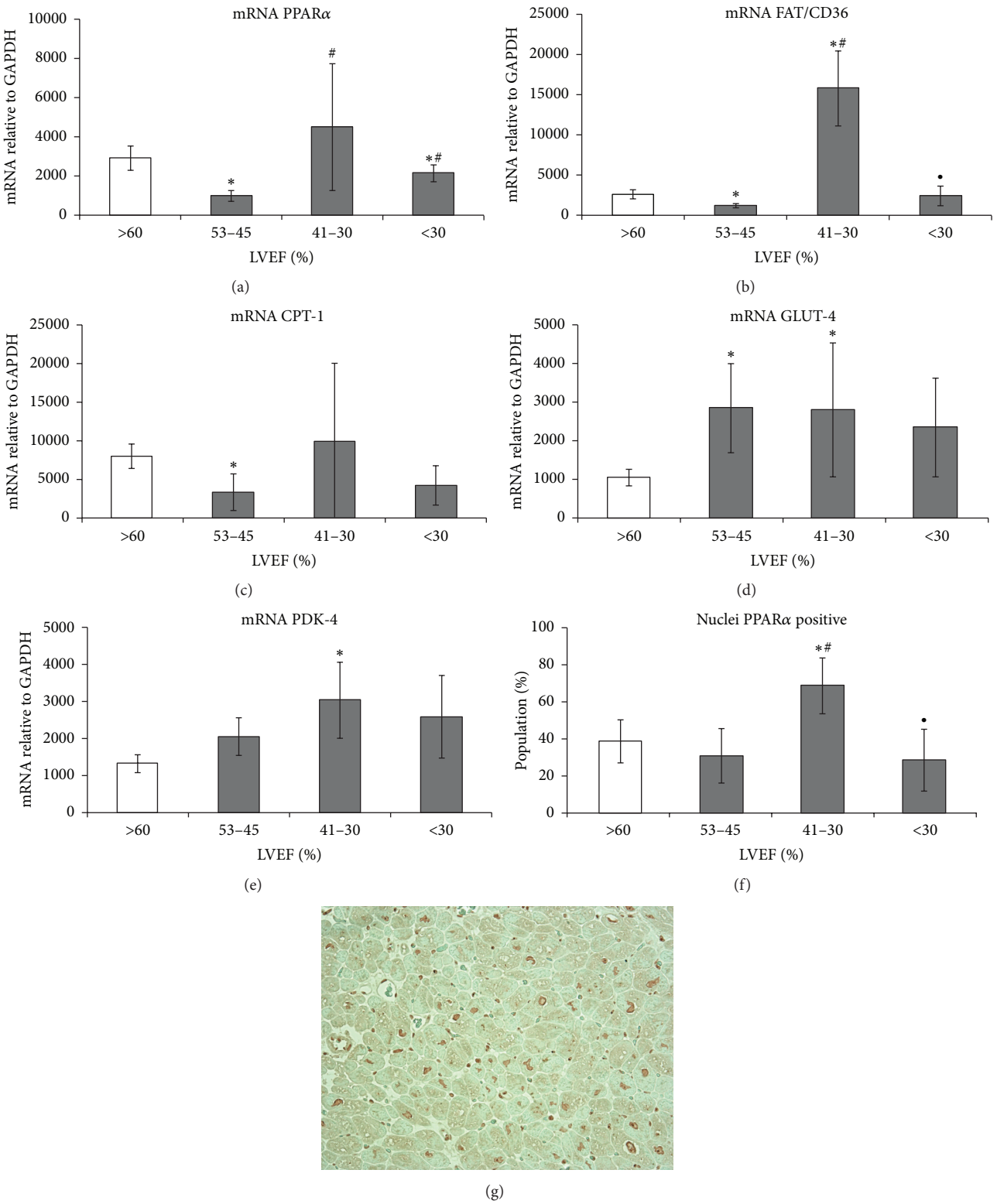


FIGURE 1: Changes in PPARα activity and in the related genes involved in fatty acid and glucose metabolism in the myocardium of the human heart during heart failure progression. Myocardial expression of (a) PPARα mRNA; (b) FAT/CD36 mRNA; (c) CPT-1 mRNA; (d) GLUT-4 mRNA; (e) PDK-4 mRNA. GPDH was used as a housekeeping gene. Results are presented as mean ± SEM; $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus Group (I); PPARα expression in myocardial tissue section. (f) Morphometric analysis of cardiomyocyte population with PPARα-positive nuclei; results are presented as mean ± SEM; $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus Group (I); $^{\bullet}p < 0.05$ versus Group (II). (g) A representative image showing the expression of PPAR in the myocardial tissue section of the heart patient from Group (II).

Table 2: Primers used for quantitative real-time polymerase chain reaction.

Gene	Primer sequence
PPARα	F: 5′-AGA-TTT-CGC-AAT-CCA-TCG-GC-3′ R: 5′-GCG-TGG-ACT-CCG-TAA-TGA-TA-3′
FAT/CD36	F: 5′-GGA-AAG-TCA-CTG-CGA-CAT-GA-3′ R: 5′-CCT-TGG-ATG-GAA-GAA-CGA-ATC-3′
CPT-1	F: 5′-GGT-GAA-CAG-CAA-CTA-TTA-TGT-C-3′ R: 5′-ATC-CTC-TGG-AAG-TGC-ATC-3′
GLUT4	F: 5′-GCT-ACC-TCT-ACA-TCA-TCC-AGA-ATC-TC-3′ R: 5′-CCA-GAA-ACA-TCG-GCC-CA-3′
PDK-4	F: 5′-TAC-TCC-ACT-GCA-CCA-ACG-C-3′ R: 5′-AAT-TGG-CAA-GCC-GTA-ACC-A-3′
Beclin-1	F: 5′-TGG-ATC-ACC-CAC-TCT-GTG-AG-3′ R: 5′-TTA-TTG-GCC-AGA-GCA-TGG-AG-3′
Caspase-3	F: 5′-AGA-ACT-GGA-CTG-TGG-CAT-TGA-G-3′ R: 5′-GCA-TTG-TCG-GCA-TAC-TGT-TTC-AG-3′
GAPDH	F: 5′-GAA-GTA-GGT-GAT-GGG-ATT-TC-3′ R: 5′-CAA-GCT-TCC-CGT-TCT-CAG-CC-3′
β-actin	F: 5′-TGC-CAT-CCT-AAA-AGC-CAC-3′ R: 5′-TCA-ACT-GGT-CTC-AAG-TCA-GTG-3′

Table 3: Morphometric data.

	Control ($n = 6$) LVEF > 60%	Group (I) ($n = 10$) LVEF 50–45%	Group (II) ($n = 15$) LVEF 41–30%	Group (III) ($n = 15$) LVEF < 30%
Myocyte cross-sectional area [μm^2]	415 ± 48	452 ± 83	548 ± 94	526 ± 127
Myocyte longitudinal area [μm^2]	1294 ± 146	1642 ± 198*	2094 ± 458*	2298 ± 477*#
Myocyte degeneration [%]	0.28 ± 0.1	1.25 ± 0.9	6.72 ± 3.5	10.23 ± 4.6
Desmin dominated pattern	I	IIA	IIB	III
Myocyte death				
Apoptotic [‰]	0	0.03 ± 0.004	0.05 ± 0.008	0.02 ± 0.001
Autophagic [‰]	0.4 ± 0.02	1.01 ± 0.8	3.5 ± 2.18	7.92 ± 2.39
Glycogen storage (final score)	Normal	Low	Severe	Moderate
Ultrastructural pathology (final score)	No	Mild	Moderate	Severe

* $P < 0.05$ versus control group, # $P < 0.05$ versus Group (I).

in all failing hearts and high expression of PDK-4 mRNA in Group (II), compared with control.

In agreement with PPARα gene expression were results of analysis of sections stained with anti-PPARα antibody showing a significant increase of cardiomyocytes with PPAR-positive nuclei in the endomyocardial tissue from hearts with moderately reduced LVEF (Figure 1).

3.2. Cardiomyocyte Hypertrophy, Structural Impairment, and Myocardial Fibrosis during HF Progression. Cardiomyocyte hypertrophy was expressed by an increase in cell cross-sectional and longitudinal areas compared with these parameters in the control group (Table 3, Figure 2). There was a significant increase in the length of cardiomyocytes in contrast to their diameter in the early stages of heart failure, while a significant increase in cardiomyocyte diameter was found in the group with moderately reduced heart function. Cellular hypertrophy was accompanied by nuclear enlargement, loss of regular striation in the contractile apparatus in HE stained sections, and appearance of abundant PAS-positive material within the cytoplasm (Figure 2). PAS-positive staining was visible in the form of discrete diffuse pattern in the group with LVEF of 50–45%, strong staining in most cardiomyocytes in the group with LVEF of 41–30%, and renewed diffuse or irregular staining in the group with severely decreased EF (Figures 2(d)-2(e)). Morphometric analysis of PAS-positive areas showed the greatest abundance of glycogen in the heart sections in Group (II).

Fibrosis sharply increased only in end-stage HF (Figure 2). The phenomenon was characterized by fine accumulation of collagen between cardiomyocytes and fascia between groups of cells in the hearts with mildly abnormal EF; however, there was no significant difference in comparison with control. Fibrosis with focal collagen accumulation

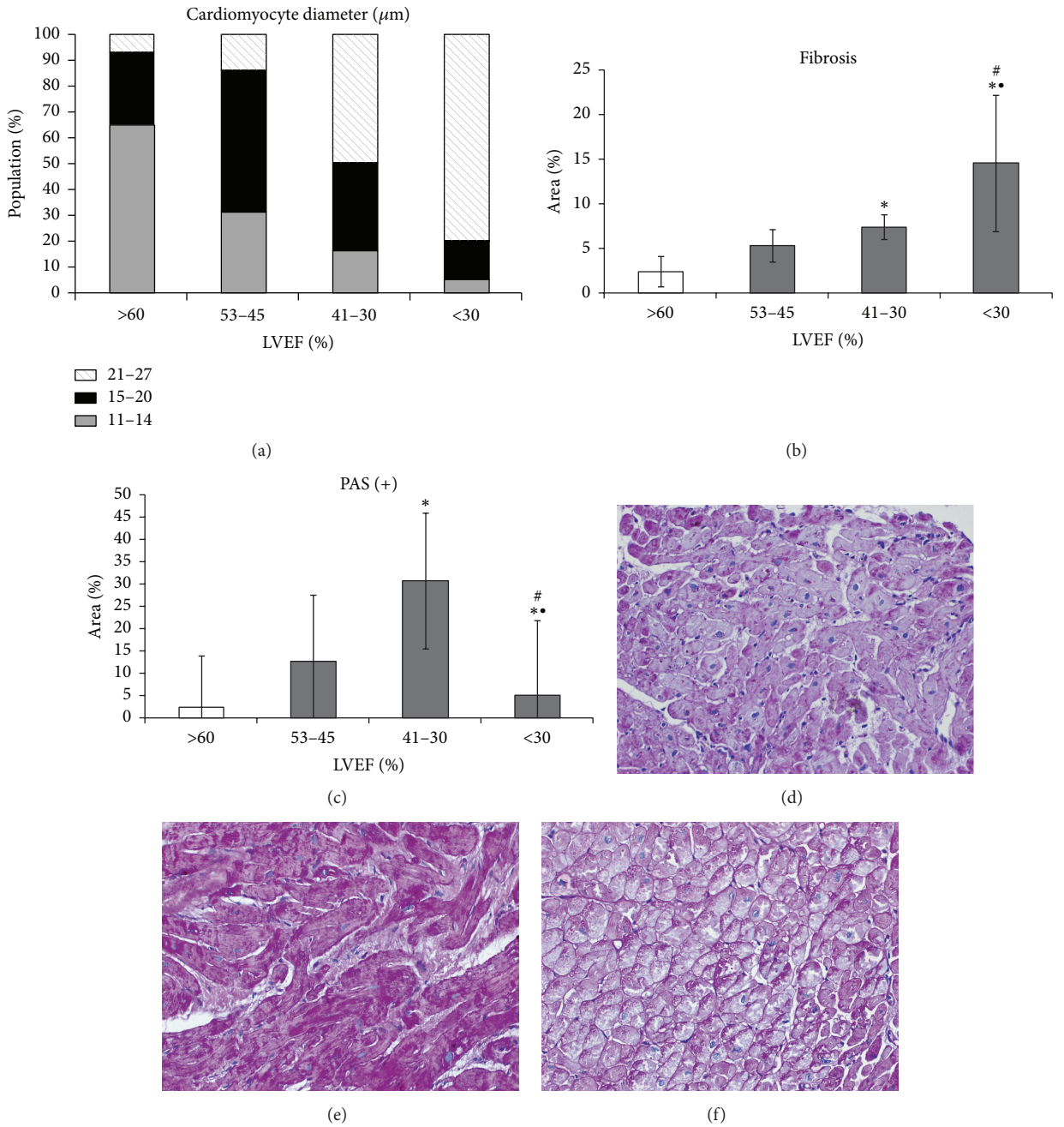


FIGURE 2: Results of morphometric analyses in tissue section: (a) cardiomyocyte hypertrophy; (b) fibrosis; (c) PAS-positive area; results as mean ± SEM; $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus Group (I); $^{\bullet}p < 0.05$ versus Group (II); representative images of PAS-stained endomyocardial tissue sections from hearts with (d) LVEF 53–45%, (e) LVEF 41–30%, and (f) LVEF < 30%.

between cardiomyocytes was seen in the hearts of Group (II) and continued further with the decrease of EF, suggesting replacement of dead cells. A small increase of fibrotic tissue was also observed around blood vessels in tissue samples from the hearts with severely reduced LVEF.

Changes of cardiomyocytes ultrastructure graded with HF progression (Figure 3). Mild ultrastructural changes appeared in cardiomyocytes in the hearts with mildly reduced EF. Significant increase in the number of mitochondria (about twofold increase in volume density compared with the control group) and low incidence of dilated T-tubules or a decreased T-tubule density or myofibril loss were characteristic features in these hearts. However, contraction bands were often seen. In contrast, in Group (II) irregular distribution and widening of sarcomeres and the T system, mitochondria polymorphism and varied size and damage of individual, and T-tubule-SR junction loss were accompanied by an abundance of glycogen in the cytoplasm, and numerous lipid

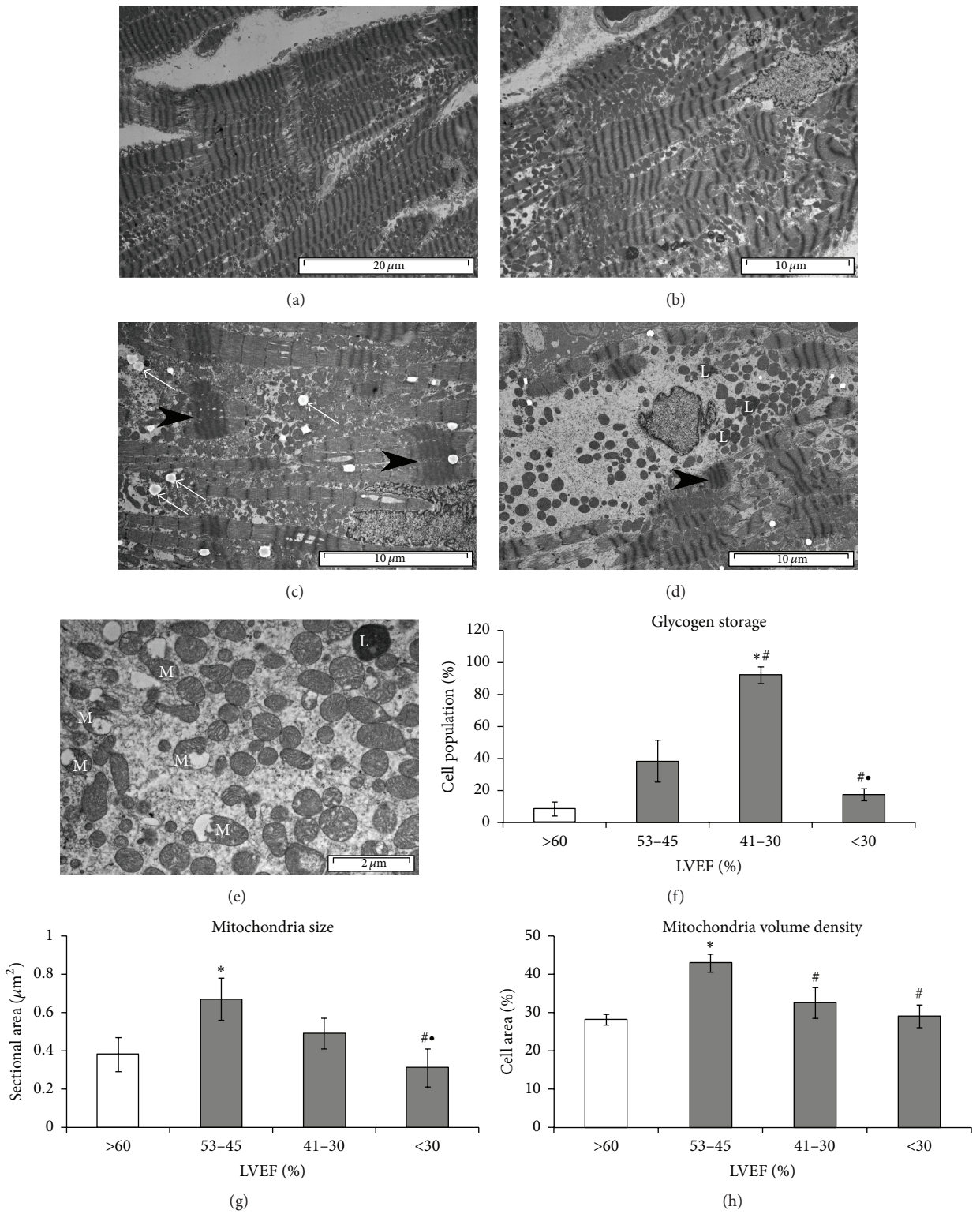
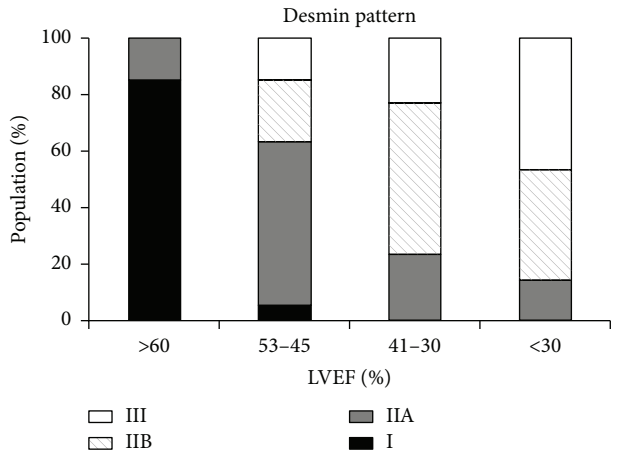
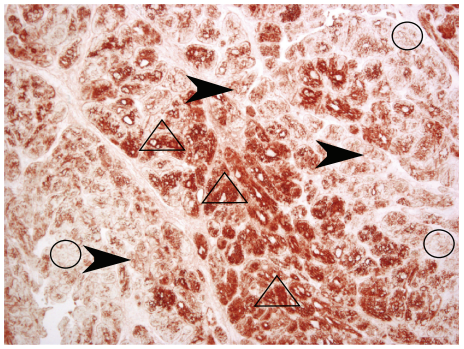


Figure 3: Cardiomyocyte ultrastructural remodeling during heart failure progression. Representative images for (a) control heart. Cardiomyocytes with normal ultrastructure and oval mitochondria arranged in rows. (b) Heart Group (I) (LVEF 53–45%). Cardiomyocyte with increased number of various size mitochondria arranged in clusters, partially missing contractile apparatus, incidence of dilated T-tubule. (c) Heart Group (II) (LVEF 41–30%). Cardiomyocyte with missing contractile apparatus and contraction bands (black arrowheads), numerous polymorphic mitochondria, lipid droplets between mitochondria (white arrows), and (d) Heart Group (III) (LVEF < 30%). Cardiomyocyte with severe loss of contractile fibrils and contraction band (black arrowhead), scattered mitochondria, and lipofuscin granules (L). (e) Impaired mitochondria (M) in hearts with LVER < 30%; results of morphometric analyses. (f) Glycogen storage. (g) Mitochondria size. (h) Mitochondria volume density. Results as mean ± SEM; $^{*}p < 0.05$ versus control; $^{\#}p < 0.05$ versus Group (I); $^{\bullet}p < 0.05$ versus Group (II).



(a)



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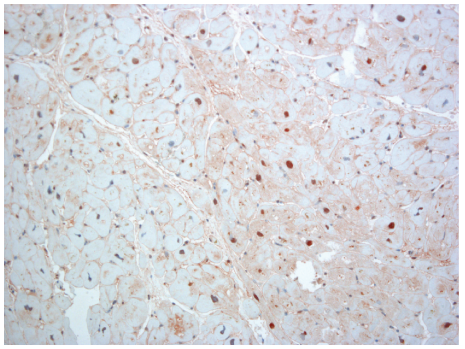

(c)

Figure 4: Desmin pattern in cardiomyocytes of failing heart. (a) Changes of desmin expression pattern in relation to the degree of heart failure. (b) A representative image showing the different types of desmin in the myocardium tissue of the patient's heart in Group (II): increased expression (arrowhead), desmin forming granular clusters (in triangles), or desmin disappearance (in circle). (c) Expression of ubiquitin in serial tissue section shown in (b).

droplets located close to mitochondria. Intensified changes, such as progressive loss of myofibrils, disorganized sarcomeres, scattered and decreased number of mitochondria, swollen mitochondria (Figure 3(d)), or loss of mitochondrial cristae and lipofuscin granules located in perinuclear area, were observed in Group (III). In this group over 30% of cardiomyocytes exhibited significant pathological changes. Important, significant differences in the accumulation of glycogen and mitochondria size and volume to the entire population of mitochondria were evident in the hearts of myocardial tissue cardiomyocytes Group (II) compared with Group (III) (Figure 3).

3.3. Cardiomyocyte Degeneration and Death. Cardiomyocyte degeneration related to desmin cytoskeleton injury. Desmin cytoskeletal anomalies manifested themselves in the form of increased appearance of desmin forming properly ordered structures (compensation pattern marked as type IIA) or granular structures (pathological pattern marked as type IIB) or showing a loss of desmin (pathological pattern marked as type III) (Figures 4(a) and 4(b)). Normal (pattern marked as type I) or compensatory desmin pattern corresponded to mild ultrastructural changes in cardiomyocytes, while pathological pattern corresponded to moderate and severe ultrastructural injury. Desmin pathological pattern was visible in increasing number of cardiomyocytes with HF progression. In addition, cardiomyocytes with a pathological IIB pattern of the desmin cytoskeleton demonstrated diffuse staining for anti-ubiquitin antibodies in the cytoplasm and nuclei (Figures 4(b) and 4(c)).

There was negligible cardiomyocyte death, autophagic and apoptotic, in Group (I); however the intensity of dying increased significantly with a reduction in LVEF (Table 3).

3.4. Association between PPARα and Metabolic and Structural Anomalies. Positive link between mRNA PPARα and GLUT-4 was observed only in Group (I), however with weak significance ($p = 0.083$) (Figure 5(a)). In contrast, we found a correlation between the expressions of PPARα mRNA and FAT/CD36 and CPT-1 mRNA for the entire study population (Figures 5(b) and 5(c)). Simultaneously, there was a strong trend in negative correlation between FAT/CD36 and LVEF (Figure 5(d)). Interestingly, a significant correlation between PPARα and cardiomyocytes degeneration (Figure 5(e)) and serum NT-proBNP level (Figure 5(f)) was found for tissue samples in Group (II) (Figure 5(e)), but not for whole failing

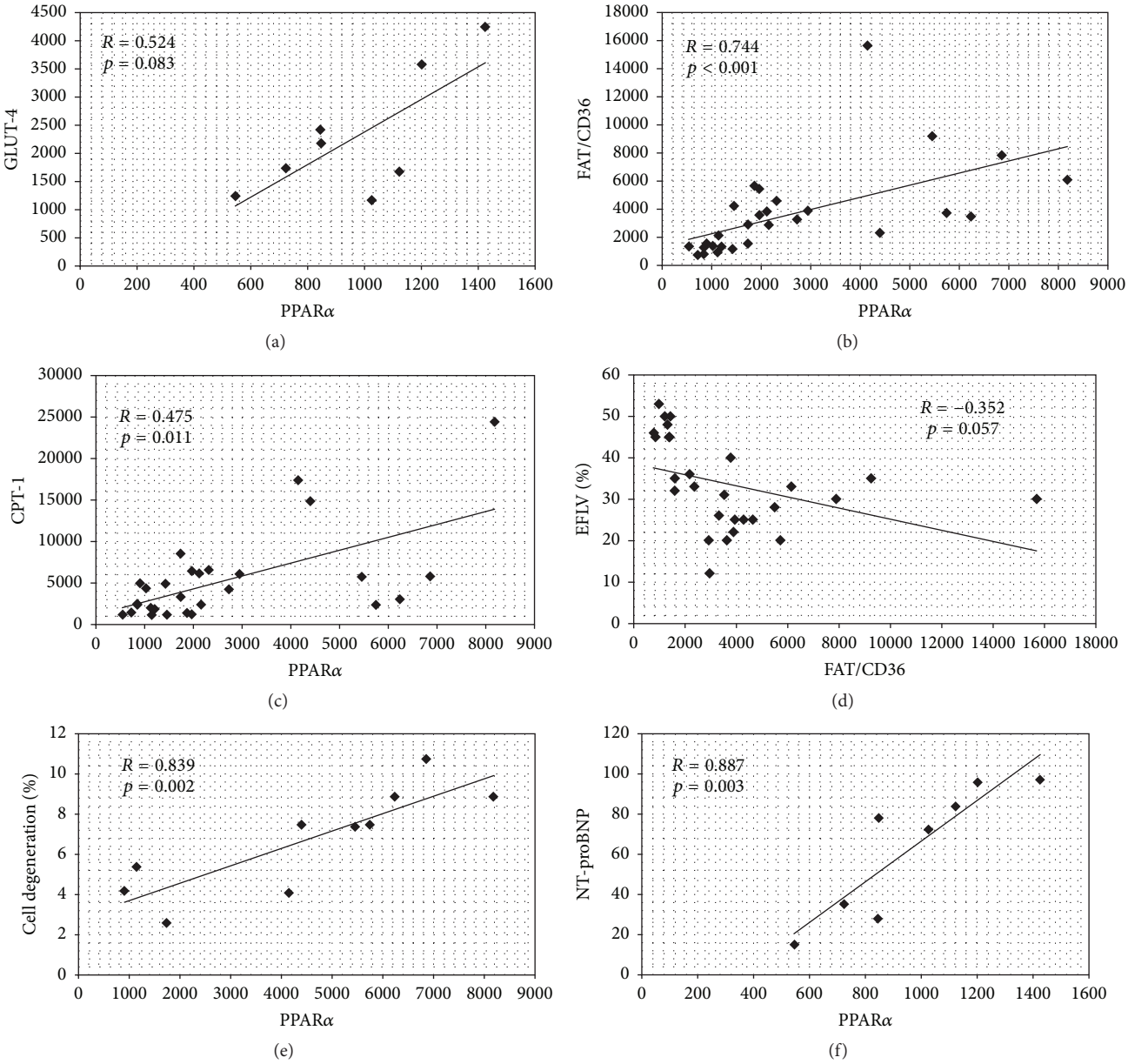

Figure 5: Association between PPARα and metabolic and structural anomalies. Results of analyses for patients Group (I): (a) PPARα and GLUT-4 mRNA for patients Group (I) and for all hearts with HF: (b) PPARα and FAT/CD36 mRNA; (c) PPARα and CPT; (d) FAT/CD36 mRNA and LVEF and for patients Group (II): (e) PPARα mRNA and cell degeneration and (f) PPARα mRNA and serum NT-proBNP level.

heart population, suggesting link PPARα overexpression and structural damage.

4. Discussion

In this study, we demonstrate for the first time the variability of PPARα mRNA levels in association with expression of genes involved in fatty acid and glucose metabolism during HF progression. Furthermore, PPARα overexpression in HF with moderately reduced LVEF suggests a maladaptive effect on cardiomyocyte structure.

Although altered PPARα expression in failing human hearts had already been reported for end-stage HF and the results, even for this stage, had been inconsistent, namely, a decreased [14], increased [16, 17, 21], or unchanged PPARα expression in comparison with that in the donor hearts [22] had been reported. In those studies the LVEF values were in range of these in patients from Groups (II) and (III) in our study. It should be emphasized that contradictory results as to PPARα expression were also reported in experimental HF models with accompanying DCM phenotype (e.g., [21, 23–25]).

Changes in PPARα expression are known to involve changes in FA and glucose metabolism, as activation of PPARα in the myocardium increases the expression of genes involved in FA uptake, mitochondrial β-oxidation, while

reduced PPARα activity leads to increased glucose uptake and use [4]. In our study, the hearts with mildly reduced LVEF (Group I) pattern of expression for genes involved in cardiac metabolism suggested an increase in intensity of glucose metabolism and decline in FA metabolism before a significant cardiomyocyte enlargement and cardiac hypertrophy was observed. Apart from an increased expression of GLUT-4, we found tendency to decrease of PDK-4 mRNA level. In this context only negligible accumulation of glycogen inside cardiomyocytes suggested an effective glycolysis and/or rapid turnover of glycogen. This metabolic pattern seems to be adaptive since cardiomyocyte death was also negligible and cardiomyocytes exhibited compensatory pattern of desmin cytoskeleton and normal ultrastructure. The increased number of mitochondria was probably a result of mitochondria biogenesis activated in order to adapt to energetic requirements in conditions of stress by the means of increasing glucose metabolism [26]. This is especially probable as the decrease in mitochondrial fatty acid oxidation (FAO) has been suggested as a predictive factor of the onset of contractile dysfunction [27, 28].

Metabolic patterns in the hearts with moderately reduced LVEF characterized by increased expression of FAT/CD36 and PDK-4 mRNA concomitant with a tendency towards increased CPT-1 levels and the accumulation of glycogen and lipid droplets in the cytoplasmic pool were linked to PPARα gene overactivation. It is known that PPARα may modify the expression of PDK-4 which phosphorylates the pyruvate dehydrogenase inhibiting the rate of glucose oxidation; therefore the resulting excess of glucose in the internal pool is stored in the form of glycogen [29]. Accumulation of lipid droplets in cardiomyocytes can be the effect of triglyceride (TG) synthesis dependent on FA uptake [30] or a decrease in FA liberation by hydrolysis [31]. The triglyceride pool has been also identified as a signaling factor for the regulation of PPARα activity [32]. Additionally, a toxic effect of lipid accumulation as well as positive correlation between lipid accumulation and cardiac dysfunction has been demonstrated in human and animal models [9, 33]. Furthermore, myocardial lipid accumulation has been demonstrated in association with mitochondrial uncoupling and ROS overproduction [34]. The observed increased FA uptake and lower glucose uptake in Group (II) hearts in our study are generally consistent with PET scan findings in human hearts with LVEF <35% [34]. Furthermore, the fact that PPARα overactivation linked with cellular structure abnormalities is consistent with studies in mice MHC-PPARα [9] and with the use of a PPARα agonist in PPARα-null mice [8]. It is known that one of the factors directly responsible for cell rebuilding process in the failing heart is the excessive production of free radicals, which is concomitant with damage to mitochondrial structure [35]. Thus, mitochondrial anomalies observed in cardiomyocytes in HF Group (II) are consistent with this observation. Additionally, glycogen storage in cardiomyocytes has been shown to be associated with cardiomyocyte apoptosis [36–38]. However our investigations not confirmed link to apoptosis but rather suggested association with autophagic death. A strong mediator of autophagy could be damaged mitochondria (demonstrated here) and mitochondria ROS overproduction (demonstrated by others). Thus cardiac FA use can be considered as unfavorable for heart at this disease stage. Additionally, PPARα may be involved in facilitating some structural features responsible for cardiac function depression, particularly as we found a strong trend of negative correlation between FAT/CD36 mRNA levels and LVEF values in failing heart. Increased cardiomyocyte degeneration (revealed by severe pathology of desmin cytoskeleton in correlation with ultrastructural cell damage) in the hearts with moderately reduced LVEF might relate to the unfavorable effects of glycogen and lipid storage suggested in literature [33, 36–38] and is consistent with the findings of Hein et al. (2003) [39]. Thus, the observed increased expression of ubiquitin in cardiomyocytes with damaged desmin cytoskeleton came as no surprise.

In end-stage HF we found that PPARα mRNA levels returned to the level found in the control group, contrary to other investigators who reported its either decrease [14, 15] or increase [17]. In this HF stage low fatty acid oxidation [40] and decreased glycolysis [41] have been reported. In our study, cardiomyocytes structural damage manifested by the loss of desmin cytoskeleton and ultrastructural pathology along with the accumulation of lipid droplets and loss of glycogen may correspond to those described by these authors changes in energy metabolism. Additionally, the magnitude of structural changes and cardiomyocyte death probably contributed to left ventricular dysfunction. Important, structural changes strongly associate with HF mortality and recurrence as has been reported recently by Saito et al. (2015) [42].

A potential mechanism of PPARα activity reduction at the early phase of HF remains unclear. From literature data comes the fact that it can be hypoxia [43] but this unlikely was not the case in our study, since we have not found evidence of significant cardiomyocyte hypertrophy or fibrosis and vascular changes in patients with mildly reduced LVEF. Thus, the role of activation of hypertrophic pathway and stress accompanying left ventricular dilatation should be taken into consideration, while increased PPARα expression during heart failure progression might be an effect of long-term adrenergic activation and the presence of lipid droplets inside cardiomyocytes, as both are characteristic for progressive HF [32, 43].

In summary the goal of this study was to show PPARα alterations during HF progression in association with cardiomyocyte metabolic and structural features in patients relatively young in contrast to other investigators, although the rigorous criteria for entry to study limited the number of patients in each group. Furthermore limited amount of tissue was a cause that we could not carry out protein expression analysis for referenced genes.

In conclusion, patients with DCM and progressive HF exhibit specific cardiac metabolism related to PPARα expression. This metabolism involves increased PPARα levels in a narrow time window that is parallel with significant cardiomyocyte injury. The observed pattern of changes during HF progression suggests other targets than modulation of cardiac metabolism as a whole, for example, mitochondria, ROS. Therefore it is not a surprise that results of FA metabolism modulation in failing heart still remain controversial delivering contrary results, while modulation of

glucose metabolism in human studies has demonstrated only short-term benefit (reviewed in [5]).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Polymorphisms of the PPAR-γ (rs1801282) and Its Coactivator (rs8192673) Have a Minor Effect on Markers of Carotid Atherosclerosis in Patients with Type 2 Diabetes Mellitus

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Background. The present study was designed to clarify whether common single nucleotide polymorphisms (SNPs) of the Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) gene (rs1801282) and the Peroxisome Proliferator-Activated Receptor-γ Coactivator-1 (PGC-1α) gene (rs8192673) are associated with markers of carotid and coronary atherosclerosis in Caucasians with type 2 diabetes mellitus (T2DM). *Patients and Methods*. 595 T2DM subjects and 200 control subjects were enrolled in the cross-sectional study. Markers of carotid atherosclerosis were assessed ultrasonographically. In 215 out of 595 subjects with T2DM, a coronary computed tomography angiography (CCTA) was performed for diagnostic purposes. Genotyping of either rs1801282 or rs8192673 was performed using KASPar assays. *Results*. In our study, we demonstrated an effect of the rs1801282 on markers of carotid atherosclerosis (presence of plaques) in Caucasians with T2DM in univariate and in multivariable linear regression analyses. Finally, we did not demonstrate any association between either rs1801282 or rs8192673 and markers of coronary atherosclerosis. *Conclusions*. In our study, we demonstrated a minor effect of the rs1801282 on markers of carotid atherosclerosis (presence of plaques) in Caucasians with T2DM. Moreover, we demonstrated a minor effect of the rs8192673 on CIMT progression in the 3.8-year follow-up in Caucasians with T2DM.

1. Introduction

Patients with diabetes mellitus have an increased risk of premature atherosclerosis [1, 2]. Type 2 diabetes mellitus (T2DM) causes more than a twofold increase in the incidence of myocardial infarction and coronary artery disease-(CAD-) related death [3].

The Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) and its coactivator, the Peroxisome Proliferator-Activated Receptor-γ Coactivator-1 (PGC-1α), are important molecules in atherogenesis because they are associated with metabolic risk factors, such as obesity and diabetes [4, 5]. PPAR-γ regulates insulin sensitivity by transcriptionally activating adipocyte-specific genes involved in insulin signaling, glucose uptake, fatty acid uptake, and lipid-storage [6]. Moreover, PPAR-γ plays an important role in adipogenesis and subcellular metabolism of arterial wall macrophage foam cells [6, 7]. Furthermore, the pharmacological PPAR-γ agonist thiazolidinedione drugs appear to be antiatherogenic at multiple levels, which include a generalized improvement of metabolism reduction of triglyceride accumulation, beneficial effects on vascular wall components (macrophages), and an improvement of the outcome of atherosclerotic disease [8–11].

Genetic polymorphisms of the PPAR-γ and PGC-1α genes have so far been reported to be associated with

metabolic and cardiovascular end points [4, 5, 12–15]. A meta-analysis of 8 case-control studies and 2 family-based studies found that the PPARG A12 allele was associated with a reduced risk of type 2 diabetes [12]. The PPARG A12 allele was also associated with a reduced risk of myocardial infarction [13].

The aim of this study was to clarify whether common single nucleotide polymorphisms (SNPs) of the Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ) gene (rs1801282) and the Peroxisome Proliferator-Activated Receptor-γ Coactivator-1 (PGC-1α) gene (rs8192673) are associated with markers of carotid atherosclerosis (carotid intima media thickness (CIMT), the number of affected segments of carotid arteries, and the sum of plaques thickness) in subjects with T2DM in the Caucasian population. The second aim of the study was to demonstrate an association between either rs1801282 or rs8192673 and the subclinical markers of CAD in the subset of patients with T2DM.

2. Methods

2.1. Patients. In this cross-sectional study 595 subjects with type 2 diabetes and 200 nondiabetic individuals were enrolled. The Slovene Medical Ethics Committee approved the study protocol. They were selected among patients admitted to the diabetes outpatient clinics of the general hospitals in Murska Sobota and Slovenj Gradec, Slovenia, and from the Cardiology Outpatient Department, MC Medicor, Ljubljana. Patients were classified as having T2DM according to the current report of the American Diabetes Association [16]. Patients were excluded if they had homozygous familial hypercholesterolaemia or a previous cardiovascular event such as myocardial infarction or a cerebral stroke. Clinical data, including smoking habits, duration and treatment of diabetes, arterial hypertension, hyperlipidemia, and consuming any other drugs were obtained from medical records and questionnaires. Patients were asked if they were smokers at the time of recruitment (current smoker).

Two experienced doctors blinded to the participants' diabetes status performed all ultrasound examinations. The CIMT, defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface, was measured, as described previously [17]. Plaques were defined as a focal intima-media thickening and divided into 5 types according to their echogenic/echolucent characteristics, as described previously [17]. The interobserver reliability for carotid plaque characterization was found to be substantial ($\kappa = 0.64$, $p < 0.001$).

Control ultrasound examination was performed on 426 patients with diabetes and 137 healthy controls after 3.8 ± 0.5 years from the first examination. We used the annual CIMT progression rate, the increase in total plaque thickness, and the number of sites with plaques as well as the presence of unstable plaques as markers of carotid atherosclerosis progression.

In 215 out of 595 subjects with T2DM, a coronary computed tomography angiography (CCTA) was performed for diagnostic purposes. In 215 subjects with T2DM, coronary calcium score was measured and the presence of CAD was determined. Four regions (left main (LM), Left anterior descending (LAD) artery, left circumflex (LCX) artery, and right coronary artery (RCA)) were analyzed for the presence of CAD and more than 50% stenotic lesions were looked for in LM, LAD, LCX, RCA regions.

2.2. Biochemical Analyses. Blood samples for biochemical analyses, total cholesterol, triglyceride levels, high-density lipoprotein (HDL), low-density lipoprotein (LDL) cholesterol level, fasting blood glucose and glycated hemoglobin (HbA1c), hsCRP, and fibrinogen, were collected after a 12-hour fasting period. All the blood biochemical analyses were determined by using standard biochemical methods in the hospital's accredited lab.

2.3. Genotyping. The genomic DNA was extracted from 100 μL of whole blood using a FlexiGene DNA isolation kit, in accordance with the recommended protocol (Qiagen GmbH, Hilden, Germany). Polymorphisms rs1801282 of the PPAR-γ gene and rs8192673 of the PGC-1α gene were determined with real-time PCR using StepOne™ (48-well) Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA).

3. Statistical Analysis

Continuous variables were expressed as means ± standard deviations, when normally distributed, and as median (interquartile range) when asymmetrically distributed. Normality of the continuous variables was examined by the Kolmogorov-Smirnov test. Continuous clinical data were compared using an unpaired Student's t-test or analysis of variance (ANOVA) when normally distributed and the Mann-Whitney U test or the Kruskal-Wallis H-test when asymmetrically distributed. The Pearson χ^2 test was used to compare discrete variables and to test whether the genotypes distribution is in Hardy-Weinberg equilibrium. Pearson's correlation was performed to examine the association between independent variables. Due to the high correlation of systolic blood pressure with the diastolic blood pressure ($r = 0.57$, $p < 0.001$) they were not included together in the same statistical model. For the same reason the body mass index (BMI) was not included in the model together with the waist circumference ($r = 0.45$, $p < 0.001$).

Multivariable linear regression analysis was performed to determine the association of the tested polymorphisms with the CIMT/annual progression of CIMT and change in number of sites with plaque/total plaque thickness. To determine the association of the tested polymorphisms with the presence of atherosclerotic plaques on the carotid arteries or the presence of unstable plaques a multivariate logistic regression analysis was performed. All the regression models were adjusted for the presence of well established cardiovascular risk factors: age, gender, hypertension, systolic blood pressure, smoking, plasma levels of LDL and HDL cholesterol, triglycerides, HbA1c, and statin treatment. The results were presented as standardized β coefficients and p values for the linear regression and by odds ratios and

Table 1: Baseline clinical and biochemical characteristics of diabetic patients and controls.

	Diabetic patients $n = 595$	Controls $n = 200$	p
Age (years)	61.38 ± 9.65	60.07 ± 9.18	0.07
Male gender (%)	338 (56.8)	92 (46.0)	**0.008**
DM duration (years)	11.25 ± 7.88	—	—
Smoking prevalence (%)	53 (8.91)	34 (17.0)	**0.002**
Statin therapy (%)	375 (63.0)	62 (31.0)	<0.001
Antihypertensive agents (%)	499 (83.8)	58 (29%)	<0.001
Waist circumference (cm)	108.65 ± 12.88	93.31 ± 13.18	<**0.001**
BMI (kg/m^2)	30.96 ± 4.74	27.90 ± 4.42	0.16
Systolic blood pressure (mm Hg)	146.98 ± 19.98	143.3 ± 16.6	0.86
Diastolic blood pressure (mm Hg)	85.75 ± 11.62	84.7 ± 11.6	0.19
Fasting glucose (mmol/L)	8.04 ± 2.57	5.27 ± 0.87	<**0.001**
HbA1c (%)	7.89 ± 3.56	4.79 ± 0.29	<**0.001**
Total cholesterol (mmol/L)	4.70 ± 1.19	5.36 ± 1.08	<**0.001**
HDL cholesterol (mmol/L)	1.19 ± 0.35	1.43 ± 0.37	<**0.001**
LDL cholesterol (mmol/L)	2.63 ± 0.94	3.24 ± 0.98	<**0.001**
Triglycerides (mmol/L)	1.9 (1.2–2.7)	1.3 (0.9–1.9)	<**0.001**
hsCRP (mg/L)	2.2 (1.0–4.3)	1.3 (0.8–2.7)	<**0.001**
CIMT (μm)	958 ± 194	890 ± 212	**0.007**

DM: diabetes mellitus; hsCRP: high sensitivity C-reactive protein.

95% CIs for the logistic regression. A two-tailed p value less than 0.05 was considered statistically significant. A statistical analysis was performed using the SPSS program for Windows version 20 (SPSS Inc., Chicago, IL).

4. Results

Patients with T2DM had a greater waist circumference and higher fasting glucose and HbA1c levels compared to controls, whereas there were no differences in BMI or systolic and diastolic blood pressure between patients with T2DM and control subjects (Table 1). Patients with T2DM had lower total, HDL, and LDL cholesterol levels and a higher triglyceride level compared to controls (Table 1). Plasma level of inflammatory marker hsCRP was higher in patients with T2DM compared to controls (Table 1). Additionally, there were a higher percentage of men, statin therapy, and antihypertensive therapy and a lower percentage of smokers in the T2DM group compared to the control group (Table 1).

No statistically significant differences in the rs1801282 and rs8192673 genotype distribution frequencies were observed between T2DM patients and controls (Table 2). The rs1801282 genotype distributions in both patients with DM2 ($\chi^2 = 0.66$; $p = 0.42$) and controls ($\chi^2 = 3.79$; $p = 0.05$) were compatible with Hardy-Weinberg expectations. The rs8192673 genotype distributions in both patients with DM2 ($\chi^2 = 1.52$; $p = 0.22$) and controls ($\chi^2 = 0.50$; $p = 0.48$) were compatible with Hardy-Weinberg expectations (Table 2).

The comparison of atherosclerosis parameters was performed with regard to different genotypes of both polymorphisms (rs1801282, rs8192673) upon enrolment (Tables 3 and 4). In our study, we demonstrated an effect of the rs1801282 on the presence of plaques on subjects with T2DM by univariate and multivariable linear regression analysis (Tables 3 and 5), but we did not demonstrate any association between either the rs1801282 or the rs8192673 and other markers of carotid atherosclerosis CIMT, the sum of plaque thickness, the presence of unstable carotid plaques (Tables 3 and 4).

Finally, we did not demonstrate any association between either rs1801282 or rs8192673 and markers of coronary

Table 2: Genotype distribution and allele frequencies of the polymorphisms rs1801282 and rs8192673 in patients with T2DM and in control subjects.

	Subjects with T2DM $n = 595$	Control subjects $n = 200$	p
rs1801282			
CC genotype	422 (70.9)	137 (68.5)	
GC genotype	155 (26.1)	52 (26.0)	0.27
GG genotype	18 (3.0)	11 (5.5)	
C allele	999 (83.9)	326 (81.5)	0.26
G allele	191 (16.1)	74 (18.5)	
rs8192673			
TT genotype	309 (52.0)	92 (46.0)	0.28
TC genotype	231 (38.8)	84 (42.0)	
CC genotype	55 (9.2)	24 (12.0)	
T allele	849 (71.4)	268 (67.0)	0.10
C allele	341 (28.6)	132 (33.0)	

Table 3: Ultrasonographic markers of carotid atherosclerosis due to rs1801282 genotypes in patients with T2DM at the time of recruitment.

Pro12AlaPPAR	CC	GC + GG	p
CIMT (μm)	1006 ± 210	1026 ± 209	0.39
Number of sites with plaque	2.56 ± 1.57	2.36 ± 1.82	0.31
Total plaque thickness (mm)	7.98 ± 4.47	7.65 ± 4.64	0.58
Presence of plaques			
+	365 (86.5)	133 (76.9)	**0.005**
−	57 (13.5)	40 (23.1)	
Presence of unstable plaques			
+	213 (58.4)	74 (55.6)	0.61
−	152 (41.6)	59 (44.4)	
Coronary calcium score*	250 ± 315	269 ± 367	0.1
Number of coronary arteries with more than 50% stenosis	0.7 ± 0.9	0.9 ± 1.2	0.4
The presence of at least 1 vessel with more than 50% stenosis*	24 (38.9%)	63 (41.1%)	0.2

*Coronary computed tomography angiography (CCTA) was performed for diagnostic purposes in 215 out of 595 subjects with T2DM.

Table 4: Ultrasonographic markers of carotid atherosclerosis due to rs8192673 genotypes in patients with T2DM at the time of recruitment.

	TT	TC	CC	p
CIMT (μm)	1007 ± 224	1012 ± 191	1012 ± 217	0.94
Number of sites with plaque	2.36 ± 1.60	2.69 ± 1.66	2.72 ± 1.67	0.13
Total plaque thickness (mm)	7.54 ± 4.54	8.06 ± 4.82	8.31 ± 4.35	0.35
Presence of plaques				
+	257 (83.2)	194 (84.0)	47 (85.5)	0.90
−	52 (16.8)	37 (16.0)	8 (14.5)	
Presence of unstable plaques				
+	149 (58.0)	108 (55.7)	29 (61.7)	0.73
−	108 (42.0)	86 (44.3)	18 (38.3)	
Coronary calcium score*	181 ± 170	344 ± 376	200 ± 304	0.2
Number of coronary arteries with more than 50% stenosis*	0.7 ± 1.1	0.9 ± 1.1	0.6 ± 1.3	0.8
The presence of at least 1 vessel with more than 50% stenosis*	7 (31.8%)	33 (39.3%)	48 (44.0%)	0.3

*Coronary computed tomography angiography (CCTA) was performed for diagnostic purposes in 215 out of 595 subjects with T2DM.

atherosclerosis obtained with CCTA (coronary calcium score, the number of coronary arteries with more than 50% stenosis and the presence of at least one vessel with more than 50% stenosis) in subjects with T2DM (Tables 3 and 4).

In our study, we demonstrated an effect of the rs8192673 on CIMT progression in the 3.8-year follow-up (Table 6). Using the multivariable linear regression analysis we demonstrated an effect of the rs1801282 on the presence of plaques in Caucasians with T2DM (Table 6).

5. Discussion

In the present study we tested the hypothesis that the rs1801282 of the PPAR-γ gene and the rs8192673 of the PGC-1α gene may be genetic markers of subclinical atherosclerosis of carotid and coronary arteries. In our study, we demonstrated an effect of the rs1801282 on markers of carotid atherosclerosis (presence of plaques) in Caucasians with T2DM in univariate and in multivariable linear regression analyses. The rs1801282 of the PPAR-γ gene was found to have a protective role against the development of atherosclerosis. Moreover, we demonstrated a minor effect of the rs8192673 on CIMT progression in Caucasians with T2DM in the 3.8-year follow-up.

In our study, we did not demonstrate any association between either the rs1801282 or the rs8192673 and CIMT, despite some previous reports on an association between the rs1801282 and CIMT [18–20]. In few populations (German population, Japanese population, and Canadian Oji-Cree Aborigines), the rs1801282 (Ala12 allele of the PPAR-γ) was reported to be associated with reduced CIMT [18–20]. Contrary to the lack of effect on CIMT, an effect of the rs8192673 on the CIMT progression rate and an effect of the rs1801282 on the presence of plaques in Caucasians with T2DM were demonstrated in univariate and in multivariable linear regression analyses. The rs1801282 of the PPAR-γ gene was found to have protective role against the development of atherosclerosis.

In the present study we pursued the hypothesis that either the rs1801282 of the PPAR-γ gene or the rs8192673 of the PGC-1α gene may be genetic markers of coronary atherosclerosis in subjects with T2DM. In our study, however, we did not demonstrate any association between either the rs1801282 or the rs8192673 and markers of coronary

Table 5: Association of the rs1801282 genotypes with the presence of plaques and presence of unstable plaques in patients with T2DM at the time of recruitment.

	Presence of plaque		Presence of unstable plaque	
	OR (95% CI)	p	OR (95% CI)	p
rs1801282				
Hypertension (0 = no; 1 = yes)	1.71 (0.93–2.58)	**0.04**	1.25 (0.88–2.64)	0.97
Systolic blood pressure (mm Hg)	1.07 (0.92–1.007)	0.17	1.11 (0.86–1.44)	0.32
LDL cholesterol (mmol/L)	1.21 (0.78–1.89)	0.40	1.08 (0.75–1.56)	0.67
HDL cholesterol (mmol/L)	0.18 (0.05–0.63)	**0.008**	0.30 (0.08–1.13)	0.08
Triglycerides (mmol/L)	1.28 (0.63–1.03)	0.09	1.09 (0.66–1.37)	0.34
Hba1c (%)	1.14 (0.64–1.54)	0.28	1.22 (0.74–1.92)	0.42
GC + GG*	0.79 (0.48–1.14)	**0.04**	0.83 (0.34–1.91)	0.65
rs8192673				
Hypertension (0 = no; 1 = yes)	1.35 (1.13–1.93)	**0.04**	1.15 (0.75–2.77)	0.79
Systolic blood pressure (mm Hg)	1.08 (0.96–1.34)	0.16	1.02 (0.97–1.25)	0.31
LDL cholesterol (mmol/L)	1.22 (0.78–1.89)	0.29	1.07 (0.74–1.54)	0.73
HDL cholesterol (mmol/L)	0.19 (0.05–0.72)	0.54	0.29 (0.08–1.05)	0.06
Triglycerides (mmol/L)	1.34 (0.63–1.90)	0.09	1.19 (0.66–1.59)	0.34
Hba1c (%)	1.16 (0.65–2.06)	0.32	1.11 (0.86–1.44)	0.42
TC**	0.97 (0.56–1.38)	0.48	1.16 (0.59–2.70)	0.55
CC**	1.08 (0.38–1.34)	0.63	1.43 (0.43–4.73)	0.56

All the models were adjusted for age, gender, smoking, and statin treatment.
*Reference group were homozygotes for allele C. **Reference group were homozygotes for allele T.

Table 6: Association of the rs1801282 genotypes with ultrasonographic markers of carotid atherosclerosis progression in patients with T2DM.

	CIMT progression rate		Δ Number of sites with plaque		Δ Total plaque thickness	
	β	p	β	p	β	p
rs1801282						
Hypertension (0 = no; 1 = yes)	0.013	0.92	0.020	0.90	0.069	0.26
Systolic blood pressure (mm Hg)	0.022	0.52	0.052	0.69	0.037	0.82
LDL cholesterol (mmol/L)	0.057	0.69	0.051	0.71	0.073	0.49
HDL cholesterol (mmol/L)	−0.211	0.19	−0.230	0.14	−0.189	0.37
Triglycerides (mmol/L)	0.249	0.13	0.343	0.78	0.359	0.44
Hba1c (%)	1.151	0.29	1.097	0.83	1.176	0.41
GC + GG*	0.818	0.93	0.728	0.18	0.684	0.16
rs8192673						
Hypertension (0 = no; 1 = yes)	0.140	0.37	0.062	0.71	0.069	0.29
Systolic blood pressure (mm Hg)	0.186	0.25	0.046	0.88	0.075	0.35
LDL cholesterol (mmol/L)	0.172	0.59	0.143	0.75	0.446	0.35
HDL cholesterol (mmol/L)	−0.203	0.18	−0.232	0.08	−0.192	0.34
Triglycerides (mmol/L)	0.168	0.28	0.117	0.43	0.127	0.21
Hba1c (%)	0.146	0.27	0.143	0.26	0.228	0.16
TC**	0.068	0.63	−0.066	0.64	0.328	0.32
CC**	0.349	**0.01**	−0.115	0.11	0.681	0.06

All the models were adjusted for age, gender, smoking, statin treatment and baseline value of dependent variable.
*Reference group were homozygotes for the allele C; **Reference group were homozygotes for the allele T.

atherosclerosis obtained with CCTA (coronary calcium score, number of coronary arteries with more than 50% stenosis, and the presence of at least one vessel with more than 50% stenosis). Our findings are in accordance with the study of Nemoto and coworkers on 91 subjects with T2DM, in which they failed to demonstrate the effect of the variability in the PPAR-γ gene on the coronary calcium score [21]. However, in several studies the effect of polymorphisms of PPARγ2/PGC-1α genes on CAD/MI risk was reported [1, 13, 15, 22–24]. In their case-control study, Galgani and coworkers

demonstrated that homozygosity for the Ala allele at codon 12 of the PPARγ2 (rs1801282) gene was associated with a reduced risk of CAD [22]. Similarly, Ridker and coworkers reported in a prospective study that the rs1801282 of the PPAR-γ (A12 allele) was associated with a 25% reduction in myocardial infarction risk [13]. Ding and coworkers, however, failed to demonstrate a significant effect of the rs1801282 of the PPAR-γ on CAD risk in their meta-analysis (74 studies with 52,998 subjects included) [23]. Cresci and coworkers reported a variant (rs1503298) in a single PPAR pathway gene (i.e., TLL1) that was associated with the extent of CAD in patients with T2DM and CAD [15].

Potential mechanisms of the effect of the variants of both genes (PPAR-γ, PGC-1α) may be speculated to affect serum/tissue levels of both proteins, other risk factors (i.e., obesity and obesity indexes) or other effects (i.e., lipid status).

In our recently published study, we demonstrated that the rs8192673 of the PGC-1α gene and the rs1801282 of the PPAR-γ gene have been associated with waist circumference in subjects with T2DM [4]. Huang and coworkers demonstrated the effect of the rs1801282 of the PPAR-γ gene in the meta-analysis (74 studies with 52,998 subjects) on lipid parameters [25]. They reported that subjects (male) with the AlaAla genotype had lower blood TG than subjects with ProPro genotype in Caucasians [25].

Strength of our study is the community-based sample and the detailed phenotypic characterization of the subjects with regard to ultrasonically determined carotid atherosclerosis, as well as having data of a rather large sample of subjects with T2DM. A limitation is the use of cross-sectional data in the analysis, restricting the possibility of causal inferences from our data and allowing for bias. An additional limitation is that while we assume that the effect of the PPAR-γ/PGC-1α gene variants on plaque is due to their influence on serum/tissue levels of the respective enzymes, we do not have any direct measure to be able to investigate this.

6. Conclusions

To conclude, in our study we demonstrated a minor effect of the rs1801282 on markers of carotid atherosclerosis (presence of plaques) in Caucasians with T2DM. Moreover, we demonstrated a minor effect of the rs8192673 on CIMT progression in the 3.8-year follow-up. Our findings suggest that the tested polymorphisms in the PPAR-γ/PGC-1α genes play a minor role in the development of subclinical atherosclerosis in subjects with T2DM.

Conflict of Interests

The authors declare no conflict of interests related to this work.

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Review Article

Peroxisome Proliferator-Activated Receptors and the Heart: Lessons from the Past and Future Directions

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Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear family of ligand activated transcriptional factors and comprise three different isoforms, PPAR-α, PPAR-β/δ, and PPAR-γ. The main role of PPARs is to regulate the expression of genes involved in lipid and glucose metabolism. Several studies have demonstrated that PPAR agonists improve dyslipidemia and glucose control in animals, supporting their potential as a promising therapeutic option to treat diabetes and dyslipidemia. However, substantial differences exist in the therapeutic or adverse effects of specific drug candidates, and clinical studies have yielded inconsistent data on their cardioprotective effects. This review summarizes the current knowledge regarding the molecular function of PPARs and the mechanisms of the PPAR regulation by posttranslational modification in the heart. We also describe the results and lessons learned from important clinical trials on PPAR agonists and discuss the potential future directions for this class of drugs.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor superfamily of ligand-activated transcription factors and include three member isoforms—α, β/δ, and γ—encoded by distinct genes located on different chromosomes with a high degree of interspecies sequence conservation [1–5]. Interestingly, while significant homology exists between PPAR proteins, they play distinct, functional roles in energy metabolism [5].

PPARs are subjected to transactivation or transrepression through distinct mechanisms, which lead to the induction or repression of target gene expression [1]. For this, PPARs dimerize with the retinoid receptor and subsequently bind sequence-specific promoter elements in their target genes to control several facets of normal cellular physiology as well as pathology. Disruption of this pathway contributes to disease progression in obesity, diabetes, and cancers. This occurs through regulation of growth and migration, apoptosis, fatty acid (FA) metabolism pathways, and oxidative stress responses. Moreover, PPARs are also known to regulate inflammatory processes that are linked to metabolic homeostasis in tissues, such as liver, adipose tissue, intestine, skeletal muscle, and cardiovascular system [1–9]. Importantly, each PPAR family member has distinct metabolic functions determined by their ligand affinity, expression, and activity, which are both tissue- and pathway-dependent [6].

All three PPAR isoforms are expressed in the heart; however, their roles in cardiac function and the outcomes of respective agonists in preclinical animal models and clinical trials vary immensely. Furthermore, studies of PPARs on myocardial fatty acid metabolism and cardiac function are currently being conducted. Thus, it is necessary to understand current PPAR research, as well as PPAR biology in the heart. In this review, we focus on the functions of PPARs in myocardial biology in addition to their regulatory effects on glucose and lipid metabolism, and we describe their potential clinical implications and future directions.

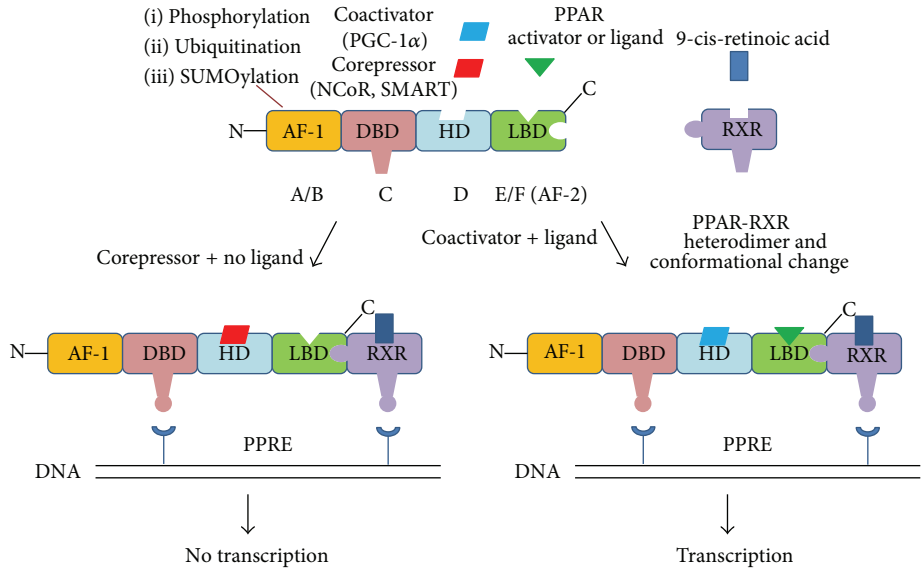

Figure 1: Structure of PPAR and its transactivation or transrepression process. In the absence of ligand, the PPAR-RXR heterodimer recruits corepressors (left process). When ligand binds, conformational changes in PPAR-RXR induce dissociation of corepressor complex. Active transcriptional complex assembles with coactivator proteins. PPAR binds to PPRE and assembles coactivator complexes (right process). PGC-1α: PPAR-γ coactivator 1α, NCoR: nuclear receptor corepressor, SMART: silencing mediator of retinoid and thyroid hormone receptor, AF: activation function, DBD: DNA-binding domain, HD: hinge domain, LBD: ligand-binding domain, RXR: retinoid X receptor, and PPRE: peroxisome proliferator response element.

2. Molecular Structure of PPARs

PPARs are orphan nuclear receptors that belong to the thyroid, steroid, and retinoid hormone receptor superfamilies of ligand-activated nuclear hormone receptors [4, 5, 10–12]. After binding with their respective ligands, PPARs translocate to the nucleus, where they undergo a conformational change, interact with transcriptional cofactors, and regulate gene transcription [13–15]. PPAR isoforms possess five or six structural regions within four functional domains, termed A/B, C, D, and E/F (Figure 1) [6, 12]. The variable N-terminal, ligand-independent transactivation domain (A/B domain) contains an activation function- (AF-) 1 motif, which is a target of kinase phosphorylation [6, 12]. The 70-amino-acid PPAR DNA-binding domain (C domain) contains two highly conserved zinc finger motifs that facilitate binding to the peroxisome proliferator response element (PPRE) [6, 12]. The hinge region (D domain) acts as a docking site for cofactors. The C-terminal or ligand-binding domain (the E/F domain) is responsible for ligand specificity and the activation of PPAR binding to the PPRE, which increases target gene expression. The E/F domain uses cofactors for the transactivation via the ligand-dependent trans-AF-2 [6, 12]. When activated by endogenous or synthetic ligands, PPARs heterodimerize with the 9-cis-retinoic acid receptor (retinoid X receptor; RXR), triggering a conformational change and their nuclear translocation [6, 12]. The PPAR-RXR heterodimer then binds the PPRE in the target gene promoter region, subsequently altering coactivator/corepressor dynamics to modulate the transcription machinery controlling gene expression [6, 16–20]. In the past 20 years, many PPAR cofactors have been identified; however, the complete physiological functions of these molecules in receptor-, gene-, and/or cell-specific transcription remain to be elucidated [21].

3. Extracardiac Function of PPARs and Their Ligands

The first PPAR isoform to be cloned, PPAR-α, was identified in 1990 and its name of PPAR originated from its activation by peroxisome proliferator chemicals [22, 23]. The PPAR-α gene is located on human chromosome 22q12.2-13.1 [24], and its expression is highest in tissues with elevated FA oxidation rates—such as liver, heart, and skeletal muscle—where it functions as a major regulator of FA homeostasis [23–27]. PPAR-α is also highly expressed in brown adipose tissue, kidney, adrenal gland, and the majority of cell types, including macrophages, smooth muscle cells, and endothelial cells [6, 26–28]. Unsaturated/saturated FAs, leukotriene (LT) derivatives, and very low-density lipoprotein (VLDL) hydrolysis products are endogenous ligands that bind PPAR-α with the greatest affinity. Moreover, PPAR-α is a major regulator of the mitochondrial and peroxisomal β-oxidation pathways, which are reported to be involved in the pathogenesis of various liver complications—such as hepatocarcinogenesis in rodent model and drug-induced liver injury [29]. PPAR-α activation inhibits proinflammatory gene expression in vascular smooth muscle cells (VSMCs) and attenuates development of atherosclerosis [30, 31].

The PPAR-β/δ gene is located on human chromosome 6p21.1-21.2 [24] and is expressed at relatively high levels in adipose tissue, liver, cardiac and skeletal muscle, brain, kidney, colon, and vasculature [28, 32, 33]. Unlike PPAR-γ and PPAR-α, PPAR-β/δ is not easily targeted by currently available drugs

Table 1: The expression of the PPARs and their gene targets. Modified from [49, 51].

Properties	PPAR-α	PPAR-β/δ	PPAR-γ
Tissue expression	*Main tissues*: tissues exhibiting high catabolic rates of FA (liver, skeletal muscle) *Other tissues*: heart, intestine, kidney, and brown adipose tissue	Ubiquitous: however, the biggest expression is in liver, esophagus, intestine, kidney, and skeletal muscle	*Main tissue*: adipose tissue (white and brown) *Other tissues*: liver, intestine, kidney, retina, immunologic system (bone marrow, lymphocytes, monocytes, and macrophages), and trace amounts in muscles
Gene targets	β-oxidation pathway (acyl-CoA oxidation, bifunctional enzyme, and thiolase) Sterol 12-hydroxylase (CYP8B1) FATP FAT/CD36 L-FABP Lipoprotein lipase apo A-I and A-II	Genes involved in lipid uptake; it represses genes implicated in lipid metabolism and efflux	FA-binding protein (aP2) Phosphoenolpyruvate carboxykinase (enzyme of the glyceroneogenesis pathway) FATP FAT/CD36

FA: fatty acid, FATP: fatty acid transport protein, L-FABP: liver cytosolic fatty acid-binding protein, and apo: apolipoprotein.

because of its ubiquitous expression. Thus, the physiological function of PPAR-β/δ is far less studied and understood [34]. Nevertheless, PPAR-β/δ activation is known to increase lipid catabolism in adipose tissue, skeletal muscle, and the heart and has been shown to improve the plasma high-density lipoprotein- (HDL-) cholesterol levels and insulin resistance. Additionally, activation has been shown to induce cell proliferation and differentiation [35] and to limit weight-gain with anti-inflammatory effects in the vessel wall through the inhibition of vascular cell adhesion molecule- (VCAM-) 1 and monocyte chemoattractant protein- (MCP-) 1 expression [36–38].

The PPAR-γ gene is located on human chromosome 3p25 [24] and is highly expressed in adipose tissue. PPAR-γ plays an essential regulatory role in glucose metabolism, adipocyte differentiation, and lipid storage by controlling the transcription of a number of genes involved in these metabolic processes [6, 15, 39–41]. Some key target genes of PPAR-γ include the fat-specific adipocyte protein 2 (aP2; FABP), lipoprotein lipase (LPL), FA translocase (FAT/CD36), FA transport, FA-binding protein, acyl-CoA synthase, glucokinase, glucose transporter type 4 (GLUT4), phosphoenolpyruvate carboxykinase, uncoupling proteins (UCP) 1, 2, and 3, and liver X receptor-α (LXR-α) [6, 39, 40]. Moreover, PPAR-γ also regulates genes involved in insulin signaling and the expression of proinflammatory cytokines, such as tumor necrosis factor- (TNF-) α [6, 41]. Most importantly, PPAR-γ is a well-recognized cellular target for the antidiabetic thiazolidinediones (TZDs), which sensitize cells to insulin and improve insulin sensitivity and activity [42–44]. However, the associated cardiac hypertrophy in response to PPAR-γ may be independent to changes in myocardial insulin signaling [45]. PPAR-γ protein stability and transcriptional activity are regulated by covalent modifications, including phosphorylation, ubiquitylation, *O*-GlcNAcylation, and SUMOylation [37, 46]. Importantly, PPAR-γ functions as a master switch in controlling adipocyte differentiation and development, and its activation plays an important role in glucose metabolism by enhancing insulin sensitivity [37, 47].

To date, many ligands have been identified that activate and modulate PPAR activity [48]. PPAR ligand-binding activities are 3-4 times greater than that of the other nuclear receptors and thus have the ability to bind a diverse set of synthetic and natural lipophilic acids, such as essential FAs (EFA) [49]. For example, endogenous lipid metabolites from saturated or unsaturated FAs bind nuclear receptors and activate or repress gene expression [48]. Another group of PPAR ligands consists of EFA lipid metabolites—such as arachidonic acid derived from lipoxygenase or cyclooxygenase activity [48]. However, both eicosanoids and EFA are required in relatively high concentrations (~100 μM) for PPAR activation [50]. In particular, the best-characterized endogenous ligands known to stimulate PPAR-α are the eicosanoids LT B4 and 8-hydroxyeicosatetraenoic acid (HETE), while 15d-prostaglandin (PG) J2 and 13-hydroxyoctadecadienoic acid (HODE) activate PPAR-γ [48]. Other essential FA metabolites, such as 15-HETE, have been suggested to activate PPAR-β/δ [48]. The physiological roles, expression, gene targets, and ligands of the various PPAR isoforms are summarized in Tables 1 and 2 and the following references [49, 51].

4. PPAR Functions in the Cardiovascular System

Many studies have reported on the complex metabolic and biological roles of PPARs in several cardiovascular diseases, including cardiac hypertrophy and heart failure [52–56]. In the cardiovascular system, PPARs have various functions outside of their characteristic roles in metabolism, including extracellular matrix remodeling, oxidative stress, inflammation, and circadian rhythm regulation [57].

Abnormalities in PPAR function have been reported in arrhythmogenic right ventricular dysplasia (ARVD), a rare

Table 2: The natural and synthetic ligands of the PPARs and their physiological roles. Modified from [49, 51].

Properties	PPAR-α	PPAR-β/δ	PPAR-γ
Natural ligands	Unsaturated FA, PG, and LT B4 8-Hydroxyeicosatetraenoic acid	Unsaturated FA Carbaprostacyclin Components of VLDL	Unsaturated FA 15-Hydroxyeicosatetraenoic acid 9- and 13-hydroxyoctadecadienoic acid 15-Hydroxy delta 12,14-PG J2 PG J2
Synthetic ligands	Clofibrate and fenofibrate Gemfibrozil	GW501516	Rosiglitazone and pioglitazone Troglitazone and ciglitazone Farglitazar, S26948, and INT131
Physiological roles	Lipid catabolism and homeostasis (stimulating β-oxidation of fatty acids), increased breakdown of TG and FA, increased cellular FA uptake, reduced TG and FA synyheis, control of inflammatory processes, and vascular integrity mediate the hypolipidemic function of fibrates *Liver*: increasing FA oxidation and uptake and increasing apoA-I, apoA-II, and HDL *Vessel*: increasing TG, HDL, ABCA1, and apoE and decreasing FFA, VLDL, cytokines, and NF-κB	Dyslipidemia? Wound healing? Increasing fat oxidation in skeletal and cardiac muscle responsible for insulin sensitivity and glucose homeostasis and vascular integrity *Adipocentric action*: decreasing cytokines, resistin, fFFA, and NF-κB and increasing ABCA1 and GLUT4 *Skeletal muscle*: increasing glucose uptake and glycogen synthesis	*Glucose homeostasis and lipid storage*: differentiation and maturation of adipocytes Increasing IS and glucose homeostasis (it prevents hyperglycemia) and vascular integrity *Skeletal muscle/liver/adipocyte*: increasing FA oxidation, UCP, and HDL and decreasing TG

FA: fatty acid; apo: apolipoprotein, PG: prostaglandin, LT: leukotriene, TG: triglyceride, HDL: high-density lipoprotein, ABCA1: ATP-binding cassette subfamily A member 1, FFA: free fatty acid, VLDL: very low-density lipoprotein, NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells, GLUT4: glucose transporter type 4, and UCP: uncoupling protein.

genetic disease characterized by a progressive fibrofatty infiltration, decreased PPAR-α, and increased PPAR-γ expression in the right ventricle. The link between PPAR dysfunction and desmosomal genetic mutations is beginning to be understood via Wnt/β-catenin pathway analyses [58–61]. PPAR-γ is a prime inducer of adipogenesis in ARVD, and the Wnt-β-catenin pathway appears to act though a similar mechanism for desmosomal abnormalities [58].

The biological functions of PPAR-α in the myocardium have been extensively investigated using PPAR-α knockout (KO) mice [62–64]. Despite a normal life span, PPAR-α KO mice exhibit progressive cardiac fibrosis with abnormal mitochondria and myofibrils [63]. Histological studies also revealed significant cardiomyocyte hypertrophy [65]. In addition, ex vivo left ventricular papillary muscle exhibits reduced shortening velocity and isometric tension, suggesting that the loss of PPAR-α is closely involved in the cardiac dysfunction induced by affecting the impairment of myosin molecule itself, targeting for oxidative stress [65–68]. This is also apparent in echocardiography studies [65]. Interestingly, the development of physiological cardiac hypertrophy, such as is seen after birth and in response to exercise, showed the increased PPAR-α expression that parallels an induction of FA utilization [69, 70]. In contrast, PPAR-α gene expression is downregulated in the heart of some pathological conditions, especially pressure overload-induced cardiac hypertrophy, that lead to the cardiac lipotoxicity as an accumulation of triglyceride and diacylglycerol [70–73].

The redox system in PPAR-α KO mice is subjected to dramatic and/or long-lasting perturbations as well as cardiac dysfunction that appear to result from the direct impairment of myosin II [65]. There is strong evidence that PPAR-α activation is necessary to prevent cellular oxidative damage that may occur during physiological cellular metabolism or under conditions of inflammation and oxidative stress, likely caused by repressing NF-κB signaling and limiting inflammatory cytokine production [74, 75]. Therefore, chronic deactivation of the PPAR-α signaling pathway may upset the normal equilibrium between oxidant production and antioxidant defenses, which can contribute to cardiac damage [58]. A recent study in PPAR-γ KO mice revealed that PPAR-γ plays a crucial protective role in cardiomyocytes and may prevent myocardial ischemia-reperfusion injury by modulating NF-κB-associated inflammatory mechanisms in the infarcted myocardium [76].

The heart responds to FA variations by activating PPARs [77]. PPAR-α can mediate diurnal variations in the responsiveness of the heart to both FAs and specific PPAR-α agonism (WY-14 643) [78]. In the normal heart, however, PPAR-α mRNA exhibits only weak circadian oscillations, although the circadian clock within the cardiomyocyte mediates diurnal variations in the responsiveness of the heart to increased workload, according to contractile function and metabolic flux levels [79, 80].

PPAR-α overexpression in the mouse myocardium attenuates glucose transporter gene expression and glucose uptake

[81]. In myocardium perfused with FA and ketone bodies, the glycolytic rate is decreased and additionally cardiomyocyte-specific PPAR-α overexpression leads to an augmentation of triglyceride-derived FAs [82]. PPAR-α interferes in pyruvate dehydrogenase kinase (PDK), phosphofructokinase, pyruvate dehydrogenase complex (PDC), and phosphofructokinase (PFK) activities, and the cellular regulation of these proteins is influenced by circadian rhythms [83, 84]. Recently it has been demonstrated that vascular PPAR-γ is a peripheral regulator of cardiovascular rhythms that controls circadian variations in blood pressure and heart rate through brain and muscle Arnt-like protein- (BMAL-) 1 [85]. PPAR-γ appears to be a main component of the vascular clock. Pioglitazone, a PPAR-γ activator, readjusts the circadian rhythm of blood pressure from nondipper to dipper in patients with type 2 diabetes [86]. Accordingly, the impairment of cardiovascular rhythmicity parallels the diurnal variations in urinary excretion of epinephrine and norepinephrine, which are suppressed in PPAR-γ mutant mice [85], similar to that observed in BMAL1 KO mice [87].

5. PPAR Regulation by Posttranslational Modification in the Myocardium

Energy utilization in heart is transcriptionally controlled in part by the PPAR family and their coreceptors/coactivators, including PPAR-α, PPAR-β/δ, PPAR-γ, RXR-α, and PPAR-γ coactivator- (PGC-) 1α. Mechanistically, PPAR-α, PPAR-β/δ, and PPAR-γ heterodimerize with the RXR-α and coactivators (e.g., PGC-1α) and repressors (e.g., nuclear receptor corepressor (NCoR)) to regulate the transcription of genes involved in energy regulation and lipid metabolism [4, 88–90]. Both PPAR and RXR-α interact with their respective ligands to enhance PPAR-DNA binding [88, 91]. In the absence of ligand binding, the unbound PPAR-RXR heterodimer remains bound to NCoR and silencing mediator of retinoid and thyroid hormone receptor (SMRT), two main corepressors within the corepressor complex [92, 93]. Both NCoR and SMRT directly interact with the Sin3 complex to form a multisubunit repressor complex [92, 94]. SMRT functions as a protein platform to promote the mobilization of histone deacetylases (HDACs) to the DNA promoters bound by specific interacting transcription factors [92, 94]. Receptor interacting protein- (RIP-) 140, also known as nuclear receptor interacting protein- (NRIP-) 1, is another corepressor that directly recruits HDAC and represses the activity of numerous nuclear receptors including PPARs by competing with their coactivators [95–97]. In the absence of ligand activation of nuclear receptor, the corepressor protein complex is known to suppress target gene transcription by causing the deacetylation of histones [92, 93].

Alterations in the cardiac expression of all three PPARs cause disturbances in glucose and FA metabolism that result in an increased susceptibility to insults or significant dysfunction [91]. While PPAR regulation is known to play a role in cardiovascular disease pathogenesis, the mechanisms regulating their expression and function at the cardiomyocyte level have not been clearly depicted. However, some progress has been made. For example, PPARs may be controlled through posttranslational modifications (PTM), such as SUMOylation and ubiquitination [88]. The conjugation of small ubiquitin-like modifier (SUMO) or ubiquitin is distinctive among PTMs in that it induces the attachment of another polypeptide, rather than the addition of a functional group [88, 98–101]. To date, SUMOylation- or ubiquitination-mediated PPAR regulation in the heart has not been reported; however, PPARs are controlled by these PTMs in other closely related muscle cell types. Other studies have established that SUMOylation of PPAR-γ1 promotes VMSC migration and proliferation. This has been demonstrated by using VSMCs transfected with a SUMOylation-defective lysine (K107R) PPAR-γ1 mutant, which results in a more potent transcriptional inhibition of inducible nitric oxide synthase when compared to cells transfected with a wild-type construct [88, 102]. These findings regarding the role of PPAR-γ SUMOylation in regulating the FA oxidation response and apoptosis in striated muscle and vascular smooth muscle, respectively, provide support for the concept that PPARs could be subjected to posttranslational regulation in the heart. Moreover, PPAR-α phosphorylation by the MAPK p38 decreases PPAR-α transcriptional activity [88, 103]. Since the p38 pathway is activated in response to cardiac stress—as found in diabetes, heart failure, and cardiac hypertrophy—this study implicates PPAR-α activation mechanism by which the heart responds to unfavorable stimuli. The broader implications of these studies indicate that the FA and glucose shifts seen in these diseases may be due to these regulatory mechanisms [88, 104].

6. Cardiac Pathophysiology in Genetic Animal Models of PPARs and Their Regulators

6.1. PPAR-α. The functions of cardiac PPAR-α have been evaluated in PPAR-α KO mice. While viable and outwardly normal, these mice exhibit mild aging-associated cardiac fibrosis [63]. The basal expression of several PPAR-α target genes and rates of FA oxidation are also diminished in hearts of PPAR-α KO mice [63, 105, 106] and fail to be induced in response to fasting or diabetes [105]. Moreover, PPAR-α KO mice exhibit increased glucose uptake, *GLUT4* expression, and reliance on glucose for cardiac ATP production [64, 107]. Notwithstanding the age-associated fibrosis, cardiac function is relatively normal in young PPAR-α KO mice; however, the response to several physiological stressors is perturbed. For example, hearts isolated from PPAR-α KO mice are unable to compensate when challenged with an increased workload [64, 108]. Furthermore, transgenic animal models overexpression of PPAR-α results in a cardiomyopathy that mimics that seen in diabetes mellitus (DM) [109] that is dependent on dietary fat. This implies that serum-free FA is an essential mediator during cardiac maladaptation [110]. Paradoxically, although chronic exposure to excess FA represses PPAR-α expression in cardiomyocytes, this downregulation of PPAR-α may result in further myocardial damage by suppressing cellular free FA oxidation on a background of excess free FAs within cells and in the circulation [111]. The PPAR-α agonist BM 17.0744 (Roche Pharmaceuticals) normalized cardiac

metabolism but was unable to improve cardiac function when given orally to type 2 DM db/db mice for 8 weeks [112]. Apoptosis plays a role in the pathophysiology of diabetic cardiomyopathy and the PPAR-α ligand, fenofibrate, was shown to suppress apoptosis. These findings support the potential role of PPAR-α ligands in diabetic cardiomyopathy [109, 113].

Cardiovascular PPAR-α expression has anti-inflammatory and antioxidative effects, and activation of inflammatory signaling pathways is important in cardiomyocyte hypertrophy [65, 114]. Accordingly, PPAR-α agonists have been useful in repressing the inflammation caused by cardiovascular disease. Pretreatment of neonatal cardiomyocytes with PPAR-α agonist significantly decreases lipopolysaccharide- (LPS-) stimulated TNF-α release, interleukin- (IL-) 1-induced IL-6 secretion, and PG and cyclooxygenase-2 expression [115, 116]. The nuclear translocation of NF-κB and apoptosis were also demonstrated to be reduced after treatment with the PPAR-α agonists in the reperfused myocardium. These findings suggest an important role of PPAR-α agonists in inhibiting inflammation in many cell types in cardiovascular disease [117, 118]. Moreover, potent PPAR-α agonist WY14643 has cardioprotective and cardiodepressive effects when used to treat encephalomyocarditis virus-induced myocarditis in diabetic mice, which may be due to its anti-inflammatory properties and its ability to increase cardiac adiponectin expression, whereas the reduced cardiac efficiency may be due to its enhancement of cardiac UCP3 mRNA expression [6, 119].

6.2. PPAR-β/δ. A decrease in cardiac expression of PPAR-β/δ was found in rats with diabetic cardiomyopathy [120, 121], and reduction in PPAR-β/δ expression during hyperglycemia is associated with increased reactive oxygen species production [121], TNF-α, IL-6, and nicotinamide-adenine dinucleotide phosphate (NADPH) activity. Further studies are needed to evaluate the precise role of PPAR-β/δ ligands in regulating diabetic cardiomyocytes [109, 120]. The selective PPAR-β/δ ligand GW501516 was evaluated for its effect on FA-induced inflammation in cardiomyocytes [122]. GW501516 was also found to reduce expression of the NF-κB target genes, MCP-1 and TNF-α in both human cardiac AC16 cells stimulated by palmitate, as well as in the hearts of mice fed with a high-fat diet. This data implies that PPAR-β/δ may counteract NF-κB activity; thus, PPAR-β/δ activation might be therapeutically useful as an anti-inflammatory agent in diabetic cardiomyopathies [122].

6.3. PPAR-γ. In contrast to the induction of the other PPAR family members, there are several studies that revealed that PPAR-γ expression is elevated in diabetic rat models [109, 120, 123, 124]. PPAR-γ leads to elevations in lipogenic enzymes, which subsequently increase triglyceride production [123]. In addition, recent evidence from animal models showed that cardiomyocyte PPAR-γ activation is associated with compromised cardiac function through its lipogenic effects, which may contribute to intracellular triglyceride accumulation and cardiac lipotoxicity [125]. The PPAR-γ ligand rosiglitazone may also have a protective role against apoptosis in diabetic cardiomyopathy, similar to the PPAR-α ligand [113]. Rosiglitazone has also been demonstrated to decrease cardiac fibrosis and improve left ventricular diastolic dysfunction through the inhibition of receptors for advanced glycated end products and connective tissue growth factor in diabetic myocardium [126]. Moreover, pioglitazone attenuated the deterioration of ischemic preconditioning against reperfusion arrhythmias in type 2 DM rats [127]. Although PPAR-γ levels are relatively low in myocardial cells, activation during inflammation might have important effects on cardiomyocytes.

The therapeutic effects of PPAR-γ ligands have been attributed primarily to their anti-inflammatory properties. Previous studies showed that both natural and synthetic PPAR-γ ligands have anti-inflammatory potentials [128]. The pretreatment of neonatal cardiomyocytes with PPAR-γ agonists significantly decreased the LPS-stimulated TNF-α release by cardiac myocytes [115]. Moreover, PPAR-γ ligands suppressed myocardial mRNA expressions of inflammatory cytokines and IL-1β in an autoimmune myocarditis model [129]. Interestingly, treatment with rosiglitazone or pioglitazone decreased the expression of proinflammatory markers and reduced accumulation of neutrophils and macrophages in reperfused myocardium [130, 131]. Nevertheless, high doses of PPAR-γ agonists were shown to induce cardiac dysfunction with marked changes in the utilization of free FA and glucose. Thus, the pathophysiological mechanisms on the cardiac effects of PPAR-γ agonists causing an increased incidence of myocardial dysfunction are yet to be elucidated [109, 132]. The model of constitutive, whole-body disruption of PPAR-γ results in embryonic lethality due to cardiac and placental defects [133], preventing the evaluation of the cardiac phenotype of these mice. However, cardiac-specific PPAR-γ (csPPAR-γ) KO mice revealed that csPPAR-γ deficiency only caused modest ventricular hypertrophy and did not impair systolic function in the unstressed condition [134]. Increased PPAR-γ expression was found in the spontaneously hypertensive rat that may have resulted from increased lipid uptake or as a compensatory response to cardiac hypertrophy and failure, thereby compromising cardiac function [124, 125].

7. Therapeutic Outcomes of PPAR Ligands in Heart Disease

7.1. PPAR-α Agonists. Synthetic PPAR-α ligands—such as clofibrate, fenofibrate, and bezafibrate—decrease triglyceride-rich lipoproteins through an increase in the gene expression of FA-β-oxidation and decrease in the expression of apolipoprotein (Apo) C-III [135, 136]. The above-noted drugs are extensively used in the treatment of hypertriglyceridemia. Such fibrates not only have a triglyceride-lowering effect, but also increase HDL-cholesterol levels resulting from the increase in the expressions of ApoA-I and ApoA-II [135–137].

Human trials with PPAR-α agonists have largely, but not uniformly, supported possible atherosclerotic benefits. In the Bezafibrate Coronary Atherosclerosis Intervention Trial

(BECAIT), bezafibrate treatment decreased angiographic evidence of coronary atherosclerosis [138, 139]. In the Helsinki Heart Study (HHT), gemfibrozil decreased cardiovascular events, especially among patients with diabetes, but an increased rate of noncoronary death was also noted [140]. In the Bezafibrate Infarction Prevention (BIP) trial, only the subgroup with the highest triglyceride levels showed a decrease in adverse cardiovascular events with fibrate therapy [141]. In the Veteran's Administration-HDL Intervention Trial (VA-HIT), gemfibrozil treatment showed a statistically significant decrease in cardiovascular events in the cohort with average LDL-cholesterol levels, history of cardiovascular disease, and modestly decreased HDL-cholesterol/elevated triglycerides [142–144]. Of note, VA-HIT subjects were not on any 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins); therefore, the outcomes of this trial may have been driven largely by the effect of gemfibrozil in patients with insulin resistance and/or diabetes [143, 144]. The Fenofibrate Intervention and Event Lowering in Diabetes (FIELD) study—a large, randomized, placebo controlled trial—investigated the effects of fenofibrate on first or recurrent cardiovascular events in patients with type 2 diabetes and found that the primary end point did not achieve a statistically significant difference between treatment groups. Several secondary end points were significantly reduced, including total cardiovascular events and nonfatal myocardial infarction. Somewhat surprising was the finding that decreases in small-vessel diseases; namely, nephropathy and retinopathy were also found. An increase in cardiovascular mortality also was noted with fenofibrate but did not reach statistical significance [145]. Comparing the positive outcomes of VA-HIT with gemfibrozil, a less potent PPAR-α agonist, to the negative results seen in FIELD achieved with fenofibrate, a more potent PPAR-α agonist might support PPAR modulation, as opposed to more powerful activation, as being clinically effective. More potent PPAR binding may not necessarily correlate with greater clinical advantage, particularly because PPAR agonists have been defined mainly in vitro [138, 146, 147].

Importantly, FIELD does not establish the impact of statin plus fibrate combination therapy on cardiovascular disease. Thus, the hypothesis that combination of a statin plus a fibrate might offer greater cardiovascular risk reduction than a statin alone implies the requirement of another clinical study such as the Action to Control Cardiometabolic Risk in Diabetes (ACCORD) trial. However, ACCORD-lipid arm in patients with DM did not demonstrate any reduction in fatal cardiovascular incidences or nonfatal myocardial infarction and stroke compared with simvastatin alone [148]. From the disappointing cardiovascular outcomes in these studies, we might expect VA-HIT and FIELD to specify advantages of fibrates in patients who are statin intolerant or for possible fibrate benefits to microvessel disease, which is a major source of morbidity in diabetes [138]. Furthermore, prespecified subgroup analysis of the ACCORD data suggested a possible benefit of fenofibrate in patients with high triglyceride and low HDL-cholesterol baseline levels. Therefore, fibrates may prove to be beneficial in treating atherogenic dyslipidemia in diabetes patients [51, 148].

7.2. PPAR-γ Agonists. PPAR-γ is a regulator of glucose and lipid metabolism; therefore, its synthetic PPAR-γ ligands—such as glitazones and TZD derivatives (such as troglitazone, rosiglitazone, and pioglitazone)—improve glucose and insulin parameters and increase whole body insulin sensitivity. Therefore, they are called insulin-sensitizers and are used in the treatment of diabetes [149]. In early human trials, PPAR-γ agonists showed decreased in-stent restenosis after coronary stent implantation [150, 151]. Furthermore, in the Carotid Intima-Media Thickness in Atherosclerosis Using Pioglitazone (CHICAGO) study, significant effects of pioglitazone on the slow progression of carotid intima-media thickness were reported in patients nearly matched for glycemic control with glimepiride [152].

Rosiglitazone and pioglitazone are used in the treatment of patients with type 2 diabetes; however, the effects of these TZDs on cardiovascular outcomes in patients with DM are different. The Prospective Pioglitazone Clinical Trial in Macrovascular Events (PROactive) trial investigated the effects of pioglitazone combined with standard contemporary antidiabetic treatment versus active, but non-TZD, antidiabetic treatment on a combined vascular end point in individuals with known macrovascular disease [153]. The purpose of the PROactive study was to achieve similar, matched hemoglobin A1c (HbA1c) levels in the TZD and non-TZD groups in order to provide more definitive insights into glucose-independent vascular effects of TZDs. In spite of the extensive in vivo and in vitro data supporting TZD effects on atherosclerosis, no statistically significant difference was noted in the primary end point between study groups. In contrast, the main secondary end point was revealed with a statistically significant 16% decrease in clinical events [153, 154]. Contrary to pioglitazone, rosiglitazone was associated with significant increases in death from cardiovascular causes and myocardial infarction after a relatively short-term of exposure [155]. Thus, the European Medicines Agency withdrew approval of rosiglitazone in 2010 due to these cardiovascular safety concerns [156]. Importantly, these divergent outcomes may result from their diverse effects on lipid subfractions [157]. Pioglitazone increases HDL-cholesterol and decreases fasting plasma free FAs and triglycerides without any influence on total cholesterol and LDL-cholesterol; however, rosiglitazone significantly augments HDL-cholesterol levels, as well as total cholesterol and the LDL-cholesterol fraction levels [156, 158, 159].

In the Diabetes Reduction Approaches with Ramipril and Rosiglitazone Medications (DREAM) study, the effects of the angiotensin-converting enzyme inhibitor ramipril and rosiglitazone on the prevention of diabetes were studied using a two-by-two placebo-controlled design [160]. Interestingly, rosiglitazone significantly reduced the progression to diabetes in a cohort with impaired fasting glucose and/or impaired glucose tolerance, whereas ramipril had no effect on this measure [161]. The Actos Now for the Prevention of Diabetes (ACT NOW) trial analyzed with a similar question to DREAM in patients with impaired glucose tolerance randomized to receive either pioglitazone (45 mg) or placebo. After a mean follow-up of 2.2 years, progression to diabetes occurred in 5% of the pioglitazone group, compared with

16.7% of the placebo group, but too few cardiovascular events occurred (pioglitazone 26, placebo 23) to draw any inferences regarding effect of treatment on cardiovascular outcomes [162, 163]. This decrease in diabetes progression with pioglitazone was consistent with previous studies, including the troglitazone arm of the Diabetes Prevention Program and women with a history of gestational diabetes [164, 165]. Additionally, the pioglitazone arm of the Pioneer study revealed significantly greater improvements in inflammatory markers—including high-sensitivity CRP, MMP-9, and MCP-1—than the glimepiride-treated group despite equivalent reductions in fasting glucose and HbA1c levels. In an additional subgroup analysis, patients with no significant glucose responses to pioglitazone still had improved surrogate markers for atherosclerosis. Despite limitation by the small numbers of patients in these subgroups, such findings continue to raise possible disassociations between TZD-mediated effects on the vasculature and inflammation versus its glycemic advantages [138, 166].

In the Cardiovascular Outcomes in Oral Agent Combination Therapy for Type 2 Diabetes (RECORD) trial, 4,447 subjects with type 2 DM poorly controlled on monotherapy with metformin or sulfonylurea, a noninferiority hypothesis was explored for rosiglitazone as second-line therapy in type 2 diabetes [163, 167]. The primary end point of RECORD was time to cardiovascular hospitalization or cardiovascular death. After a mean follow-up of 5.5 years, primary endpoint events occurred in 321 patients in the rosiglitazone group and 323 patients in the metformin/sulfonylurea group, thus meeting the requirement for noninferiority of rosiglitazone. Fatal or nonfatal HF occurred more frequently in the rosiglitazone group than in the active control group (61 versus 29 patients). Limitations of RECORD include an event rate that was substantially lower than that projected in trial design with consequent reduction of statistical power, and potential complications resulting from the differential use of statins and diuretics, and an open-labeled study design [163, 167].

Despite many beneficial features of glitazones, they also exhibit adverse effects, such as edema, heart failure, weight gain, bone fractures, and increased risk of myocardial infarctions, which have limited the use of TZDs in diabetic patients with high lipid levels [168]. In the PROactive study, an increased incidence of congestive HF was reported in the pioglitazone group, although these events were not well judged. Previous work has clearly demonstrated that TZDs can cause fluid retention, as evident from the modest decrease in hematocrit and volume expansion documented with TZD exposure [169]. The incidence of pedal edema observed with TZD monotherapy is about 3% to 5% compared with 1.2% in placebo arms [170]. The incidence of pedal edema with TZDs approaches 7.5% when combined with either metformin or sulfonylurea, compared with 2.5% and 2.1% with sulfonylurea or metformin alone, respectively [171]. The risk of pedal edema appears similar with both rosiglitazone and pioglitazone in clinical use [172]. Concomitant insulin and TZD use has been associated with a 2- to 3-fold higher rate of edema compared to insulin alone, with rates increasing from 5% to 7% with insulin alone to 13% to 15% with TZD and insulin [171]. Recent data suggest that upregulation of a specific sodium channel—sodium channel, nonvoltage gated 1 gamma subunit (SCNN1G)—in the distal nephron is a PPAR-γ-mediated mechanism for TZD-induced edema [173, 174]. Other mechanisms involved for TZD-mediated edema include altered interstitial ion transport, increased sympathetic nervous system activity, and altered endothelial permeability [175–177]. This edema is reversible and should not necessarily be equated with myocardial toxicity although some patients with DM, even absent class III or IV HF, may not tolerate this volume expansion [138].

Another clinically significant side effect of TZDs is body weight gain. This change, which likely involves both fluid retention and increases in adiposity, is typically in the range of 2 to 5 kg [178]. Some of the weight induced by TZDs may be advantageous, involving a shift from visceral to subcutaneous areas, and also track the increase in adiponectin, anti-inflammatory protein, induced by TZDs [179]. The change in fat distribution with TZDs includes a change in energy balance and possible effects on other factors and pathways influencing body weight, because a simple rearrangement in fat location would not explain an overall net increase in body mass [138, 180]. Nevertheless, the weight increase seen with PPAR-γ activation has clearly contributed to the hesitation of TZDs usage as antidiabetic drug, which may be more serious when combined with insulin [181].

7.3. PPAR-α/γ Dual Agonists. A new class of dual PPAR-α/γ agonists has been shown to have a positive influence on both glucose and lipid metabolism and are currently under development as a response to the treatment challenge of coexisting type 2 diabetes with dyslipidemia. These dual agonists not only reduce arteriosclerosis development, but also have an antidiabetic capacity. They also exhibit improvement of endothelial function, anti-inflammatory, and anticoagulant action, decrease plasma free FAs, and lower blood pressure, indicative of advantageous effects on the vasculature [49].

Until now, several attempts to develop a dual agonist for diabetes have failed due to various safety concerns: ragaglitazar, MK-0767, and naveglitazar were all found to be associated with an increased incidence of bladder cancer and hyperplasia in rodent studies [51, 182], and tesaglitazar development was discontinued due to indications that it may cause renal dysfunction [183]. The most-studied dual agonist muraglitazar was found to be effective in reducing HbA1c and triglyceride levels while increasing HDL-cholesterol levels [51, 184–188]. One randomized, double-blind trial of 1,477 drug-naive patients with type 2 diabetes found a −0.25% to −1.76% (3–17 mmol/mol) reduction in HbA1c from baseline after 24 weeks of muraglitazar treatment, compared with a reduction of −0.57% (5 mmol/mol) with pioglitazone [51, 186]. At 12 weeks, triglycerides had decreased by −4 to −41% with muraglitazar and 9% with pioglitazone and HDL-cholesterol had increased by 6–23% with muraglitazar and 10% with pioglitazone. Nevertheless, Bristol-Myers Squibb discontinued further development of this dual agonist in 2006 after Nissen and colleagues published an analysis of the available material from the clinical trial program, which revealed that muraglitazar was associated with an increased incidence of the composite end point of death, major adverse

cardiovascular events, congestive HF (relative risk: 2.62; $P = 0.04$), and excessive morbidity for all individual components of the composite endpoint when compared to placebo or pioglitazone [51, 188].

Aleglitazar (Hoffmann-La Roche) is the most recent dual PPAR-α/γ agonist that has completed in phase III trials and has a balanced affinity for both PPAR-α and PPAR-γ receptor subtypes. Preclinical and clinical trial results have been promising [51, 189–192]. Phase II study SYNCHRONY has shown a significant dose-dependent reduction in HbA1c of −0.36% (4 mmol/mol, 50 μg; $P = 0.048$) to −1.35% (15 mmol/mol, 600 μg; $P < 0.0001$) after 16 weeks of treatment with aleglitazar once daily when compared with placebo. Importantly, statistically significant beneficial effects on lipid subfractions were also found. Significant decreases in triglyceride ($P < 0.001$ for percentage changes) and increases in HDL-cholesterol ($P < 0.05$ for percentage changes) were found with all doses of aleglitazar (−43 and +21%, resp., with the 150 μg dose). In addition, significant reductions in LDL-cholesterol were found at doses of 150 μg or higher, compared with placebo ($P < 0.05$ for percentage changes): placebo-adjusted reduction in LDL-cholesterol with the 150-μg dose of aleglitazar was −15.5%. Indeed, aleglitazar, at the 150-μg dose, was associated with a greater effect on triglycerides, HDL-cholesterol, and LDL-cholesterol than pioglitazone 45 mg. Further analysis of this study data suggests that aleglitazar produces a shift from the atherogenic small dense LDL particles associated with type 2 diabetes to larger LDL particles [51, 193]. Phase III study ALECARDIO, randomized double-blind placebo-controlled clinical trial, had evaluated the hypothesis that aleglitazar (150 μg daily dose) can reduce cardiovascular mortality and morbidity in patients with type 2 DM who have suffered from a recent acute coronary syndrome (ACS) event. However, use of aleglitazar in patients with type 2 diabetes and recent ACS did not significantly reduce the incidence of cardiovascular death, myocardial infarction, or stroke. Unfortunately, aleglitazar increased the risks of HF, renal dysfunction, bone fractures, gastrointestinal hemorrhage, and hypoglycemia [194].

There are several potential explanations for why aleglitazar did not reduce cardiovascular mortality and morbidity in ALECARDIO trial. First, the magnitude of changes in HDL-cholesterol and triglyceride levels achieved with aleglitazar may not be sufficient to impart additional cardiovascular benefits when administered concurrently with statins. Second, some therapies may be unable to exert a cardioprotective effect in patients with extensive atherosclerosis and long-standing diabetes or may require a very long duration of exposure to achieve such effects. Third, favorable lipid and metabolic effects of aleglitazar may have been negated by adverse effects of the drug, including heart failure, reduced renal function, hypoglycemia, and increased LDL-cholesterol, resulting in no net cardiovascular benefit. These findings do not support the use of aleglitazar in this setting with a goal of reducing cardiovascular risk [51, 194].

8. New Modalities and Future Directions of PPAR-Directed Therapeutics

The impact of fibrates and TZDs on dyslipidemia and diabetes is linked primarily to PPAR-α and PPAR-γ activation, respectively [195, 196]. However, substantial clinical and preclinical experience has shown that individual drugs differ from one another in therapeutic and side effect properties [42, 197]. Furthermore, PPAR expression in multiple tissues raises the possible value of targeting PPAR agents in therapeutic indications of a number of other diseases (e.g., cancer and colitis) [122, 198–201]. Although many clinical studies of PPARs have demonstrated inconsistent results for cardioprotective effects [139–141, 145, 153, 167, 193, 194], the evidence reviewed above suggests that this is still a lucrative area of study. Therefore, the needs of new PPAR-directed therapeutic modalities must include pan-PPAR agonists, selective PPAR modulators, dual PPAR agonists, PPAR-γ antagonists, and nutraceuticals, all of which are being considered as possible approaches to reduce the adverse events seen with current TZDs [138, 181, 202].

8.1. Pan-PRAR Agonists. The significant structural similarity of PPAR-α, PPAR-β/δ, and PPAR-γ—particularly within their ligand-binding domains—has allowed the identification of several synthetic dual- or pan-PPAR agonists [203]. Active metabolites of fibrates, such as fenofibric acid and clofibric acid, are dual activators of PPAR-α and PPAR-γ, with about a 10-fold selectivity for PPAR-α. Another compound from this group, bezafibrate, is a broader activator because it activates all three PPAR subtypes at comparable doses to other fibrates. Therefore, bezafibrate is regarded as a pan-agonist with the potential to directly improve insulin sensitization via PPAR-γ activation [10, 15].

8.2. Selective Modulators and Partial Agonists. The intensive search for safer PPAR agonists led to the development of selective partial PPAR modulators. Currently, new selective PPAR-γ modulators are in development—including S26948 [204] and INT131 [205], which should stimulate glucose metabolism and minimize the adverse effects of full PPAR-γ agonists [49]. INT131 recruits vitamin D3 receptor interacting protein- (DRIP-) 205 and promotes its binding to a level of approximately 30% of that conferred by the full PPAR-γ agonist rosiglitazone [206]. In animal models of diabetes, INT131 caused less weight gain compared to pioglitazone or rosiglitazone while retaining efficacy to reduce plasma glucose [206, 207]. Importantly, toxicity of INT131 in cynomolgus monkeys and rats was not associated with fluid retention, changes in hematocrit, or weight gain over 6 months [207, 208]. In a phase II study, however, INT131 was associated with an increase in the incidence of edema, weight gain, and decreased hematocrit at the 10 mg dose versus placebo, highlighting the difficulty in translating promising preclinical profiles into patients [209]. While the cardiac adverse effect profile of rosiglitazone-like PPAR-γ full agonists is unfortunate, the therapeutic potential of novel pharmacological agents targeting PPAR-γ submaximal cannot be excluded. Interestingly, newly synthesized partial PPAR-γ agonists,

such as balaglitazone, MBX-102, MK-0533, PAR-1622, PAM-1616, KR-62776, and SPPAR-γM5, have a reduced tendency to cause the adverse effects associated with full PPAR-γ agonists or may be entirely devoid of such effects [6, 47].

8.3. Phosphorylation and Posttranslational Control. As noted above several compelling new mechanisms of posttranslational control of PPAR action have recently been described, including phosphorylation, SUMOylation, ubiquitination, and nitration [210]. In addition to enhancing the transcriptional activity of PPAR-γ, rosiglitazone was found to inhibit the PPAR-γ phosphorylation at Ser273 by cyclin-dependent kinase 5 (CDK5) in adipose tissue, preserving the transcription of insulin-response genes and correlating with antidiabetic activity. A second PPAR-γ agent, MRL24, was as effective as rosiglitazone at blocking phosphorylation and improving diabetes in animal models, despite being only a partial PPAR-γ agonist. Taken together, these results suggest that the insulin-sensitizing benefits of PPAR-γ agonists are due in part to their ability to block phosphorylation and not solely to their agonist activity [211].

8.4. Nongenomic Regulation. Recent evidence also suggests the potential role of nongenomic regulation of PPAR-γ and PPAR-α, mediated by interaction with cytosolic second messengers, including kinases and phosphatases [210]. The MAP/ERK kinase, MAPK kinase- (MEK-) 1, was reported to bind directly to the AF-2 domain of PPAR-γ in response to mitogenic stimulation, leading to the sequestration of PPAR-γ in the cytoplasm [212]. Selective inhibition of MEK-1/PPAR-γ interactions has recently been proposed as a concept for treatment of cancer, inflammation, and metabolic disorders but has yet to gain significant acceptance [212].

8.5. New Dual PPAR-α/γ Agonists. Saroglitazar, a PPAR agonist with predominant PPAR-α and moderate PPAR-γ activity, was launched exclusively in India for the control of dyslipidemia [213, 214]. However, limited data is available on its molecular profile, and the treatment duration and low patient number in its phase III program make it impossible to draw conclusions regarding its cardiovascular and long-term safety profiles [203].

8.6. Nutraceuticals and Life-Style Modification. As endogenous nuclear receptor ligands, dietary n-3 and n-6 polyunsaturated FAs (PUFAs) and their derivatives can upregulate PPAR-γ expression in vitro and in vivo and reduce an inflammatory response [215]. Furthermore, it has been shown that any type of regular exercise and crataegus species would improve cardiovascular function and minimizes several risk factors via stimulating lipid metabolism by acting on enzymes and genes expression such as ATP-binding cassette transporter A1 (ABCA1) and PPAR-α which are involved in this process [216]. However, though dietary PUFAs similar to synthetic ligands were able to bind to the ligand-binding domain and cause conformational changes to activate the receptor, they are considered as weak PPAR-γ ligands because of their low physiological concentrations. Another caution of nutraceuticals is that some of the flavonoids have been associated with tumor and altering pharmacodynamics and pharmacokinetics of various drugs via interacting with cytochrome P450 enzymes [202].

9. Conclusions

PPARs are critical gene regulators in cardiomyocytes, yet their functions are not fully established. PPAR agonists convey beneficial effects as therapeutic agents for diabetes and atherosclerosis by lowering blood glucose, improving insulin resistance, inflammation, and lipid metabolism; however, adverse side effects limit their clinical use. As such, the future of PPAR-directed agents in cardiometabolic therapy remains uncertain, although several late-stage molecules may still hold promise [203]. Future directions in PPAR agonist development are likely to focus on optimizing the PPAR subtype interaction profile, maximizing the inhibition of PPAR-γ phosphorylation, and screening against off-target activity. At the present time, clinicians should keep in mind the risk/benefit ratio of PPAR activators. Intensive research on this therapeutic target will likely lead to the development of safer and more effective PPAR agonists in the near future.

Abbreviations

ACS: Acute coronary syndrome
AF: Activation function
Apo: Apolipoprotein
BMAL-1: Brain and muscle Arnt-like protein-1
CDK5: Cyclin-dependent kinase 5
csPPAR-γ: Cardiac-specific PPAR-γ
DM: Diabetes mellitus
DRIP: Vitamin D3 receptor interacting protein
EFA: Essential FAs
FA: Fatty acid
GLUT4: Glucose transporter type 4
HDAC: Histone deacetylase
HDL: High-density lipoprotein
HETE: Hydroxyeicosatetraenoic acid
HF: Heart failure
HODE: Hydroxyoctadecadienoic acid
IL: Interleukin
KO: Knockout
LPS: Lipopolysaccharide
LT: Leukotriene
LXR-α: Liver X receptor-α
MCP: Monocyte chemoattractant protein
MEK: MAPK kinase
mRNA: Messenger RNA
NCoR: Nuclear receptor corepressor
NRIP: Nuclear receptor interacting protein
PG: Prostaglandin
PGC: PPAR-γ coactivator
PPAR: Peroxisome proliferator-activated receptor
PPRE: Peroxisome proliferator response element
PTM: Posttranslational modification
PUFA: Polyunsaturated fatty acid

RIP: Receptor interacting protein
RXR: Retinoid X receptor
SMRT: Silencing mediator of retinoid and thyroid hormone receptor
SUMO: Small ubiquitin-like modifier
TNF: Tumor necrosis factor
TZD: Thiazolidinedione
VLDL: Very low-density lipoprotein
VSMC: Vascular smooth muscle cell.

Conflict of Interests

The authors declare no conflict of interests.

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