Activation of Receptors δ (PPARδ) by Agonist (GW0742) may Enhance Lipid Metabolism in Heart both In Vivo and In Vitro

S.-C. Kuo1,2*, P.-M. Ku3,4*, L.-J. Chen3, H.-S. Niu6, J.-T. Cheng4,7

Affiliation addresses are listed at the end of the article

Abstract

It has been documented that cardiac agents may regulate the lipid metabolism through increased expression of PPARδ in cardiac cells. However, the effect on lipid metabolism by direct activation of PPARδ is still unknown. The present study applied specific PPARδ agonist (GW0742) to investigate this point in the heart of Wistar rats and in the primary cultured cardiomyocytes from neonatal rat. Expressions of PPARδ in the heart and cardiomyocytes after treatment with GW0742 were detected using Western blots. The fatty acid (FA) oxidation and the citric acid (TCA) cycle related genes in cardiomyocytes were also examined. In addition, PPARδ antagonist (GSK0660) and siRNA-PPARδ were employed to characterize the potential mechanisms. After a 7-day treatment with GW0742, expressions of PPARδ in the heart were markedly increased. Increased expressions of FA oxidation and TCA cycle related genes were also observed both in vivo and in vitro. This action of GW0742 was blocked by GSK0660 or by siRNA-PPARδ. The obtained results show that activation of PPARδ by GW0742 is responsible for the increase of FA oxidation and TCA cycle related genes in hearts. Role of PPARδ in the regulation of lipid metabolism in heart is then established.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcriptional factors that regulate the expression of genes involved in lipid metabolism and inflammation [1]. Three subtypes of PPARs, including PPARα, PPARγ, and PPARδ, modulate the expressions of many genes to exert various bioactivities [1]. PPARα is relatively abundant in tissues with a high oxidative capacity, such as liver and heart. PPARγ expression is confined to a limited number of tissues, primarily adipose tissue [1,2]. The ubiquitously expressed PPARδ enhances fatty acid catabolism in adipose tissue and muscle [1]. PPARδ-dependent maintenance of inotropic function and metabolic effects is crucial for cardiomyocytes [3–5]. The activation of PPARδ increases basal fatty acid (FA) oxidation to maintain the energy balance and cardiac function [6,7]. Many FA oxidation-related enzymes and mitochondrial respiratory uncoupling genes are regulated by PPARδ in cardiomyocytes, such as pyruvate dehydrogenase kinase and acyl-CoA oxidase 1 [8,9]. Deletion of cardiac PPARδ, which is accompanied by decreased contractation, increased left ventricular end-diastolic pressure, and lowered cardiac output, leads to decreased contraction and increased incidence of cardiac failure [10–12], but it remains unknown whether PPARδ is involved in the pathogenesis of cardiac disorders. Our previous study showed that cardiac agents improved cardiac contraction in STZ-diabetic rats is associated with a marked increase in cardiac PPARδ expression [13]. Also, an increase of PPARδ by digoxin is related to the regulation of FA oxidation genes [14]. GW0742 is a ligand of PPARs, which has 300–1000-fold selectivity for PPARδ vs. other PPARs [15], and shows full PPARδ agonist-like action in cell cultures and animal models [16–18]. It has been documented that activation of PPARδ by GW0742 increases cardiac contractility in rats [19]. However, the effects on cardiac lipid metabolism by GW0742 remain unclear. In the present study, we used Wistar rats and primary
neonatal cardiomyocytes of rats to investigate the effects of GW0742 on cardiac FA oxidation and TCA cycle genes in relation to PPARδ.

Materials and Methods

Materials

GW0742 (a specific PPARδ agonist) and GSK0660 (a specific PPARδ antagonist) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The TRIzol RNA extraction reagent, Opti-MEM® I Reduced Serum Medium, StealthTM Select siRNA(siRNA-PPARδ), scramble siRNA(siRNA-control), and Lipofectamine 2000™ were from Invitrogen (Carlsbad, CA, USA). Antibodies to PPARδ and actin were purchased from Abcam (Cambridge, MA, USA). The LightCycler TaqMan Master kit, primers, and universal library probes for analyzing PPARδ, fatty acid oxidation and TCA cycle related genes (Table 1) were purchased from Roche Diagnostics Corp. (Mannheim, Germany).

Animals

The male Wistar rats, weighing from 200 to 250 g, were obtained from the Animal Center of National Cheng Kung University Medical College. All experiments on rats were conducted under anesthesia with 3 % isoflurane. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Drug administration

Animals were randomly assigned into 3 groups: (I) the control group (n = 8) treated with the vehicle, saline (0.9 % sodium chloride, intravenously); (II) the GW0742 (GW) group (n = 8) treated with GW0742 at 5 mg/kg, intravenously for 7 days as described previously [20], and (III) the GW0742 + GSK0660 (GW + GSK) group (n = 8) treated with GW0742 (5 mg/kg) and GSK0660 at effective dose (3 mg/kg) [21] intravenously for 7 days. At the end of experiment, hearts of each group were dissected for detection of primary antibody reactions. Blots were incubated overnight at 4 ℃ with an immunoglobulin-G polyclonal antibody (Abcam, Cambridge, MA, USA) (1:500) in 5 % (w/v) skimmed milk powder dissolved in PBS/Tween 20. After the removal of primary antibody, the blots were washed twice, and harvested by trypsinization. Then, cells were collected and subjected to real-time reverse transcription-polymerase chain reaction or Western blotting analysis.

Western blotting analysis

Similar to our previous report [13], protein was extracted from tissue homogenates or cell lysates using ice-cold RIPA buffer supplemented with phosphatase and protease inhibitors (50 mmol/l sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 2 mg/ml aprotinin, and 0.5 mg/ml leupeptin). Protein concentrations were determined with the Bio-Rad protein assay. Protein was transferred to expanded polyvinylidene difluoride membranes (Pierce, Rockford, IL, USA) with a Bio-Rad Trans-Blot system. After transfer, the membranes were washed with PBS and blocked for 1 h at room temperature with 5 % (w/v) skimmed milk powder in PBS. The manufacturer's instructions were followed for the primary antibody reactions. Blots were incubated overnight at 4 ℃ with an immunoglobulin-G polyclonal rabbit anti-mouse antibody (Affinity BioReagents, Inc., Golden, CO, USA) (1:500) in 5 % (w/v) skimmed milk powder dissolved in PBS/Tween 20 (0.5 % by volume) to bind the PPARδ in the heart specimens. The blot was incubated with goat polyclonal antibody (1:1 000) to bind the actin serving as internal control. After the removal of primary antibody, the blots were extensively washed with PBS/Tween 20. The blots were then incubated for 2 h at room temperature with the appropriate peroxidase-conjugated secondary antibody diluted in 5 % (w/v) in skimmed milk powder and dissolved in PBS/Tween 20. The blots were developed by autoradiography using the ECL-Western blotting system (Amersham International, Buckinghamshire, UK). The immune blot of PPARδ (49 kDa) was quantified with a laser densitometer.

Table 1 Real-time PCR primers and universal library probes (UPL) of target genes.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward</th>
<th>Reverse</th>
<th>UPL Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-coenzyme A dehydrogenase, very long chain (VLCAD)</td>
<td>ggggttgggtcctcttca</td>
<td>ggaacggtaaccaaggg</td>
<td>53</td>
</tr>
<tr>
<td>Pyruvate dehydrogenase kinase, isoenzyme 4 (PDK4)</td>
<td>gagctcctccctcgacag</td>
<td>ttctcactgacattcctg</td>
<td>120</td>
</tr>
<tr>
<td>Uncoupling protein 3 (UCP3)</td>
<td>cccccacctagtcttgag</td>
<td>aagaaagggagctgaacct</td>
<td>79</td>
</tr>
<tr>
<td>Malonyl-coenzyme A decarboxylase (MCD)</td>
<td>bbtctgtgagggtatc</td>
<td>ggttgtgggccttg</td>
<td>158</td>
</tr>
<tr>
<td>Acetyl-coenzyme A dehydrogenase, long chain (LCAD)</td>
<td>gacgtagctgggaacagca</td>
<td>ggtatcagatctggaagga</td>
<td>81</td>
</tr>
<tr>
<td>Acetyl-coenzyme A oxidase 1, palmitoyl (ACOX1)</td>
<td>caccctggaggaaacaca</td>
<td>cpacagtgtcagtttt</td>
<td>112</td>
</tr>
<tr>
<td>Hydroxymethylbilane synthase (HMBS)</td>
<td>tccctgagtggtcctac</td>
<td>aagggtttccgttg</td>
<td>79</td>
</tr>
</tbody>
</table>

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Real-time reverse transcription-polymerase chain reaction
Total RNA was extracted from heart ventricles and cell lysates with TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA). The web-based assay-design software from the Universal Probe Library Assay Design Center ([http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp](http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp)) was used to design TaqMan primer pairs and to select appropriate hybridization probes. All the PCR experiments were performed using a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany). Reactions were performed in 20 μl of a mixture consisting of 13.4 μl of PCR buffer, 0.2 μl of each of the Universal Probe Library probes (10 mmol/l) ([Table 1](#)), 0.2 μl of each primer (20 μmol/l), 4 μl of LightCycler TaqMan Master (Roche Diagnostics GmbH) and 2 μl of template cDNA. The thermal cycling conditions consisted of an initial denaturation step at 95 °C for 10 min, followed by 45 cycles at 94 °C for 10 s, 60 °C for 20 s, and 72 °C for 1 s. The crossing point for each amplification was calculated relative to the respective standard curve with the aid of the LightCycler software. Relative gene expression was expressed as the ratio of the concentration of the target gene to that of a housekeeping gene hydroxymethylbilane synthase (HBMS).

Small interfering RNA (siRNA)
Duplexed RNA oligonucleotides for rat PPARδ (Stealth RNAi™) were synthesized from Invitrogen. According to our previous method [13], cardiomyocytes were transfected with 40 pmol of PPARδ-specific siRNAs (siRNA-PPARδ) or scramble siRNA using Lipofectamine 2000 (Invitrogen) and they were treated 48 h post-transfection. The sequences of the siRNA-PPARδ are UUUGCAGAUCCGAUCGCACUCGU (sense strand) and ACCGAGAUGUGCAGAUCCGACAA (antisense strand).

Statistical analysis
Data are expressed as the mean ± SEM for the number (n) of animals in 1 group as indicated. Statistical analysis was carried out using repeated measures analysis of variance (ANOVA) and Newman-Keuls post-hoc analysis. Bonferroni’s correction was applied to the data, which were obtained from relatively small groups. A p-value of 0.05 or less was considered significant.

Results

Effects of GW0742 on PPARδ expression in the heart of rats and in the cardiomyocytes
The level of PPARδ protein was significantly increased in the heart of rats, which received GW0742-treatment as compared with the control rats ([Fig. 1a](#)). Also, a significant induction of the expression of PPARδ protein was observed in GW0742-treated neonatal rat cardiomyocytes ([Fig. 1b](#)).

Effects of GW0742 on the levels of fatty acid β-oxidation related genes in the heart of rats
We examined the transcription levels of fatty acid β-oxidation genes using real-time PCR [3]. The relative levels of gene expression were compared to the internal control with the LightCycler software 4.05. Long-chain acyl-CoA dehydrogenase (LCAD), very long-chain acyl-CoA dehydrogenase (VLCAD), and acyl-CoA oxidase 1 (ACOX1) were increased in the heart of GW0742-treated rats. As compared to the control rats treated with vehicle, the transcription levels were increased by 122 % for LCAD, 113 % for VLCAD, and 161 % for ACOX1, respectively ([Fig. 2](#)). In addition, effects of GW0742 on these genes were blocked by co-treatment with GSK0660 ([Fig. 2](#)).

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**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Pair</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCAD</td>
<td>Forward: 5'-TCGAGCTGAGGTTGAGCTA-3'</td>
<td>Probe: 5'-FAM-CCACGCACTGACGATTGACCTT-MGB</td>
</tr>
<tr>
<td>VLCAD</td>
<td>Forward: 5'-CAGAGGTCTGCTCTCTCTCGCTG-3'</td>
<td>Probe: 5'-FAM-CACGCCGCGCGGGTGTGTTCAG-MGB</td>
</tr>
<tr>
<td>ACOX1</td>
<td>Forward: 5'-TGGTGTTCTGCTCAGGATCGCTG-3'</td>
<td>Probe: 5'-FAM-CGGTTCGCTCCTCCTCCTCCTT-MGB</td>
</tr>
</tbody>
</table>
Effects of GW0742 on the levels of TCA cycle related genes in the heart of rats
We examined the transcription levels of TCA cycle genes using real-time PCR [3]. The relative levels of gene expression were compared to the internal control with the LightCycler software 4.05. Uncoupling protein 3 (UCP3), malonyl-CoA decarboxylase (MCD) and pyruvate dehydrogenase kinase 4 (PDK4) were raised in the heart of GW0742-treated rats. As compared to control rats treated with vehicle, the transcription levels were increased by 208% for PDK4, 333% for UCP3 and 118% for MCD, respectively (Fig. 3). Also, effects of GW0742 on these genes were blocked by the co-treatment with GSK0660 (Fig. 3).

Effects of GW0742 on the levels of fatty acid β-oxidation related genes in the neonatal rat cardiomyocytes
The transcription levels of fatty acid β-oxidation genes were further examined in neonatal rat cardiomyocytes using real-time PCR [3]. The mRNA levels of uncoupling protein 3 (UCP3), malonyl-CoA dehydrogenase (MCD) and pyruvate dehydrogenase kinase 4 (PDK4) were raised in the heart of GW0742-treated rats. As compared to control rats treated with vehicle, the transcription levels were increased by 208% for PDK4, 333% for UCP3 and 118% for MCD, respectively (Fig. 3). Also, effects of GW0742 on these genes were blocked by the co-treatment with GSK0660 (Fig. 3).

Discussion
The present study showed that administration of GW0742 causes an increase of PPARδ expression in the heart of rats and neonatal rat cardiomyocytes. We also demonstrated the fatty acid β-oxidation and TCA cycle related genes could be upregulated by GW0742 both in the heart of rats and neonatal rat cardiomyocytes. Furthermore, the increased expressions of these genes by GW0742 were suppressed by GSK0660 or siRNA-PPARδ in the heart of rats or neonatal rat cardiomyocytes. Thus, it can be identified that an activation of PPARδ by GW0742 is related to...
the increase of lipid metabolism in heart; this view has not been mentioned before.

It has been established that PPARδ plays an important role in the regulation of cardiac performance [24–26]. In our previous study, an activation of PPARδ using the selective agonist GW0742 enhanced the cardiac contractility in isolated hearts and the hemodynamic dP/dt max in rats; both actions of GW0742 were blocked by GSK0660 at a concentration sufficient to block PPARδ [27,28]. In the present study, we have found that a 7-day treatment of GW0742 not only increased the level of PPARδ expression but also upregulated the fatty acid β-oxidation and TCA cycle related genes in the hearts and cardiomyocytes. Moreover, PPARδ activation in muscle is responsible for the lowering of plasma triglycerides in obese monkeys [29] and diabetic mice [30]. This supports the view that PPARδ activation in muscle seems beneficial in the metabolism of lipids [31] by increasing the catabolism of lipids and decreasing lipid accumulation [31]. The enhancing effects of cardiac agent on lipid accumulation in heart appear to be related to the increased expression of PPARδ [25]. In this report, we have demonstrated that GSK0660 and siRNA-PPARδ suppressed the GW0742-induced actions regarding the increase in both expressions of PPARδ and lipid metabolism related genes. These results suggest the mediation of PPARδ in GW0742-induced actions for increased expressions of fatty acid β-oxidation and TCA cycle related genes in the heart.

The expression of PPARδ is more ubiquitous, with relatively high levels in metabolically active tissues, such as muscle, liver, and adipose tissue [32]. Selective activation of PPARδ by agonists has been shown to improve glucose metabolism and insulin sensitivity in mouse models of obesity and insulin resistance, and these results are mainly related to the agonists’ capacity for activation of fatty acid transport and oxidation [33]. Activation of PPARδ in skeletal muscle increases the expression of regulatory genes involved in FA metabolism and mitochondrial oxidative phosphorylation, such as CPT-1 and the uncoupling proteins [34,35]. In the present study, transfection with siRNA-PPARδ suppressed the GW0742-induced PPARδ expression and lipid metabolic genes in cardiomyocytes. Thus, it shows that expressions of FA oxidation and mitochondrial respiratory uncoupling genes are regulated by PPARδ activated by GW0742.

PPARδ activation reduced adiposity by decreasing intracellular triglyceride accumulation in mouse adipose tissue and liver. PPARδ also enhanced β-oxidation in mouse preadipocytes [36]. PPARδ mRNA is expressed at 10 and 50 times the concentrations of PPARα and PPARγ mRNA [32], respectively, in skeletal muscle, and administration of PPARδ agonists results in an increase in expression of genes involved in fatty acid oxidation, mitochondrial respiration, and oxidative metabolism, decreasing muscle fatigability. In addition, activation of PPARδ may increase the mitochondrial gene expression and function. It has been reported...
that TCA cycle related genes could be upregulated in skeletal muscle of rats after administration of PPARδ agonist, such as acyl-CoA dehydrogenase long chain, acyl-CoA synthetase long-chain family member, carnitine palmitoyltransferase, inositol(myo)-1 (or 4)-monophosphatase, 2,4-dienoyl CoA reductase, hormone-sensitive lipase, high-density lipoprotein-binding protein, mitochondrial acyl-CoA thioesterase 1, pyruvate dehydrogenase kinase, peroxisomal D3,D2-enoyl-CoA isomerase, uncoupling protein, dithiol-cysteine peroxisomal acyl-CoA thioesterase 1, pyruvate dehydrogenase kinase, mitochondrial acyl-CoA thioesterase 1, pyruvate dehydrogenase kinase, peroxisomal D3,D2-enoyl-CoA isomerase, uncoupling protein, etc. [34]. Concordantly, PPARδ transgenic mice display the enhanced exercise endurance as compared with the wild-type mice and showing more fatigue resistant of skeletal muscle Type I fibers [37,38]. In the current study, GW0742 increases the expression of TCA cycle related genes. Moreover, GSK0660 and siRNA-PPARδ suppressed this action of GW0742. Relation of PPARδ activation of TCA cycle related genes. Moreover, GSK0660 and siRNA-PPARδ suppressed this action of GW0742. The authors demonstrate that they have no conflicts of interest in the authorship or publication of this contribution.

Conflicts of Interest

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

Affiliations

1 Department of Ophthalmology, Chi-Mei Medical Center, Yong Kang, Tainan City, Taiwan
2 Department of Optometry, Chung Hwa University of Medical Technology, Jen-Feh, Tainan City, Taiwan
3 Department of Cardiology, Chi-Mei Medical Center, Yong Kang, Tainan City, Taiwan
4 Department of Medical Research, Chi-Mei Medical Center, Yong Kang, Tainan City, Taiwan
5 Department of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan City, Taiwan
6 Department of Nursing, Tzu Chi College of Technology, Hualien City, Taiwan
7 Department of Nutrition and Institute of Medical Science, College of Health Science, Chang Jung Christian University, Guei-Ren, Tainan City, Taiwan

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