Activating PPARα Prevents Post–Ischemic Contractile Dysfunction in Hypertrophied Neonatal Hearts

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Rationale: Post–ischemic contractile dysfunction is a contributor to morbidity and mortality after the surgical correction of congenital heart defects in neonatal patients. Pre-existing hypertrophy in the newborn heart can exacerbate these ischemic injuries, which may partly be due to a decreased energy supply to the heart resulting from low fatty acid β-oxidation rates.

Objective: We determined whether stimulating fatty acid β-oxidation with GW7647, a peroxisome proliferator–activated receptor-α (PPARα) activator, would improve cardiac energy production and post–ischemic functional recovery in neonatal rabbit hearts subjected to volume overload-induced cardiac hypertrophy.

Methods and Results: Volume-overload cardiac hypertrophy was produced in 7-day-old rabbits via an aorto-caval shunt, after which, the rabbits were treated with or without GW7647 (3 mg/kg per day) for 14 days. Biventricular working hearts were subjected to 35 minutes of aerobic perfusion, 25 minutes of global no-flow ischemia, and 30 minutes of aerobic reperfusion. GW7647 treatment did not prevent the development of cardiac hypertrophy, but did prevent the decline in left ventricular ejection fraction in vivo. GW7647 treatment increased cardiac fatty acid β-oxidation rates before and after ischemia, which resulted in a significant increase in overall ATP production and an improved in vitro post–ischemic functional recovery. A decrease in post–ischemic proton production and endoplasmic reticulum stress, as well as an activation of sarcoplasmic reticulum calcium ATPase isoform 2 and citrate synthase, was evident in GW7647-treated hearts.

Conclusions: Stimulating fatty acid β-oxidation in neonatal hearts may present a novel cardioprotective intervention to limit post–ischemic contractile dysfunction. (Circ Res. 2015;117:41-51. DOI: 10.1161/CIRCRESAHA.117.306585.)

Key Words: heart failure ▪ hypertrophy ▪ metabolism ▪ therapy

The myocardium undergoes several dramatic adaptations in energy substrate metabolism during the transition from the fetal/immediate newborn to the neonatal period. During the immediate newborn period, the heart relies predominantly on glycolysis and lactate oxidation as sources of ATP,6,7 while fatty acid β-oxidation contributes only a small fraction of total myocardial ATP production.7 Shortly after birth, glycolysis decreases, which accounts for <15% of total ATP production, while fatty acid β-oxidation rapidly matures and meets the majority of myocardial ATP demand.7,9 These maturational alterations in energy metabolism result from dramatic alterations in the allosteric and post-translational control of fatty acid β-oxidation,10,11 as well as alterations in key transcriptional regulators of energy metabolism.12-16 One such regulator is peroxisome proliferator–activated receptor-α (PPARα), of...
which, the expression increases in the postnatal period, and promotes the expression of genes involved in fatty acid metabolism, including carnitine palmitoyltransferase I (CPT-I), and malonyl-CoA decarboxylase.

Our previous reports demonstrate that volume-overload cardiac hypertrophy prevents the maturational increase in fatty acid oxidation seen postpartum partly due to higher malonyl-CoA levels, a potent endogenous CPT-I inhibitor, in neonatal pig and rabbit hearts. As such, the hypertrophied myocardium retains a metabolic profile characteristic of the fetal heart. Importantly, these alterations in energy substrate metabolism decrease the post-ischemic function, whereas enhancing fatty acid β-oxidation by increasing substrate supply is cardioprotective in neonatal rabbit hearts. One potential approach to increasing fatty acid oxidation in the neonatal heart is through activation of PPARα by GW7647, a potent and selective PPARα activator. We sought to determine whether enhancing fatty acid β-oxidation by PPARα activation using GW7647 in hypertrophied neonatal rabbit hearts could restore post-ischemic functional recovery, reducing some of the negative impact of hypertrophy in this setting, and to determine the underlying mechanisms responsible for such an effect.

Methods

Animals

The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication, 8th edition, 2011) and to the guidelines of the Canadian Council on Animal Care. All animal protocols were approved by the University of Alberta Health Sciences Animal Welfare Committee.

Assessment of Myocardial Function

Newborn New Zealand White rabbits of either sex (7 days old, 90–200 g) were anesthetized with inhaled isoflurane (2%), and were subjected to an aorto-caval shunt to induce volume-overload cardiac hypertrophy as described previously. The presence of a successful shunt was verified at postsurgical days 7 and 13 by color flow doppler that visualizes a physical shunt between the abdominal aorta and the inferior vena cava in both an axial and transverse plane. This is further validated by an enlarged inferior vena cava (an expanded Methods section is available in the Online Data Supplement). After validation, the animals in shunt group were randomly assigned to receive an intraperitoneal injection of vehicle (dimethyl sulfoxide, the solvent of GW7647) or GW7647 (3 mg/kg per day; EC₅₀=6 nmol/L for PPARα, Cayman Chemical) twice a day for 14 days. Animals that underwent surgery to create shunt, but consequently the shunt either did not exhibit or closed, were excluded from the study. Left ventricular ejection fraction (%) and other cardiac parameters were assessed by transthoracic echocardiography at postsurgical days 7 and 13 as described previously.

At 21 days of age (14 days post surgery), all animals were euthanized with Na⁺ pentobarbital, and hearts were removed for isolated biventricular working heart perfusions.

Isolated Biventricular Heart Perfusion and Ischemia-Reperfusion Protocol

Biventricular working heart perfusions used a modified Krebs–Henseleit solution containing 2.5 mmol/L Ca²⁺, 5.5 mmol/L glucose, 1.2 mmol/L palmitate prebound to 3% bovine serum albumin, 0.5 mmol/L lactate, and 100 μU/mL insulin as described previously. The hearts were subjected to 35 minutes aerobic perfusion, followed by 25 minute period of normothermic global (no flow) ischemia, and a 30 minute period of aerobic reperfusion. Cardiac function was assessed in biventricular working hearts as described previously. Glycolysis, glucose oxidation, and palmitate oxidation rates were measured in the aerobic and reperfusion periods using radiolabeled [5-³H]glucose, [U-¹⁴C]glucose, or [9,10-³H]palmitate, respectively, in the perfusate as described previously. The ATP and acetyl-CoA production rates from glycolysis, glucose oxidation, and fatty acid oxidation were calculated as described previously.

Malonyl-CoA and Triacylglycerol Measurements

Measurements of malonyl-CoA levels in ventricular tissue were performed using a modified high pressure liquid chromatography procedure as described previously. Total triacylglycerol content was determined using an enzymatic assay kit (Wako Pure Chemical Industries), whereas the incorporation of palmitoyl-CoA into triacylglycerol was calculated based on the specific activity of radiolabeled palmitate in the triacylglycerol pool as described previously.

Immunoblot Analysis and mRNA Expression

Heart tissue homogenates were subjected to SDS-PAGE and transferred to nitrocellulose membranes as previously described. Membranes were probed with the following antibodies: malonyl-CoA decarboxylase (University of Alberta); sarcoplasmic reticulum calcium ATPase isoform 2 (SERCA2), phospho-phospholamban at serine site-16, IκB protein-α, BCL2 adenovirus E1B 19kDa interacting protein 3 (BNIP3), tubulin, and lamin A (Cell Signaling Technologies); adipose triglyceride lipase (ATGL) and phosphorylated acetyl-CoA carboxylase (Millipore); serine-palmitoyltransferase (SPT1/2), and glucose transporter 4 (Santa Cruz); and glucose-regulated protein 78 (GRP78; Abcam). The content of nuclear PPARγ coactivator-1α (PGC-1α), and nuclear factor-κB subunit p65 were assessed relative to lamin A in the nuclear fractions (an expanded Methods section is available in the Online Data Supplement) as described previously. Membranes were quantified using Image J or Quantity One (4.4.0) software (Bio-Rad Laboratories). Levels of PPARα mRNA were determined by reverse transcription of total RNA followed by quantitative polymerase chain reaction analysis. The results were normalized by 18S mRNA.

Measurement of Glycerol-3-Phosphate Dehydrogenase, Citrate Synthase, CPT-I, and Complex I/II Activities

Glycerol-3-phosphate dehydrogenase (GPD) was measured in ventricular lysates, involving a reaction done at 30°C in a buffer containing 20 mmol/L Tris (pH 8.5), 100 mmol/L glycine, 1 mmol/L dithiothreitol, 0.5 mmol/L EDTA, 1 mmol/L nicotinamide adenine dinucleotide, and 1 mmol/L dihydroxyacetone phosphate. The reaction was monitored at 340 nm for 3 to 5 minutes for the formation of

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ATGL</td>
<td>Adipose triglyceride lipase</td>
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<tr>
<td>BINP3</td>
<td>BCL2/adenovirus E1B 19kDa interacting protein 3</td>
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<tr>
<td>CPT-I</td>
<td>Carnitine palmitoyltransferase I</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>GPD</td>
<td>Glycerol-3-phosphate dehydrogenase</td>
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<tr>
<td>GRP78</td>
<td>Glucose-regulated protein 78</td>
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<tr>
<td>LV</td>
<td>Left ventricle</td>
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<tr>
<td>NFXB</td>
<td>Nuclear factor-xB</td>
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<tr>
<td>PGC-1α</td>
<td>Peroxisome proliferator-activated receptor γ coactivator-1α</td>
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<td>PPARα</td>
<td>Peroxisome proliferator-activated receptor-α</td>
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<tr>
<td>RV</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>SERCA2</td>
<td>Sarcoplasmic reticulum calcium ATPase isoform 2</td>
</tr>
<tr>
<td>SPT1/2</td>
<td>Serine-palmitoyltransferase</td>
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NAD⁺. Citrate synthase activity was measured using tissue homogenates as described previously.²¹ CPT-I activity was assessed on the mitochondrial fraction (an expanded Methods section is available in the Online Data Supplement) based on formation of radiolabeled palmitoylcarnitine as described previously.²¹ Activities of complex I/II were spectrophotometrically quantified using mitochondrial fractions by recording time-dependent changes of absorption at 600 nm for complex I/II. The activity was expressed as μmol/mg mitochondrial protein/min.²¹

Statistical Analyses
Data are expressed as mean±SE. Samples sizes are indicated in the Table and Figures 1 to 5. The significance of differences for multiple comparisons was estimated by 1-way ANOVA. If ANOVA revealed differences, selected data sets were compared by Tukey multiple-comparison test. The significance of differences between 2 groups was estimated by unpaired, 2-tailed Student t test where appropriate. Differences were considered significant at *P<0.05.

Results
GW7647 Treatment Improves In Vivo and In Vitro Contractile Function in Hypertrophied Neonatal Rabbit Hearts
As expected, neonatal rabbits subjected to the aorto-caval fistula surgery were found to have an increase in inferior vena cava diameter (an index of aorto-caval fistula formation) compared with sham-operated rabbits (Table). This was accompanied by an increase in both left ventricular (LV) and right ventricular (RV) internal diastolic diameter by 14 days in the aorto-caval fistula rabbits. GW7647 treatment did not alter either the inferior vena cava diameter or LV and RV internal diastolic diameter in the aorto-caval fistula rabbits.

Although the LV posterior wall thickness was unaltered between sham-operated and volume-overload groups (Table), both LV and RV internal diastolic diameter were increased in vehicle-treated hypertrophied hearts relative to sham hearts, as was the ratio of heart weight to body weight. This is indicative of global volume overload-induced cardiac hypertrophy. Interestingly, despite a similar degree of hypertrophy in vehicle- and GW7647-treated rabbits subjected to an aorto-caval fistula, the hypertrophy-induced decrease in left ventricular ejection fraction was prevented by GW7647 treatment to the levels comparable with sham hearts (Table).

To evaluate whether GW7647-treated hypertrophied hearts could better withstand an ischemic insult, isolated biventricular working hearts from the experimental groups were subjected to an ischemia-reperfusion protocol (Figure 1A). Comparable with what was seen in vivo, cardiac function in the initial aerobic perfusion was similar in the sham and hypertrophy+GW7647 group, and was slightly depressed in the hypertrophy+vehicle group (Figure 1B). During reperfusion after ischemia, recovery of cardiac function was significantly less in the vehicle-treated hypertrophied hearts compared with sham hearts, confirming our previous observations.²¹ Of interest, hypertrophied hearts from GW7647-treated rabbits showed a marked improvement in cardiac function during reperfusion, and recovered to similar levels as sham hearts (Figure 1B and 1C).

GW7647 Treatment Prevents the Hypertrophy-Induced Shift in Myocardial Energy Metabolism
In vehicle-treated hypertrophied hearts, a decrease in fatty acid β-oxidation rates was seen compared with sham hearts during the preischemic period (Figure 2A), confirming our previous findings.²¹ GW7647 treatment prevented this deficit in fatty acid β-oxidation in hypertrophied hearts (Figure 2A). During post–ischemic reperfusion, fatty acid β-oxidation was also decreased in the hypertrophied hearts compared with sham hearts, an effect that was prevented in the GW7647-treated hypertrophied hearts.

Glucose oxidation rates were similar during the preischemic aerobic period between the experimental groups, and recovered to similar levels after ischemia (Figure 2B).

| Table. Physical and Cardiac Parameters in Vehicle- or GW7647-Treated Rabbits Subjected to a Sham or Aorto-Caval Fistula Operation |
|------------------|------------------|------------------|
|                  | Sham             | Hypertrophy+Vehicle | Hypertrophy+GW7647 |
| n                | 9                | 13               | 7                |
| IVC, mm          | 2.42±0.15        | 3.38±0.11*       | 3.53±0.09*       |
| RVDD, mm         | 3.15±0.16        | 3.42±0.11*       | 3.71±0.13*       |
| LVDD, mm         | 7.83±0.31        | 8.74±0.28*       | 8.79±0.31*       |
| LVPW, mm         | 1.76±0.09        | 1.66±0.07        | 1.84±0.11        |
| LVEF, %          | 75.40±1.10       | 67.40±3.00*      | 76.50±2.50†      |
| Body weight, g   | 422±13           | 385±28           | 422±11           |
| HW:BW (10⁻³)     | 4.20±0.10        | 4.70±0.30*       | 4.80±0.20*       |
| LV:BW (10⁻³)     | 1.85±0.07        | 1.93±0.11        | 2.12±0.08*       |
| SEP:BW (10⁻³)    | 1.52±0.07        | 1.60±0.11        | 1.66±0.06        |
| RV:BW (10⁻³)     | 0.86±0.03        | 1.07±0.09*       | 1.02±0.04*       |

IVC indicates vena cava diameter; HW, heart weight; LV, left ventricle; LVEF, left ventricular ejection fraction; LVDD, left ventricular internal diameter in diastole; LVPW; LV posterior wall thickness; RVDD, right ventricular internal diameter in diastole; RV, right ventricle; and SEP, septum

*P<0.05 vs sham; †P<0.05 vs hypertrophy.
In contrast, glycolysis rates were significantly increased in vehicle-treated hypertrophied hearts during the preischemic period compared with sham hearts, and were dramatically elevated in the post–ischemic period relative to sham hearts (Figure 2C). This post–ischemic rise in glycolysis was significantly less in the GW7647-treated hypertrophied hearts.

![Figure 1](http://circres.ahajournals.org/content/44/6/4464/f1.large.jpg)

**Figure 1.** Effect of GW7647 on the reperfusion recovery of ischemic hypertrophied hearts from neonatal rabbits. Hearts from sham, hypertrophied, and GW7647-treated hypertrophied neonatal rabbits were subjected to an isolated biventricular working heart perfusion with buffer containing 5.5 mmol/L glucose, 1.2 mmol/L palmitate prebound to 3% bovine serum albumin, 0.5 mmol/L lactate, and 100 μU/mL insulin. A. Ischemia-reperfusion protocol. B. Normalized cardiac function (heart rate [HR] × aortic peak systolic pressure [AoPSP] + pulmonary vein peak systolic pressure [PVPSP]) from biventricular perfusion from sham (n=9), hypertrophied hearts with GW7647 (n=8), or vehicle (n=13). *P<0.05, significant difference vs hypertrophy group. C. Recovery of myocardial function (the product of heart rate and developed pressure: RPP) after ischemia. Values represent mean±SEM. *P<0.05, significant difference. LV indicates left ventricle.

![Figure 2](http://circres.ahajournals.org/content/44/6/4464/f2.large.jpg)

**Figure 2.** Effect of GW7647 treatment on cardiac energy metabolism in hypertrophied neonatal hearts. A. Palmitate oxidation rates during pre- and post-ischemia from sham (n=11), hypertrophied hearts with GW7647 (n=24) or vehicle (n=16). B. Glucose oxidation rates during pre- and post-ischemia from sham (n=7), hypertrophied hearts with GW7647 (n=5) or vehicle (n=11). C. Glycolysis rates during pre- and post-ischemia from sham (n=16), hypertrophied hearts with GW7647 (n=9) or vehicle (n=11). D. Proton production rates during pre- and post-ischemia from sham (n=7), hypertrophied hearts with GW7647 (n=5) or vehicle (n=11). E. ATP production rates during pre- and post-ischemia from sham (n=16), hypertrophied hearts with GW7647 (n=9) or vehicle (n=11). F. Tricarboxylic acid cycle activity measured as an acetyl-CoA production during pre- and post-ischemia from sham (n=16), hypertrophied hearts with GW7647 (n=9) or vehicle (n=11). Values represent mean±SEM. *P<0.05, significant difference.
Glycolysis uncoupled from glucose oxidation is a major source of \( H^+ \) production in the heart, and contributes to post–ischemic injury. We, therefore, calculated myocardial proton production in pre–ischemic hearts and post–ischemic hearts. In hypertrophied hearts, an increase in proton production was observed compared with sham hearts, both before and after ischemia (Figure 2D). Proton production was significantly decreased in GW7647-treated hypertrophied hearts, especially during the post–ischemic period (Figure 2D).

GW7647 Treatment Enhances Rates of ATP Production and Tricarboxylic Acid Cycle Activity in Hypertrophied Hearts

The GW7647 mediated increase in fatty acid \( \beta \)-oxidation resulted in an accelerated rate of total ATP production both before and after the ischemic period when compared with vehicle-treated hypertrophied hearts (Figure 2E). Rates of tricarboxylic acid cycle activity measured by an acetyl-CoA production in hypertrophied hearts were also increased by GW7647 treatment (Figure 2F).

GW7647 Treatment Increases Mitochondrial Biogenesis and Fatty Acid Oxidative Capacity

PPAR\( \alpha \) is an important transcriptional factor involved in the control of many enzymes involved in fatty acid oxidation. The mRNA expression of PPAR\( \alpha \) tended to be decreased in the LV of vehicle treated hypertrophied hearts (Figure 3A). Interestingly, cardiac hypertrophy increased PPAR\( \alpha \) mRNA expression in the RV, which was further increased by GW7647 treatment (Figure 3A). Because GW7647 is a PPAR\( \alpha \) agonist, we examined what effect GW7647 had on downstream enzymes controlled by PPAR\( \alpha \). CPT-I is a key enzyme involved in mitochondrial fatty acid uptake, and is under PPAR\( \alpha \) transcriptional control. GW7647 treatment did not change CPT-I activity in either the right or LV of hypertrophied hearts (Figure 3B). However, malonyl-CoA, a potent inhibitor of CPT-I in the heart, was decreased in GW7647-treated hypertrophied hearts in both the right and LVs (Figure 3C). The expression of malonyl-CoA decarboxylase, which degrades malonyl-CoA and is regulated by PPAR\( \alpha \), was also decreased in the RV.

Figure 3. Effect of GW7647 treatment on peroxisome proliferator–activated receptor–\( \alpha \) (PPAR\( \alpha \)) expression, carnitine palmitoyltransferase I (CPT-I) activity, malonyl-CoA levels, peroxisome proliferator–activated receptor \( \gamma \) coactivator–1\( \alpha \) (PGC-1\( \alpha \)) expression and citrate synthase activity in hypertrophied neonatal hearts. A, Expression of PPAR\( \alpha \) mRNA (n=5). B, Activity of CPT-I after ischemia (n=5). C, Content of malonyl-CoA after ischemia (n=4–5). D, Expression of nuclear PGC-1\( \alpha \) relative to lamin A (n=6). E, Activities of citrate synthase (n=5–6). Values represent mean±SEM. *P<0.05, significant difference. LV indicates left ventricle; and RV, right ventricle.
of hypertrophied hearts, and its expression was increased by GW7647 treatment (Online Figure 1A). The expression of acetyl-CoA carboxylase, which synthesizes malonyl-CoA, was unchanged in hypertrophied hearts with and without GW7647 treatment (Online Figure 1B). Combined, a GW7647-mediated increase in malonyl-CoA decarboxylase content (and activity) could explain the decrease in malonyl-CoA levels observed in GW7647-treated hypertrophied hearts (Figure 3C).

PGC-1α, a transcriptional factor involved in mitochondrial biogenesis, was not decreased in hypertrophied hearts (Figure 3D). However, GW7647 treatment tended to increase PGC-1α expression in the LV of hypertrophied hearts. This was accompanied by an increase in mitochondrial citrate synthase activity in the LV (Figure 3E), consistent with an increase in PGC-1α activity. In contrast, GW7647 treatment did not alter the activity of the mitochondrial complex activities in hypertrophied hearts (Online Figure 1C), which was also not altered by hypertrophy itself.

To understand what contributed to the increase in glycolysis in hypertrophied hearts (Figure 2C), we measured glucose transporter 4 expression (Online Figure 1D). Although glycolysis was increased in the hypertrophied hearts, glucose transporter 4 expression was not increased in hypertrophied hearts (Online Figure 1D). In GW7647-treated hypertrophied hearts, the decrease in glycolysis was accompanied by an increased expression of glucose transporter 4, relative to vehicle-treated hypertrophied hearts (Online Figure 1D).

GW7647 Treatment Reduces Myocardial Triacylglycerol Content by Enhancing Its Turnover

Myocardial triacylglycerol stores are an important source of fatty acids for myocardial fatty acid β-oxidation. As shown in Figure 4A, the amount of radiolabeled palmitate incorporated into myocardial triacylglycerol throughout the perfusion was significantly decreased in both the right and LV of hypertrophied hearts compared with sham hearts. Despite this decreased labeling of triacylglycerol, total triacylglycerol stores were unchanged in the hypertrophied hearts compared with sham hearts (Figure 4B). This suggests a decreased triacylglycerol turnover in the hypertrophied hearts. In support of this, expression of ATGL, a major triacylglycerol lipase in the heart, was decreased in hypertrophied hearts compared with sham hearts (Figure 4C). Interestingly, it has been demonstrated that provision of fatty acids for PPARα activation in mouse heart requires ATGL-mediated lipolysis that is essential for the activation of PPARα and PGC-1α complex and mitochondrial biogenesis. 23 As a result, the decreased ATGL expression and triacylglycerol turnover in hypertrophied hearts is consistent with the decrease in fatty acid β-oxidation in these hearts.

GW7647 treatment of hypertrophied hearts resulted in significantly increased incorporation of radiolabel in the triacylglycerol pool of hypertrophied hearts (Figure 4A). Despite this increased labeling, total triacylglycerol levels decreased in the GW7647-treated hypertrophied hearts (Figure 4B), suggestive of an increased triacylglycerol turnover in these hearts. Supportive of this finding was an increased ATGL expression in the LV of GW7647-treated hypertrophied hearts (Figure 4C). These data are consistent with a GW7647-mediated increase in PPARα activation and an increase in fatty acid supply for mitochondrial fatty acid β-oxidation.

As cardiomyocytes display a low capacity for glycerol uptake,24 most of glycerol-3-phosphate used for triacylglycerol biosynthesis is generated from dihydroxyacetone phosphate, [46]
an intermediate of glycolysis, produced via GPD. In hypertrophied hearts with a low triacylglycerol turnover, GPD activity was decreased in the LV (Figure 4D). In GW7647-treated hypertrophied hearts, both LV and RV GPD activities were increased.

**GW7647 Treatment Activates Calcium-Handling Proteins, Reduces Ceramide Synthesis, Endoplasmic Reticulum Stress, and Reduces Inflammation**

Inactivation of SERCA2 occurs during myocardial remodeling seen in systolic heart failure, which contributes to mitochondrial dysfunction. Inactivation of SERCA2 occurs during myocardial remodeling seen in systolic heart failure, which contributes to mitochondrial dysfunction.25 We, therefore, examined the effect of hypertrophy and GW7647 treatment on SERCA2 levels in neonatal hearts. A hypertrophy-mediated decrease in SERCA2 protein expression was seen in the LV, but not in the RV (Figure 5A). This decrease was significantly prevented by GW7647 treatment (Figure 5A). In addition, phosphorylated phospholamban (p-PLN), an active form of a regulatory protein for SERCA2 activity, was decreased in hypertrophied hearts at the Ser-16 site (Figure 5B). GW7647 treatment increased p-PLN in hypertrophied hearts, similar to its effects on the expression of SERCA2.

Endoplasmic reticulum (ER) stress is associated with increased proapoptotic proteins and subsequent poor functional

**Figure 5.** Effect of GW7647 on calcium-handling proteins and endoplasmic reticulum stress in hypertrophied neonatal hearts. **A and B,** Expression of sarcoplasmic reticulum calcium ATPase isoform 2 (SERCA2) and phosphorylated phospholamban (p-PLN) at serine16 (PLN-s16) relative to tubulin (n=4–5). **C–F,** Expression of glucose-regulated protein 78 (GRP78), BNIP3, serine-palmitoyltransferase (SPT) 1, and SPT2 relative to tubulin (n=4–5). **G,** Expression of nuclear factor-κB subunit p65 (p65 NF-κB) relative to lamin A (n=6). **H,** Expression of cytosolic total IκBα (T-IκBα) relative to tubulin (n=4–5). Values represent mean±SEM. *P<0.05, significant difference.
recovery in ischemic hearts secondary to induced cell death. In our study, hypertrophy increased the expression of GRP78, a marker of ER stress, in the LV. This increase in GRP78 was prevented by GW7647 treatment (Figure 5C). BNIP3, a hypoxia-inducible member of the Bcl-2 family of apoptotic regulators, was also increased in the LV of hypertrophied hearts (Figure 5D). Although GW7647 treatment did not alter BNIP3 expression in the LV of hypertrophied hearts, BNIP3 did decrease in the RV of hypertrophied hearts. ER stress is also associated with alterations in ceramide levels and synthesis. Since SPT, the rate-limiting enzyme for de novo ceramide synthesis, and is located in the ER membrane, we investigated whether SPT expression was changed concomitant with altered ER stress. Indeed, increases in the expression of SPT1 and SPT2 were seen in the RV of hypertrophied hearts (Figure 5E and 5F), and these effects were prevented by GW7647 treatment (Figure 5E and 5F).

The inducible transcription factor NF-kB plays a central role in ischemia-reperfusion injury. Cardiac hypertrophy did not alter the expression of nuclear p65 NF-kB per se, but GW7647 treatment did reduce the nuclear retention of NF-kB-p65 in the RV of hypertrophied hearts (Figure 5G). This was accompanied by an enhanced cytosolic expression of total IκB protein-α, an inhibitor of NF-kB (Figure 5H).

Discussion

Pathological hypertrophy in the neonatal heart delays the normal maturation of fatty acid β-oxidation, thereby decreasing overall energy production of the heart. In this study, we show that increasing fatty acid β-oxidation, via chronic stimulation of PPARα, increases overall energy production in the hypertrophied neonatal heart. Interestingly, the PPARα-induced stimulation of fatty acid β-oxidation did not prevent the development of cardiac hypertrophy per se, but rather prevented its negative effects. This included a decrease in hypertrophy-induced in vivo contractile dysfunction, and a significant improvement in reperfusion recovery after ischemia. Part of this cardioprotection may result from the increased oxidative metabolism by GW7647 stimulation of PPARα and a concurrent attenuation in the hypertrophy-induced increase in glycolysis. This decrease in glycolysis alleviated the increased uncoupling of myocardial glycolysis and glucose oxidation seen in the hypertrophied hearts, resulting in a reduced rate of proton production. In addition, GW7647 treatment reduced ER stress and inflammation associated with hypertrophy, thus further contributing to cardioprotection. Combined, our data suggest that stimulating mitochondrial oxidative metabolism by increasing fatty acid β-oxidation has beneficial effects on the hypertrophied neonatal heart.

In both the fetal and the immediate newborn period, glycolysis is a major source of ATP production for the heart. Shortly after birth, glycolysis rates decrease dramatically, whereas fatty acid β-oxidation rates rapidly increase and become the major source of ATP production for the neonatal heart. This normal maturation process provides an important source of energy for the heart of the suckling infant. This postnatal increase in myocardial fatty acid β-oxidation occurs because of both a rise in circulating plasma fatty acid levels in the neonatal and a decrease in allosteric inhibition of myocardial fatty acid oxidation, as well as an increase in transcriptional control of myocardial enzymes involved in fatty acid β-oxidation. However, the presence of cardiac hypertrophy inhibits this normal maturation of fatty acid β-oxidation, decreasing overall energy production of the neonatal heart. As is observed in adult hearts, cardiac hypertrophy results in the heart maintaining a more fetal phenotype, including being more reliant on glycolysis as a source of energy production (Figure 2). We propose that this increase in glycolysis and decrease in fatty acid β-oxidation increases the susceptibility of the neonatal hypertrophied heart to ischemia-reperfusion injury, primarily by decreasing overall energy supply during reperfusion.

In support of this concept, we show that stimulation of PPARα can overcome the hypertrophy-induced decrease in fatty acid β-oxidation (Figure 2), resulting in a significant improvement of cardiac function during reperfusion of ischemic hearts (Figure 1). In the adult heart, high rates of fatty acid β-oxidation contribute the severity of ischemic injury, and contractile dysfunction of hearts subjected to pressure overload. This is primarily because of a fatty acid-induced decrease in glucose oxidation, which can lead to an uncoupling of glycolysis from glucose oxidation and an increase in proton production. However, in the neonatal period, myocardial glucose oxidation rates are low, and do not increase until weaning. Indeed, high levels of fatty acids do not dramatically inhibit post–ischemic glucose oxidation in the neonatal heart, and have actually been shown to be beneficial to reperfusion recovery of function in the neonatal heart. We, therefore, propose that stimulating fatty acid β-oxidation in the neonatal heart is beneficial, secondary to increasing overall ATP production in the stressed heart. We also propose that a delayed maturation of fatty acid β-oxidation because of hypertrophy of the neonatal heart contributes to ischemic injury by decreasing ATP supply and activating cellular death signaling. In support of this, we show that stimulating fatty acid β-oxidation with chronic PPARα stimulation, marked benefits post–ischemic functional recovery of hypertrophied neonatal hearts. This is accompanied by a significant increase in overall energy production (Figure 2). In addition, glycolysis rates are decreased in the hypertrophied hearts treated with the PPARα agonist, resulting in a significant reduction in proton production from glycolysis uncoupled from glucose oxidation during reperfusion after ischemia (Figure 2). This, combined with the increase in overall ATP production, may be responsible for the beneficial effects of fatty acid β-oxidation stimulation in the hypertrophied neonatal hearts.

GW7647-treated hypertrophied hearts showed not only increased β-oxidation of exogenous fatty acids but also evidence of increased triacylglycerol turnover. Indeed, GW7647 treatment increases fatty acid incorporation into triacylglycerol, despite an overall decrease in triacylglycerol pool size (Figure 4). This combined with an increase in myocardial ATGL expression in hypertrophied hearts from GW7647-treated rabbits is consistent with an increase
in triacylglycerol synthesis and turnover. Recent studies have suggested that ATGL-mediated triacylglycerol lipolysis provides an important source of fatty acids as a ligand for activation of PPARs. As a result, the increase in triacylglycerol lipolysis may have also served as a stimulus to increase the rates of fatty acid β-oxidation in the hypertrophied neonatal hearts.

In addition to enhancing glycerolipid biosynthesis, it is also possible that GW7647 treatment in hypertrophied neonatal hearts was decreasing biosynthesis of ceramide, a cardiotoxic molecule. This notion is supported by our observations that hypertrophy-mediated increases in the protein expression of SPT1 and SPT2, the rate-limiting enzyme for ceramide synthesis, are attenuated on GW7647 treatment. The mechanisms underlying the regulation of SPT activity is unknown. Some studies suggest that SPT mRNA and activity levels increase in response to inflammation and stress stimuli. It is unknown to what extent an increase in ceramide biosynthesis contributes to cardiac dysfunction in hypertrophied hearts. A reduction of ceramide biosynthesis may play an indirect role in facilitating mitochondrial fatty acid uptake, as reflected by simultaneous increased ATGL expression on GW7647 treatment. This phenomenon indicates that GW7647 increases the mobilization of endogenous fatty acids from the triacylglycerol pool for subsequent mitochondrial uptake and oxidation.

As SPT is localized in the ER, we suggest that the upregulation of SPT expression could contribute to the increase in ER stress seen in the hypertrophied hearts. Although GRP78 and SPT are both upregulated in the hypertrophied hearts, this upregulation is prevented in GW7647-treated hypertrophied hearts. Similarly, an association between increased ER stress and ceramide accumulation has been demonstrated in the infarcted area of pig hearts and mouse hearts. Calcium homeostasis can be the nidus for ER stress and cellular death and injury. We found that enzymes involved myocardial calcium homeostasis (SERCA2 and p-PLN) are downregulated in hypertrophied neonatal hearts compared with sham-operated neonatal hearts (Figure 5). GW7647 treatment reverses the changes in expression of these calcium-handling enzymes in hypertrophied neonatal hearts and simultaneously decreases the magnitude of the ER stress and release of proapoptotic proteins.

Endothelial nitric oxide synthase (eNOS) is a molecule that signals several pathways including that which produce nitric oxide to regulate myocardial glucose uptake. Its phosphorylation protects cardiac muscles from post–ischemic contractile dysfunction, and eNOS also inhibits cytochrome-c oxidase activity, thereby blocking the NF-κB/hypoxia-inducible factor-1α pathway to reduce glycolysis. We propose that the mechanism underlying GW7647-mediated cardioprotection is associated with an increase in SERCA2, which may be a result of reduced glycolysis, thereby alleviating ER stress and inactivating SPT to reduce ceramide synthesis and reducing its cardiotoxic burden in the myocardium. In concert with the elevated GPD activity, GW7647-mediated activation of ATGL ensures the supply of mitochondrial uptake of fatty acids for β-oxidation. Overexpression of SERCA2 in heart failure attenuates BNIP3-mediated mitochondrial destruction by shuttling cytoplasmic Ca2+ into the ER to prevent calcium overload. In our study, where GW7647 reduces BNIP3 expression, citrate synthase activity may be restored. Combined with the accelerated ATP production, restored SERCA2 activity is able to sustain myocardial contractility during ischemia-reperfusion. This notion is further supported by the observation that preserving SERCA2 activity attenuates ER stress and prevents myocardial ischemic injury.

Activation of PPARα in the adult can decrease circulating free fatty acid levels in vivo by promoting fatty acid β-oxidation in peripheral tissues, thereby limiting the delivery of fatty acids to the myocardium. A similar effect on fatty acid supply in the hypertrophied neonatal heart may not be desirable because accelerating fatty acid β-oxidation in the neonatal hypertrophied heart is associated with reduced ischemic injury. As a result, we propose that the primary beneficial effect of PPARα agonist in our studies is via direct activation of fatty acid β-oxidation in the hypertrophied newborn heart.

Study Limitations
GW7647 probably reduced plasma triacylglycerol levels by increasing the rate of hepatic fatty acid use and reducing very low-density lipoprotein production. The levels of serum free fatty acid and lipoproteins, however, were not measured in this study. It is, therefore, unclear what contribution enhanced fatty acid β-oxidation in the heart alone versus that from peripheral tissues has on myocardial inflammation. In addition, the GW7647-mediated decrease in coronary flow alters the influx of Ca2+ during reperfusion in the hearts was not determined. Overexpression of very low-density lipoprotein receptor enhances ER stress and apoptosis in mouse heart. Thus studies about GW7647-mediated expression of very low-density lipoprotein receptor and myocardial uptake of cholesteryl esters are warranted.

Summary
The presence of hypertrophy markedly decreases the normal maturation of myocardial fatty acid β-oxidation that occurs in neonatal heart. This contributes to a decrease in energy supply, a decrease in contractile function, and an increase in sensitivity to ischemic injury. PPARα activation of fatty acid β-oxidation in hypertrophied neonatal hearts can increase myocardial energy production, improve heart function, and decrease ischemic injury, while decreasing ER stress and inflammation. This suggests that stimulation of fatty acid β-oxidation may be a novel cardioprotective intervention to limit post–ischemic contractile dysfunction in neonatal patients requiring the surgical correction of congenital heart defects.

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Disclosures
None.

References


What Is Known?

- Immediately after birth, the heart relies predominantly on glycolysis and lactate oxidation as sources of ATP. Shortly after birth, glycolysis decreases, whereas fatty acid β-oxidation meets the majority of myocardial ATP demand.
- The expression of peroxisome proliferator–activated receptor-α increases in the postnatal period, and promotes the expression of genes involved in fatty acid metabolism, including carnitine palmitoyltransferase I, and malonyl-CoA decarboxylase.
- Volume-overload cardiac hypertrophy prevents the maturational increase in fatty acid oxidation seen postbirth partly due to higher malonyl-CoA levels, a potent endogenous carnitine palmitoyltransferase I inhibitor. These alterations decrease the recovery of post–ischemic function.

What New Information Does This Article Contribute?

- Treatment of neonatal rabbits with a peroxisome proliferator–activated receptor-α agonist promotes the maturation of fatty acid β-oxidation in neonatal rabbit hearts subjected to volume overload-induced cardiac hypertrophy, and improves cardiac energy production.
- Promoting the maturation of fatty acid β-oxidation in hypertrophied neonatal rabbit hearts improves functional recovery after myocardial ischemia.
- Treatment of hypertrophied hearts with peroxisome proliferator–activated receptor-α decreases post–ischemic proton production and endoplasmic reticulum stress, and activates sarcoplasmic reticulum calcium ATPase isoform 2 and citrate synthase.

In this study, we determined whether stimulating fatty acid β-oxidation with GW7647, a peroxisome proliferator–activated receptor-α activator, would improve cardiac energy production and post–ischemic functional recovery in neonatal rabbit hearts subjected to volume overload-induced cardiac hypertrophy. Treatment of neonatal rabbits with GW7647 did not prevent the development of cardiac hypertrophy per se, but did increase overall energy production in the hypertrophied neonatal hearts. This resulted in a decrease in the hypertrophy-induced in vivo contractile dysfunction, and an improvement in reperfusion recovery of hearts subjected to ischemia. The cardioprotection was associated with increased fatty acid β-oxidation rates, resulting in a concurrent attenuation in the hypertrophy-induced increase in glycolysis. This reduced rates of proton production via preventing the uncoupling of myocardial glycolysis and glucose oxidation in hypertrophied hearts. In addition, reduced endoplasmic reticulum stress and inflammation by GW7647 treatment may also contribute to this cardioprotection. Our data suggest that stimulating mitochondrial energy production by increasing fatty acid β-oxidation has beneficial effects on the hypertrophied neonatal heart.

**Novelty and Significance**

- Treatment of neonatal rabbits with a peroxisome proliferator–activated receptor-α agonist promotes the maturation of fatty acid β-oxidation in neonatal rabbit hearts subjected to volume overload-induced cardiac hypertrophy, and improves cardiac energy production.
- Promoting the maturation of fatty acid β-oxidation in hypertrophied neonatal rabbit hearts improves functional recovery after myocardial ischemia.
- Treatment of hypertrophied hearts with peroxisome proliferator–activated receptor-α decreases post–ischemic proton production and endoplasmic reticulum stress, and activates sarcoplasmic reticulum calcium ATPase isoform 2 and citrate synthase.
Activating PPARα Prevents Post–Ischemic Contractile Dysfunction in Hypertrophied Neonatal Hearts

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Activating PPARα Prevents Post-Ischemic Contractile Dysfunction in Hypertrophied Neonatal Hearts

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Detailed Methods

An expanded Methods section is available in the online Data Supplement at http://circres.ahajournals.org.

Assessment of myocardial function in neonatal rabbit hypertrophied heart induced by volume-overload: Newborn New Zealand White rabbits of either sex (7-days old, 90-200 g) were anesthetised with inhaled isofluorane (2%), and were subjected to an aorto-caval shunt to induce volume-overloaded cardiac hypertrophy as described previously. The presence of a successful fistula was verified at post-surgical day 7 and 13 by color flow doppler that visualizes a physical shunt between the abdominal aorta and the IVC in both an axial and transverse plane. This is further validated by an enlarged IVC. The size of the IVC enlargement is compared to control animal IVC size attained during our model development stage. Post-operative day 13 IVCs sizes are reported in table 1. We therefore further validate the effects of the shunt using echo to assess for ventricular dilation (LVIDD and RVIDD). None of the animals deemed to have a shunt present on day 7th, by the criteria above, had conflicting ultrasonographic data on post-op day 13th. Thus, the animals in shunt group were randomly assigned to receive an intraperitoneal injection of vehicle (DMSO, the solvent of GW7647) or GW7647 (3 mg/kg/day; EC₅₀ = 6 nmol/L for PPARα, Cayman Chemical) twice a day for 14-days. Animals which were performed surgery to create shunt, but consequently the shunt either did not exhibit or closed, were excluded from the study. Left ventricular ejection fraction (LVEF, %) and other cardiac parameters were assessed by transthoracic echocardiography at post-surgical days 7 and 13 as described previously. At 21-days of age (14-days post-surgery) all animals (n=29) were euthanized with NaCl pentobarbital, and hearts were removed for isolated bi-ventricular working heart perfusions.

Isolated bi-ventricular heart perfusions and ischemia-reperfusion protocol: Bi-ventricular working heart perfusions used a modified Krebs-Henseleit solution containing...
2.5 mmol/L Ca\textsuperscript{2+}, 5.5 mmol/L glucose, 1.2 mmol/L palmitate prebound to 3% bovine serum albumin, 0.5 mmol/L lactate, and 100 µU/mL insulin as described previously.\textsuperscript{1} The hearts were subjected to a 35 min-aerobic perfusion, followed by a 25 min period of normothermic global (no-flow) ischemia, and a 30 min period of aerobic reperfusion. Cardiac function was assessed in the bi-ventricular working hearts as described previously.\textsuperscript{1} Glycolysis, glucose oxidation, and palmitate oxidation rates were measured in the aerobic and reperfusion periods using radiolabelled [5-\textsuperscript{3}H]glucose, [U-\textsuperscript{14}C]glucose, or [9,10-\textsuperscript{3}H]palmitate, respectively, in the perfusate as described previously.\textsuperscript{1} The ATP and acetyl CoA production rates from glycolysis, glucose oxidation and fatty acid oxidation were calculated as previously described.\textsuperscript{1} Proton production from glucose metabolism is derived from hydrolysis of glycolysis-derived ATP (2 ATP per 1 glucose molecule produce 2 protons) while TCA cycle utilizes 1 proton for each pyruvate molecule (2 protons for each glucose molecule oxidized) at the pyruvate carboxylation step. Thus, if the rate of glycolysis and glucose oxidation are mismatched, there is a net proton production, the rate of which is 2 X (glycolysis rate – glucose oxidation rate).

**Malonyl CoA, and triacylglycerol measurements:** Measurements of malonyl CoA levels in ventricular tissue were performed using a modified high pressure liquid chromatography procedure as described previously.\textsuperscript{4} Approximately 10 mg heart tissue was homogenated in a solution containing (volume 2:1) chloroform: methanol mixture. The extracted lipids were dissolved in 3:2 tert-butyl alcohol : triton X-100/methyl alcohol (1:1) mixture. Total triacylglycerol (TG) content was determined using an enzymatic assay kit (Wako Pure Chemical Industries), while the incorporation of palmitate-CoA into TG was counted with scintillation fluid and calculated based on the specific activity of radiolabelled palmitate in the triacylglycerol pool as previously described.\textsuperscript{4}

**Immunoblot analysis:** Heart tissue homogenates were subjected to SDS-PAGE and transferred to nitrocellulose membranes as previously described.\textsuperscript{21} Membranes were blocked in 10% fat-free milk for 1 hr and probed with the following antibodies: MCD (University of Alberta); sarcoplasmic reticulum calcium ATPase isoform 2 (SERCA2), phospho-phospholamban (p-PLB-serine-16), I kappa B protein-α (IkBα), BNIP3, tubulin, and Lamin A (Cell Signaling Technologies); adipocyte TG lipase (ATGL) and phosphorylated acetyl-CoA carboxylase (P-ACC) (Millipore); serine-palmitoyltransferase (SPT1/2), and glucose transporter 4 (GLUT4) (Santa Cruz); glucose-regulated protein 78 (GRP78) (Abcam). To isolate the nuclear fraction, heart tissue homogenates were centrifuged at 1000 g for 30 minutes at 4 °C. The resulting pellets were suspended in a cold buffer containing 10 mmol/L Hepes (pH 7.8), 25% glycerol, 1.5 mmol/L MgCl\textsubscript{2}, 0.42 mol/L NaCl and 0.2 mmol/L EDTA. The suspension was shaken for 0.5 hr at 4 °C, then followed by centrifugation at 12,000 g for 15 minutes at 4 °C. The resulting supernatants were applied for western blots to assess the
content of nuclear peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), and NF-κB subunit p65 (p65 NFκB) relative to Lamin A as described previously. Membranes were quantified using Image J or Quantity One (4.4.0) software (Bio-Rad Laboratories).

**Quantitative reverse transcriptase PCR:** Total RNA was extracted from flash-frozen hearts using TRIzol extraction protocol according to the manufacturer's instructions. TaqMan quantitative PCR was performed with the 7700 Sequence Detection System. All samples were run in triplicates in 384 well plates. 18S rRNA was used as an endogenous control.

**Glycerol-3-phosphate dehydrogenase (GPD), citrate synthase, CPT1, and complex I/II activities measurements:** GPD was measured in ventricular lysate, involving a reaction done at 30°C in a buffer containing 20 mmol/L Tris (pH 8.5), 100 mmol/L glycine, 1 mmol/L DTT, 0.5 mmol/L EDTA, 1 mmol/L NADH, and 1 mmol/L dihydroxyacetone-phosphate. The reaction was monitored at 340 nm for 3-5 min for the formation of NAD⁺. Citrate synthase activity was measured using tissue homogenates. The reactions were done in the assay buffer containing 100 mmol/L Tris, 1 mmol/L EDTA, 1 mmol/L MgCl₂, 0.3 mmol/L acetyl CoA, 0.1 mmol/L 5-5′-dithio-bis-2-nitrobenzoic acid and 0.5 mmol/L oxaloacetic acid. The reaction was recorded at 30 °C for 3 minutes at 412 nm as described previously. To isolate mitochondrial fraction, the supernatants from tissue homogenates after initial centrifugation at 1000 g for 30 minutes at 4 °C were subjected to another centrifugation at 8000 g for 30 minutes at 4 °C. The resulting pellets were suspended in a buffer containing 70 mmol/L sucrose, 1 mmol/L EDTA, 25 mmol/L K2HPO4 (pH 7.8) with protease inhibitor. CPT-I activity was assessed on the mitochondrial fraction based on formation of radiolabeled palmitoyl-carnitine as described previously. Activities of complex I/II were spectrophotometrically quantified using mitochondrial fractions in a buffer containing 25 mmol/L K2HPO4 (pH 7.8), 5 mmol/L MgCl₂, 3.5 g/L BSA, 60 μmol/L 2,6-dichlorophenolinodophenol, 70 μmol/L decylibiquinone and 3.6 μmol/L antimycine A with additional 0.2 mmol/L NADH for complex I or 0.2 mmol/L ATP, 5 μmol/L rotenone and 10 mmol/L succinate for complex II. The activity was recorded as the time-dependent changes of absorptions at 600 nm. The activity was expressed as μmol/mg mitochondrial protein/min.
Online Supplemental Figures and Figure Legends

Online Figure I. Effect of GW7647 on the expression of the key enzymes for fatty acid oxidation.

A) Expression of MCD. B) Expression of total ACC and phosphorylation of ACC. C) Mitochondrial complex I/II activity. D) Expression of GLUT4. Values represent means ± SEM. (n=4-6) *p < 0.05, significant difference.
Supplemental References


