Peroxisome Proliferator Activated Receptor-α Association With Silent Information Regulator 1 Suppresses Cardiac Fatty Acid Metabolism in the Failing Heart

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Background—Heart failure is accompanied by changes in cardiac metabolism characterized by reduced fatty acid (FA) utilization. However, the underlying mechanism and its causative involvement in the progression of heart failure are poorly understood. The peroxisome proliferator activated receptor-α (PPARα)/retinoid X receptor (RXR) heterodimer promotes transcription of genes involved in FA metabolism through binding to the PPAR response element, called direct repeat 1 (DR1). Silent information regulator 1 (Sir1) is a histone deacetylase, which interacts with PPARα.

Methods and Results—To investigate the role of PPARα in the impaired FA utilization observed during heart failure, genetically altered mice were subjected to pressure overload. The DNA binding of PPARα, RXRα, and Sir1 to DR1 was evaluated with oligonucleotide pull-down and chromatin immunoprecipitation assays. Although the binding of PPARα to DR1 was enhanced in response to pressure overload, that of RXRα was attenuated. Sir1 competes with RXRα to dimerize with PPARα, thereby suppressing FA utilization in the failing heart. DR1 sequence analysis indicated that the typical DR1 sequence favors PPARα/RXRα heterodimerization, whereas the switch from RXRα to Sir1 takes place on degenerate DR1s. Sir1 bound to PPARα through a region homologous to the PPARα binding domain in RXRα. A short peptide corresponding to the RXRα domain not only inhibited the interaction between PPARα and Sir1 but also improved FA metabolism and ameliorated cardiac dysfunction.

Conclusions—A change in the heterodimeric partner of PPARα from RXRα to Sir1 is responsible for the impaired FA metabolism and cardiac dysfunction in the failing heart. (Circ Heart Fail. 2015;8:1123-1132. DOI: 10.1161/CIRCHEARTFAILURE.115.002216.)

Key Words: DNA ■ fatty acids ■ heart failure ■ metabolism ■ peroxisome proliferator activated receptor—α

The healthy heart produces and consumes a large amount of energy to maintain cardiac output. To satisfy the high-energy demand, cardiomyocytes harbor a large number of mitochondria and use fatty acids (FAs) as their primary substrate for energy production. FAs are catabolized to acetyl-coenzyme A through β-oxidation in mitochondria and peroxisomes. The resultant acetyl-coenzyme A is then used for ATP production through oxidative phosphorylation in the tricarboxylic acid cycle and the electron transport chain in mitochondria. However, the FA utilization capability declines in the failing heart.1 Associated with this decline is a broad downregulation of metabolic genes throughout the FA consumption pathway, from FA uptake to the electron transport chain.2 Nevertheless, neither the mechanism responsible for this downregulation nor its functional significance is well understood.

Clinical Perspective on p 1132

Peroxisome proliferator activated receptor-α (PPARα), a member of the PPAR nuclear receptor subfamily, plays an important role in cardiac FA utilization.3 PPARα heterodimerizes with retinoid X receptor (RXR) to activate transcription through binding to the PPAR response element (PPRE), also called direct repeat 1 (DR1). The consensus sequence of the PPRE/DR1 comprises 2 AGGTCA sequences separated by 1 nucleotide, AGGTCAAGGTCA, with PPARα and RXR binding to the 5′ and 3′ AGGTCA sequences, respectively.4 PPARα/RXR target genes harboring a functional PPRE/DR1 are enriched in the upstream steps of the FA metabolic pathway, including FA uptake, transport, and β-oxidation.5 Loss of PPARα in mice results in impaired PPARα target gene expression, FA utilization, and contractile function, indicating that PPARα is essential for cardiac function and FA utilization in the healthy heart.6 However, overexpression of PPARα in the heart induces a mild failing heart phenotype.7 Thus, PPARα may also play a role in the pathological development of heart failure (HF).
Silent information regulator 1 (Sirt1) is a nicotinamide adenine dinucleotide–dependent protein deacetylase that plays an important role in metabolic adaptation to starvation conditions. Sirt1 is a functional binding partner of both PPARα and PPARγ. However, modulation of PPAR function by Sirt1 is complex. PPARα target gene expression and FA utilization are enhanced by Sirt1 in the liver. Sirt1 also protects against hypertrophy and metabolic dysregulation through PPARα activation in cultured cardiomyocytes. However, Sirt1 promotes PPARα-induced failing heart phenotypes in vivo. Likewise, Sirt1 inhibits PPARγ in white adipocytes, leading to release of FAs during starvation, but enhances its function in brown adipocytes.

Sirt1 is upregulated during HF induced by pressure overload (PO) and exacerbates the failing heart phenotypes. Furthermore, cardiac-specific overexpression of Sirt1 exacerbates cardiac dysfunction under PO conditions. We showed previously that PPARα recruits Sirt1 to the estrogen-related receptor (ERR) response element (ERRE) and suppresses ERR target gene expression in the failing heart. PPARα can bind to the ERRE because its consensus sequence, TNAAGGTC, contains a core hexad binding sequence for PPARα as a monomeric unit. ERR targets are enriched in the mitochondrial tricarboxylic acid cycle and electron transport chain, and PPARα thus suppresses genes mediating the downstream metabolic pathway of FA consumption. This raises the question of whether PPARα is also responsible for downregulation of canonical PPARα target genes harboring a PPRE/DR1, namely, those mediating the upstream FA metabolism, in the failing heart. We here show that PPARα is required not only for PPAR target gene expression in the healthy heart but also for gene suppression in the failing heart. We propose that inhibition of PPARα/Sirt1 heterodimerization has therapeutic potential for HF treatment.

Methods

Animal Experiments
Cardiac-specific PPARα transgenic, PPARα knockout, and cardiac-specific Sirt1 knockout mice have been described previously. All procedures involving animals were performed in accordance with protocols approved by Rutgers Biomedical and Health Sciences.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction
Total RNA was prepared from left ventricles using the RNeasy Fibrous Tissue Mini Kit (Qiagen), and cDNA was then generated using M-MLV Reverse transcriptase (Promega). Real-time reverse transcriptase-polymerase chain reaction was performed using Maxima SYBR Green quantitative polymerase chain reaction master mix (Fermentas).

In Vitro Binding Assays
Recombinant glutathione S-transferase–fused proteins and double-stranded DNA were incubated at 4°C for 2 hours. Precipitation was performed with streptavidin beads or glutathione beads.

Human Heart Samples
Heart lysates from explanted hearts were obtained from patients who received heart transplants and from age-matched donors. All patients or their families expressed their willingness to participate through an informed consent form. Detailed information on the human samples was provided previously.

Statistical Analysis
Detailed statistical methods are described in the Data Supplement. P<0.05 was defined as statistically significant and indicated by a filled asterisk. NS represents not significant. All error bars represent SEM. The Biostatistics Laboratory of Rutgers New Jersey Medical School independently reviewed all data sets and applied the appropriate statistical methods.

Results

PPRE/DR1 Sequence Affects PPARα/RXR Heterodimerization and Target Gene Expression
To examine the role of PPARα in PPARα target gene expression in the failing heart, we used a murine model of HF in which surgical constriction of the aorta (transverse aortic constriction [TAC]) induces PO, leading to cardiac dysfunction and metabolic remodeling. PPARα heterozygous and homozygous knockout (PPARα−/− and PPARα+−) mice were subjected to 4 weeks of TAC, whereas PPARα-overexpressing (Tg-PPARα) mice were subjected to 2 weeks of TAC because of their high mortality rate (Figure I in the Data Supplement). As expected, many PPAR target genes were downregulated in PPARα−/− mice even at baseline, confirming that PPARα is essential for their basal gene expression (Figure 1A; Figure IIA in the Data Supplement). Conversely, several PPAR targets, such as Acx1, Cd36, Cpt1b, Acsl3, Cpt1a, Hmgcs2, Pcx, and Plin2, were upregulated in Tg-PPARα mice and were not significantly suppressed below the wild-type (WT) baseline even in the presence of PO. Interestingly, however, some PPAR targets were not upregulated in Tg-PPARα at baseline and were downregulated by TAC in WT mice, including Cpt2, Ech1, Fabp3, Mcad, Vldlr, Abcd2, Acaa1b, Fatp1, Mlycd, Slc22a5, Txnip and Lpl. This TAC-induced downregulation was partially normalized in PPARα+/−, whereas it was exacerbated in Tg-PPARα mice (Figure 1A and 1B; Figure IIA and IIB in the Data Supplement). Thus, PPARα negatively regulates a subset of PPAR target genes under PO.

PPARα regulates expression of its targets primarily through PPRE/DR1. To understand the mechanism by which PPARα differentially regulates its downstream targets, we examined the sequences of known PPREs/DR1s. Although the consensus sequence of the PPRE/DR1 has been reported as RGSWVANAGGTCA17 (R=A or G, S=G or C, W=A or T, V=C, G, or A), the actual nucleotide sequence exhibits considerable variation from gene to gene. We hypothesized that the diversity in the PPRE/DR1 sequences is a critical factor in the differential responses of PPARα targets. Of the 9 PPAR target genes, we examined that were upregulated under basal conditions in Tg-PPARα, all 9 harbored well-conserved typical PPRE/DR1 sequences with ≤1 nucleotide mismatch from the reported consensus in each 5′ and 3′ hexad sequence (Figure 1C; Figure IIB and IIC in the Data Supplement), which we designated as perfect DR1s. On the contrary, 11 of 14 PPAR target genes that were not significantly upregulated in Tg-PPARα harbored unique PPRE/DR1 sequences comprising 1 well-conserved AGGTC sequence with ≤1 nucleotide mismatch from the consensus and 1 relatively degenerate...
hexad sequence with ≥2 nucleotide mismatch, which we designated as imperfect DR1s. Fisher exact test indicates that the perfect DR1 correlates with upregulation, whereas the imperfect DR1 correlates with either no change or downregulation of PPARα targets in Tg-PPARα mice under basal conditions (P=0.014; Figure IID in the Data Supplement). Several PPAR target genes, including Acaa1b, Ehhadh, Fatp1, and Slc22a5, harbor both perfect and imperfect DR1s (Figure IIC in the Data Supplement) and were therefore included in both the perfect DR1 and the imperfect DR1 groups. The number of genes harboring both perfect and imperfect DR1s is indicated in parentheses (Figure IID and IIE in the Data Supplement).

We next investigated whether PO differentially regulates PPARα targets with perfect and imperfect DR1s. Of the 13 PPAR targets suppressed under PO in WT mice, 11 harbored an imperfect DR1. In contrast, all 10 of the PPAR target genes not significantly suppressed under PO harbored perfect DR1s. The perfect DR1 correlates with resistance against PO-induced downregulation, whereas the imperfect DR1 correlates with PO-induced downregulation (P=0.0047; Figure IIE in the Data Supplement). Because the downregulation of the imperfect DR1 group of genes was partly normalized in PPARα+/−, these results suggest that PPARα negatively regulates target genes harboring an imperfect DR1, whereas genes with a perfect DR1 are resistant to PPARα-mediated downregulation during PO.

RXR is an essential heterodimerization partner for PPARα-mediated transcription and directly binds to the 3′ hexad sequence of the PPRE/DR1. We hypothesized that the extent of similarity of an individual PPRE/DR1 to the perfect DR1 affects the level of PPARα/RXR heterodimerization, which, in turn, may determine the response of each PPARα.
target. To test this hypothesis, recombinant PPARα and RXRα were incubated with double-stranded DNA comprising intrinsic DR1s. PPARα bound to all DR1s we tested (Figure 1D). Recruitment of RXRα to PPARα was readily observed in the presence of either the DR1 consensus sequence (DR1) or perfect DR1s derived from genes upregulated in Tg-PPARα, including Acox1, Cd36, and Cpt1b. However, it was much weaker in the presence of imperfect DR1s derived from genes downregulated during PO, including Cpt2, Mcad, and Vldlr (Figure 1E). Thus, intrinsic DR1s differ in their abilities to promote PPARα/RXR heterodimerization. Specifically, perfect DR1s with 2 typical AGGTCA-like sequences strongly recruit both PPARα and RXR, whereas imperfect DR1s strongly recruit PPARα but only weakly recruit RXR. These results suggest that the extent of nucleotide mismatch from the consensus in the DR1 affects the level of PPARα/RXR heterodimerization, which, in turn, may affect the pattern of gene expression in response to PO or in Tg-PPARα.

Functional Significance of Imperfect DR1 Group of Genes in FA Utilization and Cardiac Function in the Failing Heart

To investigate the functional significance of the imperfect DR1 group of genes, FA utilization and cardiac function were examined in PPARα gene-manipulated mice. As expected, PPARα−/− mice showed impaired FA utilization both at baseline and under PO. However, PO-induced impairment of FA utilization was attenuated in PPARα+/− mice (Figure 2A), in which PO-induced downregulation of the imperfect DR1 group of genes was normalized (Figure 1A; Figure IIA in the Data Supplement). PO induced cardiac systolic dysfunction, characterized by a decrease in the LV ejection fraction, in WT mice. However, the ejection fraction under PO was normalized in PPARα−/− mice, whereas PPARα+/− mice exhibited cardiac dysfunction even at baseline (Figure 2B). PO-induced lung congestion and LV hypertrophy were normalized in PPARα−/−, but not in PPARα+/− mice (Figure 2C and 2D). Both the impairment of FA utilization (Figure 2E) and the cardiac dysfunction (Figure 2F–2H) induced by PO were exacerbated in Tg-PPARα mice, in which the imperfect DR1, but not the perfect DR1, group of genes was further suppressed (Figure 1B; Figure IIB in the Data Supplement). Even without PO, there was a trend toward reduced FA utilization in Tg-PPARα mice. Indeed, many genes belonging to the imperfect DR1 group showed a trend of downregulation. These results suggest that suppression of the imperfect DR1 group of genes results in a decline in FA utilization and cardiac function.

An Essential Role of Sirt1 in PPARα-Mediated Gene Suppression

As in PPARα-mediated ERR target gene suppression, Sirt1 may have a role in PPARα-mediated suppression of PPARα target genes harboring an imperfect DR1. Sirt1 was increased, whereas RXR was decreased, in response to PO in the mouse heart (Figure 3A). Similarly, there was a trend toward upregulation of Sirt1 and downregulation of RXR in failing human hearts (Figure 3B; Figure III in the Data Supplement). Furthermore, Sirt1 binding to PPARα increased, whereas RXR binding to PPARα decreased, during PO in the mouse heart (Figure 3C; Figure IV in the Data Supplement). In vitro binding assays demonstrated that Sirt1 binds to PPARα more strongly than to RXRα, suggesting that Sirt1 interacts with PPARα directly rather than through RXR (Figure 3D). Sirt1 does not have a typical DNA-binding domain. To investigate whether PPARα recruits Sirt1 to DNA, recombinant PPARα and Sirt1 were incubated with biotin-labeled double-stranded DNA containing 3 repeats of the PPAR-binding sequence (AGGTCA) separated by 7 bp spacers. Sirt1 was pulled down with the AGGTCA sequences in a PPARα-dependent manner, suggesting that Sirt1 interacts with DNA through PPARα (Figure 3E) and that neither RXR nor the perfect DR1 element is necessary for PPARα DNA binding and Sirt1 recruitment. Chromatin immunoprecipitation assays showed that, during PO, Sirt1 was recruited to imperfect DR1s, such as in the Mcad and Vldlr promoters, in WT but not in PPARα−/− mice, suggesting that PPARα recruits Sirt1 to imperfect DR1s during...
PO in vivo (Figure 3F). Furthermore, PO-induced downregulation of the PPAR target genes harboring imperfect DR1s was partly normalized in Sirt1−/− mice (Figure 3G). Reporter gene assays showed that co-overexpression of PPARα and Sirt1 significantly suppressed reporter activity driven by an imperfect DR1, such as those in the Mcad and Vldlr promoters, whereas PPARα-induced suppression was normalized by knockdown of Sirt1 (Figure 3I). Thus, Sirt1 recruited to imperfect DR1s by PPARα seems to be crucial for PPARα-mediated transcriptional suppression. To investigate whether the imperfect DR1 is important for suppression by PPARα/Sirt1, the degenerate hexad sequences of intrinsic promoters were mutated to typical AGGTCA sequences to form perfect DR1s (Mcad(DR1) and Vldlr(DR1); Figure 3H). PPARα/Sirt1 failed to suppress reporter activity driven by these sequences (Figure 3J). These results suggest that PPARα/Sirt1 suppresses transcription through direct interaction with an imperfect DR1, but that the perfect DR1 resists PPARα/Sirt1-induced suppression.

To further evaluate whether PPARα and Sirt1 suppress a subset of the known PPARα targets, and, if so, whether the sequence of the PPRE/DR1 contributes to this mechanism, we conducted a microarray analysis using transgenic mice with combined cardiac-specific overexpression of both PPARα and Sirt1 (Tg-PPARα/Sirt1).12 We examined 33 PPARα target genes that were upregulated or downregulated >1.2-fold in Tg-PPARα/Sirt1 mice and have been reported or suggested to contain a PPRE. Although many known PPARα target genes were upregulated, a subset of the known targets of PPARα was downregulated in Tg-PPARα/Sirt1 (Figure 4A). Among the 33 PPARα target genes that were upregulated or downregulated >1.2-fold in Tg-PPARα/Sirt1 mice, 13 of the 15 genes that were upregulated in Tg-PPARα/Sirt1 mice harbored perfect DR1s (Figure 4B and 4C), whereas 16 of the 18 genes that were downregulated in Tg-PPARα/Sirt1 mice harbored imperfect DR1s (Figure 4B and 4D). Thus, the perfect DR1 correlates with upregulation, whereas the imperfect DR1 correlates with downregulation, of PPARα targets (P=0.0028; Figure 4E). These results suggest that crosregulation of PPARα and Sirt1 suppresses PPARα target genes harboring imperfect DR1s but not those with perfect DR1s.

Figure 3. Silent information regulator 1 (Sirt1) plays an important role in peroxisome proliferator activated receptor (PPAR)-α-mediated transcriptional suppression. A, Expression levels of Sirt1, retinoid X receptor (RXR), and PPARα in mouse hearts. B, Expression levels of Sirt1, RXR, and PPARα in healthy (donor) and failing (recipient) human hearts. C, Reciprocal binding of RXR and Sirt1 to PPARα in the heart. Transgenic (Tg)-Flag-PPARα mice were subjected to transverse aortic constriction (TAC). The Flag-PPARα was immunoprecipitated with anti-Flag antibody. D, Direct interaction between PPARα and Sirt1. Recombinant Sirt1 was incubated with glutathione S-transferase (GST)-fused PPARα and RXRα. E, PPARα recruits Sirt1 to the DNA. Recombinant PPARα and Sirt1 were incubated with biotin-labeled DNA containing 3 repeats of the PPARα binding sequence (AGGTCA) or a mutant sequence (GAATCA). F and G, Sirt1 recruited by PPARα suppresses gene expression. F, PPARα is required for recruitment of Sirt1 to imperfect DR1s in the heart in vivo. Chromatin immunoprecipitation assays were performed with wild-type and PPARα−/− hearts subjected to TAC (n=6–10). G, Sirt1 suppresses PPAR target genes harboring imperfect DR1s. Quantitative polymerase chain reaction was performed using cDNA prepared from Sirt1−/− mice (n=5–8). H, A schematic representation of the PPAR response element constructs. I and J, Luciferase assays were performed (n=6–12). I, Reporter gene activity driven by imperfect direct repeat 1 (DR1) was suppressed by PPARα/Sirt1. J, PPARα failed to suppress perfect DR1 reporter activity.
Switching PPARα Heterodimerization Partner
From RXR to Sirt1 Suppresses FA Utilization and Cardiac Function

Given that the perfect DR1 more effectively recruits RXR to PPARα (Figure 1E), RXR may prevent transcriptional suppression by PPARα/Sirt1. Forced expression of RXRα normalized the reporter gene activity suppressed by PPARα/Sirt1 (Figure 5A), suggesting that decreased heterodimerization of RXR with PPARα promotes the transcriptional suppression in the failing heart. In vitro binding assays showed that RXRα inhibited the interaction between Sirt1 and PPARα in a dose-dependent manner (Figure 5B), whereas Sirt1 did not significantly inhibit the interaction between RXRα and PPARα (Figure V in the Data Supplement). Thus, Sirt1 and RXRα seem to compete for heterodimerization with PPARα, with RXRα having a higher affinity for PPARα than for Sirt1. Incubation with a perfect DR1, but not with an imperfect DR1 from the Mcad promoter (DR1(Mcad)) or a mutated DR1 (DR1m5/3’), enhanced both PPARα/RXRα heterodimerization and dissociation of Sirt1 from PPARα (Figure 5C). These results suggest that RXR competitively inhibits the interaction between Sirt1 and
PPARα, and that Sirt1 recruitment is inhibited on a perfect DR1 because of enhancement of PPARα/RXR heterodimerization. As shown in Figure 5D, co-occupancy of PPARα/Sirt1 on imperfect DR1s, such as those in Mcad and Vldlr, was increased under PO conditions, whereas that of PPARα/RXR was decreased. Furthermore, deacetylation of histone H3 at lysine 9 by Sirt1 generally leads to suppression of transcriptional activity, and, under PO, acetylation of histone H3 at lysine 9 was decreased in the imperfect DR1 promoter regions. These results suggest that, during PO, PPARα/RXR
is replaced by PPARα/Sirt1, which, in turn, suppresses transcription in correlation with histone deacetylation.

The competitive binding of RXR and Sirt1 to PPARα could be because of structural similarity between the proteins. A comparison of the protein sequences of Sirt1 and RXR showed that Sirt1 contains regions homologous to the DNA-binding domain of RXRα (RXRα (DBD)) that binds to PPAR (Figure 5E). PPARα was effectively pulled down with Sirt1(184–409), which includes the regions homologous to RXR, but not with Sirt1(199–379), which lacks the RXR-homologous regions (Figure 5F). Conversely, RXRα (DBD) inhibited the interaction of PPARα with Sirt1 (Figure 5F and 5G). Thus, the RXR-homologous regions in Sirt1 are binding sites for PPARα. RXRα (DBD) also attenuated PPARα/Sirt1-induced transcriptional suppression (Figure 5H).

Furthermore, PO-induced impairment of FA utilization and cardiac dysfunction was partially rescued by RXRα (DBD) expressed via adenovirus injection into the heart (Figure 5I; Figure VI in the Data Supplement ). Thus, disruption of the PPARα/Sirt1 dimer ameliorates impairment of FA utilization and cardiac dysfunction.

**Discussion**

We here show a transcriptional regulatory mechanism leading to downregulation of PPARα target gene expression and impairment of FA utilization during HF. PPARα typically heterodimerizes with RXRα, thereby promoting transcription of PPARα targets. However, PPARα can also heterodimerize with Sirt1, which suppresses transcription of PPARα targets. Using Sirt1 heterozygous knockout mice and a minigene approach that interferes with the specific interaction between the RXR-homologous region of Sirt1 and PPARα, we show that there is a switch from PPARα/RXR to PPARα/Sirt1 during PO, leading to downregulation of genes controlled by imperfect DR1s and inhibition of FA utilization.

Downregulation of genes involved in FA utilization is commonly observed during HF, and the substrate switch from FAs to glucose has a great impact on cardiac metabolism, including ATP production, oxidative stress, and mitochondrial function. Previous works have suggested that reduced levels of PPARα primarily explain the downregulation of genes involved in FA metabolism. However, neither decreased nuclear PPARα expression nor downregulation of certain well-established targets of PPARα such as Cpt1b is uniformly observed in the failing heart. The PPARα level may vary depending on the severity and the timing of PO. Thus, alternative mechanisms may mediate the downregulation of genes involved in FA metabolism, in a context-dependent manner. Our results provide an alternative mechanism through which a subset of genes controlled by PPARα and involved in FA utilization is downregulated without obvious downregulation of PPARα, but instead through a mechanism dependent on Sirt1, which is upregulated in failing hearts. We have previously reported that acetylation of PPARα is not detectable even in Sirt1+/− mice. We speculate that the level of PPARα acetylation may be below the detection limit or that Sirt1 may not be a major deacetylase of PPARα. However, whether Sirt1 deacetylates PPARα, and if so, whether deacetylation of PPARα rather than histone deacetylation is involved in the suppression of PPARα targets remain to be elucidated.

Our previous work showed that the PPARα/Sirt1 complex suppresses ERR target genes involved in the mitochondrial electron transport chain and the tricarboxylic acid cycle through direct interaction with the ERR target during fasting and PO. The consensus sequence of the ERR is TNAAGGTCA, providing a binding sequence for monomeric PPARα, which recruits Sirt1, but lacking an additional AGGTCA-like sequence for RXR binding. We proposed that this mechanism may allow the heart to reduce nutrient consumption in the presence of energy stress. Here, we extended this observation from the ERR to the PPRE/DR1 and showed that PPARα/Sirt1 binding to a subset of DR1s plays an important role in mediating metabolic remodeling during HF. Importantly, this study demonstrates for the first time that (1) the dimerization partner of PPARα on a given DR1 can switch from RXR to Sirt1 during HF, depending on the DNA sequence of the DR1, (2) the switch causes a change from upregulation to downregulation of the genes involved in FA utilization, the metabolic hallmark of HF, and (3) PPARα/Sirt1-mediated transcriptional suppression promotes both impaired FA utilization and cardiac dysfunction in response to PO.

Although Sirt1 and RXRα are competitive heterodimerization partners of PPARα, RXRα has a stronger affinity for PPARα than Sirt1. This suggests that PPARα preferentially heterodimerizes with RXR rather than Sirt1 and that Sirt1 can bind to PPARα only when PPARα exists in excess, making available PPARα not dimerized with RXR. This may explain why PO-induced decreases in FA oxidation are normalized by heterozygous downregulation of PPARα (Figure 2B). Partial knockdown of PPARα in PPARα+/− mice preferentially reduces PPARα/Sirt1 heterodimerization rather than PPARα/RXR, which prevents transcriptional suppression of genes involved in FA utilization.

Although never explicitly stated, close inspection of previously published reports led us to speculate that the function of PPARs is partially controlled by the sequence of their DNA-binding sites. Chromatin immunoprecipitation–on-Chip and Chromatin immunoprecipitation–sequencing analyses showed that, in fact, single isolated AGGTCA and imperfect DR1
sequences comprise the majority of DNA-binding sites for all PPARs, suggesting that the perfect DR1 type of sequence is not necessarily the predominant binding site for PPARs in vivo and that the diversity of DNA-binding sites may explain the functional diversity of PPARs. For example, suppression of the phospholipid transfer protein gene by PPARα is mediated through isolated AGGTCA sequences in the promoter.26 We here show the critical involvement of Sirt1 in mediating the molecular mechanism by which PPARα downregulates its targets through DR1. Importantly, Sirt1 and RXR both bind to PPARα through homologous amino acid stretches. This property allows Sirt1 to displace RXR as the binding partner of PPARα, especially when RXR is downregulated and where RXR has a low affinity for binding to the DR1, as in the case of imperfect DR1s.

Our data suggest that the PPARα/Sirt1 heterodimer primarily forms on imperfect DR1s because of the weak binding of RXR to the imperfect DR1s. In contrast because RXR has a low affinity for binding to the DR1, as in the case of imperfect DR1s that have not been reported. Further investigation is needed to clarify this issue.

Although the decline in FA metabolism is a hallmark of the metabolic change that takes place during HF, whether the decline in FA utilization is adaptive or maladaptive in the metabolic change that takes place during HF, whether the decrease in FA utilization and cardiac dysfunction during PO is adaptive or maladaptive in mediating cardiac dysfunction in response to PO but also suggest that downregulation of FA utilization through this Sirt1-dependent mechanism plays a causative role in mediating PO-induced HF.

In conclusion, PPARα targets regulated by imperfect DR1s are flexibly regulated by dimer transition between PPARα/RXR and PPARα/Sirt1, leading to metabolic remodeling in the heart in response to PO. Preventing PPARα/Sirt1 or restoring PPARα/RXR heterodimerization may have therapeutic potential in combating HF.

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Disclosures
None.


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**CLINICAL PERSPECTIVE**

The downregulation of genes involved in fatty acid metabolism is a hallmark of heart failure. This work investigated underlying molecular mechanisms by which downregulation of fatty acid oxidation occurs and also addressed its functional significance. We here show, for the first time, that the function of PPARα changes from a positive to negative regulator of genes involved in fatty acid metabolism and that the switch is directed by the balance between RXR and Sirt1, the nuclear binding partners of PPARα. Although whether metabolic remodeling observed in patients with heart failure is adaptive or maladaptive has been a long-standing issue of debate and can be context dependent, we show here that preventing downregulation of fatty acid oxidation by inhibiting heterodimerization of PPARα with Sirt1 ameliorates cardiac dysfunction during pressure overload at least in our mouse model of heart failure. Thus, interventions to modulate the endogenous level of PPARs and its interaction with RXR or Sirt1 may alleviate the progression of heart failure through modulation of metabolic remodeling.
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SUPPLEMENTARY INFORMATION

PPARα association with Sirt1 suppresses cardiac fatty acid metabolism.

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Expanded Methods

Transverse aortic constriction

Transverse aortic constriction (TAC) was performed by ligation of the innominate artery and left common carotid artery \(^1\). Two-dimension guided M-mode measurements of left ventricular (LV) internal diameter were taken to estimate LV ejection fraction.

Fatty acid oxidation

Fatty acid oxidation was performed as described previously\(^2,^3\). Dissected ventricular tissue was weighed and washed with PBS. The ventricular tissue was homogenized in 400 µl [0.25 M Sucrose, 1 mM EDTA]/100 mg tissue weight. Forty µl of the heart homogenate was dispensed per tube and centrifuged at 800g for 1.5 minutes. The precipitates were incubated with 200 µl Reaction buffer [10 mM Hepes pH 7.4, 150 mM KCl, 0.1 mM EDTA, 1 mM K\(_2\)HPO\(_4\), 10 mM MgCl\(_2\), 1 mM Malate, 10 mM Glucose, 1 mCi/20 pmol \(^3\)H-Oleic acid] at 37 °C for 10 minutes. The reactions were terminated by addition of 1 ml chloroform/methanol (2:1) and the samples were mixed well. The samples were kept at -20 °C for 2 to 24 hours and centrifuged at 12,000g at room temperature for 10 minutes. The supernatants (approximately 400 µl) containing \(^3\)H\(_2\)O were mixed with 4 ml scintillation fluid and the radioactivity was measured with a scintillation counter. The radioactivity was subtracted by background radioactivity which was obtained from the same reaction without myocardium.

Gene transfer to the mouse heart

Adenovirus vectors expressing LacZ and RXR\(_{\alpha(DBD)}\) were purified using the Adeno-X Maxi Purification Kit (Clontech). The left chest of C57BL/6 mice was opened, after anesthesia with pentobarbital sodium. The vectors were injected with an insulin syringe
at a concentration corresponding to $7.5 \times 10^{12}$ Optical Particle Units, and then the chest was closed. Two days after vector injection, the TAC operation was conducted. The expression of RXR$_\alpha$ (DBD) was confirmed with qPCR using the following primers which detect exogenous RXR$_\alpha$ (DBD) but not endogenous RXR$_\alpha$:

GGGAATTCCGCCAAGCGGACGGTGCAG and GTAACCATTATAAGCTGCAATAAAACAAGTTG.

**Quantitative RT-PCR**

Total RNA was prepared from left ventricles using the RNeasy Fibrous Tissue Mini Kit (Qiagen), and then cDNA was generated using M-MLV Reverse transcriptase (Promega). Real-time RT-PCR was performed using the Maxima SYBR Green qPCR master mix (Fermentas). Ribosomal protein S15 (RPS15) was used as an internal control. The mean value from non-Tg mice was expressed as 1. PCRs were carried out using the following oligonucleotide primers (5' to 3'); Acox1, ATGAATCCCGATCTGCAG and TTCTCGATTCTCGACGGCG; Cd36, GAATCTGAAGACCTTACATTGTACC and CACTCCAATCCCCAAGTAAGGCCAT; Cpt1b, TTTGGGAACCACAATCCCGCAAA and TTATGCCTGTGAGCTGGCCAC; Cpt2, GGCCAGCTGACCAAAAGAGCAG and GGTGGACAGATGTTGTTGTTTATC; Ech1, CTGACGAGGCGCTGGACAG and TGATTTTGGACCCCCTGCACAGCC; Fabp3, AAGGAGGCCGTGACCTGGCTG and ACCTTGAGCACCCTTTTGGATACA; Mcad, GAAGCTGATGAGGGACGCCA and GCTTGGAGCTTAGTTACACGAGG; Vldlr, GCCTGGGCCATCCTCCTCCTC and GTATGTGTGCTCTACAGGCAGC; RPS15, TTCGCAAGTTTACCTACC and CGGGGCCCATGCTTTTACCTTTTACCTTTT.
AGTGTCCACTGTTCAAAGCGCA; Acaa1b, CTGGGGGTGCAATAGCCCT and
GGCCTCAGTTCCCCAGGGGTATT; Acs1, GAAGAACTGTGCAGGAACAAGGATATC
and TTGTCAATAGAAAAATAATTCCGGGTGCAC; Ehhadh,
AGTGGTCAACTCCCTCAGGAGC and CGATGCCTCGGCCCATCGTT; Fatp1,
ATCTTCTGCGTCTTTCTGCC and TGCGGGCATGGACTCTCTCATC; Mlycd,
GAGTGGTCAGGAGCTGAGGAA and AGGCCCAGCACCTTGGT; Slc22a5,
ACAGCTATCCCTACCTTGTCTTCC and GGTGGTGAAGGCTGTCGTCTCTTTAG;
Txnip, GAGCTGGATAGACCTTACATCCA and
GTTGTTGTTGTTGTTAAGGAGC; Acsl3, ACTTGGGAAGAGCTGTGAACAGC
and AGTCCATGGTACCGCTAAAA; Cpt1a, GGGGTTTGGGGCCGGTG and
GTGAGTGGCCACTCTGTTAAGGAA; Dbi, GACCTCAAGGGCAAAGGAGT and
GTGAGGGTCTGAA; Hmgcs2, CCCCTGAAGATTTACAGAATAATGGA
and GCATCTCATCCACTGTTTCAAGG; Lpl, AGTGAAAGGCCAGGAGACAGCT
and GACTTCTCAGAGACTTTGTGTCGGCATT; Pcx, TGGCAAGGGGAGGTTCTCTT
and CCAATTTGGGCTACCATCCTTC; Plin2, TGTCTCTGGGTGAGTGGAA and
TCTTGAATGTCTTGTGGTACCGCTT.
intrinsic PPRE/DR1 sequences were taken from published papers. The following are the sequences of the double-stranded DNAs used for the pulldown assays (5' to 3'): DR1, ACCAAAAAGGTCAAAGGTCAAACCAC; DR1m3', ACCAAAAAGGTCAAATACTCAAACCAC; DR1m3'5', ACCAAAAATCAAATCAAACCAC; DR1m3'5', ACCAAAAATCAAATCAAACCAC; 3xAGGTCA, TTTTGTGAGGTCACTTTGTGAGGTCACTTTGTGAGTCATTTT; 3xGAATCA, TTTTGTGGAATCCTTTGTGGAATCCTTTGTGGAATCATT; Acox1, AAAGCAAGTGTTAAGCTAGCTGCTGAGGCA4; Cd36, CCAAGTAAGTCAGAGGCCAGAGAAC5; Cpt1b, GAATGTAGGGAAAAGGTCACCAGGA6; Cpt2, TGTCGCCCGCCTGAGGTCAGCGGCC7; Ech1, TTCAGGAAGCCCTGAGGTCAGCTCTCCAGG8; Fabp3, GGCACAAGCTCAGAGGTCTCCAGGG9; Mcad, AAGTAAAGGTCACAGCTGACTGCTA10; Vldlr, GTTTACAGGTCAGATGCGCAGCACA11.

**Plasmids**

The shuttle and mammalian expression vectors for PPARα (pDC316-PPARα), Myc-tagged PPARα (pDC316-Myc-PPARα), RXR (pDC316-RXRα) and Sirt1 (pDC316-Sirt1), and the bacterial expression vector for GST-fused PPARα (pCold-GST-PPARα) were described previously12. The shuttle and mammalian expression vector for the DNA binding domain of RXRα (RXRα(DBD)) was generated by insertion of RXRα cDNA corresponding to amino acids 160-212 into pDC316-Myc. The bacterial expression vectors for GST-fused RXRα (pCold-GST-RXRα) and Sirt1 (pCold-GST-Sirt1) were
generated by insertion of their cDNA into pCold-GST. Sirt1 cDNAs corresponding to the full length protein and amino acids 184-409 and 199-179 were inserted into pCold-GST-HA, which contains a DNA fragment encoding the HA tag (TATGTACCCCATACGATGTTCCGGATTACGCTAGTCTCGGGGTAC; YPYDVPDYASL), to generate pCold-GST-HA-Sirt1, pCold-GST-HA-Sirt1(184-409) and pCold-GST-HA-Sirt1(199-379). RXRα cDNA corresponding to amino acids 156-212 was inserted into pCold-GST-HA to generate pCold-GST-HA-RXRα(156-212). The minimal promoter luciferase reporter was generated by insertion of 32 bp of minimal promoter (CTCGAGTAGAGGTATATAATGGAAGCTCGACTTCCAG) between the XhoI and HindIII sites of pGL3Basic (Promega). Artificial and intrinsic DR1s with proximal ± 6bp sequences were inserted between NheI and XhoI to generate a series of reporter constructs.

**Chromatin immunoprecipitation (ChIP)**
Heart tissue was crosslinked with formaldehyde. The nuclear fraction was isolated and sonicated to generate a chromatin solution that was then used for immunoprecipitation. Antibodies used for ChIP assays were PPARα (Cayman 101710), RXR (Santa Cruz △N197), Sirt1 (Millipore 09-544) and Acetyl-Histone H3 at lysine 9 (Cell Signaling Technology 9649). PCRs were carried out using the following oligonucleotide primers (5’ to 3’): Mcad, ATCTAGCCAGAATTTGTTGTTCCAGTG and TCTAGGCCAGAGCGGCAGCAG, and Vldlr, CATGTGGGTTTTCTGATTTCAGTTTAC and ACTGCCTCTGCCTCTGTGTAAC.

**Primary cultures of neonatal rat ventricular myocytes**
Primary cultures of ventricular cardiac myocytes were prepared from 1-day-old Crl : (WI) BR-Wistar rats (Harlan). A discontinuous Percoll gradient was employed to isolate a cardiac myocyte-enriched fraction. Cells were cultured with complete medium containing Dulbecco’s modified Eagle medium/F12 supplemented with 5% horse serum, 4 μg/ml transferrin, 0.7 ng/ml sodium selenite, 2 g/l bovine serum albumin (fraction V), 3 mM pyruvate, 15 mM Hepes pH 7.1, 100 μM ascorbate, 100 mg/l ampicillin, 5 mg/l linoleic acid and 100 μM 5-bromo-2'-deoxyuridine (Sigma). Culture dishes were coated with 0.3% gelatin.

**Luciferase assay**

Luciferase activity was measured with a luciferase assay system (Promega). Reporter and expression plasmids were transfected into neonatal primary myocytes (12 well plate) using LipofectAmine 2000 (Invitrogen). Unless otherwise indicated, the amounts of expression vectors transfected into cells per well were 0.3 μg PPARα (pDC316-PPARα), 0.3 μg Myc-PPARα (pDC316-Myc-PPARα), 0.1 μg Sirt1 (pDC316-Sirt1), 0.3 μg RXRα (pDC316-RXRα) and 0.3 μg luciferase reporter plasmids. The total amount of plasmid vectors was kept constant (1 to 1.3 μg) using the control vector (pDC316). Cells were lysed with 100 μl Reporter lysis buffer 24 hours after transfection. Alternatively, after overnight culture, cells were transduced with adenovirus vectors carrying siRNA-Sirt1 (10 MOI) or siRNA-scramble (10 MOI). Cells were lysed with 100 μl Reporter lysis buffer 72-96 hours after transduction.

**Microarray analyses**

The accession number of the microarray data used for this study is GSE33101 at the Gene Expression Omnibus. The clustering and visualization of PPAR target genes,
whose PPREs have been reported in the literature (see below), were carried out by the
MultiExperiment Viewer (MeV) (http://www.tm4.org/mev.html). Sequence logos were
created by WebLogo3 (http://weblogo.threeplusone.com/create.cgi).

PPRE sequences

All PPRE sequences used in this study have been described in the literature and they
exist in the promoters of 60 genes, including Abcd213, Acs114, Fatp115, G0s216,
Hmgcs217, Mlycd18, Mttp19, Pcx20, and Txnip21. We designated them as PPAR target
genes in this work. Among them, 27 genes are involved in fatty acid catabolism.

Statistical analysis

Log2-transformed relative mRNA expression data were analyzed using LIMMA in R. All
pairwise comparisons among treatments were examined after ascertaining the
significance of the treatment effect (P < 0.05). The false discovery rate was controlled
for comparisons made across all genes and all treatments for each model using the
method of Benjamini and Hochberg. The number of genes upregulated/downregulated
with respect to imperfect and perfect DR1 was compared using Fisher's exact test. All
other analyses were performed using Welch's ANOVA performed on rank-transformed
data with ties handled by average ranks (PROC GLM, SAS version 9.4 for Windows). If
the treatment effect was significant (P < 0.05), all pairwise comparisons were made
using Welch's t tests on the ranked data (PROC TTEST, SAS version 9.4 for
Windows). The false discovery rate was controlled across all comparisons made within
each experiment. Statistical significance (P < 0.05) is indicated in the figures by a filled
asterisk. NS indicates non-significance. All error bars represent S.E.M. The
Biostatistics Laboratory in Rutgers New Jersey Medical School independently reviewed all data sets and applied the appropriated statistical methods.
Supplementary Figures

Supplementary Figure 1

Supplementary Figure 1. High mortality rate in Tg-PPARα mice under PO conditions. Tg-PPARα mice were subjected to TAC. Statistical analysis was performed with the Kaplan-Meier log rank test.
Supplementary Figure 2

A

B

C

D

E

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<th>Perfect DR1</th>
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p=0.014

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<td>5(3)</td>
</tr>
<tr>
<td>Not-Downregulated</td>
<td>1(1)</td>
<td>10(1)</td>
</tr>
</tbody>
</table>

p=0.0047
Supplementary Figure 2.
PPAR target gene expression in PPARα gene-manipulated mice under PO conditions. After 4 (A) or 2 weeks (B) of TAC, quantitative PCR was performed using cDNA prepared from the indicated transgenic mice. At least 4 mice were used for each group. (C) Intrinsic PPRE/DR1 sequences harbored by the indicated PPAR target genes. 5' PPARα and 3’ RXR binding hexad sequences that have no more than 1 nucleotide mismatch from the reported consensus RGSWVANAGGTCA (R=A or G, S=G or C, W=A or T, V=C, G or A) are shown in red, and those with more than 1 nucleotide mismatch are shown in blue. (D) Contingency table of PPAR target gene expression in Tg-PPARα mice. PPAR target genes that are significantly upregulated in Tg-PPARα compared to wild type mice are defined as upregulated genes. (E) Contingency table of PPAR target gene expression under PO conditions. PPAR target genes that are significantly downregulated by PO in wild type mice are defined as downregulated genes. (D-E) The number of genes counted as having both perfect and imperfect DR1s is indicated in parentheses. The p value was determined using Fisher’s exact test.
Supplementary Figure 3

Supplementary Figure 3. Densitometric analysis of RXR and Sirt1 in human heart failure patients. The graphs correspond to Fig. 3B. n=4-6. Signal densities were measured using the ImageJ program (n=4).
Supplementary Figure 4

Supplementary Figure 4. Densitometeric analysis of RXR and Sirt1 bound to PPARα. The graphs correspond to Fig. 3C. Signal densities were measured using the ImageJ program (n=4-6)
Supplementary Figure 5

Supplementary Figure 5. Sirt1 does not effectively inhibit the interaction between PPARα and RXRα.
Supplementary Figure 6. The expression of RXRα(DBD) in the heart *in vivo*. Adenovirus harboring RXRα(DBD) was injected into the heart. After 4 weeks, expression of RXRα(DBD) was detected by qPCR.
Supplemental references


