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Cardiac lipin 1 expression is regulated by the peroxisome proliferator activated receptor γ coactivator 1α/estrogen related receptor axis

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Abstract

Lipin family proteins (lipin 1, 2, and 3) are bifunctional intracellular proteins that regulate metabolism by acting as coregulators of DNA-bound transcription factors and also dephosphorylate phosphatidate to form diacylglycerol [phosphatidate phosphohydrolase activity] in the triglyceride synthesis pathway. Herein, we report that lipin 1 is enriched in heart and that hearts of mice lacking lipin 1 (fld mice) exhibit accumulation of phosphatidate. We also demonstrate that the expression of the gene encoding lipin 1 (*Lpin1*) is under the control of the estrogen-related receptors (ERRs) and their coactivator the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α). PGC-1α, ERRα, or ERRγ overexpression increased *Lpin1* transcription in cultured ventricular myocytes and the ERRs were associated with response elements in the first intron of the *Lpin1* gene. Concomitant RNAi-mediated knockdown of ERRα and ERRγ abrogated the induction of lipin 1 expression by PGC-1α overexpression. Consistent with these data, 3-fold overexpression of PGC-1α in intact myocardium of transgenic mice increased cardiac lipin 1 and ERRα/γ expression. Similarly, injection of the β2-adrenergic agonist clenbuterol induced PGC-1α and lipin 1 expression, and the induction in lipin 1 after clenbuterol occurred in a PGC-1α-dependent manner. In contrast, expression of PGC-1α, ERRα, ERRγ, and lipin 1 was down-regulated in failing heart. Cardiac *phosphatidic acid phosphohydrolase* activity was also diminished, while cardiac phosphatidate content was increased, in failing heart. Collectively, these data suggest that lipin 1 is the principal lipin protein in the myocardium and is regulated in response to physiologic and pathologic stimuli that impact cardiac metabolism.

Keywords

lipin; PGC-1α; metabolism; heart failure

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1. Introduction

Abnormalities in myocardial energy metabolism occur in several acquired forms of pathologic cardiac remodeling [1] and recent evidence suggests that impairments in myocardial metabolism are not only a consequence of disease, but also may play a role in pathologic cardiac remodeling. For example, therapies designed to modulate cardiac metabolism have shown promise in treating heart failure [2], while specific heritable deficiencies of single genes encoding metabolic enzymes are associated with cardiomyopathy and or acute cardiac failure [3]. A comprehensive understanding of the factors that control cardiac energy metabolism could lead to development of novel therapeutic approaches to modulate these pathways and treat cardiomyopathic remodeling.

Lipin 1 is an intracellular protein that was first identified by using a positional cloning approach to localize the causative mutation in the fatty liver dystrophic (*fld*) mouse [4, 5]. *Fld* mice exhibit neonatal hepatic steatosis, life-long deficiencies in adipose tissue development, insulin resistance, and increased susceptibility to developing atherosclerotic lesions [6]. Based on sequence homology, additional genes encoding lipin proteins (lipin 2 and lipin 3) were also identified. The three lipin proteins exhibit dissimilar patterns of tissue-specific expression [7]. Two distinct molecular functions for lipin proteins have emerged. Lipin 1 acts in the nucleus as a transcriptional coregulatory protein by interacting with DNA-bound transcription factors and recruiting other transcriptional regulators that possess histone modifying activity [8, 9]. For example, lipin 1 interacts with the peroxisome proliferator-activated receptor α (PPARα) and its coactivator protein PPARγ coactivator-1α (PGC-1α) and increases expression of genes involved in fatty acid oxidation (FAO) in liver [8]. Surprisingly, lipin proteins also possess enzymatic activity as a Mg^{2+}-dependent phosphatidic acid phosphohydrolase (PAP) enzyme [7, 10, 11]. PAP enzymes catalyze the formation of diacylglycerol from phosphatidic acid (PA); the penultimate step in triglyceride synthesis.

In the myocardium, PAP activity is known to be dynamically-regulated. For example, prior to the cloning of lipins as PAP enzymes, it was demonstrated that myocardial PAP activity was increased in streptozotocin-induced diabetic rats, but decreased in JCR:LA corpulent rats [12, 13]. More recently, the expression of lipin 1 and cardiac PAP activity was found to be decreased in Zucker diabetic fatty rats and human subjects with type 2 diabetes mellitus [14]. We have hypothesized that lipin 1 is the cardiac-enriched lipin protein and that lipin 1 expression and activity is regulated in the myocardium in response to physiologic and pathophysiologic stimuli. We also sought to test our hypothesis that the PGC-1α coactivator was regulating the inducible expression of lipin 1 in myocardium and aimed to identify transcription factor partners for PGC-1α in this response.

In this work, we addressed these hypotheses using a variety of model systems. We found that [1] lipin 1 is the primary lipin protein in the heart, [2] lack of lipin 1 in the fld mouse hearts leads to increased accumulation of cardiac PA, [3] the expression of lipin 1 is decreased in failing heart and increased in response to β-adrenergic signaling, and [4] that lipin 1 is a target gene of estrogen related receptors (ERRs) and PGC-1α in cardiac myocytes through a novel intronic promoter. These studies provide new information regarding the regulation of this pathway in myocardium.

2. Materials and methods

2.1. Mouse studies

Mice constitutively deficient in lipin 1 (*fld* mice), were compared to wild-type (+/+) littermate control mice (Balb/cByJ strain). The PGC-1α knockout (PGC-1α $^{-/-}$) mice [15]

were extensively backcrossed (>10 generations) into C57BL/6J background mice and were compared to C57BL/6J wild-type (WT) controls. Cardiac-specific PGC-1α (MHC-tetON-PGC-1α) [16] overexpressing double transgenic mice are purebred FVB/N and were compared to littermate mice singly transgenic for the MHC-rtTA construct. FVB/N mice expressing extremely high levels of Cre-recombinase in a cardiac-specific manner (TG9 mice) develop dilated cardiomyopathy and death from congestive heart failure as previously described [17]. TG9 mice were compared to littermate non-transgenic mice to determine the effects of heart failure and lipin 1 expression. All studies were conducted with age- and sex-matched controls.

To determine the effects of PGC-1α on lipin 1 expression in vivo, we used the inducible MHC-TetON-PGC-1α overexpressing transgenic mice model [16]. These mice use a dual transgenic system (MHC-rtTA X TRE-PGC-1α), which is regulated by tetracycline or its analog doxycycline (DOX) (Supplemental Fig. 1). At two months of age, the transgene was induced by administration of chow containing DOX (200 mg/kg of the chow) for 2 days to the transgenic mice (TetON-PGC-1α) and their sex matched littermate non-transgenic (NTG) mice.

For the β-adrenergic agonist studies, two month old C57BL/6 wild-type mice and PGC-1α deficient (PGC-1$\alpha^{-/-}$) mice were administered a single dose of β1-agonist dobutamine (10 μg/g body weight, i.p.) or β2-agonist clenbuterol (250 ng/g body weight, i.p.), respectively, and mRNA and protein isolated after 6 hr.

For heart failure studies, TG9 and sex-matched littermate NTG control mice were sacrificed on day 78 (during decompensated cardiac hypertrophy) of life and cardiac mRNA and protein isolated [17].

All animal experiments were approved by the Animal Studies Committee of Washington University School of Medicine.

2.2. Western blot analysis

For Western blotting studies to quantify lipin proteins, frozen tissues were homogenized in ice cold RIPA buffer containing: 1% NP-40, 0.1% SDS, and protease inhibitors using a sonicator. Homogenates were incubated on ice for 30 minutes followed by centrifugation for 15 min at 15,000 × g.

Nuclear fractions were isolated for quantifying PGC-1α and acetyl-histone H3. Briefly, tissues were sonicated in sucrose homogenization buffer [0.255 M sucrose, 1mM EDTA, and 20 mM Tris (pH 7.4)] and centrifuged at 1000g for 20 min. The nuclear pellet was resuspended in TNET buffer [1% triton-X 100, 150 mM Nacl, 50 MM Tris (pH 7.4), and 2 mM EDTA], centrifuged and the nuclear pellet was resuspended in RIPA buffer. The tissue extracts were collected and the protein concentrations determined.

Total tissue protein homogenate (25 μg) was prepared in XT loading buffer and subjected to SDS-PAGE using Criterion XT precast gels. Proteins were then transferred from the gel to nitrocellulose membrane. Membranes were blocked for 1 hour in TBS containing 0.1% Tween 20 (TBS-T) and 5% non-fat milk. Subsequently, the blots were probed using antibodies against lipin 1 (gift of Dr. Thurl Harris, University of Virginia), lipin 2 [18], lipin 3 (Alpha Diagnostic), actin (Sigma), and alpha-tubulin (AA4.3, Developmental Studies Hybridoma Bank, Iowa City, IA) acetylated-histone H3 (Upstate Biotechnology) and PGC-1α (Calbiochem) overnight at 4°C in TBS-T containing 0.05% SDS. Blots were then washed in TBS-T and then incubated with goat anti-rabbit IRDye 800 or anti-mouse IRDye 700 conjugated IgG secondary antibodies (Licor Biosciences) for 1 hour at room

temperature in TBS-T. After again washing in TBS-T, bands were visualized by Odessey Imaging System (LI-COR Biosciences, Lincoln, NE).

2.3. Neonatal Rat Ventricular Myocytes Isolation, Adenoviral Infection, and TG synthesis assay

Primary cultures of neonatal rat ventricular myocytes were isolated from 1-day old Sprague-Dawley rat pups using kit from Worthington Biochemical Corporation (Lakewood, NJ) according to the manufacturer's protocol. NRVM were infected with the adenoviral constructs at the time of plating the cells. PGC-1α, ERRα, or ERRγ was overexpressed in NRVM using previously described adenoviruses [8, 19]. Adenovirus driving the expression of GFP (Ad-GFP) was used as a control. To knockdown ERRγ *or* lipin 1, adenovirus expressing ERRγ shRNA (kindly provided by A. Kralli) or lipin 1 [8] was used and compared to cells expressing shRNA against LacZ as a control. ERRα was knocked down by using siRNA directed towards rat ERRα mRNA (Supplemental Table 1).

Triglyceride synthesis rates were determined in isolated NRVM 48 hr after infection with shRNA (sh-lipin 1) adenoviral vector to knockdown lipin 1. To measure rates of TG synthesis NRVM were plated on 12-well plates and incubated with 2-^{3}H-glycerol-containing DMEM (10 μCi/ml) for 3 hr in the presence or absence of exogenously added fatty acids (0.5 mM). ^{3}H-labelled TG was isolated from cells and ^{3}H radioactivity determined as previously described [20].

2.4. Plasmid Constructs

i. *Expression vectors:* pcDNA 3.1, pcDNA-PGC-1α, pcDNA-PGC-1α constructs harboring mutations in LXXLL motifs, pcDNA-ERRα, and pSG5-HA-ERRγ have been described previously [21].

ii. *Luciferase reporter constructs:* The mouse −2045-*Lpin1*.luc, −421-*Lpin1*.luc, and −285-*Lpin1*.luc luciferase promoters were generously provided by Dr. Karen Reue, UCLA [22]. The intronic mouse lipin 1 genomic region (+2293-*Lpin1*.luc) was cloned by PCR amplification from −393 to +2293 relative to the transcriptional start site of the mouse *Lpin1* gene using C57BL/6 mouse genomic DNA as a template. The resulting product was cloned into the promoterless pGL3.basic firefly luciferase reporter plasmid using SacI and XhoI sites (Promega, Madison, WI). Cloning primer sequence in Supplemental Table 1.

2.5. Mass spectrometric analyses of cardiac PA levels

Lipids were extracted in the presence of PA (12:0/13:0) (20 pmol/mg wet wt.) as previously described [23]. Cardiac tissue samples were weighed and lipid extracts were dried under a stream of nitrogen and dissolved in chloroform/methanol/ammonium hydroxide (80/19.5/0.5) prior to LC/MS analysis. Samples were injected into Ascentis Silica chromatography column (100 mm × 2.1 mm, 3 μm ID) on an Agilent 1100 LC system connected to an Agilent 6400 triple quadrupole mass spectrometer operated in electrospray ionization and negative mode. A gradient elution with two mobile phases was used: chloroform/methanol/ammonium hydroxide (80/19.5/0.5) (A) and chloroform/methanol/water/ammonia (60/34.5/5/0.5) (B). The quantification of PA species was performed as described [24].

2.6. Dual-luciferase Reporter Assay

Transient transfections were performed by the calcium phosphate co-precipitation method. Firefly and renilla luciferase was assessed using Dual-Glo (Promega) according to the

manufacturer's instructions. All transfection data are presented as means ± standard errors (SE) for at least three separate transfection experiments done in triplicate.

2.7. PAP Activity

Phosphatidic acid phosphatase (PAP) activity was determined by using a modification of the method of Carman and Lin [25] to measure phosphate release from Triton X-100/phosphatidic acid mixed micelles. Briefly, labelled phosphatidic acid was purified by thin layer chromatography after using [γ-^{32}P]ATP and *Escherichia coli* diacylglycerol kinase to phosphorylate 1,2-dioleoyl-*sn*-glycerol. Samples of tissue extracts (5 μl) were added to reaction mixtures (50 μl) containing a final concentration of 1 mM Triton X-100, 10 mM β-mercaptoethanol, 0.2 mM [^{32}P]phosphatidic acid (5000 cpm/nmol), 50 mM Tris-maleate (pH 7.0), and either no added Mg^{2+} or 2 mM $MgCl_2$. After 20 min at 30°C the reactions were terminated by adding 0.25 ml of 0.1 N HCl in methanol. [^{32}P]Phosphate was extracted by vigorous mixing after adding 0.5 ml of chloroform and 0.5 ml of 1 M $MgCl_2$. The amount of soluble [^{32}P] inorganic phosphate in 0.5 ml of the aqueous phase was measured by scintillation counting. Mg^{2+}-dependent PAP activity (PAP) was determined by subtracting activity obtained in the absence of $MgCl_2$ from activity measured in the presence of 2 mM $MgCl_2$.

2.8. Chromatin Immunoprecipitation (ChIP)

C2C12 cells were differentiated for 5 days, and then infected with adenovirus overexpressing GFP, HA-tagged ERRγ, or ERRα for 72 hr before harvesting. Cells were subsequently washed with ice cold PBS and crosslinked with 1% formaldehyde solution. ChIP was performed using kit from Upstate (Cat # 17–295, Temecula, CA) according to manufacturer's instructions using anti-HA (Sigma) or anti-ERRα [26] antibodies. PCR primers were designed to flank two putative nuclear receptor response elements in the first intron of the *Lpin1* gene (Supplemental Table 1).

2.9. Statistical Analysis

Statistical comparisons were made using analysis of variance or t-test. All data are presented as the means ± S.E., with a statistically significant difference defined as a p-value < 0.05.

3. Results

3.1. Lipin 1 protein is abundantly expressed in the heart and regulates cardiac PA levels

We first examined the expression of lipin family proteins in myocardium. Lipin 1 protein was highly expressed in the heart of WT mice and was expressed at levels comparable to the expression in white adipose tissue and skeletal muscle, which are enriched in lipin 1 (Fig. 1). Lipin 2, which is up-regulated in lipin 1-deficient *fld* liver [18], was undetectable in the hearts of WT and *fld* mice, but was readily detected in liver lysates (Fig. 1A). Lipin 3 was only detected in the myocardium of both WT and *fld* mice after prolonged exposure and when 75 μg protein was loaded (compared to 25 μg protein for lipin 1 Western blots). Lipin 3 expression was also observed in lung, but we did not find robust expression of lipin 3 protein in any tissue we examined (data not shown). Consistent with high expression of lipin 1 in myocardium, cardiac PAP activity was markedly diminished in fld mice (Fig. 1B). Given the abundant lipin 1 expression in the heart, the effects of lipin 1 deletion on myocardial phosphatidic acid (PA) levels were determined. The PA (34:1) and PA (34:2) species tended to be increased in the hearts of the fld mice, and total PA content was significantly increased (more than 2-fold) in fld mouse hearts compared to the wild-type (WT) control littermates (Fig. 1C). However, cardiac TG was not different between the WT and fld mice (Fig. 1D). These studies show that lipin 1 is abundantly expressed in the

myocardium, *fld* mice exhibit marked decrements in cardiac PAP activity, which is consistent with a previous report [11], and loss of lipin 1 leads to accumulation of PA in the myocardium.

To determine the effects of diminished lipin 1 activity on TG synthesis, we infected isolated neonatal rat ventricular myocytes (NRVM) with a previously-described [8] adenoviral vector driving the expression of a shRNA to knock-down lipin 1 (sh-lipin 1). Lipin 1 shRNA down-regulated lipin 1 mRNA 60% compared to LacZ shRNA control (data not shown). Unexpectedly, basal rates of TG synthesis, which were very low in NRVMs, were actually increased in the lipin 1 knock-down group compared to the controls (Fig. 1E). Exogenous oleate administration increased the rates of TG synthesis by more that 30-fold, but knock-down of lipin 1 significantly attenuated this effect (Fig. 1E). These data suggest that, similar to our previous observations in hepatocytes [18], PAP activity is not rate-limiting during basal TG synthesis in cardiac myocytes. In the context of high levels of fatty acids, however, loss of lipin-mediated PAP activity impairs TG synthesis.

3.2. PGC-1α regulates lipin 1 in myocytes through an intronic promoter

Previous work revealed that lipin 1 is a target gene of PGC-1α coactivator in liver [8], but whether lipin 1 is regulated by PGC-1α in other tissues and the transcriptional mechanisms involved are not well understood. To address this, we overexpressed PGC-1α in NRVMs by using an adenovirus and found that PGC-1α significantly induced lipin 1 expression (Fig. 2A). Next, a luciferase reporter construct controlled by 2045 bp of the 5′ flanking region of lipin 1 gene (−2045-*Lpin1*.luc) [22] was co-transfected into NRVMs with a PGC-1α expression construct to determine whether PGC-1α was acting via a direct transcriptional mechanism. Surprisingly, PGC-1α did not activate this luciferase reporter construct driven by the characterized lipin 1 promoter [22] (Fig. 2B). Nor did PGC-1α affect the activity of a series of progressively shorter lipin 1 promoter-driven constructs (−421-*Lpin1*.luc and −285-*Lpin1*.luc) (Supplemental Fig. 2A). The basal activity of these luciferase promoters was quite high in NRVMs and varied as previously described [22] (Supplemental Fig. 2B). Two previous ChIP-seq approaches had suggested that known transcription factor partners of PGC-1α might bind to DNA in the first intron of the *Lpin1* gene [27, 28]. Therefore, we cloned a DNA fragment containing 393 nucleotides of the 5′ flanking sequence, the first *Lpin1* exon (53 nucleotides), and 2,240 bp of the first intron of the mouse lipin 1 gene into a promoterless luciferase reporter vector (+2293-*Lpin1*.luc). The basal activity of this construct was roughly equivalent to that of the −2045 bp construct (Supplemental Fig. 2B). Co-transfection of an expression vector for PGC-1α significantly activated +2293-*Lpin1*.luc reporter activity (Fig. 2B), suggesting that PGC-1α regulates lipin 1 gene transcription through the first intron of the *Lpin1* gene.

PGC-1α coactivates a number of nuclear receptor and non-nuclear receptor transcription factors that are expressed in the myocardium [29]. To gain insight into transcription factor partners that might be coactivated by PGC-1α on the lipin promoter, we overexpressed PGC-1α proteins with site-directed mutations in 3 key leucine-rich LXXLL domains that mediate interactions with nuclear receptors. Mutation of any of the three LXXLL motifs alone, including the L2 motif that mediates the interaction with PPARα and several other nuclear receptors [30], did not affect the ability of PGC-1α to induce *Lpin1* promoter activity (Fig. 2C). However, dual mutation of both L2 and L3 LXXLL motifs (mL2/3) completely abolished the induction of +2293-*Lpin1*.luc activity in NRVMs (Fig. 2C). In contrast, concomitant L1/L3 mutation did not block the induction of Lpin1 promoter activity. This indicates that PGC-1α mediates its effects on lipin 1 transcription through a nuclear receptor partner *that can utilize either the L2 or L3 domains for interaction*.

3.3. PGC-1α cooperates with ERR proteins to regulate lipin 1 in myocytes

To our knowledge, members of the estrogen related receptor (ERR) family of proteins are the only transcription factors *that can utilize both the L2 and L3 domains of* PGC-1α for interaction and coactivation [21]. Consistent with this idea, adenovirus-mediated overexpression of ERRα or ERRγ induced lipin 1 mRNA expression in NRVMs (Fig. 3A). ERRγ also activated the +2293-*Lpin1*.luc reporter in an additive manner with PGC-1α (Fig. 3B). Our search for nuclear receptor response elements in the first intron of *Lpin1* gene revealed two putative half sites (site 1 and site 2) (Fig. 3C). Chromatin immunoprecipitation studies demonstrated that overexpressed ERRα and ERRγ proteins were associated with both site 1 and site 2 of the first intron of *Lpin1* gene (Fig. 3D). These collective data indicate that ERRα and ERRγ act through the first intron of the *Lpin1* gene to regulate its transcription.

Next, we tested whether knocking down ERR expression would prevent the induction of lipin 1 by PGC-1α. Interestingly, PGC-1α overexpression led to a marked increase in ERRα and ERRγ expression in NRVMs (Supplemental Fig. 3). Although ERRγ shRNA knocked down ERRγ in the NRVMs (Supplemental Fig. 3), this did not abrogate PGC-1α-induced increases of lipin 1 expression (Fig. 3E). Similarly, knockdown of ERRα alone did not affect lipin 1 expression. However, combined ERRα/ERRγ knockdown abolished the ability of PGC-1α to induce lipin 1 expression (Fig. 3E). Dual knockdown of ERRα/ERRγ also led to a significant decrease in lipin 1 expression at baseline (GFP expressing cells). These findings suggest that either ERRα or ERRγ is sufficient to allow PGC-1α to induce lipin 1 and that combined ERRα/γ deficiency blocks this effect of PGC-1α.

3.4. Lipin 1 expression and activity is induced by PGC-1α overexpression in the mouse heart

We next assessed whether induction of PGC-1α at physiologic levels in intact mouse hearts was sufficient to induce lipin 1 expression. We used transgenic mice with inducible, cardiac-specific PGC-1α (TetON-PGC-1α) overexpression [16, 31]. Doxycycline administration to double transgenic mice for 2 days led to induction of PGC-1α (3-fold, Fig. 4A) mRNA in the TetON-PGC-1α mice compared to their respective non-transgenic (NTG) littermates. Although chronic induction of PGC-1α in myocardium leads to dysfunction and heart failure, the acute increase in PGC-1α activity did not affect cardiac systolic function (data not shown and [31]). PGC-1α overexpressing mice exhibited an increase in the expression ERRα, and ERRγ. Lipin 1 was also up-regulated greater than 2-fold in TetON-PGC-1α mice and this was accompanied with a corresponding increase in lipin 1 protein levels (Fig. 4A). PGC-1α overexpression led to a significant (~50 %) increase in PAP activity in the PGC-1α overexpressing mice (Fig. 4B), but Mg^{2+}-independent dephosphorylation of PA (LPP activity) was unaffected. These data indicate that lipin 1 is a PGC-1α target gene in vivo.

3.5. PGC-1α is required for the physiologic induction of lipin 1 in the heart

Cardiac PGC-1α activity is known to be regulated by pathophysiologic and physiologic stimuli. We sought to evaluate whether lipin 1 expression was regulated similarly. PGC-1α is known to be induced in response to β-adrenergic agonism [32]. We sought to determine whether lipin 1 was regulated similarly, and if so, whether PGC-1α was required for this induction. We used dobutamine and clenbuterol, β1 and β2-adrenergic agonists, respectively, to activate PGC-1α in the heart. Quantitative RT-PCR analysis showed that β1-agonism by dobutamine administration did not induce either PGC-1α or lipin 1 expression. However, β2-agonism by clenbuterol administration induced PGC-1α and lipin 1 mRNA and protein expression 6 hr post-injection in the WT C57BL/6J mice (Fig. 5A). In contrast, expression of PGC-1β, ERRα, and ERRγ was not altered by clenbuterol (Fig. 5B and Supplemental Fig. 4). Furthermore, cardiac lipin 1 gene expression was not induced

following clenbuterol injection in the PGC-1α deficient mice (Fig. 5B), indicating that the induction of lipin 1 by the β2-adrenergic agonist is dependent on the presence of PGC-1α.

3.6. Cardiac lipin 1 is down-regulated in the failing heart

The activity of PGC-1α and the ERRs is known to be deactivated in the failing heart [26, 33, 34]. We examined lipin 1 expression in a mouse model of heart failure (TG9 mice) [17]. Predictably, the expression of markers of cardiac hypertrophy (ANF and BNP) were increased in the TG9 mice compared to NTG controls (Fig. 6A), while cardiac PGC-1α, ERRα, and ERRγ expression was downregulated in these mice (Fig. 6A). This was associated with a significant reduction in lipin 1 mRNA and protein expression in the failing hearts compared to the WT littermates (Fig. 6A). Down-regulation of lipin1 mRNA and protein was also accompanied by reduced PAP activity during end-stage cardiac failure (Fig. 6B). As might be predicted by diminished PAP activity, quantitation of PA species by LC-MS/MS revealed myocardial accumulation of the palmitoyloleoyl (34:1) PA specie in failing hearts compared to littermate controls (Fig. 6C). These data suggest that lipin 1 expression and activity is downregulated in compensated pathologic cardiac hypertrophy and decompensated heart failure.

4. Discussion

In this study, we determined that lipin 1 is highly expressed in myocardium and that cardiac lipin 1 expression is altered in response to physiologic and pathophysiologic stimuli that impact fatty acid metabolism. The expression of lipin 1 was determined to be under the control of ERRα/γ and PGC-1α, which are known to be important regulators of cardiac metabolic homeostasis. These findings are consistent with the emerging and important roles that lipin 1 plays in FA homeostasis and the partnership between PGC-1α and lipin 1 pathways.

The ERR family (α, β, and γ) of nuclear receptor transcription factors exhibit sequence and structural homology to the estrogen receptors, but are not bound by estrogen and are considered “orphans” (no known endogenous ligands). It is generally believed that ERR activity is primarily regulated by associations with transcriptional regulatory proteins, including PGC-1α, that coactivate ERRα and ERRγ [21]. There are now several studies indicating that the ERRs work with PGC-1α to control expression of genes involved in FAO in myocardium [26, 35]. Mice lacking ERRα display modest changes in cardiac gene expression at baseline, but exhibit marked defects in energy homeostasis after chronic pressure overload [26]. ERRγ nullizygosity in mice is lethal within 1 week of birth due, at least in part, to cardiac defects [35]. Hearts of mice null for ERRγ are metabolically abnormal and are unable to switch from anaerobic carbohydrate utilization in the fetal state to high level FAO post-natally [35]. Similarly, mice doubly null for PGC-1α and PGC-1β in heart also die soon after birth due to metabolic and mitochondrial deficiencies of the myocardium [19]. We found that overexpression of ERRs or PGC-1α in NRVMs was sufficient to induce lipin 1 gene expression. When taken with the results of RNAi-mediated knockdown studies which showed that knockdown of both ERRα and ERRα was required to block the PGC-1α-mediated induction of lipin 1, our data indicate that both cardiac-enriched members of the ERR family are novel regulators of lipin 1 in the heart that work with PGC-1α to control its expression (Fig. 7).

The myocardium is a tissue that does not normally store TG in large quantities. However, several recent studies have suggested that a great deal of the fatty acid entering the cardiac myocyte fluxes through the intracellular TG pool before its fate in mitochondrial β-oxidation [36, 37]. We found little evidence that PAP activity was related to steady-state TG content of the myocardium. Fld mice, which have marked reductions in PAP activity, have normal

cardiac TG levels, while inducing lipin 1 by PGC-1α overexpression actually led to a decrease in cardiac TG content (data not shown). On the other hand, myocardial TG content is increased in PGC-1α$^{-/-}$ mice [38], which have reduced lipin 1 under stimulated conditions. Why are steady-state TG levels not reflective of changes in PAP activity? Steady-state TG content is also strongly influenced by rates of lipolysis and catabolism and it is possible that alterations in the lipolytic rate explain the observed disconnect between lipin 1 and TG levels. It was recently shown that the increase in TG content in PGC-1α$^{-/-}$ mice was due primarily to a defect in recruiting FA from TG stores for β-oxidation [38]. PAP activity may not be rate-limiting for TG synthesis under basal conditions. We only observed an effect of lipin 1 knockdown on TG synthesis rates when oleate was added to boost TG synthesis. Additional studies to evaluate the overall effects of lipin 1 on TG flux are warranted.

Our findings suggested that altered lipin 1 activity is better correlated with the concentration of the substrate of lipin 1, phosphatidate. Fld mouse heart exhibited PA accumulation and we, along with previous studies [39, 40], have shown that cardiac PA levels are elevated in cardiac hypertrophy and heart failure, when lipin 1 is down-regulated. PA is not the product of DAG lipolysis (that would be monoacylglycerol), and thus, steady state PA levels might more accurately reflect lipin 1 activity compared to TG or DAG content. We believe that these alterations in PA content are quite significant. The membrane content of PA is normally very low, but fluctuations in PA content have a variety of cellular effects. For example, intracellular PA concentration can impact the activity of multiple signaling cascades. PA is known to activate mammalian target of rapamycin signaling and mitogen-activated protein kinase cascades [39–41]. Additionally, PA is also converted to various phospholipids including cardiolipin, a critical component of mitochondrial membranes. Changes in the cellular PA pool caused by altered PAP activity could impact these signaling pathways, the synthesis of phospholipids and glycerolipids, or have other cellular functions.

The other molecular function of lipin 1 as a transcriptional regulatory protein could also play a significant role in regulating cardiac fatty acid metabolism. We have previously shown that lipin 1 partners with PPARα and PGC-1α to regulate expression of various genes involved in FAO in liver [8]. Interestingly, in this study, the expression of cardiac lipin 1 was induced by a physiologic stimulus that increases FAO gene expression (β-adrenergic agonism) and downregulated by a pathophysiologic stimulus (heart failure) associated with reduced FAO gene expression [1]. Mice deficient in either PPARα or PGC-1α display significant defects in mitochondrial FAO (PPARα deficient) [42] or electron transport chain functions (PGC-1α deficient) [15]. Conversely, PPARα or PGC-1α overexpression increases the expression of multiple enzymes involved in these pathways [16, 43]. Our initial examinations of *fld* mice have revealed no detectable changes in the cardiac expression of genes encoding enzymes in these oxidative pathways. The myriad metabolic abnormalities of *fld* mice may confound the interpretation of these observations. We believe that cardiac specific deletion of lipin 1 will be needed to answer these questions convincingly.

In summary, this work provides new information regarding the transcriptional regulatory mechanisms controlling expression of lipin 1 in myocardium. The present data that lipin expression and activity is regulated by these physiological and pathophysiologic stimuli suggest that lipin 1-mediated regulation of FA metabolism could play a role in the metabolic changes that occur in these states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PAP	phosphatidic acid phosphatase
LPP	lipid phosphate phosphatase
PPAR	peroxisome proliferator-activated receptor
PGC-1α	PPARγ coactivator 1α
ERR	estrogen related receptor
TG	triglyceride
FAO	fatty acid oxidation
MHC	myosin heavy chain
rtTA	reverse tetracycline transactivator
NRVM	neonatal rat ventricular myocytes
WT	wild-type
TG	transgenic
NTG	non-transgenic
fld	fatty liver dystrophic
TAC	trans-aortic constriction

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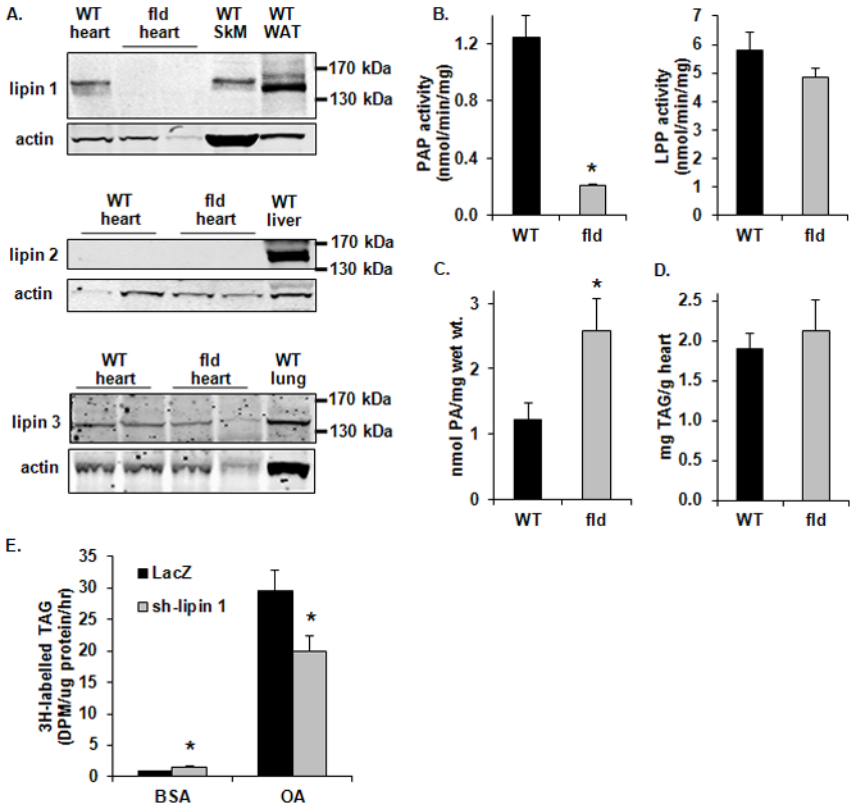

Fig. 1.

Lipin 1 is abundantly-expressed in the heart. (top) Results of Western blot analyses for lipin 1 with 25 μg protein isolated from cardiac tissue, skeletal muscle (SkM), and white adipose tissue (WAT) of mice. (middle) Results of Western blot analyses for lipin 2 with 25 μg protein isolated from cardiac tissue of adult WT and *fld* mice. Protein from WT mouse liver is used as positive control for lipin 2. (bottom) Seventy five μg cardiac protein from WT and *fld* mouse or 75 μg protein from WT mouse lung (positive control) blotted for lipin 3. B. Result of PAP activity in the hearts of WT and *fld* mice (n=3/group). C. PA levels in the hearts of WT and *fld* mice (n=4/group). D. Cardiac TG levels in the WT and fld mice (n=7/group). E. Graphs depict mean rates of TG synthesis under basal (BSA) or oleate-stimulated (OA, 0.5 mM for 3 hr) conditions (n=6/group) in the NRVMs. *p<0.05 versus wild-type group.

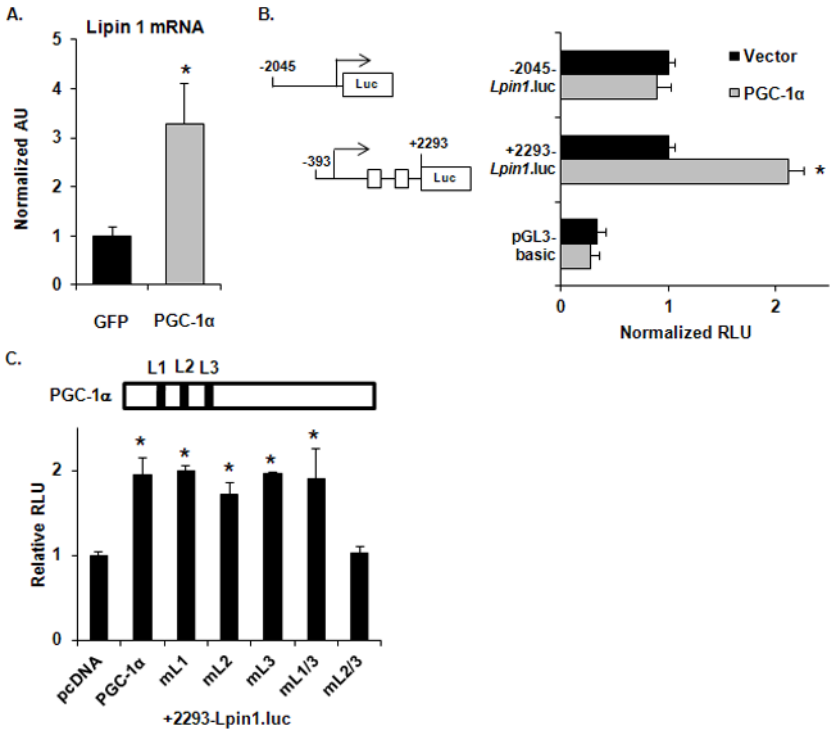

Fig. 2.

PGC-1α induces *Lpin1* gene expression in NRVMs through an intronic promoter element. A. RT-PCR analyses to quantify lipin 1 in mRNA isolated from NRVM infected with Ad-GFP and Ad-PGC-1α (n=6). Values are normalized (=1.0) to Ad-GFP. B. Graph depicts results from luciferase reporter assays using lysates from NRVMs co-transfected with 2045 bp of 5′ flanking sequence or 2293 bp 3′ from the transcriptional start site of the *Lpin1* gene and a PGC-1α expression vector (n=6). The vector values are normalized (=1.0). C. Inset at top; a schematic depicting PGC-1α protein and its functional interaction domains: *L1*, *L2*, and *L3* indicate conserved L*XX*LL motifs. The graph shows results from luciferase assays from NRVMs cotransfected with the +2293-*Lpin1*.luc luciferase reporter construct and PGC-1α, PGC-1α-mL1, PGC-1α-mL2, PGC-1α-mL3, PGC-1α-mL1/3 or the PGC-1α-mL2/3 expression constructs (n=6).

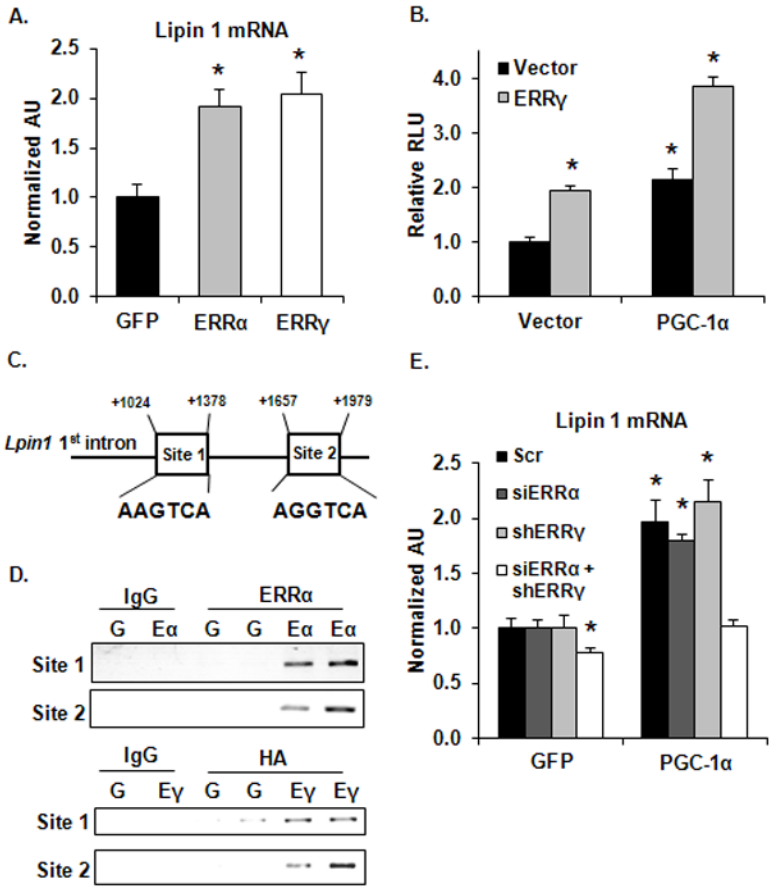

Fig. 3.

ERRs regulate lipin 1 expression and are required for the induction in lipin 1 expression after PGC-1α overexpression. A. RT-PCR analyses to quantify lipin 1 in mRNA isolated from NRVM infected with Ad-GFP, Ad-ERRα, or Ad-ERRγ (n=6). Values are normalized (=1.0) to Ad-GFP. *p<0.05 versus control group. B. The graph shows results from luciferase assays from NRVMs cotransfected with the +2293-*Lpin1*.luc luciferase reporter construct, expression vectors for ERRγ, in the presence or absence of PGC-1α expression vector. The vector values are normalized (=1.0). C. Schematic representation of the putative nuclear receptor response elements on site 1 and site 2 in the first intron of *Lpin1* gene. D. The images depict the results of chromatin immunoprecipitation studies using chromatin from C2C12 cells infected with adenovirus to overexpress Ad-GFP (G) or Ad-ERRα (Eα) (top two images) or Ad-GFP (G) or HA-tagged Ad-ERRγ (Eγ) (bottom two images). Crosslinked proteins were IP'ed with ERRα antibody, HA antibody, or IgG controls. E. RT-PCR analyses to quantify lipin 1 in mRNA isolated from NRVM treated with scrambled siRNA, siERRα, shERRγ, siERRα + shERRγ with or without Ad-PGC-1α (n=6). Values are normalized (=1.0) to Ad-GFP scrambled control. *p<0.05 versus Ad-GFP scrambled control group.

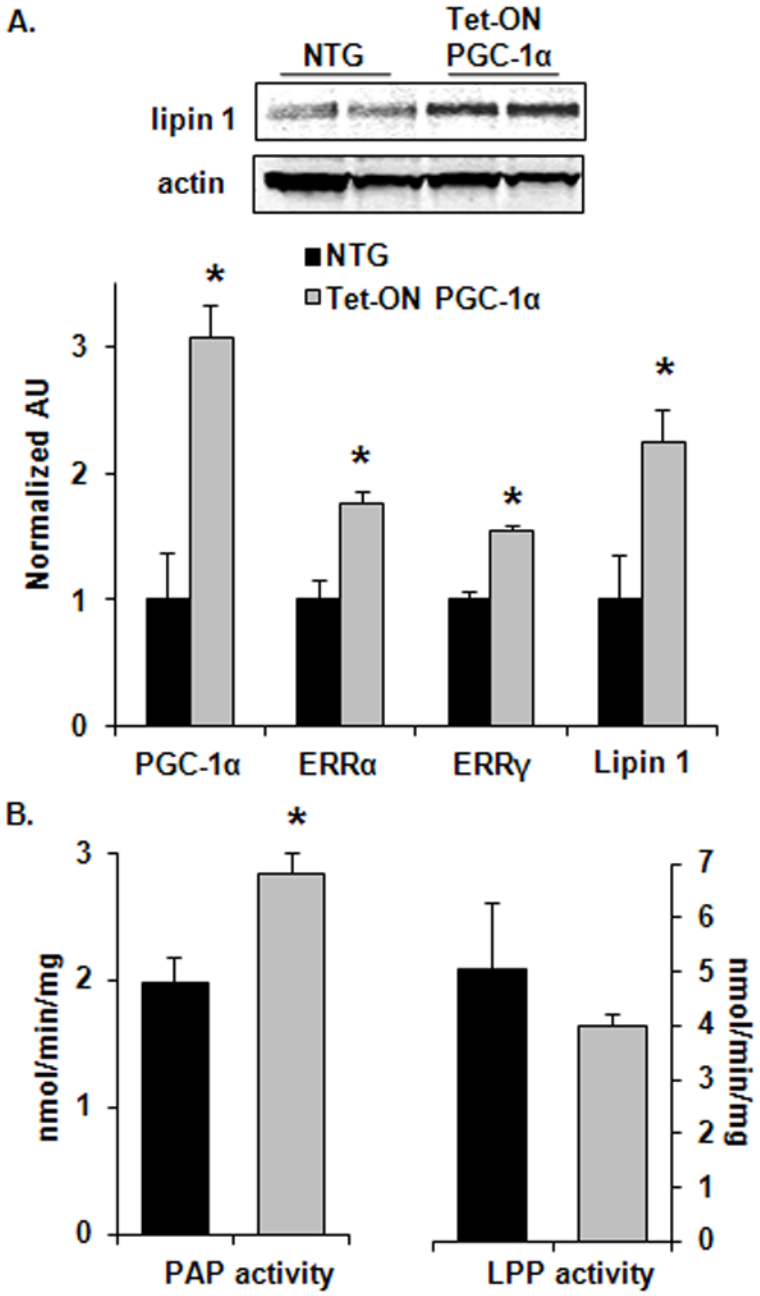

Fig. 4.

Cardiac lipin 1 is a PGC-1α target gene in the intact mouse heart. A. Results of RT-PCR and Western blot analyses to quantify PGC-1α, ERRα, ERRγ, and lipin 1 mRNA and protein in cardiac tissue from control NTG and Tet-ON PGC-1α (n=8) mice fed doxycycline (200 mg/kg of the chow) for 2 days. Control group values are normalized (=1.0). Representative Western Blot images are inserted and were probed with antibodies directed against lipin 1 or actin (as a loading control). B. Results of PAP and LPP activity in cardiac tissue from NTG and Tet-ON PGC-1α (n=8) mice fed doxycycline (200 mg/kg of the chow) for 2 days. *$p<0.05$ versus all control groups.

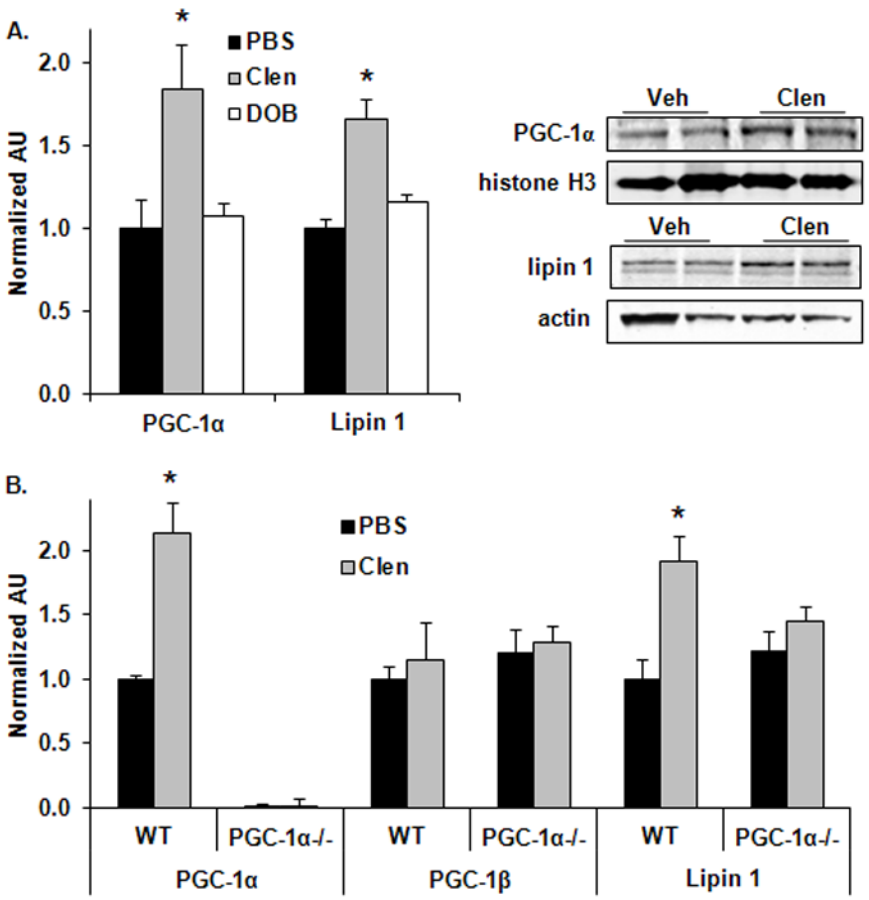

Fig. 5.

Cardiac lipin 1 expression is induced by β2-adrenergic agonism in a PGC-1α dependent manner. A. Results of RT-PCR analyses for PGC-1α and lipin 1 in mRNA isolated from cardiac tissues harvested from mice treated with PBS, dobutamine, and clenbuterol. Results of western blot analyses for PGC-1α and histone H3 in nuclear protein fractions (*top*) and lipin 1 and actin from total tissue homogenates (*bottom*) from cardiac tissue isolated from mice injected with PBS or clenbuterol. Cardiac tissues were harvested 6 hr after pharmacological treatments. B. Results of RT-PCR analyses for PGC-1α and lipin 1 in mRNA isolated from cardiac tissues harvested from WT and PGC-1α $^{-/-}$ mice treated with vehicle and clenbuterol (250 ng/g body weight, i.p.). Cardiac tissues were harvested 6 hr after clenbuterol administration (n=7). *p<0.05 versus all vehicle treated control groups. #p<0.05 versus WT mice in the same treatment group.

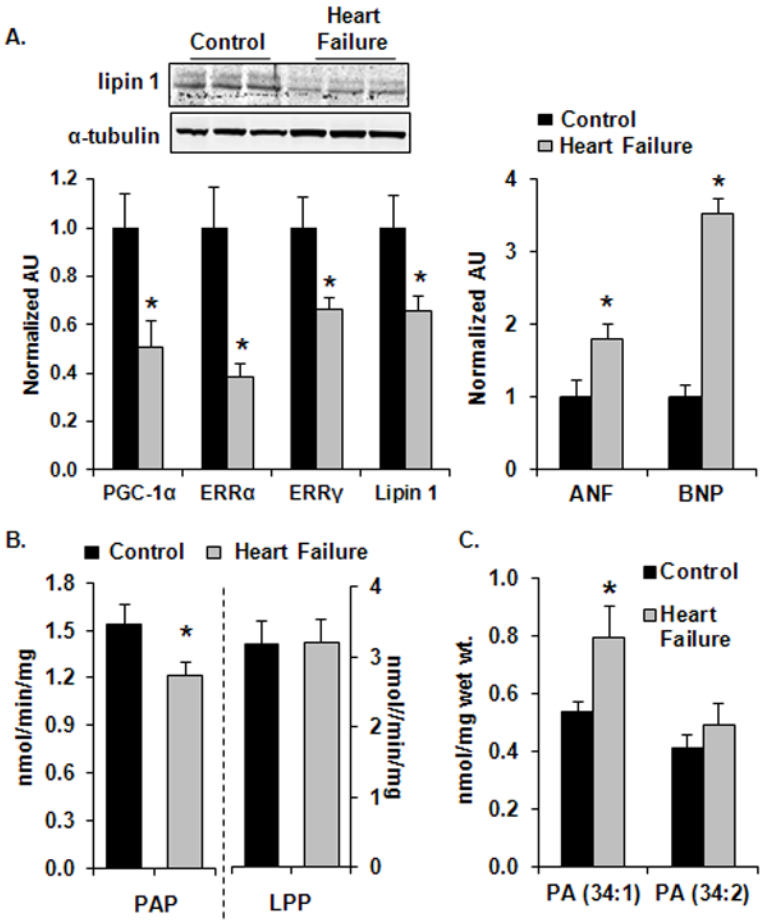

Fig. 6.

Lipin 1 mRNA, protein, and PAP activity are downregulated in failing heart. A. Results of Western blot and RT-PCR analyses to quantify the expression of the indicated genes in control and TG9 mice. B. PAP and LPP activity in control and failing hearts (n=6). C. PA levels in the hearts of control and TG9 mice (n=8/group). *p<0.05 versus all control groups.

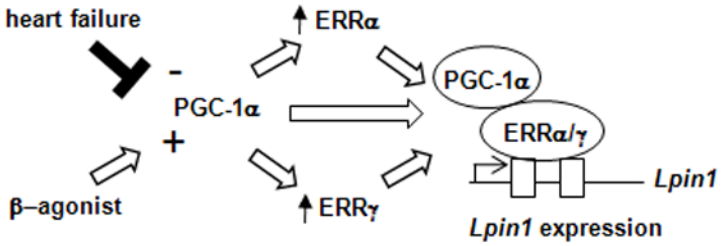

Fig 7.

Schematic representation of cardiac *Lpin1* transcriptional regulation by PGC-1α, ERRα, and ERRγ by pathologic cardiac hypertrophy and β-adrenergic agonism.