Rescue of Cardiomyopathy in Peroxisome Proliferator-Activated Receptor-α Transgenic Mice by Deletion of Lipoprotein Lipase Identifies Sources of Cardiac Lipids and Peroxisome Proliferator-Activated Receptor-α Activators

Jennifer G. Duncan, MD; Kalyani G. Bharadwaj, PhD; Juliet L. Fong, BA; Riddhi Mitra, MS; Nandakumar Sambandam, PhD; Michael R. Courtois, MA; Kory J. Lavine, PhD, MD; Ira J. Goldberg, MD; Daniel P. Kelly, MD

Background—Emerging evidence in obesity and diabetes mellitus demonstrates that excessive myocardial fatty acid uptake and oxidation contribute to cardiac dysfunction. Transgenic mice with cardiac-specific overexpression of the fatty acid–activated nuclear receptor peroxisome proliferator-activated receptor-α (myosin heavy chain [MHC]-PPARα mice) exhibit phenotypic features of the diabetic heart, which are rescued by deletion of CD36, a fatty acid transporter, despite persistent activation of PPARα gene targets involved in fatty acid oxidation.

Methods and Results—To further define the source of fatty acid that leads to cardiomyopathy associated with lipid excess, we crossed MHC-PPARα mice with mice deficient for cardiac lipoprotein lipase (hsLpLko). MHC-PPARα/hsLpLko mice exhibit improved cardiac function and reduced myocardial triglyceride content compared with MHC-PPARα mice. Surprisingly, in contrast to MHC-PPARα/CD36ko mice, the activity of the cardiac PPARα gene regulatory pathway is normalized in MHC-PPARα/hsLpLko mice, suggesting that PPARα ligand activity exists in the lipoprotein particle. Indeed, LpL mediated hydrolysis of very-low-density lipoprotein activated PPARα in cardiac myocytes in culture. The rescue of cardiac function in both models was associated with improved mitochondrial ultrastructure and reactivation of transcriptional regulators of mitochondrial function.

Conclusions—MHC-PPARα mouse hearts acquire excess lipoprotein-derived lipids. LpL deficiency rescues myocyte triglyceride accumulation, mitochondrial gene regulatory derangements, and contractile function in MHC-PPARα mice. Finally, LpL serves as a source of activating ligand for PPARα in the cardiomyocyte. (Circulation. 2010;121:426-435.)

Key Words: cardiomyopathy ■ lipids ■ diabetes mellitus

Type 2 diabetes mellitus and its associated cardiovascular complications are a worldwide health threat.1,2 Although patients with obesity-related diabetes mellitus have an increased incidence of heart failure after myocardial infarction,3 they are also prone to develop heart failure in the absence of significant coronary artery disease.4 These observations suggest that myocardial dysfunction in diabetes mellitus, metabolic syndrome, and obesity has distinct pathogenic features.

Clinical Perspective on p 435

Multiple mechanisms have been proposed to drive diabetes mellitus–associated heart dysfunction, including glucose toxicity (advanced glycation end products),5 microvascular disease,6 mitochondrial dysfunction,7,8 and lipid toxicity.9,10 Evidence supports a role for lipid metabolic derangements in the development of cardiomyocyte dysfunction in the insulin-resistant and diabetic heart9,10; both lipid accumulation and excessive fatty acid (FA) oxidation (FAO) are postulated to cause cardiomyocyte toxicity. Human studies and animal models demonstrate that diabetes mellitus and obesity are associated with accumulation of myocyte fat.11,12 Additionally, the insulin-resistant heart is unable to fully use glucose, forcing the organ to rely on FAs, leading to a vicious cycle of increased myocyte FA import, oxidation, and triglyceride accumulation,10 signatures of a metabolic cardiomyopathy called lipotoxic cardiomyopathy.

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From the Center for Cardiovascular Research (J.G.D., N.S., M.R.C., K.J.L.) and Departments of Medicine (N.S., M.R.C., K.J.L., D.P.K.) and Pediatrics (J.G.D., R.M.) Washington University School of Medicine, St Louis, Mo; Division of Preventive Medicine and Nutrition, Columbia University, New York, NY (K.G.B.; I.J.G.); and Burnham Institute for Medical Research, Lake Nona, Fla (J.L.F., D.P.K.).

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.109.888735/DC1. Correspondence to Daniel P. Kelly, MD, Burnham Institute for Medical Research–Lake Nona, 6400 Sanger Rd, Orlando, FL 32827. E-mail dkelly@burnham.org

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Reprogramming of the insulin-resistant heart toward FA use involves gene regulatory mechanisms. Peroxisome proliferator-activated receptor-α (PPARα), a fasting-induced nuclear receptor, is chronically activated in the insulin-resistant and diabetic heart, 8, 13 coordinately upregulating genes involved in cellular FA uptake and oxidation. 14 PPARs are a ligand-activated transcription factor, but the endogenous ligand(s) has not been fully determined. It is well established that FA moieties and fibrates activate PPARs. 15 Transgenic mice with cardiac-specific overexpression of PPARα (myosin heavy chain [MHC]-PPARα mice) exhibit a phenotype similar to that of the diabetic heart: ventricular dysfunction associated with increased FA uptake and FAO, myocyte triglyceride deposition, and reduced glucose use. 13 This cardiomyopathy of MHC-PPARα mice is exacerbated by a high-fat (HF) diet, consistent with the importance of FA overload in driving lipotoxic effects.

FAs are delivered to cardiomyocytes from 2 major sources: albumin-bound free FA (FFA) released from adipose tissue and FA incorporated into triglycerides within very-low-density lipoprotein (VLDL) or from intestines within chylomicrons. Both FFA and lipoprotein-derived FA are substrates for human hearts via receptor-mediated uptake. Although there are several FA transporters, it is well established that for human hearts via receptor-mediated uptake. Although there are several FA transporters, it is well established that CD36 mediates FFA uptake into rodent 16 and human hearts. 17

Previously, we crossed MHC-PPARα mice with CD36-deficient mice (MHC-PPARα/CD36ko mice). 18 CD36 deficiency prevented development of cardiomyopathy and myocardial triglyceride overload and normalized glucose oxidation rates in MHC-PPARα hearts. 18 However, FAO rates and expression of PPARα gene targets involved in FAO remained elevated in the MHC-PPARα/CD36ko mice, suggesting that PPARα ligand was still being delivered to the MHC-PPARα hearts.

Lipoprotein lipase (LpL) associated with the capillary endothelium is primarily responsible for liberation of triglyceride-derived FA. 19 Cardiac-specific deletion of this enzyme reduced heart lipoprotein-derived FA uptake without altering FFA uptake. 20 To further define the FA source that drives lipotoxic cardiomyopathy, MHC-PPARα mice were crossed with heart-specific LpL knockout (hsLpLko) mice. 20 We found that LpL deficiency rescues the lipotoxic cardiomyopathy and reverses the chronic activation of PPARα, identifying a source of PPARα ligand, which must be delivered via a CD36-independent pathway. Additionally, we found that the rescue of cardiomyopathy by either CD36 or LpL deficiency correlates with reactivation of genes involved in regulating mitochondrial function.

Methods

Animal Generation and Experiments

The generation of the MHC-PPARα/hsLpLko mice is described in the Methods section of the online-only Data Supplement. MHC-PPARα/CD36ko mice have been described elsewhere. 18 For HF diet studies, 1-month-old male and female mice were fed a diet with 43% calories from fat containing triglycerides composed of long-chain FA (TD01381, Harlan Teklad, Madison, Wis) for 4 weeks. All animal experiments were conducted in accordance with National Institutes of Health guidelines for humane treatment of animals and reviewed by the Animal Studies Committee of the Washington University School of Medicine.

Histological Analyses

Oil Red O staining was performed as described by the Morphology Core of the Digestive Diseases Research Core Center (DDRCC) at the Washington University School of Medicine. 18

Myocardial Triglyceride Quantification and Plasma Chemistries

Tissue triglyceride and serum FA, triglyceride, and cholesterol levels were determined by enzymatic, colorimetric assays (Thermo Scientific, Waltham, Mass, and Wako Pure Chemical Industries, Ltd. Osaka, Japan) by the Clinical Research Nutrition Unit Core at the Washington University School of Medicine.

Echocardiographic Studies

Transthoracic M-mode and 2-dimensional echocardiography was performed on conscious mice (n = 3 to 4 males and 3 to 4 females per group) with an Acuson Sequoia 256 Echocardiography system (Acuson Corp, Mountain View, Calif) as described. 21

Electron Microscopy

Papillary muscle was dissected from the left ventricle (LV) of the heart, fixed as described, 22 and sectioned for electron microscopy.

RNA Isolation and Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction Analyses

Total RNA was isolated from male and female hearts (n = 7 to 10 per group) and subjected to quantitative real-time reverse-transcription polymerase chain reaction (Q-rtPCR) using mouse-specific primer-probe sets as previously described. 8, 23, 24

Mouse Isolated Working Heart Preparation

Low-expressing MHC-PPARα/hsLpLko animals were used for isolated working heart studies. Eight-week-old male and female mice (n = 6 to 9 per group) on standard diet had isolated working heart preparations performed as described. 25 The Krebs-Henseleit perfusate solution contained 5 mmol/L glucose, 100 μU/mL insulin, and 0.4 mmol/L palmitate.

VLDL Uptake Studies

VLDL was isolated from normolipidemic human donors by ultracentrifugation at d = 1.006 g/mL for 22 hours at 39,000 rpm and subjected to labeling as previously described. 26 Details of VLDL preparation and injection are given in the Methods section of the online-only Data Supplement.

Neonatal Rat Ventricular Myocyte Preparation

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1-day-old Sprague-Dawley rat pups with a kit from Worthington Biochemical Corp (Lakewood, NJ). Cells were resuspended in medium (10% horse serum, 5% FCS, 100 μg/mL BrdU, 2 mmol/L 1-glutamine in DMEM containing 4.5 g/L glucose).

Transient Transfection Assays

NRVMs were plated at a concentration of ~3 000 000 cells/mL in 12-well gelatin-coated (Millipore, Billerica, Mass) plates. At the time of plating, cells were transfected with plasmid DNA using the calcium-phosphate method as described. 27 A detailed description of plasmid constructs and concentrations, reagents, and cell harvest can be found in the Materials section of the online-only Data Supplement.

Statistics

For quantitative data, statistical comparisons were made with ANOVA coupled to the Tukey test or Student t test assuming normally distributed and homoscedastic variances. In the case of non-parametric data, the non-parametric Mann-Whitney U test was used.
unequal variances. All data are presented as mean±SE, with a statistically significant difference defined as P<0.05.

Results

LpL Deficiency Rescues Cardiomyopathy in MHC-PPARα Mice

Four experimental groups were evaluated: LpLWT (wild type [WT]), LpLhsLpLko, LpLMHC-PPARα (MHC-PPARα), and LpL/hsLpLko (MHC-PPA-MHC-Cre/MHC-PPARα/hsLpLko). Similar levels of PPARα gene expression were confirmed among the MHC-PPARα genotypes, and the absence of LpL was confirmed in the LpLhsLpLko genotypes by Q-rtPCR and assessment of myocardial LpL enzymatic activity (Figure II of the online-only Data Supplement). Plasma levels of FFA, triglycerides, and cholesterol were not significantly different between any genotype after HF diet (Table I of the online-only Data Supplement). Although plasma triglyceride trended toward being higher in hsLpLko animals, it was not further altered in the setting of the MHC-PPARα transgene.

As expected, MHC-PPARα mice exhibited increased LV mass index, reduced LV fractional shortening, and increased LV systolic and diastolic diameters (Figure I and Table II of the online-only Data Supplement), a phenotype that worsened after the HF diet. In contrast, MHC-PPARα/hsLpLko mice had normal ventricular function, LV mass index, and chamber size on both diets. Thus, LpL is necessary for the development of cardiomyopathy in MHC-PPARα mice.

LpL Deficiency Rescues the Myocardial Metabolic Derangements of MHC-PPARα Mice

After 4 weeks of HF diet, Oil Red O staining, a marker of neutral lipid overload, demonstrated abnormal lipid accumulation in myocardial sections from MHC-PPARα mice compared with WT controls (Figure 2A). In striking contrast, lipid staining was similar in hearts from MHC-PPARα/hsLpLko and WT mice. Consistent with these results, triglyceride levels in the hearts of MHC-PPARα mice were 15-fold greater than those of WT controls but were normal in MHC-PPARα/hsLpLko hearts (Figure 2B).

To evaluate the effects of LpL deficiency on myocardial fuel use, we used an isolated working heart preparation. Low-expressing MHC-PPARα mice were chosen for these experiments because, in the absence of HF diet, this line does not develop ventricular dysfunction, which can independently influence myocardial substrate use rates. As previously shown, MHC-PPARα mice exhibited high rates of palmitate oxidation and reduced glucose oxidation rates (Figure 3). In striking contrast, myocardial glucose and palmitate oxidation rates of MHC-PPARα/hsLpLko mice were not different from those of WT animals, indicating a complete reversal of the cardiac fuel use abnormalities characteristic of MHC-PPARα mice. Taken together with the results of the myocardial triglyceride measurements, these results indicate that cardiac-specific LpL deficiency corrects elevated myocardial triglyceride levels and fuel use derangements in MHC-PPARα mice.

Rescue of the Cardiomyopathy Phenotype of MHC-PPARα Mice Correlates With Normalization of Mitochondrial Ultrastructural Abnormalities

The above results, together with our previously published work, demonstrate that the cardiomyopathy associated with MHC-PPARα mice is rescued by either CD36 deficiency or LpL deficiency. The metabolic phenotypes of the 2 rescue models display both overlapping and distinct features, potentially providing a clue about the basis for lipotoxic forms of cardiomyopathy. In both cases, triglyceride accumulation is reversed, but only LpL deficiency reverses the high FAO rates driven by PPARα. These results suggest that triglyceride accumulation rather than increased FAO rates is linked to cardiac dysfunction. Recent data demonstrate an association
between cellular lipid accumulation and mitochondrial dys-
function. In addition, balanced fuel use requires appro-
priately matched mitochondrial capacity to oxidize fuels via
the tricarboxylic acid cycle and oxidative phosphorylation
pathways. Evaluation of electron micrographs prepared
from the LVs of MHC-PPAR\textsuperscript{α}/H\textsubscript{9251} hearts demonstrated disorga-
nized mitochondria with altered cristae density and archite-
ture (black arrows in Figure 4A). Increased mitochondrial
volume density was also noted. These changes were strikingly
absent in electron micrographs of MHC-PPAR\textsuperscript{α}/hsLpLko heart
muscle (Figure 4A). The transcriptional coactivator PPAR\textsubscript{α}
coactivator-1\textsubscript{α} (PGC-1\textsubscript{α}) serves a critical role in controlling
mitochondrial biogenesis and function in the postnatal
heart. The expression of PGC-1\textsubscript{α} is dysregulated in
several mouse models of heart failure. We found that
levels of PGC-1\textsubscript{α} messenger RNA were significantly reduced
in the MHC-PPAR\textsuperscript{α} hearts (Figure 4B). Both LpL deficiency
and CD36 deficiency reversed the downregulation of PGC-1\textsubscript{α}
expression (Figure 4C). Furthermore, the expression of addi-
tional genes involved in mitochondrial metabolism, including
the tricarboxylic acid cycle (succinate dehydrogenase-\textsubscript{α}) and
oxidative phosphorylation (ATP synthase \textsubscript{β}), was reduced in
hearts from MHC-PPAR\textsuperscript{α} mice but normalized in MHC-
PPAR\textsubscript{α}/hsLpLko hearts (Figure 4D). Taken together, these data
suggest that myocardial lipid accumulation leads to mitochon-
drial dysfunction related, at least in part, to suppression of
PGC-1\textsubscript{α} gene expression.

LpL Is Required for the Delivery of PPAR\textsubscript{α}
Ligand to the Cardiac Myocyte
LpL deficiency rescued both glucose and FA use abnormal-
ities in MHC-PPAR\textsubscript{α} mice, unveiling a striking phenotypic
difference compared with CD36 deficiency. Specifically,
CD36 deficiency did not reverse the high FAO rates and the

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** Reversal of myocardial lipid accumulation in MHC-PPAR\textsubscript{α}/hsLpLko mice on HF diet. A, Representative photomicrograph depicting Oil Red O–stained ventricular tissue prepared from all genotypes after 4 weeks of HF diet. Red droplets indicate neutral lipid staining. B, Mean (±SE) myocardial triglyceride levels for each genotype after HF diet (n=3 to 5 per group). NTG indicated nontrans-
genric; TG, transgenic. *P<0.05 vs WT and hsLpLko; †P<0.05 vs MHC-PPARα.

![Figure 3](http://circ.ahajournals.org/)

**Figure 3.** LpL deficiency normalizes fuel use of MHC-PPAR\textsubscript{α} hearts. The oxidation rates of [U-\textsuperscript{14}C]glucose (left) and [9,10-\textsuperscript{3}H]palmitate (right) were assessed in isolated working hearts (WT, n=8; hsLpLko, n=6; MHC-PPARα, n=9; MHC-PPARα/hsLpLko, n=6). Bars represent mean (±SE) oxidation rates expressed as nanomoles of substrate oxidized per minute per gram dry weight. *P<0.05 vs WT and hsLpLko; †P<0.05 vs MHC-PPARα.
increased expression PPARα FAO target genes in MHC-PPARα hearts, indicating that PPARα is still chronically stimulated in this model.18 In contrast, cardiac expression of PPARα target genes in the MHC-PPARα/hsLpLko mice was not different from that in WT mice. Specifically, the expression of PPARα target genes involved in FAO (muscle-type carnitine palmitoyl-transferase-1, uncoupling protein 3, and acyl-coA oxidase), FA uptake (FA transport protein 1 [FATP1]), and glucose metabolism (pyruvate dehydrogenase kinase 4 and glucose transporter 4) in hearts from HF-fed MHC-PPARα/hsLpLko mice was normalized to the level in WT animals (Figure 5). CD36 expression was not significantly different between genotypes (Figure III of the online-only Data Supplement). PPARβ also plays a role in regulating cardiac metabolism, and we have found that the ratio of PPARα to PPARβ drives lipid accumulation and cardiomyopathy.35 Gene expression for PPARβ was mildly increased in MHC-PPARα hearts (2.2-fold) but was unchanged in MHC-PPARα/hsLpLko hearts compared with WT hearts (data not shown).

The gene expression data demonstrating that LpL deficiency normalizes the expression of PPARα metabolic gene targets suggest that LpL plays a more important role than
CD36 in delivering FA or other PPARα ligands to cardiomyocytes. To assess the impact on lipoprotein-derived FA uptake in the 2 models, radiolabeled lipid uptake studies were performed in WT, MHC-PPARα, MHC-PPARα/hsLpLko, and MHC-PPARα/CD36ko mice using double-labeled VLDL; triglycerides were labeled with 14C; and cholesteryl ester (CE) was labeled with 3H. Eight- to 10-week-old animals were injected with the dual-labeled VLDL, and tissue was harvested 30 minutes after injection. Cardiac uptake of 14C-labeled triglycerides by MHC-PPARα mouse hearts was increased compared with WT animals. Triglyceride-derived lipid uptake was normalized in both LpL- and CD36-deficient MHC-PPARα mice (Figure 6). In contrast, 3H-CE uptake was dramatically reduced in LpL-deficient MHC-PPARα mice but not in CD36-deficient hearts (Figure 6). A similar reduction in CE uptake was seen in hsLpLko animals compared with WT animals (data not shown).

LpL- and CD36-deficient hearts differed in the uptake of “core lipids” that are not hydrolyzed and remain associated with the VLDL remnant. Thus, we postulated that the lipid contained in lipoprotein remnants activates PPARα in heart and that the ligand enters the cardiomyocyte independently of CD36 in an LpL-dependent manner. To test this hypothesis, a cell culture assay was used in which the Gal4-PPARα-LBD

![Figure 5](http://circ.ahajournals.org/)

**Figure 5.** Cardiac metabolic PPARα target gene expression is normalized in LpL-deficient MHC-PPARα hearts. Messenger RNA levels of FA metabolism gene targets (A) and glucose metabolism gene targets (B; see text) as determined by Q-rtPCR using RNA from hearts of 2-month-old mice after HF diet (n=7 to 9 per genotype). Bars represent mean (±SE) arbitrary units (AU) normalized to the WT value (1.0). *P<0.05 vs WT and hsLpLko; †P<0.05 vs MHC-PPARα.

![Figure 6](http://circ.ahajournals.org/)

**Figure 6.** Heart uptake of VLDL triglyceride and CE is altered in MHC-PPARα/hsLpLko hearts. Bars represent mean (±SE) cardiac lipid uptake as represented by radioactive count (dpm/g tissue), normalized to liver radioactive counts, for triglycerides (14C-TG; left) and CE (3H-CE; right) in WT (n=6), MHC-PPARα (n=7), MHC-PPARα/hsLpLko (n=8), and MHC-PPARα/CD36ko (n=8) animals. *P<0.05 vs all other groups.
fusion reporter was used in primary NRVMs. As expected, the FA oleate activated Gal4-PPARα-LBD to a modest (3-fold) but significant extent (Figure 7A). Docohexanoic acid (DHA) had an effect similar to that of oleate, but palmitate was unable to activate the Gal4-PPARα-LBD (Figure V of the online-only Data Supplement). VLDL alone had no effect on Gal4-PPARα-LBD activation. However, in the presence of LpL, VLDL resulted in a striking 15-fold activation of Gal4-PPARα-LBD, consistent with the hypothesis that LpL is necessary for VLDL-mediated PPARα activation. Studies done using higher concentrations of oleate resulted in a slight additional effect at 400 µmol/L, but the VLDL/LpL effect was still much greater (data not shown). To assess the necessity of LpL enzymatic activity in VLDL activation of PPARα, the experiments were repeated using VLDL that had been depleted of triglycerides by pretreatment with LpL or in the presence of the LpL inhibitor tetrahydro-lipostatin (THL). Both the triglyceride-depleted VLDL remnant (which contains core lipids) and VLDL+THL+LpL were able to activate the reporter only to a level similar to oleate (Figure 7B). These results indicate that the mechanism whereby LpL increases PPARα activation requires triglyceride lipolysis and intact LpL enzymatic activity.

Our results indicated that the LpL-mediated delivery of PPARα ligand was CD36 independent. Thus, the NRVM experiments were repeated after “knockdown” of CD36. CD36 small interfering RNA (siCD36) reduced CD36 messenger RNA expression by >90% compared with cells transfected with control small interfering RNA (siCtrl) (Figure IV of the online-only Data Supplement). NRVMs were transfected with either siCtrl or siCD36 in the presence of oleate or VLDL/LpL. Cells treated with oleate or VLDL/LpL and siCD36 had a visible decrease in intracellular lipid droplets compared with cells transfected with siCtrl, providing evidence for a reduction in CD36 function (data not shown). However, even in the presence of siCD36, oleate or VLDL/LpL activated the Gal4-PPARα-LBD (Figure 7C). These data are consistent with the in vivo data and strongly suggest that ligands generated by LpL-mediated hydrolysis of VLDL are transported into the myocyte independently of CD36.

**Discussion**

To understand the contributions of increased FA use and lipid accumulation to diabetic cardiac dysfunction, several transgenic mouse models have been generated, including cardiac-specific LpL,36 acyl-CoA-synthetase and37 FATP1 overexpression,38 and the MHC-PPARα model used in this study.28 MHC-PPARα mice reproduce the diabetic metabolic signatures of increased FAO and cardiac triglyceride stores combined with reduced glucose use. Characterization of these models has led to new questions relevant to the role of lipotoxicity in the development of cardiomyopathy including: What is the relative contribution of FAO and triglyceride accumulation to the cardiomyopathy? Why, despite greater FAO rates, do triglycerides accumulate in the myocyte? Finally, what is the source of excess lipid (FFA or lipoproteins), and does it also activate PPARα?
We have previously shown that cardiac function can be restored in MHC-PPARα mice bred into a CD36-deficient background. CD36 deficiency reduces uptake of both FFA and lipoprotein-derived FA. The present study addressed the specific contribution of LpL to lipotoxic cardiomyopathy, when FA transporters remained intact. The study revealed 3 key findings: cardiac LpL deficiency rescues contractile dysfunction and myocardial triglyceride accumulation in MHC-PPARα mice; lipoprotein-derived FA is a major source of lipid for this form of cardiomyopathy; and delivery of PPARα ligand is dependent on LpL-mediated hydrolysis of VLDL and independent of CD36.

Many mechanisms have been proposed for cardiac dysfunction associated with excessive lipid uptake, including direct toxicity caused by FAs, triglycerides, or other lipids; excessive FAO resulting in reactive oxygen species and/or increased oxygen consumption; decreased glucose oxidation; and altered mitochondrial function. The current rescue with LpL deficiency is similar to our previous work in MHC-PPARα/CD36ko animals in the rescue of triglyceride accumulation, confirming that excess lipid contributes to contractile dysfunction. Correction of the lipotoxicity by LpL deletion is further proof that lipoprotein triglycerides, and not FFA, is the primary culprit.

Our data also point to derangements in mitochondrial function, mediated by downregulation of PGC-1α, as a potential determinant of cardiomyopathy in this lipotoxic model. Consistent with this notion, we found that LpL deficiency corrected the reduction in mitochondrial metabolic target gene expression and mitochondrial ultrastructural derangements in hearts of MHC-PPARα mice. From these results, we speculate that myocyte lipid overload leads to decreased glucose oxidation, and altered mitochondrial function. The current rescue with LpL deficiency is similar to our previous work in MHC-PPARα/CD36ko animals in the rescue of triglyceride accumulation, confirming that excess lipid contributes to contractile dysfunction. Correction of the lipotoxicity by LpL deletion is further proof that lipoprotein triglycerides, and not FFA, is the primary culprit.

Because CD36 reduces uptake of both FFA and lipoprotein triglycerides, the reduction in total FA uptake should be greater in MCH-PPARα/CD36ko; our data implicate a ligand other than FFA in PPARα activation. Previous in vitro studies have shown that VLDL/LpL can activate PPARs in endothelial cells and macrophages. Our data extend these findings to an in vivo model and suggest that myocardial LpL participates in PPARα ligand generation. NRVM studies confirmed that VLDL, in the presence of LpL, leads to robust activation of a PPARα reporter. Furthermore, activation of PPARα by VLDL/LpL or oleate occurs in the absence of CD36, confirming that the ligand delivery pathway is independent of CD36. It is possible that mechanisms such as diffusion or alternative transporters (e.g., FATP family members) are important for transporting and targeting PPARα ligand in the CD36-deficient animals. Notably, LpL deficiency does not eliminate PPARα activation, reflected by target gene expression and FAO rates similar to those of WT, suggesting that some ligand is available for basal PPARα activation. In addition, the lipid uptake studies demonstrate that MHC-PPARα/hsLpLko animals have triglyceride uptake similar to WT, suggesting that basal VLDL-triglyceride uptake is preserved. This may occur via several mechanisms, including noncardiomyocyte LpL activity (e.g., macrophages), peripheral lipolysis leading to FA uptake by non-LpL-mediated pathways, or uptake of VLDL "remnant" lipoproteins generated via peripheral lipolysis.

The specific cardiac PPARα ligand was not defined in this study, but several clues were provided. The VLDL uptake studies performed in vivo revealed a dramatic difference in CE uptake between CD36-deficient and LpL-deficient MHC-PPARα animals. CE is a marker of whole particle uptake, suggesting that the ligand activity could reside in lipid moieties other than triglyceride-derived FA. However, the NRVM experiments using triglyceride-depleted VLDL demonstrated a decreased ability to activate the PPARα-LBD, suggesting that triglyceride lipolysis is necessary for the full PPARα-activating effect; providing remnant-like lipoproteins to the cells alone is not sufficient. These latter results are consistent with previous findings suggesting that intact LpL enzymatic activity was necessary for PPARα activation. Ligand generation from VLDL particle components may be a multistep process requiring triglyceride hydrolysis and components of the core lipid. LpL hydrolysis results in the elaboration of a number of other lipid species, including oxidized lipids (e.g., HODEs) that could activate PPARα. It should also be noted that we have not excluded the possibility that activating ligand(s) for related PPARs (PPARδ, PPARγ) may be carried by the VLDL particle. Further studies are warranted to evaluate the specific components of VLDL lipolyse that are capable of activating PPARα and their downstream effect on the cardiomyocyte. Identification of the specific PPARα ligand could lead to tissue-specific strategies to block chronic activation of the cardiac PPARα pathway and thus prevent or therapeutic strategies for lipotoxic cardiomyopathy in humans.

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Disclosures
Dr Kelly serves on the Scientific Advisory Boards for Lilly and Johnson and Johnson. The other authors report no conflicts.

References


CLINICAL PERSPECTIVE

Metabolic syndrome and type 2 diabetes mellitus are dramatically increasing in prevalence worldwide. Cardiovascular disease is the leading cause of death among patients with diabetes mellitus, including a unique form of heart failure that is independent of coronary risk factors. Accumulation of myocardial lipid and alterations in cardiac fuel metabolism likely play an important role in cardiomyopathy in diabetic patients, a problem referred to as lipotoxic cardiomyopathy. This article explores several key questions related to the cause of diabetic heart disease such as, What is the contribution of excess fatty acid burning to contractile dysfunction? What is the source of excess fat, and does it activate peroxisome proliferator-activated receptor-α (PPAR-α), an important transcriptional mediator of increased fatty acid uptake and use in the diabetic heart? We demonstrate in an animal model of lipotoxic cardiomyopathy that lipoprotein-triglycerides rather than free fatty acid are the primary contributor to cardiomyopathy. Furthermore, we link lipoprotein lipase hydrolysis of very-low-density lipoproteins to activation of PPARα, suggesting that lipoprotein lipase plays a critical role in both driving lipid excess and generating ligand for activation of PPARα, thus promoting excess FA utilization pathways. Finally, our data suggest that lipid excess is associated with mitochondrial dysfunction, a likely mechanism for cardiac failure in patients with lipotoxic cardiomyopathy. Future studies to identify the specific PPARα ligand generated by lipoprotein lipase may help guide therapeutic strategies for diabetic heart failure.
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SUPPLEMENTAL MATERIALS

METHODS

Animal Generation

A high-expressing MHC-PPARα transgenic mouse line (404-3) in C57Bl/6 was crossed with LpL-deficient mice and subsequently crossed with mice overexpressing Cre recombinase driven by the αMHC promoter (MHC-Cre) (in a FVB background that had been back-crossed twice to C57Bl/6) (see Supplemental Figure 1 for complete breeding strategy), generating MHC-PPARα/hsLpLko mice (approximately 90% C57Bl/6). Mice with floxed LpL alleles but without LpL excision due to lack of Cre expression are denoted LpL$. Additional lines of non-floxed LpL-MHC-PPARα Cre+ and Cre mice were also generated for controls in some experiments. A second line of low-expressing MHC-PPARα mice were subjected to the same breeding strategy to generate an independent line of animals with LpL deficiency.

LpL Activity Assay

Frozen hearts obtained from mice after a 4-hour fast were homogenized and used for LpL activity assays as described by Hocquette et al.4

VLDL Uptake Studies

Isolated VLDL was labeled with [C¹⁴]-triolein (C¹⁴-TG) (Perkin Elmer, Boston, MA) or [1α,2α(n)-³H] cholesteryl oleoyl ether (³H-CE) (Amersham Pharmacia Biotech, UK). Labeled VLDL preparations were dialyzed against saline containing 1mg/ml EDTA and filtered through 0.45μm syringe filters. The filtered [C¹⁴]- and [³H]-labeled VLDL preparations were mixed at a 1:10 ratio such that each mouse received a dose of 1 x 10⁵ dpm [C¹⁴] VLDL and 1 x 10⁶ dpm
\[^{3}\text{H}\] VLDL via the tail vein. Blood was collected via retro orbital bleeding at 0.5, 10, and 30 min after injection. At 30 min, the heart was perfused with heparinized PBS (1000 U/kg) and the tissues were harvested, flash frozen, and homogenized in PBS. Radioactive counts were obtained in plasma and tissue homogenates using a scintillation counter (Beckman LS6000IC).

**Plasmid Constructs and Expression Vectors**

The previously described Gal4–PPARα ligand binding domain (Gal4-PPARα-LBD) construct was obtained from Jonathan Brown at Harvard Medical School. The UAS\textsubscript{3}tk.luc and simian virus 40 promoter-driven *Renilla* luciferase promoter have been described previously. Silencer Select™ siRNAs against CD3 (s130863) and the control siRNA (catalog # 4390843) were purchased from Applied Biosystems (Foster City, CA).

**Cell Culture Reagents**

VLDL was isolated by FPLC from pooled plasma obtained from Ob/Ob mice. Protein content of VLDL was determined using a Pierce BCA assay kit (Rockford, IL). Oleate and LpL were obtained from Sigma (St. Louis, MO). DHA and palmitate were obtained from Nu-Check Prep (Elysian, MN) and complexed to BSA as previously described.

**Transient Transfection Assays**

UAS\textsubscript{3}tk.luc reporter plasmid (4µg/mL) was cotransfected with the Gal4-PPARα-LBD (0.5µg/mL) and simian virus 40 promoter-driven *Renilla* luciferase (600 ng/mL), to control for transfection efficiency. All transfection data are presented as means ± standard errors (SE) for at least three separate transfection experiments done in triplicate. 14-16 hours following
transfection, cells were washed 4 times with HBSS and serum-free differentiation medium (100\(\mu\)mol/L BrdU, 2mmol/L L-glutamine, 10\(\mu\)g/mL transferrin, 10ng/mL insulin, 0.5mg/mL FA free BSA in DMEM containing 1g/L glucose) was added to the cells. After 4 hours, cells were washed again and fresh differentiation medium was added. 12 hours later, media was replaced with differentiation medium containing either BSA (10% solution of FA free BSA), oleate (100\(\mu\)mol/L), VLDL (10\(\mu\)g protein/mL), LpL (5 units/mL) or VLDL + LpL. The complexed FAs were added to differentiation medium to achieve final concentrations of 400\(\mu\)mol/L for DHA and 500\(\mu\)mol/L for palmitate. For experiments with tetrahydrolipostatin (THL), THL was initially dissolved in DMSO (0.4mmol/L) and then added to differentiation medium to a final concentration of 2\(\mu\)mol/L.

For TG depleted VLDL experiments, original VLDL was incubated at 37 degrees for 3 hours in DMEM (1g/L glucose) containing 3% FA free BSA with LpL (TG-depleted) and without LpL (control) at a ratio of 5units LpL/10ug VLDL protein. VLDL and TG-depleted lipoprotein particles were then re-isolated by ultracentrifugation (18hr at 200,000g) after adjusting the density of the solution to 1.21g/L with KBr. The KBr was then removed from the sample by dialysis against saline. Importantly, the FFA released from VLDL during LpL incubation were bound to the albumin so they were not floating with the lipoproteins during re-isolation. Cholesterol and TG content of the original, control and TG-depleted VLDL were quantified. Equivalent amounts of VLDL cholesterol were added to the cells for original, control and TG-depleted VLDL samples. Cells were harvested 12-14 hours after stimulation with different FA or lipid containing media and luciferase assays were performed using Dual-glo (Promega Corporation) and read on a BioTek Clarity plate reader per manufacturer’s instructions.
REFERENCES


Supplemental Table 1. Plasma lipid levels.

<table>
<thead>
<tr>
<th></th>
<th>Free Fatty Acids</th>
<th>Triacylglycerol</th>
<th>Total Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.85 ± 0.07</td>
<td>169 ± 23</td>
<td>143 ± 25</td>
</tr>
<tr>
<td>hsLPLko</td>
<td>1.18 ± 0.06</td>
<td>226 ± 50</td>
<td>181 ± 11</td>
</tr>
<tr>
<td>MHC-PPARα</td>
<td>1.12 ± 0.08</td>
<td>215 ± 80</td>
<td>178 ± 24</td>
</tr>
<tr>
<td>MHC-PPARα/hsLPLko</td>
<td>1.11 ± 0.14</td>
<td>230 ± 33</td>
<td>148 ± 19</td>
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</table>

High fat fed mice were fasted for 4 hours and blood was taken from the inferior vena cava for analysis. Values are mean ± SEM. N=4-7/group
Supplemental Table 2. Transthoracic Echocardiography Measurements on High Fat Diet.

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>hsLpLkko</th>
<th>MHC-PPARα</th>
<th>MHC-PPARα/hsLpLkko</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HR</strong></td>
<td>745±12.1</td>
<td>736±18.9</td>
<td>584±29.7*</td>
<td>684±19</td>
</tr>
<tr>
<td>LVPWd</td>
<td>0.77±0.05</td>
<td>0.72±0.03</td>
<td>0.71±0.05†</td>
<td>0.72±0.03</td>
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<tr>
<td>IVSd</td>
<td>0.81±0.03</td>
<td>0.66±0.04</td>
<td>0.92±0.07†</td>
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<td>LVPWs</td>
<td>1.51±0.05</td>
<td>1.53±0.05</td>
<td>1.29±0.09</td>
<td>1.48±0.1</td>
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<tr>
<td>IVSs</td>
<td>1.70±0.06</td>
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<td>1.59±0.1</td>
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<tr>
<td>LVIDs</td>
<td>1.25±0.2</td>
<td>1.13±0.08</td>
<td>3.13±0.3†</td>
<td>2.00±0.4</td>
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<tr>
<td>LVIDd</td>
<td>3.31±0.2</td>
<td>3.22±0.1</td>
<td>4.59±0.2†</td>
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<td>LVMI</td>
<td>2.45±0.09</td>
<td>2.44±0.07</td>
<td>4.17±0.2*</td>
<td>3.25±0.4</td>
</tr>
</tbody>
</table>

Value represent mean ± SEM. * p < 0.05 vs all other genotypes, † p < 0.05 vs WT; n= 7-8 per group. HR, indicates heart rate; LVPWd, left ventricular posterior wall thickness at diastole; IVSd, interventricular septal wall thickness at diastole; LVPWs, left ventricular posterior wall thickness at systole; IVSs, interventricular septal wall thickness at systole; LVIDs, left ventricular internal diameter at systole; LVIDd, left ventricular internal diameter at diastole; LVMI, left ventricular mass indexed to body weight.
SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1: Schematic of breeding strategy for generating MHC-PPARα/hsLPLko animals

Supplemental Figure 2: LpL and PPARα expression in hearts of experimental animal groups. A) Quantitative real-time RT-PCR analysis of cardiac transcripts encoding LpL or PPARα in WT, MHC-PPARα, hsLpLko, MHC-PPARα and MHC-PPARα/hsLpLko (n=7-9/group). All bars represent mean (± SE) arbitrary unit (AU) normalized to the WT value (=1.0) in each case. *p<0.05 vs WT. B) LpL activity in MHCPPARα and MHCPPARα/hsLPLko animals. Bars represent mean (± SE) umoles of FFA/gm heart tissue/hour

Supplemental Figure 3: CD36 mRNA expression is not significantly different between groups. Bars represent mean (± SE) mRNA levels of CD36 by quantitative real-time RT-PCR normalized to the WT value (=1.0).

Supplemental Figure 4: CD36 expression levels in NRVM after transfection with siRNA. Quantitative real-time RT-PCR analysis from RNA extracted from NRVMs plated in 6-well plates and co-transfected with Gal4-PPARα-LBD and siRNA for either control (siCtrl) or CD36 (siCD36), (n=6/group). All bars represent mean (± SE) arbitrary unit (AU) normalized to the siCtrl value (=1.0), *p<0.05 vs siCtrl.
Supplemental Figure 5: DHA and oleate activate PPARα similarly. A) Transient co-transfections using the UAS₃tk.luc heterologous reporter and the Gal4-PPARα-LBD performed in NRVM. Cells were stimulated for the last 12-14 hours with either BSA, 100 µmol/L oleate, 400 µmol/L DHA, 500 µmol/L palmitate. The bars represent mean (±SE) relative light units (RLU) for three experiments each done in triplicate, corrected for Renilla luciferase activity, and normalized to the activity of cells stimulated with BSA (= 1.0).
Experimental Groups:

- LPL<sup>ff</sup> MHC-PPAR<sup>TG</sup> Cre<sup>+</sup>
- LPL<sup>ff</sup> MHC-PPAR<sup>TG</sup> Cre<sup>-</sup>
- LPL<sup>ff</sup> MHC-PPAR<sup>WT</sup> Cre<sup>+</sup>
- LPL<sup>ff</sup> MHC-PPAR<sup>WT</sup> Cre<sup>-</sup>
Supplemental Figure 2

A

![Graph showing normalized AU for PPARα and LPL](image)

B

![Graph showing umole FFA/g heart/hour](image)
Supplemental Figure 3

CD36

- WT
- hsLPLko
- MHC-PPARα
- MHCP-PPARα/hsLPLko

Normalized AU