Long Chain Fatty Acid Storage Dynamics and Nuclear Receptor Activation in Failing Hearts

BY

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THESIS

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“Life’s most persistent and urgent question is: ‘What are you doing for others?’”
[Dr. Martin Luther King, Jr.]

This work is dedicated to my loving parents. Thank you for fostering my intellectual curiosity over the years and for teaching me, through words and example, the importance of humility and selflessness.

RL
ACKNOWLEDGMENTS

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Significant portions of this thesis, including text, figures, and tables, have been published as a manuscript in the journal *Circulation* and are reproduced here in Chapters I-III with permission of the publisher (Appendix A). The full citation is included below as an acknowledgement.

PREFACE

The research presented in this dissertation is just a small piece of a substantial body of work produced by the lab of Dr. E. Douglas Lewandowski, elucidating the mechanisms of maladaptive metabolic changes known to occur in the failing heart.
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<td>-dP/dt</td>
<td>Rate of relaxation</td>
</tr>
<tr>
<td>+dP/dt</td>
<td>Rate of pressure development</td>
</tr>
<tr>
<td>$^{13}$C</td>
<td>Carbon-13</td>
</tr>
<tr>
<td>$^{31}$P</td>
<td>Phosphorus-31</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Ago2</td>
<td>Argonaute-2</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATGL</td>
<td>Adipose triglyceride lipase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchininic acid</td>
</tr>
<tr>
<td>bpm</td>
<td>Beats per minute</td>
</tr>
<tr>
<td>CALSEQ</td>
<td>Calsequestrin</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>CPT1a</td>
<td>Carnitine palmitoyltransferase 1a (liver isoform)</td>
</tr>
<tr>
<td>CPT1b</td>
<td>Carnitine palmitoyltransferase 1b (muscle isoform)</td>
</tr>
<tr>
<td>CT</td>
<td>Treshold cycle</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DCA</td>
<td>Dichloroacetate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>DGAT1</td>
<td>Diacylglycerol acyltransferase 1</td>
</tr>
<tr>
<td>DGCR8</td>
<td>DiGeorge syndrome critical region 8</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithio-bis(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>dw</td>
<td>Dry weight</td>
</tr>
<tr>
<td>ERR</td>
<td>Estrogen-related receptor</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FATP1</td>
<td>Fatty acid transport protein 1</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>GOT</td>
<td>Glutamate-oxaloacetate transaminase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione (reduced glutathione)</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide (oxidized glutathione)</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>HSD</td>
<td>Honestly standard difference</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long-chain fatty acid</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>LVDP</td>
<td>Left ventricular developed pressure</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>MAG</td>
<td>Monoacylglycerol</td>
</tr>
<tr>
<td>MDH</td>
<td>Malate dehydrogenase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ME1</td>
<td>Malic enzyme 1</td>
</tr>
<tr>
<td>MED13</td>
<td>Mediator complex subunit 13</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NRF1</td>
<td>Nuclear respiratory factor 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCr</td>
<td>Phosphocreatine</td>
</tr>
<tr>
<td>PDC</td>
<td>Pyruvate dehydrogenase complex</td>
</tr>
<tr>
<td>PFK</td>
<td>Phosphofructokinase</td>
</tr>
<tr>
<td>PGC-1</td>
<td>Peroxisome proliferator-activated receptor-gamma coactivator-1</td>
</tr>
<tr>
<td>P_i</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>pre-miRNA</td>
<td>Precursor miRNA</td>
</tr>
<tr>
<td>pri-mRNA</td>
<td>Primary miRNA</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
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<tr>
<td>RPP</td>
<td>Rate-pressure product</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>rt-PCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoic X receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>T</td>
<td>Tesla</td>
</tr>
<tr>
<td>TAC</td>
<td>Transverse aortic constriction</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TG</td>
<td>Triglyceride</td>
</tr>
<tr>
<td>TNB</td>
<td>5'-thio-2-nitrobenzoic acid</td>
</tr>
<tr>
<td>TRBP</td>
<td>TAR (Trans-activation response) RNA binding protein</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinidione</td>
</tr>
<tr>
<td>VAD</td>
<td>Ventricular assist device</td>
</tr>
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</table>
Intramyocardial triglyceride (TG) turnover is reduced in hypertrophied, failing hearts, limiting availability of this rich source of stored long-chain fatty acids (LCFAs) for mitochondrial oxidation and nuclear receptor activation. The work presented here demonstrates that the two major dietary LCFAs, palmitate and oleate, differentially influence TG turnover in normal and hypertrophied rat hearts with dramatic effects on contractility and transcription. In contrast to palmitate, oleate induced normal TG content and elevated turnover rates in decompensated, hypertrophic hearts. Normalized lipid dynamics with oleate in the hypertrophied heart resulted in normalized peroxisome proliferator-activated receptor-α (PPAR-α) target gene expression and mitochondrial oxidation of TG. Oleate supply to failing hearts also averted an increase in the lipotoxic acyl intermediate C16 ceramide, associated with improved cardiac contractility. The findings link reduced intracellular lipid storage dynamics to impaired PPAR-α signaling and contractility in diseased hearts and indicate a rate-dependent lipolytic activation of PPAR-α. In decompensated hearts, oleate may serve as a beneficial energy substrate versus palmitate, to confer improved TG dynamics and nuclear receptor signaling.

As part of an overall programmatic shift in lipid handling enzymes, hypertrophied hearts demonstrate increased cytosolic malic enzyme 1 (ME1) expression which upregulates anaplerotic flux into the second span of the cycle by carboxylating glycolytic pyruvate, forming malate. ME1 is known to be lipogenic in other tissue types, generating NADPH and pyruvate from malate. However, reverse flux that consumes pyruvate and NADPH in hypertrophied myocardium appears to be maladaptive, as this alternative route of
entry into the TCA cycle is inefficient compared to pyruvate decarboxylation into acetyl-CoA by pyruvate dehydrogenase complex (PDC). Numerous NADH-producing reactions are bypassed, contributing to a net loss of ATP per glycolytic carbon. Furthermore, the carboxylation reaction consumes NADPH, depleting the cardiomyocyte of a critical cofactor for maintaining TG stores and intracellular redox state. We tested the effects of cardiac-specific knockdown of ME1 in hypertrophied hearts via delivery of adenovirus containing non-native microRNA targeted to ME1 mRNA. Knockdown of ME1 returned anaplerosis to normal low levels and increased glutathione content in hypertrophy. Ongoing work in this study will elucidate further beneficial effects of ME1 knockdown on intramyocardial lipid dynamics, glucose oxidation, and function in failing hearts.
I. INTRODUCTION

A. Heart failure

Heart failure is a complex, multifactorial syndrome in which cardiac output is insufficient to support tissue oxygen and nutrient demands. Heart failure is a significant cause of morbidity and mortality in the United States, affecting over 5 million people. The 5-year survival rate is roughly 50% (Go et al., 2013), worse than many types of cancer. Indeed, about a third of heart failure patients will die within one year of diagnosis (McMurray and Pfeffer, 2005). At a cost of $32 billion to the nation annually (Heidenreich et al., 2011), heart failure imposes a significant financial burden that is widely expected to increase in the future due to aging population and the obesity epidemic.

In the United States, the most common risk factors for developing heart failure are ischemic heart disease, cigarette smoking, uncontrolled hypertension, obesity and type II diabetes (He et al., 2001). Diagnosis is often made through clinical evaluation, including patient history and physical exam, and supported with findings from imaging studies such as chest X-ray and echocardiography. Treatment of heart failure is multifaceted, involving lifestyle modification, medication, and implantable pacing devices.

Heart failure is a chronic illness that rarely goes away completely. In patients with advanced, end-stage heart failure, heart transplantation is the preferred treatment option (Kittleson and Kobashigawa, 2011). Patients who are not candidates to receive
a transplant, or for whom a suitable transplant is not available, may be implanted with a ventricular assist device (VAD) to aid the heart in pumping blood. In the time since the first VAD was successfully used in 1984 (Portner et al., 1985), significant technological advances have made VADs effective therapy as bridge to transplant, bridge to recovery, or destination therapy (Osaki et al., 2008). Despite major progress in treatment, heart failure is still associated with 1 in 9 deaths annually in the United States (Go et al., 2013), and the search for more effective treatments is a constant challenge in the field of cardiology and cardiovascular sciences.

B. **The heart is one of the largest consumers of energy in the body**

In performing its main function of circulating blood throughout the body, the heart will beat on average 100,000 times per day, with a daily consumption of ATP equal to 20 times its own weight (Neubauer, 2007). In order to satisfy its energy requirements, the heart has a highly regulated metabolism that is tightly coupled to rapidly changing energy demands to adequately regenerate ATP from ADP and AMP. The heart is an omnivore, and will use any available fuel source to meet its energy demands. Under normal conditions, the heart derives the majority of its energy from long-chain fatty acids (LCFAs) through β-oxidation, with minor contributions from glucose, lactate, and ketone utilization making up the balance (O’Donnell et al., 2008; Pound et al., 2009).

C. **The failing heart is energy-starved**

The concept of the failing heart as an energy-starved organ was first proposed in 1939 by two physicians from University of Texas, George Hermann and George
Decherd, in an article entitled “The Chemical Nature of Heart Failure” (Herrmann and Decherd, 1939). These two physicians were the first to describe decreased creatine content in the failing myocardium. Indeed, it has since been observed that increased workload and adrenergic tone lead to decreased levels of ATP and the reserve pool of high-energy phosphate, phosphocreatine (PCr) (Ingwall and Weiss, 2004). However, the pathogenic metabolic changes occurring in stressed myocardium extend far beyond ATP synthesis. Limitations in substrate utilization, disruptions in tricarboxylic acid (TCA) cycle flux, and shifts in metabolic gene expression all contribute to impaired metabolic reserve and the progression towards overt failure (Carley et al. 2014). The study of metabolism in the heart is a topic of considerable research interest because, as opposed to tissue that has been infarcted, the myocardium of failing hearts is viable, and can be rescued by correcting the deranged metabolism.

D. **Inefficient energy production in pressure overload hypertrophy**

As a response to chronically increased systemic vascular resistance, the heart undergoes an initial, compensatory hypertrophy that progresses to a state of decompensation and eventually overt failure characterized by insufficient ATP levels to maintain cardiac function (Ingwall, 2009). The failing heart has been broadly characterized as reverting to a metabolic profile resembling that of the fetal heart, with reduced fatty acid oxidation and increased carbohydrate utilization (Sack et al., 1996). The metabolic remodeling in the hypertrophied myocardium has dramatic effects on carbon flux into and out of the TCA cycle and on the dynamic processes of lipid storage
and utilization that lead to impaired cardiac function (Chiu et al., 2005; O'Donnell et al., 2008; Sorokina et al., 2007; Pound et al., 2009).

In the absence of impaired oxygen delivery to tissue in the hypertrophied myocardium (Murakami, et al., 1999), LCFAs are a more efficient fuel source than carbohydrate for oxidative ATP production, generating more ATP per mole of substrate. Furthermore, compared to fat, carbohydrate is highly hydrated; 1 g of glycogen binds about 2 g of water. Consequently, 1 g of stored fat can be used to produce nearly six times the amount of ATP as 1 g of stored carbohydrate (Berg et al., 2002). Thus, reduced fatty acid contribution to mitochondrial oxidative ATP production in hypertrophied hearts represents a net loss of ATP per mole of oxidized substrate. Importantly, our lab was the first to expose several key metabolic inefficiencies in hypertrophied hearts discussed below.

E. Impaired triglyceride turnover in pressure overload hypertrophy

In the normal heart, LCFA oxidation provides more than 70% of the ATP that supports cardiac function (Sorokina et al., 2007; O'Donnell et al., 2008; Pound et al., 2009). Alternatively, following transport into the cell, LCFAs may be incorporated into the endogenous lipid storage pool as triglyceride (TG) by action of the rate-limiting enzyme for TG synthesis in myocardium, diacylglycerol acyltransferase 1 (DGAT1) (Goldberg et al., 2012; Carley et al., 2013). TG turnover, the dynamic reciprocal processes of LCFA esterification into and release from TG, is reduced in hypertrophied rat hearts supplied palmitate (16:0) (O'Donnell et al., 2008), a finding not obvious from
TG content alone. Interestingly, a cell culture study comparing the two major dietary fats, palmitate (16-carbon, saturated) and oleate (18-carbon, monounsaturated) demonstrated that the intracellular fate (storage versus oxidation) of exogenous LCFAs depends upon chain length and saturation (Listenberger et al., 2003). Whether such differential handling of palmitate and oleate occurs in the intact heart, and whether it can be exploited to affect TG pool size and turnover in hypertrophy, is a major focus of the work presented in this dissertation.

1. Elimination of triglyceride oxidation

Given the established role of TG-derived LCFA to support mitochondrial β-oxidation (Banke et al., 2010), a portion of the reduced LCFA oxidation rates in hypertrophied hearts is caused by a marked reduction in the oxidation of stored TG which cannot be restored by adrenergic stimulation (O'Donnell et al., 2008). Thus, TG as an energy source becomes essentially unavailable, coinciding with reduced LCFA trafficking into and out of the TG pool.

2. Consequences for nuclear receptor activation

TG turnover rate is augmented by activation of the nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α), a transcription factor whose target genes support TG synthesis and hydrolysis (Banke et al., 2010) and fatty acid utilization (Barger et al., 2000). Coinciding with reduced rates of TG turnover (O'Donnell et al., 2008), hypertrophied hearts demonstrate decreased signaling through PPAR-α (Sack et al., 1996; Barger et al., 2000; Planavila et al., 2005; Luptak et al.,
2005; Chokshi et al., 2012). Interestingly, recent evidence implicates TG lipolysis, mediated in myocardium by the rate-limiting TG lipase adipose triglyceride lipase (ATGL), to play a major role in supplying ligand for nuclear receptor activation (Haemmerle et al., 2011). Thus, whether reduced TG turnover in the hypertrophic heart is a cause or a consequence of impaired PPAR-α signaling is critical in determining etiology of the low-energy state that contributes to progressive decompensation of the pressure overloaded heart toward overt failure (Ingwall and Weiss, 2004; Neubauer, 2007).

3. **Lipotoxicity**

The potential exists for any limitations in LCFA oxidation and esterification into TG to contribute to lipotoxicity, with consequences for the pathogenesis of myocardial dysfunction (Listenberger et al., 2003; Chiu et al., 2005). In particular, hindered incorporation of palmitate into TG may increase its availability for other intracellular biosynthetic reactions such as formation of potentially lipotoxic acyl derivatives like ceramide, which has been shown to induce apoptotic signaling (Aflaki et al., 2012) and influence contractility by acting on the sarcomere (Chiu et al., 2001; Relling et al., 2003; Guinamard et al., 2004; Landeen et al., 2007).

F. **Inefficient glucose metabolism in pressure overload hypertrophy**

Hypertrophied hearts demonstrate an apparent uncoupling between glycolysis and oxidation of glycolytic end products: pyruvate decarboxylation to form acetyl-CoA, mediated by the pyruvate dehydrogenase complex (PDC), does not keep pace with
increased glycolytic flux (Lydell et al., 2002; Leong et al., 2003). This discrepancy was recently accounted for by studies in our lab demonstrating that hypertrophied hearts shift carbohydrate oxidation away from PDC-mediated entry into the TCA cycle and toward anaplerosis via cytosolic NADPH-dependent malic enzyme 1 (ME1) (Sorokina et al., 2007). ME1 carboxylates pyruvate, forming malate, which enters the second span of the TCA cycle. Increased anaplerotic flux in hypertrophied hearts serves to counter decreased carbon flux into the TCA cycle due to reduced fatty acid oxidation.

Compared to oxidation via PDC, the alternative route for carbon entry into the TCA cycle facilitated by ME1 is inefficient, bypassing numerous reactions that would otherwise generate NADH for oxidative production of ATP. In addition, carboxylation of pyruvate by ME1 consumes NADPH, a cofactor for lipid synthesis and glutathione reduction to maintain cellular redox state.

In adipocytes, ME1 expression increases with high fat feeding (Zabala et al., 2006). In this context, ME1 is a lipogenic enzyme catalyzing NADPH production through the conversion of malate into pyruvate. NADPH then serves as a reducing agent for conversion of acyl-dihydroxyacetone phosphate into acyl-glycerophosphate, an initial step in TG formation. In hypertrophied hearts, reverse flux through ME1 (pyruvate to malate) consumes NADPH and may be a contributing factor to impaired TG content and formation (O'Donnell et al., 2008; Chokshi et al., 2012). Indeed, treating perfused hypertrophied hearts with dichloroacetate (DCA), an activator of PDC, induced
competition with ME1, partially attenuated increased anaplerosis, and restored TG content (Pound et al., 2009).

Increased conversion of pyruvate to malate by ME1 in heart may hold consequences for other cellular processes that require NADPH such as maintenance of cellular redox state. NADPH is used to maintain glutathione in its reduced form (GSH) and the ratio of reduced-to-oxidized glutathione (GSH:GSSG) reflects the overall redox state of the cell (Jain et al., 2003; Jain et al., 2004). Imbalanced cellular redox state causes contractile dysfunction and is reported to be a contributing factor to the pathophysiology of heart failure (Giordano, 2005), potentially linking increased ME1-mediated consumption of NADPH with myocardial oxidative stress in hypertrophy.

G. Peroxisome proliferator-activated receptors

Transcriptional regulation of myocardial energy metabolism is carried out by a family of transcription factors known as peroxisome proliferator-activated receptors (PPARs), which drive the expression of a number of genes related to fatty acid and carbohydrate metabolism. Activation of PPARs occurs through binding of ligand, dimerization with the retinoic X receptor (RXR) and coactivation with PPAR-γ coactivator-1α or β (PGC-1α or PGC-1β) (Lehmann et al., 2000). The receptor then translocates to the nucleus and binds to PPAR response elements in the promoter regions of target genes to increase transcription (Lopaschuk et al., 2010). The PPARs are activated by binding fatty acids as well as leukotrienes and eicosanoids (Huss and Kelly, 2004) and occur as three different isoforms: PPAR-α, PPAR-β/δ, and PPAR-γ.
1. **Peroxisome proliferator-activated receptor alpha**

PPAR-α is an important regulator of fatty acid metabolism and highly expressed in oxidative tissues, including heart (Lefebvre et al., 2006). Its target genes are involved in fatty acid uptake (CD36), mitochondrial β-oxidation (medium-chain, long-chain and very-long-chain acyl CoA dehydrogenases, carnitine palmitoyltransferase 1b), fatty acid storage, and glucose metabolism (pyruvate dehydrogenase kinase 4) (Huss and Kelly, 2004). Cardiac-specific overexpression of PPAR-α resembles the phenotype of the diabetic heart, demonstrating increased fatty acid uptake and oxidation and marked intracellular lipid accumulation (Finck et al., 2002). PPAR-α overexpression augments TG turnover rate, leading to greater oxidation of endogenous TG (Banke et al., 2010). While the precise endogenous ligand for PPAR-α activation has yet to be identified, it was recently shown that hydrolysis of intracellular TG, mediated in heart by the enzyme adipose triglyceride lipase (ATGL), is required for biological activity of PPAR-α target gene expression. In the pathologic setting of chronic pressure overload, hearts demonstrate decreased signaling through nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α) (Sack et al., 1996; Barger et al., 2000; Planavila et al., 2005; Luptak et al., 2005; Chokshi et al., 2012), coinciding with reduced rates of fatty acid oxidation (Sorokina et al., 2007; Lewandowski et al., 2013) and lipid turnover (O'Donnell et al., 2008).

2. **Peroxisome proliferator-activated receptor beta**

PPAR-β (also known as PPAR-δ) is ubiquitously expressed and exists at high levels in cardiomyocytes. Studies in cultured cardiomyocytes showed that PPAR-β
activation induces LCFA oxidation and regulates many of the same genes as PPAR-α (Gilde et al., 2003). In contrast to cardiac-specific overexpression of PPAR-α, mouse hearts overexpressing PPAR-β do not accumulate lipid or develop cardiomyopathy (Burkart et al., 2007). Furthermore, PPAR-β overexpressors demonstrated increased mRNA levels of glucose transporter GLUT4 and phosphofructokinase (PFK), while PPAR-α overexpressors had decreased levels of these genes (Burkart et al., 2007). Therefore, PPAR-β may have a metabolic regulatory role distinct from PPAR-α, yet little is known about any potential role of PPAR-β in cardiac hypertrophy.

3. **Peroxisome proliferator-activated receptor gamma**

PPAR-γ is highly expressed in white adipose tissue and is involved in coordinating gene expression for fatty acid storage and lipogenesis (Finck, 2007). PPAR-γ is expressed at very low levels in myocardium, and is thought to play an indirect role in heart metabolism by nature of its activity in adipose tissue (Gilde et al., 2003). A class of PPAR-γ agonist drugs, thiazolidinediones (TZDs), which reduce insulin resistance and circulating fatty acids are approved for the treatment of type II diabetes mellitus. The use of TZDs is contraindicated in the presence of heart failure because a common side effect is weight gain and fluid retention.

4. **Peroxisome proliferator-activated receptor gamma coactivator 1-alpha**

PGC-1α, a transcriptional coactivator of PPARs, is highly expressed in oxidative tissues, including brown adipose tissue, kidney, and heart (Puigserver et al.,
1998; Lehman et al., 2000). It is rapidly induced in response to exercise (Baar et al., 2002), and given its wide range of biological targets, PGC-1α is considered to be a master regulator of enhanced ATP production and mitochondrial biogenesis. Through interactions with PPAR-RXR heterodimer, PGC-1α augments transcription of PPAR target genes involved in fatty acid oxidation (Finck and Kelly, 2007). PGC-1α also acts as a coactivator of estrogen-related receptor α (ERRα) to induce expression of genes involved in oxidative phosphorylation as well as nuclear respiratory factor 1 (NRF1) to induce mitochondrial gene transcription and replication (Finck and Kelly, 2007). PGC-1α expression is decreased in animal models of cardiomyopathy (Garnier et al., 2003; Sano et al., 2004), evidently impairing the activity of PPARα-RXR complex and contributing to the overall reduced rates of fatty acid oxidation and other metabolic abnormalities characteristic of the failing heart.

5. **Peroxisome proliferator-activated receptor gamma coactivator 1-beta**

The role of PGC-1β in normal and pathophysiological states has not been as extensively studied as PGC-1α, although it likely shares many of the same properties (Rowe et al., 2010). A rather intriguing study identified two microRNAs (miRNAs) encoded on the PGC-1β gene that counteract the metabolic activity of PGC-1β and inhibit MED13, a subunit of the Mediator complex (Carrer et al., 2012). MED13 has been shown to control a metabolic gene program in the heart, through which the heart can regulate whole-body adiposity (Grueter et al., 2012). These studies are part of a growing body of evidence that implicates the heart as a central regulator of systemic
energy homeostasis with endocrine function to influence activity of liver, adipose, and other insulin-sensitive tissues (Taegtmeyer and Rodriguez, 2012).

H. Nuclear magnetic resonance spectroscopy as a technique for studying metabolic flux

$^{13}$C nuclear magnetic resonance (NMR) spectroscopy is an indispensable tool for studying metabolic processes in the intact beating heart. $^{13}$C nuclei have a low natural abundance of 1.1% and demonstrate a wide range of chemical shifts, enabling exogenous delivery of $^{13}$C-enriched substrate, such as glucose or fatty acid, for studying intermediary metabolism. $^{13}$C NMR has been used to study a variety of processes such as transporter activity, compartmentation of metabolic intermediates, and oxidation rates under both physiologic and pathophysiologic conditions. In the work presented here, $^{13}$C NMR spectroscopy is used to assess intramyocardial lipid storage dynamics by monitoring the progressive incorporation of exogenous LCFA into the steady-state TG pool and TCA cycle flux in normal and hypertrophic hearts.
II. LIPID STORAGE DYNAMICS AND OXIDATION IN THE FAILING HEART ARE INFLUENCED BY SUPPLY OF DIETARY FAT

A. Introduction

Long-chain fatty acids (LCFAs) are the preferred and most carbon-efficient substrates for oxidative ATP production in the heart in the absence of impaired oxygen delivery (Saddik and Lopaschuk, 1991; Gong et al., 2003; Sorokina et al., 2007; O’Donnell et al., 2008; Ingwall, 2009). The pressure overloaded failing heart has been well-characterized as having reduced LCFA oxidation and elevated, albeit impaired and NADH-inefficient, reliance on carbohydrate metabolism (Sorokina et al., 2007; O’Donnell et al., 2008; Pound et al., 2009). The intracellular lipid, stored as triglyceride (TG), has recently been identified as a significant source of fuel for mitochondrial oxidative metabolism in the heart (Banke et al., 2010) and has also been implicated as a dynamic source of lipolytic signaling for transcriptional activation of metabolic gene expression (Haemmerle et al., 2011). In failing hearts of non-obese human patients, TG content is reduced (Chokshi et al., 2012), and in animal models of pressure overload-induced heart failure the reduced TG becomes static, no longer supporting oxidative metabolism (O’Donnell et al., 2008). This study compared TG dynamics and oxidation in failing rat hearts as supported by the two most common dietary fats, palmitate (16:1) and oleate (18:0).

Among dietary fats that fuel the heart, palmitate and oleate together comprise approximately 60% of the circulating LCFAs in serum from healthy human subjects (Püttman et al., 1993). Following uptake into the cardiomyocyte, LCFAs may be
oxidized by mitochondria or incorporated into the endogenous lipid stores as TG (Goldberg et al., 2009; Carley and Kleinfeld, 2011; Goldberg et al., 2012; Carley et al., 2013). Interestingly, studies in cell culture suggest palmitate and oleate may be channeled to distinct metabolic fates (Listenberger et al., 2003). Given the reduced lipid content and turnover reported in failing heart from both human patients (Chokshi et al., 2012) and animal models (O’Donnell et al., 2008), these major dietary fats hold potential to affect TG pool size and dynamics in diseased myocardium.

B. Methods

1. Animal model of pressure overload-induced hypertrophy

Cardiac hypertrophy by chronic pressure overload was induced by constricting the transverse aorta (hemoclip) of three-week-old male Sprague Dawley rats, as previously described (Sorokina et al., 2007; O’Donnell et al., 2008; Pound et al., 2009; Lewandowski et al., 2013). This transverse aortic constriction (TAC) procedure relies on the natural growth of the animal to produce a gradually increasing degree of aortic constriction. The rats develop a concentric hypertrophy and increased heart weight, heart weight-to-body weight ratio, and heart weight-to-tibia length ratio (Figure 1) associated with short-term improvement in the systolic function of the heart (Vatner and Ingwall, 1986; Sack et al., 1996; Ribeiro et al., 2003; O’Donnell et al., 2008). At 12 weeks post-surgery, the animals enter a decompensated stage with depressed left ventricular developed pressure (LVDP) and rate of pressure development (dP/dt). In this model of left ventricular hypertrophy, no systemic activation of the sympathetic nervous system or of the renin-angiotensin-aldosterone system occurs (Ribeiro et al.,
Consequently, there are no signs of cardiac lesions, peripheral arteritis, myocardial necrosis, or extensive fibrosis. The rats progress to a dilated cardiac hypertrophy with acute end-stage heart failure at 4-6 months post-surgery. The sham groups underwent similar surgery without placement of the aortic band (SHAM). Rats had free access to food and water while being housed under controlled temperature and lighting. All experimental procedures were approved by the University of Illinois at Chicago Animal Care and Use Committee.

**Figure 1.** Chronic pressure overload induces cardiac hypertrophy in male Sprague-Dawley rat hearts. Transverse aortic constriction (TAC) induced an increase in (A) heart weight, (B) heart weight-to-body weight ratio, and (C) heart weight-to-tibia length ratio 12 weeks after surgery. White bar, SHAM; black bar, TAC. (n=13 for all groups). Error bars indicate mean ± standard error of the mean (SEM). *P<0.05 versus SHAM, †P<0.05 versus TAC Oleate.
2. **Isolated heart perfusion protocol**

12 weeks post-surgery, animals were heparinized (1000 IU, intraperitoneal injection) and anesthetized (100 mg/kg pentobarbital, intraperitoneal injection). Hearts were excised and retrogradely perfused with modified Krebs-Henseleit buffer (in mmol/L: 116 NaCl, 4 KCl, 1.5 CaCl$_2$, 1.2 MgSO$_4$ and 1.2 NaH$_2$PO$_4$) equilibrated with 95% O$_2$/5% CO$_2$ and containing 0.4 mmol/L $^{12}$C palmitate or 0.4 mmol/L $^{12}$C oleate complexed to bovine serum albumin in a 3:1 molar ratio, 5 mmol/L $^{12}$C glucose and 1 mmol/L $^{12}$C sodium lactate. Buffer temperature was maintained at 37°C. A water-filled latex balloon, connected to a force transducer, was fitted into the left ventricle (LV) and set to a diastolic pressure of 5 mmHg. LV developed pressure (LVDP) data was continuously acquired during perfusion with Powerlab (ADInstruments, Dunedin, New Zealand). Rate-pressure product (RPP) was calculated as heart rate (HR) × LVDP, and mean peak rates of pressure development and relaxation (+ and -dP/dt) were calculated from the first derivative of the LVDP trace. Functional data are reported at midpoint of perfusion and were determined to be not significantly different over the entire protocol using repeated measures ANOVA.

3. **Nuclear magnetic resonance spectroscopy**

Perfused hearts were situated in a 20 mm broadband NMR probe within a 9.4-T, vertical bore (89 mm) NMR magnet interfaced to a spectroscopy console (Avance series, Bruker, Billerica, MA). Magnetic field homogeneity was optimized by shimming to a proton line width of 20-30 Hz. The bioenergetic status of perfused hearts was determined by the relative content of phosphocreatine to ATP (PCr:ATP) (Figures 2 and
3) from $^{31}$P NMR as per methods extensively described elsewhere (Sorokina et al., 2007; O'Donnell et al., 2008). Prior to switching to a supply of isotope-enriched buffer to the heart, a $^{13}$C NMR spectrum of background signal from naturally abundant $^{13}$C (1.1%) was collected. Isotopic enrichment was initiated by switching the perfusate supply to buffer containing either 0.4 mmol/L [4,6,8,10,12,14,16-$^{13}$C$_7$] palmitate or 0.4 mmol/L [4,6,8,10,12,14,16,18-$^{13}$C$_8$] oleate, 5 mmol/L unlabeled glucose, and 1 mmol/L unlabeled sodium lactate. Enrichment continued for 40 minutes while sequential proton decoupled $^{13}$C NMR spectra (2 second interpulse interval, averaged over 2 minutes, 20 spectra total) (Figure 4) were acquired to detect the progressive isotopic enrichment of myocardial triglyceride (TG) and glutamate isotopomers (Sorokina et al., 2007; O'Donnell et al., 2008; Carley et al., 2013; Lewandowski et al., 2013). At endpoint of the enrichment protocol, hearts were removed from the magnet and oxygen consumption was determined from pulmonary artery effluent with a blood gas analyzer (GEM Premier 300, Instrumentation Laboratory) prior to freeze clamping hearts with liquid N$_2$-cooled tongs for subsequent in vitro biochemical analysis. The protocol was repeated with 5 mmol/L [1,6-$^{13}$C$_2$] glucose and 1 mmol/L sodium [3-$^{13}$C] lactate and 0.4 mmol/L unlabeled fatty acid to assess carbohydrate oxidation. Experimental groups perfused with $^{13}$C-enriched LCFA and unenriched glucose and sodium lactate were as follows: sham-operated hearts (SHAM) supplied oleate ($n=6$) or palmitate ($n=6$); hypertrophied hearts (TAC) supplied oleate ($n=6$) or palmitate ($n=6$). Experimental groups perfused with unenriched LCFA and $^{13}$C-enriched glucose and sodium lactate were as follows: sham-operated hearts (SHAM) supplied oleate ($n=3$) or palmitate ($n=3$); hypertrophied hearts (TAC) supplied oleate ($n=3$) or palmitate ($n=3$).
Figure 2. $^{31}$P NMR spectroscopy assesses energetic state of the intact perfused heart.

$^{31}$P NMR spectrum was acquired over 2 minutes and shows myocardial inorganic phosphate (P$_i$), phosphocreatine (PCr) and α-, β-, and γ-phosphate resonances of ATP. The arrow indicates the resonance peak of the intracellular P$_i$, which is shifted upstream from the buffer P$_i$ peak due to differences in pH between the buffer and intracellular environment. Spectrum is from a normal SHAM heart.
Figure 3. Phosphocreatine:ATP ratios of perfused hearts as determined by $^{31}$P NMR spectroscopy.

Hypertrophied hearts (TAC) demonstrated impaired bioenergetic state consistent with previous reports on this model (Sorokina et al., 2007; O’Donnell et al., 2008) that was unaffected by fatty acid supply. *P<0.05 versus corresponding SHAM ($n$=6-8 in each group; error bars indicate mean ± SEM).
Figure 4. Dynamic-mode $^{13}$C NMR spectroscopy. Sequential $^{13}$C spectra (with signal from the 1.1% naturally-abundant $^{13}$C subtracted) show the progressive accumulation of $^{13}$C nuclei into the methylene groups of TG (chemical shift 30.5 parts per million, ppm) as well as the 2-, 4-, and 3-carbon positions of glutamate. Spectra were acquired over 2 minutes for a total of 20 spectra during the 40 minute enrichment protocol. Spectra shown are from a normal SHAM heart perfused with [4,6,8,10,12,14,16-$^{13}$C$_7$] palmitate.
4. **Lipid extraction**

Lipids were extracted from frozen perfused heart samples as previously described (O’Donnell et al., 2006). 30-50 mg of frozen heart tissue was homogenized in 0.5 mL of PBS and extracted in 10 mL of chloroform:methanol (2:1), followed by an addition of 2 mL of methanol. Samples were vortexed and placed on ice. After 30 min, the samples were centrifuged and the pellets discarded. 2 mL of 4% CaCl$_2$ was added to the supernatant and centrifuged to separate upper and lower phases. The lower phase was washed three times with solvent (1.5 mL chloroform, 24 mL methanol, 23.5 mL H$_2$O) and then dried under vacuum at 50°C (RapidVac, Labconco, Kansas City, MO). Lipids were resuspended in 0.5 mL chloroform and divided for subsequent assay of TG content, LC/MS, and diacylglycerol (DAG) content.

5. **Triglyceride content**

Lipids were resuspended in 0.5 mL tert-butyl alcohol:Triton X-100 (3:2) and TG was quantified by calorimetric assay (Wako Pure Chemical Industries). TG content was normalized to tissue dry weight (dw).

6. **Liquid chromatography/mass spectrometry**

TG was saponified and analyzed using liquid chromatography/mass spectrometry (LC/MS) for determination of $^{13}$C fractional enrichment as previously described (Carley et al., 2013). TG, DAG, and monoacylglycerol (MAG) were separated from phospholipids by passage through Bond Elut NH2 solid-phase extraction column (Agilent). The collected effluent was dried under vacuum at 50°C (RapidVac, Labconco,
Kansas City, MO) and resuspended in 470 µL methanol 30 µL 1 M KOH for saponification at 70°C for 1 h. Samples were neutralized with 10 µL 10% formic acid and LCFAs were extracted with two washes of 500 µL hexane. LCFAs were dried under vacuum at 50°C (RapidVac, Labconco, Kansas City, MO) and resuspended in chloroform:methanol (1:1) (Optima grade).

Fractional enrichment of the LCFAs in TG was analyzed by LC/MS (Waters X Terra MS C18 3.5 µm 2.1×100 mm column; MS: scan m/z 100-600 Fragmentor 75 V Negative ESI) as previously described (O'Donnell et al., 2008; Banke et al., 2010; Carley et al., 2013) (Figure 5).
Figure 5. Endpoint LC/MS allows for determination of $^{13}$C fractional enrichment of myocardial triglyceride (TG).

(A) The chromatogram demonstrates elution of palmitate (indicated with arrow) and other fatty acid constituents of TG from the column. (B) The mass spectrum reveals that the eluted palmitate is comprised of endogenous, unenriched palmitate with mass-to-charge ($m/z$) ratio of 255, as well as exogenous $^{13}$C-enriched $[4,6,8,10,12,14,16^{-13}$C$_7]$ palmitate, with $m/z$ ratio of 262.

7. **Quantification of triglyceride turnover**

$^{13}$C enrichment of TG in intact hearts was detected from the NMR signal at 30.5 parts per million (ppm), corresponding to the methylene groups of exogenous $^{13}$C-enriched LCFAs that were incorporated into the TG pool (O'Donnell et al., 2008; Carley...
et al., 2013). The integral of the peak at 30.5 ppm plotted over time yielded an enrichment curve with biphasic kinetics: an early exponential phase, previously shown to be dependent on transporter-mediated LCFA uptake (Figure 6) (Carley et al., 2013), and a linear phase reflecting the rate of incorporation of exogenous $^{13}$C-enriched LCFA into TG. Exponential phase time constants and linear phase slopes were determined by regression analysis. The rate of LCFA incorporation into TG was calculated as previously described (Goodwin et al., 1998; Carley et al., 2013):

\[
\text{Rate of incorporation} = (\text{slope of linear phase}) \times (\text{TG content}) \times (^{13}\text{C fractional enrichment})
\]

Because myocardial TG content remained constant during the enrichment protocol (Figure 7), influx of LCFA into TG was balanced by efflux of LCFA from TG, allowing the rate of incorporation to reflect the overall rate of TG turnover (Goodwin et al., 1998).
Figure 6. TG enrichment profile from a heart perfused with $^{13}$C-enriched palmitate. Blue square data points represent the integrals of the peak at 30.5 parts per million over the course of perfusion, yielding an enrichment profile reflecting incorporation of exogenous $^{13}$C-enriched palmitate into the TG pool. The incorporation curve displays two distinct kinetic components: an initial exponential component (red line) directly related to the process of fatty acid uptake, and a delayed linear component (black line) reflecting TG turnover. Red and black lines are determined from monoexponential and linear best fit, respectively, of the individual data points.
Figure 7. Steady-state triglyceride (TG) content was established prior to supplying hearts with $^{13}$C-enriched long-chain fatty acids (LCFAs) and did not change significantly over the course of perfusion. Unchanged TG content in each experimental group ($P>0.05$) demonstrates that incorporation of exogenous LCFA into TG was balanced by an equivalent efflux of LCFA from TG. Endpoint TG content of TAC hearts supplied palmitate was significantly lower than all other groups. White bars indicate TG content of perfused hearts prior to initiation of $^{13}$C enrichment protocol; black bars indicate TG content of hearts at the endpoint of the enrichment protocol. Values are reported as $\mu$mol TG / g dry weight (dw); *$P<0.05$ versus endpoint SHAM ($n=7-9$ in each group; error bars indicate mean ± SEM).

8. Diacylglycerol content

DAG was separated from TG and MAG from frozen perfused heart samples as previously described (Carley et al., 2013). Total myocardial lipids suspended in chloroform were applied to an NH2 column (Agilent) conditioned with hexane. TG was released from the column with 5 mL of diethyl ether:methylene chloride:hexane (1:10:89) and discarded. DAG was released from the column with 5
mL ethyl acetate:hexane (15:85). MAG was retained on the column and discarded. Effluent containing DAG was dried and resuspended in 470 µL methanol 30 µL 1 M KOH for saponification at 70°C for 1 h. Samples were neutralized with 10 µL 10% formic acid and dried. DAG content was determined using a free glycerol kit (Abcam) and normalized to tissue dry weight (dw).

9. **Ceramide content**

Individual ceramides (C16, C18, C20, C22, C24, C24:1) were isolated from frozen perfused hearts and quantified by precursor-product scans followed by LC-ESI-MS/MS with multiple reaction monitoring as described previously (Kasumov et al., 2010; Sullards et al., 2011; Carley et al., 2013) with some modifications. Extracted ceramides were dissolved in 300 µL of mobile phase A (60:40:0.2 isopropanol:acetonitrile:formic acid with 1 mmol/L ammonium formate), as were all ceramide standards. Extracted samples (10 µL) and standards were injected into a 125/2 Nucleodur 100-3 C8sec column (Macherey-Nagel). Ceramides were separated via reverse phase chromatography starting from a gradient of 75% mobile phase A:25% mobile phase B (8:1:1:0.2 H2O:isopropanol:acetonitrile:formic acid with 2 mmol/L ammonium formate) held for 2 min, 2 to 15 min a linear gradient to 90% mobile phase A, 15 to 18 min a linear gradient to 100% mobile phase A, 18 to 20 a linear gradient to 75% mobile phase A, then held from 20 to 23 min. The flow rate was 0.3 mL/min. Ceramide content was normalized to protein content, as determined by bicinchoninic acid (BCA) protein assay kit (Thermo Scientific).
10. **Tissue metabolite content**

Tricarboxylic acid (TCA) cycle intermediates and related metabolites were extracted from frozen heart tissue by grinding with 7% perchloric acid and neutralized with KOH. Extracts were analyzed spectrophotometrically and fluorometrically according to methods previously established (Williamson and Corkey, 1969). Glutamate content was determined with glutamate dehydrogenase and diaphorase (L-Glutamic acid colorimetric kit, Roche). α-Ketoglutarate content was measured by coupling glutamate-oxaloacetate transaminase (GOT, Roche) with malate dehydrogenase (MDH, Roche) in the presence of excess L-aspartate. Aspartate content was measured by coupling GOT with MDH similar to α-ketoglutarate, with the exception of excess α-ketoglutarate. Citrate content was determined with citrate lyase (Roche) and MDH. Metabolite content was normalized to tissue dry weight (dw) and is reported in Table I.

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<th>Table I</th>
<th>Steady-state metabolite content in intact perfused hearts</th>
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<tr>
<td>Group</td>
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Values are reported as µmol / g dry weight (n=5 for each group; error bars indicate mean ± SEM).
11. **In vitro nuclear magnetic resonance spectroscopy and substrate oxidation**

*In vitro* high-resolution $^{13}$C NMR spectra of lyophilized tissue perchloric acid extracts reconstituted with 0.5 mL of $^2$H$_2$O were collected with a 5 mm $^{13}$C probe (Bruker Instruments, Billerica, MA). Analysis was performed to determine fractional enrichment of [2-$^{13}$C] acetyl CoA (Malloy et al., 1988; Yu et al., 1995). The fractional contribution of $^{13}$C-enriched LCFA, $^{13}$C-enriched glucose, $^{13}$C-enriched lactate, and endogenous unenriched glycogen to mitochondrial acetyl-CoA formation was determined from the isotopomer distribution at the 4- and 3-carbon positions of glutamate as previously described (Malloy et al., 1988; Lewandowski et al., 1996; O'Donnell et al., 2008; Pound et al., 2009) (Figure 8). Fractional contribution of endogenous, unenriched TG to mitochondrial acetyl-CoA production was calculated as 100% minus the fractional contributions from carbohydrate and exogenous LCFA. Fractional contribution of TG was corrected to account for oxidation of $^{13}$C LCFA that had entered the TG pool and originated from lipolysis of TG prior to β-oxidation, using the fractional enrichment of TG determined by LC/MS.
Figure 8. *In vitro* high resolution $^{13}$C NMR spectrum of tissue extract. Multiplet structures of the $^{13}$C resonance signal at glutamate 4-carbon (34.6 ppm) and 3-carbon (28.3 ppm) positions. Extract was from a hypertrophied heart perfused with [4,6,8,10,12,14,16-$^{13}$C$_7$] palmitate.

12. **Tricarboxylic acid cycle flux**

The progressive isotopic enrichment of the 4-, then 2- and 3-carbon positions of glutamate (chemical shifts 56, 34.6, and 28.3 ppm, respectively) reflect the oxidation of $^{13}$C-enriched LCFA by mitochondria (Sorokina et al., 2007; O'Donnell et al., 2008; Lewandowski et al., 2013). Metabolic flux through citrate synthase was determined using TCA cycle metabolite content (Table I) and kinetic modeling applied to
progressive enrichment of 2- and 4-carbon positions of glutamate acquired using dynamic-mode $^{13}$C NMR during perfusion (see Figure 4) (Yu et al., 1995; Yu et al., 1997).

13. **Western blot**

Diacylglycerol acyltransferase 1 (DGAT1, Abcam), adipose triglyceride lipase (ATGL, Cell Signaling), and carnitine palmitoyltransferase 1b (CTP1b, Alpha Diagnostic) expression were measured in whole-tissue lysates, with calsequestrin (CALSEQ, Thermal Scientific) as a loading control. CPT1a (primary antibody generously provided by Dr. Charles Hoppel, Case Western Reserve University, Cleveland, Ohio) and CD36 (Cascade Bioscience) were assayed from cardiac membranes isolated as previously described (Sorokina et al., 2007; Carley et al., 2013) with the $\alpha_1$ subunit of the Na$^+$/K$^+$ ATPase as a loading control. Western band intensity, normalized to loading control, was analyzed by NIH Image software.

14. **Statistical analysis**

Results are presented as mean ± standard error of the mean (SEM). Comparisons of two means were performed using Student’s unpaired $t$ test. Comparisons of more than two means were performed using ANOVA and Tukey's honestly significant difference (HSD) post hoc test. Significant differences between means were determined at the 5% probability level ($P<0.05$).
C. Results

1. **Contractility in decompensated hearts is influenced by fatty acid source**

   Compared to age-matched control rats given a sham surgery (SHAM), rats given TAC showed 30% increase in heart weight, 39% increase in heart weight-to-body weight ratio, and 35% increase in heart weight-to-tibia length ratio (Figure 1) ($P<0.05$). The degree of hypertrophy was similar in TAC hearts perfused with either oleate or palmitate. Post-surgery animals were enrolled randomly for isolated heart perfusions supplied either palmitate or oleate.

   Consistent with previous reports (Sorokina et al., 2007; O'Donnell et al., 2008; Pound et al., 2009) RPP, an index of cardiac work output, and LVDP were impaired in TAC hearts by 21% and 26%, respectively, as compared to SHAM hearts (Figure 9A-B) ($P<0.05$). LV contractility and relaxation, as assessed by $+dP/dt$ and $-dP/dt$, respectively, were impaired in TAC hearts metabolizing palmitate (Figure 9C-D) ($P<0.05$). In contrast, oleate supported normal contractility in TAC hearts, as determined from both $+dP/dt$ and $-dP/dt$, indicating that LCFA source impacts LV contraction and relaxation rates, despite having no effect on RPP or LVDP.
Figure 9. Left ventricular (LV) function of sham-operated (SHAM) and hypertrophic (TAC) isolated perfused hearts. (A) Rate-pressure product (RPP), an index of cardiac work output, and (B) left ventricular developed pressure (LVDP) were similarly reduced compared to SHAM in TAC hearts metabolizing oleate or palmitate. (C,D) Oleate maintained LV contractility and relaxation (+ and -dP/dt, respectively) in TAC hearts, while TAC hearts metabolizing palmitate demonstrated impaired contractility and relaxation compared to SHAM and TAC oleate groups. White bar, SHAM; black bar, TAC. (n=13 for all groups). Error bars indicate mean ± SEM. *P<0.05 versus SHAM, †P<0.05 versus TAC Oleate.

2. Fatty acid-dependent regulation of triglyceride content and turnover

TG turnover rates were detected from sequential $^{13}$C NMR spectra (Figure 10A). Due to the potential for confounding kinetic contributions from different substrate
affinities for synthase and lipase enzymes, this formative study focused on the individual TG incorporation kinetics of oleate and palmitate in isolation. LCFA incorporation into the TG pool displayed two distinct kinetic components: an initial saturable exponential component dependent upon carrier mediate LCFA transport across the sarcolemma (Carley et al., 2013) and a linear component from the rate of TG turnover (Figure 10B) (Goodwin et al., 1998; Carley et al., 2013). Palmitate incorporation into TG stores was much reduced in the hypertrophied TAC hearts versus SHAM. In contrast, oleate restored TG enrichment in TAC hearts.

Figure 10. Incorporation of $^{13}$C-oleate and $^{13}$C-palmitate into triglyceride (TG). (A) Representative, sequential $^{13}$C NMR spectra (from bottom to top, 2 min acquisition each) from a SHAM heart perfused with $^{13}$C-oleate. Signal at chemical shift 30.5 parts per million (ppm) reflects the $^{13}$C-enriched methylene (-CH$_2$-) groups as $^{13}$C-oleate is esterified into TG. Signal at chemical shifts 56, 34.6, and 28.3 ppm reflects $^{13}$C enrichment of glutamate at the 2-, 4-, and 3-carbon positions, respectively. (B) TG enrichment profiles reflect incorporation of $^{13}$C-enriched LCFA throughout perfusion. White circle, SHAM oleate; black circle, TAC oleate; white square, SHAM palmitate; black square, TAC palmitate. (n=6 for all groups). Error bars indicate mean ± SEM.
In SHAM hearts, oleate supported a rate of TG turnover that was 71% faster compared to palmitate (Figure 11A) ($P<0.05$). Consistent with data previously published by O'Donnell et al, palmitate failed to maintain normal lipid storage dynamics in the hypertrophic heart (O'Donnell et al., 2008). TG turnover rates in TAC hearts perfused with palmitate were reduced by 47% compared with SHAM ($P<0.05$). However, we found that oleate preserved normal TG turnover rates in the hypertrophic heart. The steady-state condition enables these TG turnover rates to also reflect elevated TG lipolysis in hearts metabolizing oleate. These data suggest that oleate rescues lipid dynamics in the failing heart that are otherwise reduced in the presence of palmitate.

Oleate supported normal TG content in decompensated hearts (Figure 11B) ($P<0.05$). Elevated TG turnover induced by oleate also demonstrates that oleate is more readily incorporated into TG than palmitate, as was evidenced by increased $^{13}$C fractional enrichment of TG with oleate (Figure 11C) ($P<0.05$).
Figure 11. Myocardial triglyceride (TG) turnover, content, and $^{13}$C fractional enrichment of TG.

(A) Oleate supported elevated turnover in normal (SHAM) hearts and attenuated the drop in turnover in hypertrophied (TAC) hearts versus palmitate ($n=6$ for each group).

(B) Palmitate failed to maintain normal levels of TG in TAC hearts, while oleate supported normal TG similar SHAM hearts ($n=6$ for each group).

(C) Oleate induced greater $^{13}$C fractional enrichment of the TG pool than palmitate on both SHAM and TAC hearts, indicating that oleate is more readily incorporated into TG ($n=6$ for each group).

White bar, SHAM; black bar, TAC. Error bars indicate mean ± SEM. *$P<0.05$, versus SHAM Oleate; †$P<0.05$, versus TAC Oleate; ‡$P<0.05$, versus SHAM Palmitate.

Consistent with the elevated TG turnover rates that were supported by oleate, in comparison to palmitate, the composition of esterified LCFA within the TG pool was also affected. Hearts displaying more rapid TG turnover in response to oleate held lower esterified stearate (18:0) and linoleate (18:2), as a consequence of the more rapid replacement by oleate (Figure 12).
Figure 12. Constituent long-chain fatty acids (LCFAs) in the intramyocardial triglyceride (TG) pool as determined by LC/MS.
Consistent with the elevated TG turnover rates that were supported by oleate, hearts held lower esterified stearate and linoleate, as a consequence of the more rapid replacement by oleate. Values are reported as % of LCFAs in TG (n=6-8 in each group). White bar, SHAM oleate; black bar, SHAM palmitate; light grey bar, TAC oleate; dark grey bar, TAC palmitate. Error bars indicate mean ± SEM. *P<0.05.

3. **Diacylglycerol and ceramide content**

TAC hearts perfused with palmitate demonstrated depleted levels of myocardial DAG (Figure 13A) (P<0.05), a substrate for diacylglycerol acyltransferase 1 (DGAT1) for TG formation, and increased content of potentially lipotoxic C16 ceramide compared with SHAM (Figure 13B) (P<0.05). A potential cardioprotective consequence
of oleate is the competitive displacement of TG-derived LCFA away from ceramide due to rapid turnover within the TG pool formation and increased oxidation of TG-derived LCFA, both supported by oleate and consistent with elevated DAG. Total ceramide was not different between the four experimental groups (Figure 14A), although we detected shifts in composition of the individual ceramide species (Figure 14B).

**Figure 13.** Myocardial diacylglycerol (DAG) and C16 ceramide content. (A) TAC hearts supplied palmitate contained lower levels of DAG, versus SHAM (n=3 for each group). (B) C16 ceramide content was higher in TAC hearts supplied palmitate, versus SHAM Palmitate and TAC Oleate (n=5 for each group). White bar, SHAM; black bar, TAC. Error bars indicate mean ± SEM. †P<0.05, versus TAC Oleate; ‡P<0.05, versus SHAM Palmitate.
Figure 14. Ceramide content of perfused hearts.

(A) Total ceramide content was not different between groups (n=5 in each group; error bars indicated mean ± SEM).

(B) Content of individual ceramide species; *P<0.05 versus corresponding SHAM, †P<0.05 versus all other groups (n=5 in each group; error bars indicate mean ± SEM).

(C) Chromatograms from LC/MS depicting elution of total ceramide (C, top left) and individual ceramide species. C17 ceramide, which does not occur naturally, was added as an internal standard (IS) for quantification of individual species.
4. **Enzymes involved in triglyceride synthesis and lipolysis**

Despite evidence for differential effects on transcriptional activation (discussed later in Chapter III), the differences in TG content and turnover among experimental groups occurred despite no detectable difference in protein levels of the enzymes DGAT1 or adipose triglyceride lipase (ATGL) (Figure 15), suggesting that the rate-limiting TG synthase and lipase in myocardium have a higher affinity for shuttling oleate through TG stores than palmitate.

**Figure 15.** Protein levels of enzymes involved in TG synthesis and hydrolysis.  
(A) Western Blot analysis of rate-limiting enzymes of TG synthesis, diacylglycerol acyltransferase 1 (DGAT1), and hydrolysis, adipose triglyceride lipase (ATGL) from whole tissue lysate. Calsequestrin (CALSEQ) served as a loading control. (B,C) Protein content of DGAT1 and ATGL was not different across groups (n=3 for each group; P=0.14 versus all other groups). White bar, SHAM; black bar, TAC. Error bars indicate mean ± SEM. “n.s.”, not statistically significant.
5. **Fatty acid uptake kinetics**

Time constants of the exponential phase were not significantly different between the experimental groups (Figure 16A), consistent with similar protein content of sarcolemmal fatty acid transporter CD36 (Figure 16B). Although rates of LCFA uptake were not measured directly, these data indicate that the physical characteristics of the fatty acid transport process were not different for either LCFA and, moreover, were unaffected by the presence of decompensated hypertrophy (Carley et al., 2013). The findings are consistent with previous *in vitro* studies demonstrating CD36 to have similar affinities for palmitate and oleate across a range of physiologic concentrations (Baillie et al., 1996).

![Figure 16. Exponential time constants and protein levels of fatty acid transporter CD36.](image)

**Figure 16. Exponential time constants and protein levels of fatty acid transporter CD36.**

*(A)* Mean time constants characterizing the saturable exponential phase of $^{13}$C TG enrichment ($n=6$ for each group). *(B)* Western Blot analysis of CD36 from isolated total cardiac membranes. Na$^+$/K$^+$ ATPase served as a loading control ($n=3$ for each group). White bar, SHAM; black bar, TAC. Error bars indicate mean ± SEM.
6. Lipolytic rate determines mitochondrial oxidation of triglyceride

Oleate induced greater utilization of endogenous TG for mitochondrial β-oxidation and ATP production in normal hearts and attenuated the decline in lipolytic support of mitochondrial oxidation in TAC hearts metabolizing palmitate (O’Donnell et al., 2008). TCA cycle flux rate was not different between SHAM groups and was similarly reduced with either palmitate or oleate in TAC (Figure 17A), correlating with work output as determined by RPP (Figure 9A) without influence from LCFA source. Substrate utilization in both TAC groups reflected an increased contribution of carbohydrate (glucose, lactate, and glycogen) to oxidative ATP production and a drop in fatty acid oxidation versus SHAM hearts (Figure 17B).

![Figure 17. Tricarboxylic acid (TCA) cycle flux and substrate contribution to oxidative ATP production.](image_url)

- **(A)** TCA cycle flux, as determined from kinetic analysis of $^{13}$C enrichment of glutamate. ($n=5$ for each group).
- **(B)** Carbohydrate oxidation (white bar) includes exogenous glucose, lactate, and endogenous glycogen; exogenous long-chain fatty acid (LCFA) oxidation (black bar); oxidation of endogenous triglyceride (TG, gray bar). Elevated TG turnover driven by oleate corresponded with increased contribution of TG to oxidative ATP production in both sham-operated (SHAM) and hypertrophied (TAC) hearts ($n=3$ for each group). Error bars indicate mean ± SEM. *P<0.05 versus SHAM, †P<0.05 versus SHAM Palmitate, ‡P<0.05 versus TAC Palmitate.
The change in substrate utilization coincided with increased expression of the liver isoform of carnitine palmitoyltransferase 1 (CPT1a) (Lewandowski et al., 2013), and unchanged expression of the muscle isoform (CPT1b) (Figure 18). While the fractional contribution of carbohydrate and fat (either exogenous or endogenous) to oxidative ATP production was not influenced by exogenous LCFA, the increased TG turnover driven by oleate increased TG oxidation in both SHAM and TAC groups ($P<0.05$). Notably, lipolytic support of $\beta$-oxidation in TAC hearts metabolizing palmitate was nearly undetectable, consistent with the dramatically reduced rates of TG turnover supported by palmitate. In contrast, increased lipolysis of TG driven by oleate in TAC hearts resulted in significantly more TG oxidation compared to TAC palmitate ($P<0.05$). These data imply that TG turnover directly determines the extent to which LCFAs stored in TG contribute to mitochondrial $\beta$-oxidation.

**Figure 18.** Western Blot analysis of rate-limiting enzyme carnitine palmitoyltransferase 1 (CPT1) catalyzing entry of activated LCFA-CoA into mitochondria for $\beta$-oxidation. (A) Western blot of CPT1b and CPT1a isoforms. (B) Muscle isoform CPT1b protein expression was unchanged, while (C) CPT1a was increased in TAC groups ($n=3$ for each group). White bar, SHAM; black bar, TAC. Error bars indicate mean ± SEM. *$P<0.05$ versus SHAM.
D. **Discussion**

This study is the first to demonstrate in the intact heart that the intracellular fate of exogenous LCFA, including storage dynamics and mitochondrial β-oxidation, is dependent upon chain composition. By comparing the two major dietary fats oleate and palmitate, we found that oleate is more readily esterified into intramyocardial TG than palmitate. In the normal heart, oleate drove elevated rates of TG turnover compared with palmitate. In the diseased heart, the previously observed drop in TG content and turnover with provision of palmitate (O'Donnell et al., 2008) was attenuated by oleate. Indeed, oleate supported lipid content, turnover and oxidation in hypertrophic myocardium that were indistinguishable from those of the healthy heart. Clearly, oleate supported greater contribution of TG to mitochondrial β-oxidation, and aided cardiac contractility in decompensated hearts in comparison to palmitate.

The kinetics of LCFA storage over the relatively short protocol were greatly affected by the presence of oleate versus palmitate despite no changes in protein levels of DGAT1 or ATGL. Therefore, these two LCFAs hold different affinities for the key rate-limiting TG synthase and lipase activities. Indeed, in TG extracted from both SHAM and TAC hearts, exogenous oleate constituted a significantly greater proportion of the acyl chains esterified to glycerol backbone than palmitate, indicating that oleate is more readily esterified than palmitate. Listenberger et al demonstrated differential fates of palmitate and oleate in cultured Chinese hamster ovary cells (Listenberger et al., 2003). We show here for the first time that such distinctions occur in the intact heart. In the absence of any difference in protein content of DGAT1, known to be the rate-limiting
enzyme of TG formation (Goldberg et al., 2012), the data are consistent with a higher affinity of DGAT1 for esterifying oleate. A limitation of this study is that we may not draw any conclusions about the substrate affinity of ATGL, since our methods cannot discern the LCFAs being released from TG, only that the influx of exogenous oleate or palmitate is balance by efflux of LCFA. Limited studies have examined substrate preference of ATGL in vitro (Eichmann et al., 2012), but whether ATGL preferentially hydrolyzes TG based upon its acyl moieties in the intact heart has yet to be explored.

The rate-limiting step of LCFA oxidation is catalyzed by CPT1, which transports acylcarnitines across the outer mitochondrial membrane. In the normal adult heart, the muscle isoform CPT1b is predominately expressed with a minor contribution of liver isoform CPT1a (Weis et al., 1994). In the development of heart failure, the heart reverts to a fetal metabolic profile with reduced fatty acid oxidation and greater reliance on carbohydrate metabolism (Sack et al., 1996). Our lab was the first to report a shift in CPT1 isoform expression in the hypertrophic heart (Sorokina et al., 2007), which demonstrates a marked increase in CPT1a protein content, and link the isoform shift with reduced rates of LCFA oxidation (Lewandowski et al., 2013). In the current study, we observed increased expression of CPT1a in TAC hearts, which occurred alongside decreased fractional contribution of LCFAs (both endogenous and exogenous) and increased contribution of carbohydrate to mitochondrial ATP production. Consistent with previous reports (O’Donnell et al., 2008), TG oxidation in TAC hearts supplied palmitate was nearly undetectable while TAC hearts supplied oleate demonstrated significantly greater TG oxidation. Although the CPT1 isoform shift may determine the
fractional contributions of fat and carbohydrate to oxidative ATP synthesis, accelerated TG turnover, as supported by oleate, enabled a supply of energy-rich endogenous lipid to the mitochondria.

Oleate also improved the rates of pressure development (+dP/dt) and relaxation (-dP/dt), suggesting that TG dynamics are important factors contributing to LV contractile performance. The beneficial effect of oleate on contractility in TAC may be partially explained by averting an increase in C16 ceramide (Figure 13B), the ceramide species associated with lipotoxicity, cardiac dysfunction and apoptotic signaling (Aflaki et al., 2012; Goldberg et al., 2012). As a consequence of reduced TG turnover and LCFA oxidation in TAC hearts, palmitate may become increasingly available as substrate for synthesis of C16 ceramide in the decompensated heart. Thus, a potential cardioprotective consequence of oleate is the competitive displacement of TG-derived LCFA away from C16 ceramide synthesis due to rapid turnover within the TG pool and increased oxidation of TG-derived LCFA, both supported by oleate and consistent with elevated DAG. Interestingly, we detected no difference in total ceramide content (Figure 14A) alongside shifted composition of individual ceramide species (Figure 14B). The various ceramides are known to have distinct bioactivities, not all of which are implicated in lipotoxicity (Hannun and Obeid, 2008). Ultimately, it may be that the composition of the ceramide pool, not merely total ceramide content, is the determining factor contributing to lipotoxicity and impaired LV function.
Dynamic mode $^{13}$C NMR spectroscopy of intact rat hearts allows for non-invasive study of cardiomyocytes producing physiologic work output and actively metabolizing substrate. Importantly, our experiments provide real-time rates of dynamic metabolic processes occurring within the cell, which otherwise would be difficult to detect or quantify based upon static measurements of endpoint metabolite content. Incorporation of exogenous LCFA into the myocardial TG pool displayed biphasic kinetics, an initial exponential component followed by a linear component. Previous work from our lab attributes the exponential component to the saturable process of LCFA uptake at the sarcolemma, where increasing the amount of membrane-bound LCFA transporter CD36 sped up the exponential phase as reflected by a decreased time constant (Carley et al., 2013). In the present study, we detected no difference in exponential time constants or CD36 expression between the four experimental groups, indicating that while the net flux of LCFA into the cell may be increased or decreased, the physical characteristics of the transport process were unaltered. Thus, the observed changes in TG turnover are not explained by differences in uptake between the two dietary fats. The slope of the linear phase, in contrast, was affected by type of LCFA provided to the heart. Differences in slope occurred independent of altered protein levels of enzymes catalyzing the rate-limiting steps of TG synthesis (DGAT1) and lipolysis (ATGL), suggesting that these two enzymes have a greater affinity for shuttling oleate in and out of TG than palmitate. Therefore, the difference in TG turnover observed between the two fats must be explained by TG synthase and lipase affinity.
III. TRANSCRIPTIONAL REGULATION IS MEDIATED BY RATE-DEPENDENT LIPOLYTIC SIGNALING

A. Introduction

Commensurate with shifting substrate utilization, hypertrophied hearts demonstrate decreased signaling through nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α) (Sack et al., 1996; Barger et al., 2000; Planavila et al., 2005; Luptak et al., 2005; Chokshi et al., 2012) a transcription factor for target genes that drive TG turnover (Banke et al., 2010) and fatty acid utilization (Barger et al., 2000) in the cardiomyocyte. Given recent evidence for TG lipolysis to play a major role in supplying ligand for nuclear receptor activation (Haemmerle et al., 2011), whether reduced TG turnover in the hypertrophic heart is a cause or a consequence of impaired PPAR-α signaling becomes an important component in determining etiology of the low-energy state that contributes to progressive decompensation of the pressure overloaded heart toward overt failure (Ingwall and Weiss, 2004; Neubauer, 2007).

Therefore, we hypothesized that the turnover of TG within the cardiomyocyte serves as a fundamental process for lipolytic signaling to the nucleus and that in the normal and failing heart such lipid dynamics are dependent on the LCFA chain composition. Changes in LCFA-mediated lipid dynamics may then hold consequences for substrate utilization, nuclear receptor activation, and cardiac function. To test our hypothesis, we compared metabolic gene expression in normal and decompensated rat hearts perfused with palmitate and oleate as sources for lipid storage. Our findings implicate TG turnover as a mediator of deficient PPAR-α activation in failing hearts, and
show that in comparison to palmitate, oleate confers normal nuclear signaling in decompensated hearts. This study also presents the first evidence for rate-dependent activation of transcriptional regulation and cardiac energy metabolism in diseased hearts to be mediated by common dietary fats.

B. Methods

1. Animal model of pressure-overload hypertrophy

Cardiac hypertrophy by chronic pressure-overload was induced by constricting the transverse aorta (hemoclip) of three-week-old male Sprague Dawley rats, as previously described (Sorokina et al., 2007; O'Donnell et al., 2008a; O'Donnell et al., 2008b; Lewandowski et al., 2013). This banding procedure relies on the natural growth of the animal to produce a gradually increasing degree of aortic constriction. The rats develop a concentric hypertrophy and increased heart weight, heart weight-to-body weight ratio, and heart weight-to-tibia length ratio (see Figure 1A-C) that is associated with short-term improvement in the systolic function of the heart (Vatner and Ingwall, 1986; Sack et al., 1996; Ribeiro et al., 2003; O'Donnell et al., 2008). At 12 weeks post-banding, the animals enter a decompensated stage with depressed left ventricular developed pressure (LVDP) and rate of pressure development (dP/dt). In this model of left ventricular hypertrophy, no systemic activation of the sympathetic nervous system or of the renin-angiotensin-aldosterone system occurs (Ribeiro et al., 2003). Consequently, there are no signs of cardiac lesions, peripheral arteritis, myocardial necrosis, or extensive fibrosis. The rats progress to a dilated cardiac hypertrophy with acute end-stage heart failure at 4-6 months post-banding. The sham
groups underwent similar surgery without placement of the aortic band. Rats had free access to food and water while being housed under controlled temperature and lighting. All experimental procedures were approved by the University of Illinois at Chicago Animal Care and Use Committee.

2. **Isolated heart perfusion protocol**

12 weeks post-surgery, animals were heparinized (1000 IU, intraperitoneal injection) and anesthetized (100 mg/kg pentobarbital, intraperitoneal injection). Hearts were excised and retrogradely perfused with modified Krebs-Henseleit buffer (in mmol/L: 116 NaCl, 4 KCl, 1.5 CaCl2, 1.2 MgSO4 and 1.2 NaH2PO4) equilibrated with 95% O₂/5% CO₂ and containing 0.4 mmol/L ¹³C palmitate or 0.4 mmol/L ¹³C oleate complexed to bovine serum albumin in a 3:1 molar ratio, 5 mmol/L ¹³C glucose and 1 mmol/L ¹³C sodium lactate. Buffer temperature was maintained at 37°C. A water-filled latex balloon, connected to a force transducer, was fitted into the left ventricle (LV) and set to a diastolic pressure of 5 mmHg. LV developed pressure (LVDP) data was continuously acquired during perfusion with Powerlab (ADInstruments, Dunedin, New Zealand). Rate-pressure product (RPP) was calculated as heart rate (HR)×LVDP, and mean peak rates of pressure development and relaxation (+ and -dP/dt) were calculated from the first derivative of the LVDP trace. Functional data are reported at midpoint of perfusion and were determined to be not significantly different over the entire protocol using repeated measures ANOVA.
Experimental groups perfused with unenriched LCFA and unenriched glucose and sodium lactate were as follows: sham-operated hearts (SHAM) supplied oleate \( (n=5) \) or palmitate \( (n=5) \); hypertrophied hearts (TAC) supplied oleate \( (n=5) \) or palmitate \( (n=5) \).

Additional groups of isolated hearts were perfused with an unenriched substrate media containing a 1:1 mix of albumin-bound palmitate and oleate (similar total LCFA concentration of 0.4 mmol/L), 5 mmol/L glucose, and 1 mmol/L lactate as follows: sham-operated hearts (SHAM) supplied mix \( (n=4) \); hypertrophied hearts (TAC) supplied mix \( (n=5) \).

3. **Lipid extraction**

Lipids were extracted from frozen perfused heart samples as previously described (O’Donnell et al., 2006). 30-50 mg of frozen heart tissue was homogenized in 0.5 mL of PBS and extracted in 10 mL of chloroform:methanol \( (2:1) \), followed by an addition of 2 mL of methanol. Samples were vortexed and placed on ice. After 30 min, the samples were centrifuged and the pellets discarded. 2 mL of 4% \( \text{CaCl}_2 \) was added to the supernatant and centrifuged to separate upper and lower phases. The lower phase was washed three times with solvent \( (1.5 \text{ mL chloroform, 24 mL methanol, 23.5 mL H}_2\text{O}) \) and then dried under vacuum at 50°C (RapidVac, Labconco, Kansas City, MO).
4. **Triglyceride content**

Lipids were resuspended in 0.5 mL tert-butyl alcohol:Triton X-100 (3:2) and TG was quantified by calorimetric assay (Wako Pure Chemical Industries). TG content was normalized to tissue dry weight (dw).

5. **Western blot**

Peroxisome proliferator-activated receptor-α (PPAR-α, Abcam) expression was measured in whole-tissue lysates, with calsequestrin (CALSEQ, Thermal Scientific) as a loading control. Western band intensity, normalized to loading control, was analyzed by NIH Image software.

6. **Quantitative real-time polymerase chain reaction**

Total RNA was extracted from frozen heart tissue by using an RNeasy Lipid Tissue kit (Qiagen), according to the manufacturer's instructions. RNA quantity was determined at 260 nm (NanoDrop 1000 Spectrometer, Thermal Scientific). Single-stranded cDNA was synthesized from the prepared RNA by using High Capacity cDNA Reverse Transcription kit (Applied Biosystems), and gene products were determined by quantitative real-time polymerase chain reaction, using Fast SYBR Green Master Mix (Applied Biosystems) with an ABI ViiA7 instrument. The cycle profile was: 1 cycle at 95°C for 20 sec, 40 cycles of 95°C for 1 sec, 60°C for 20 sec. The mRNA levels were determined by a comparative CT method, normalized to ribosomal protein S29. Primer sequences are provided in Table II.
### Table II

**Primer sequences used in quantification of mRNA levels by quantitative real-time PCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene symbol</th>
<th>Primer sequences (5’ to 3’)</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| Medium-chain acyl-CoA dehydrogenase       | *Acadm*     | Sense: GTATTGACGCGATCTAAACCAG  
|                                           |             | Antisense: TAGAGGCAAAGTACGTGTTCCG                                                          | Lemitsu et al., 2008  |
| Carnitine palmitoyl-transferase 1b        | *Cpt1b*     | Sense: GCAGGCAACACAGGCAGTA  
|                                           |             | Antisense: ATGTTGGGAAGCTATAGAGCA                                                          | Leone et al., 1999    |
| Pyruvate dehydrogenase kinase 4           | *Pdhk4*     | Sense: CCTTTGGCTGGTTTGGTTTA  
|                                           |             | Antisense: CACCAGTCATCGCCTCAG                                                          | Puthanveetil et al., 2010 |
| Adipose triglyceride lipase               | *Atgl*      | Sense: AGTTCAACCTTGCCTCCTCTC  
|                                           |             | Antisense: GTACCCAATTTCCTGTTG                                                        | Ogasawara et al., 2012 |
| Diacylglycerol acyltransferase 1          | *Dgat1*     | Sense: CACCAGGATGCTACTCTGA  
|                                           |             | Antisense: ACTCTTTGTTCAGCAG                                                          |                      |
| Peroxisome proliferator-activated receptor-γ coactivator 1α | *Ppargc1a* | Sense: ACCCACAAGAAGAAACAG  
|                                           |             | Antisense: GGTCAGAGGAGGATAAGTTG                                                          |                      |
| Peroxisome proliferator-activated receptor-γ coactivator 1-β | *Ppargc1b* | Sense: AACTTCAGACTTGAGGAGCAGAG  
|                                           |             | Antisense: GTATACCACACAGCCTTCACC                                                          |                      |
| Ribosomal protein S29                     | *S29*       | Sense: TCTGATCCGTAATACGGGC  
|                                           |             | Antisense: CTGTGTGCAGAAGACTAG                                                          | Weitzel et al., 2001  |

* : Primer sequences were custom designed for this study by Integrated DNA Technologies (Coralville, IA).
7. **Statistical analysis**

Results are presented as mean ± standard error of the mean (SEM). Comparisons of two means were performed using Student's unpaired t test. Comparisons of more than two means were performed using ANOVA and Tukey's honestly significant difference (HSD) post hoc test. Significant differences between means were determined at the 5% probability level (P<0.05).

C. **Results**

1. **Improved triglyceride dynamics restores nuclear signaling in failing hearts**

   Transcript levels of PPAR-α target genes provided insights into the link between TG turnover (see Figure 11A) and PPAR-α signaling: carnitine palmitoyltransferase 1b (Cpt1b), pyruvate dehydrogenase kinase 4 (Pdhk4), and medium-chain acyl-CoA dehydrogenase (Acadm). Consistent with previous reports (Sack et al., 1996), PPAR-α protein was reduced by TAC (Figure 19). Transcripts of all three target genes were significantly reduced in TAC hearts perfused with palmitate compared to SHAM (Cpt1b: -61%, Pdhk4: -83%, Acadm: -63%) (P<0.01) (Figure 19). In contrast, improved TG dynamics in TAC hearts conferred by oleate were associated with near-normal levels of PPAR-α target gene mRNA.
Figure 19. PPAR-α expression in SHAM and TAC hearts. (A,B) Western blot analysis detected decreased protein levels of PPAR-α in TAC hearts (n=3 for each group). White bar, SHAM oleate; black bar, TAC oleate; light gray bar, SHAM palmitate; dark gray bar, TAC palmitate. Error bars indicate mean ± SEM. **P<0.01 versus SHAM.

2. Transcription of genes for triglyceride storage and mitochondrial biogenesis

Additional reductions in mRNA for DGAT1, ATGL, PPAR-γ-coactivator-1α (PGC-1α) and PGC-1β occurred in TAC hearts supplied palmitate (Figure 20). The data indicate that dramatically reduced rates of lipolysis in TAC hearts supplied palmitate were not sufficient to maintain transcription of genes involved in LCFA metabolism. In contrast, oleate restored normal PPAR-α signaling via enhanced rates of ATGL-mediated TG lipolysis.
Figure 20. Expression of peroxisome proliferator-activated receptor-α (PPAR-α) target genes and diacylglycerol acyltransferase 1 (DGAT1), adipose triglyceride lipase (ATGL), and PPAR-γ coactivator-1α (PGC-1α) and PGC-1β genes in perfused sham-operated (SHAM) and hypertrophied (TAC) heart tissue. Transcript levels of select PPAR-α target genes (Cpt1b, Pdhk4, Acadm) were decreased in TAC hearts metabolizing palmitate. mRNA levels of genes encoding DGAT1 (Dgat1), ATGL (Atgl), PGC-1α (Ppargc1a) and PGC-1β (Ppargc1b) were reduced in TAC hearts metabolizing palmitate, while oleate maintained transcript levels in TAC hearts (n=5 for each group). White bar, SHAM oleate; black bar, TAC oleate; light gray bar, SHAM palmitate; dark gray bar, TAC palmitate. Error bars indicate mean ± SEM. *P<0.05 versus all other groups, ** P<0.01 versus all other groups.

3. **Perfusion with a mix of palmitate and oleate**

Additional hearts perfused with a 1:1 mix of oleate and palmitate demonstrated intermediate transcript levels of PPAR-α targets, that were generally mid-range of those observed in TAC hearts supplied either of the individual fatty acids (Figure 21A). Similarly, the response to the palmitate:oleate mix resulted in a mean TG content that was also mid-range of values observed in the TAC palmitate and TAC oleate groups (Figure 21B).
Figure 21. Expression of peroxisome proliferator-activated receptor-α (PPAR-α) target genes and diacylglycerol acyltransferase 1 (DGAT1), adipose triglyceride lipase (ATGL), and PPAR-γ coactivator-1α (PGC-1α) and PGC-1β genes in sham-operated (SHAM) and hypertrophied (TAC) hearts perfused with a 1:1 mix of oleate and palmitate. (A) Perfusing TAC hearts with 1:1 mix of oleate:palmitate supported transcript levels of Cpt1b, Pdhk4, Acadm and Dgat1 that were mid-range of values observed in TAC oleate and TAC palmitate (see Figure 20), while levels Atgl, Ppargc1a, and Ppargc1b were the same as SHAM (n=4 for SHAM Mix, white bar; n=5 for TAC Mix, black bar). (B) TG content in TAC hearts perfused with oleate (white bar), 1:1 oleate:palmitate mix (gray bar), and palmitate (black bar) (n=5-6 for each group). *P<0.05 versus SHAM Mix, **P<0.01 versus SHAM Mix. Error bars indicate mean ± SEM.

D. Discussion

While it has long been appreciated that dietary LCFAs can regulate gene transcription in cardiomyocytes by serving as ligands for activating the various PPARs (van der Lee et al., 2000; Stanley et al., 2005; Georgiadi et al., 2010), recent work by Haemmerle et al demonstrated that ATGL-mediated TG lipolysis was required for nuclear signaling via PPAR-α (Haemmerle et al., 2011), suggesting that LCFAs must first traverse the TG pool in order to activate transcription. The evidence presented here indicates that lipolytic support of nuclear signaling via PPAR-α and mitochondrial
biogenesis via PGC-1 complex is a rate-dependent process that can be rescued in the hypertrophied failing heart by inducing TG turnover with oleate as the source of esterification. Consequentially, the enhanced ability of oleate to support lipid dynamics had profound impact on mRNA levels of PPAR-α target genes.

In the decompensated, failing heart, the dramatically impaired rate of TG turnover supported by palmitate provided insufficient lipolytic flux through ATGL to support PPAR-α activation. This drop in activation occurred despite no detectable change in ATGL protein content. The importance of myocardial lipid dynamics supporting transcriptional regulation of fatty acid metabolism are underscored by recent studies in non-obese human heart failure patients. Reduced TG content in tissue from failing human hearts was linked with decreased transcription of Cpt1b, Pdhk4, and Ppargc1a (Chokshi et al., 2012), consistent with impaired lipolytic signaling to the nucleus due to decreased rates of TG turnover that we observed here and in previous studies (O’Donnell et al., 2008) with rodent models of chronic pressure overload hypertrophy.

Interestingly, TAC hearts supplied a 1:1 mix of palmitate and oleate demonstrated transcript levels that were higher than supplying palmitate alone, yet generally lower than supplying oleate alone. These intermediate mRNA levels suggest that the concentration of oleate may be a determining factor in attenuating the reduction in nuclear signaling. Alternatively, palmitate may have an inhibitory effect on transcription of certain PPAR-α target genes in pressure-overload hypertrophy. These
data indicate that dietary studies aimed at increasing oleate in the blood might hold promise for rescuing the otherwise impaired lipid dynamics and PPAR-α activation in decompensated hearts. However, examination of the kinetic features of the TG pool in the presence of a mixed supply of exogenous LCFA actually introduces additional complexities owing to potential differences in the affinity of the intracellular lipases, namely ATGL, for each of the specific LCFA species (Eichmann et al., 2012). Therefore, further investigation into the affinities of TG synthase and lipase for individual LCFA species is necessary to fully elucidate the precise mechanisms of transcriptional regulation.

Taken together with results presented in the previous chapter, our study provides evidence of a regulatory role for dietary fats on TG turnover to dynamically balance the reciprocal processes of energy production by mitochondrial β-oxidation, supplying substrate via lipolysis, and target gene expression via PPAR-α activation (Figure 22). Although the relatively brief protocols did not accommodate immediate differences in protein content, additional implications for the responses of the endogenous TG content in TAC hearts includes potential LCFA effects on the function of the perilipin proteins involved in TG formation and lipolysis in the lipid droplet (Wang et al., 2013). Additionally, since it is known that micro RNAs (miRNAs) located on the PGC-1β gene act to regulate adiposity (Carrer et al., 2012), data of oleate normalizing PGC-1β message levels in TAC hearts hold implications for mechanisms by which dietary fats affect the cardiac-adipose axis. Prolonged increase in PPAR-α signaling supported by oleate in TAC, beyond the time course of our protocol, may be sufficient to restore
protein levels of target genes involved in fatty acid oxidation and attenuate the maladaptive shift in substrate utilization away from LCFA oxidation. These findings provide the impetus for future studies supplying diets artificially enriched with either palmitate or oleate following TAC procedure, to assess long-term effects of dietary fats on cardiac function and metabolism. Thus, this study provides the necessary, direct comparison of each LCFA in isolation of the other.
Figure 22. Summary scheme depicting the proposed rate-dependence of peroxisome proliferator-activated receptor-α (PPAR-α) activation and triglyceride (TG) oxidation on TG turnover.

(A) In normal hearts, oleate supports a faster rate of TG turnover than palmitate, resulting in increased oxidation of TG by mitochondria. At baseline, either substrate supports sufficient TG turnover to maintain PPAR-α signaling. (B) In failing hearts, TG turnover supported by palmitate is reduced; TG becomes unavailable as a source of substrate for PPAR-α activation and mitochondrial ATP production. Oleate maintains normal TG content and turnover in failing hearts, with normal PPAR-α target gene expression and oxidation of TG.
In summary, chronic hypertension, as a consequence of poor management or lack of treatment, is one of the leading causes of heart failure (McMurray and Pfeffer, 2005). While the benefits of diets rich in unsaturated fats and low in saturated fats are well documented for prevention of coronary artery disease (Appel et al., 2005; Estruch et al., 2013), the potential benefits of unsaturated fats on the diseased myocardium itself are largely unexplored. TG stores in the failing heart become static, no longer supporting oxidative metabolism and lipolytic signaling (O’Donnell et al., 2008; Chokshi et al., 2012). With palmitate (16:0) as a saturated fat source, lipid dynamics and gene activation were greatly reduced in failing hearts. In contrast, oleate (18:1), a monounsaturated fat, supported normal lipid content, turnover, and oxidation, along with normal metabolic gene activation. The responses of decompensated rat hearts to oleate included improved cardiac contractility. These findings indicate oleate as a beneficial energy substrate for the decompensated heart and present strong evidence for further study on the potential benefits of dietary supplementation with oleate in patients at risk of developing congestive heart failure.
IV. CARDIAC-SPECIFIC KNOCKDOWN OF MALIC ENZYME 1 IN PRESSURE OVERLOADED HEARTS

A. Introduction

The hypertrophied failing heart has been well-characterized as having both reduced fatty acid oxidation and increased glucose utilization, a reversion to the so-called “fetal metabolic profile” (Sack et al., 1996). However, such broad characterization of shifting substrate utilization by the failing heart ignores the overall balance of influx and efflux of carbon to and from the tricarboxylic acid (TCA) cycle. Hypertrophied hearts demonstrate increased glycolytic rate without a corresponding increase in glucose oxidation rate, mediated by the pyruvate dehydrogenase complex (PDC) that converts pyruvate into acetyl-CoA (Lydell et al., 2002; Leong et al., 2003). This discrepancy was recently accounted for by studies in our lab demonstrating that hypertrophied hearts have increased protein content of the cytosolic enzyme malic enzyme 1 (ME1), which redirects pyruvate metabolism away from oxidative decarboxylation by PDC (Sorokina et al., 2007). Alternatively, ME1 carboxylates pyruvate, forming malate, which enters the second span of the TCA cycle, a process called anaplerosis. Increased anaplerotic flux in hypertrophied hearts serves to compensate for decreased carbon flux into the TCA cycle due to reduced fatty acid oxidation.

Compared to oxidation via PDC, the alternative route for carbon entry into the TCA cycle facilitated by ME1 is inefficient, bypassing numerous reactions that would otherwise generate reducing equivalents for oxidative production of ATP (Figure 23). In
addition, carboxylation of pyruvate by ME1 consumes NADPH, a cofactor for lipid synthesis and glutathione reduction to maintain cellular redox state. Increased anaplerotic flux through ME1 may lead to impaired TG formation in cardiac hypertrophy (O’Donnell et al., 2008; Chokshi et al., 2012) as well as imbalanced cellular redox state that reportedly contributes to the pathophysiology of heart failure (Giordano, 2005).

Figure 23. Increased anaplerosis in cardiac hypertrophy induces inefficiencies in the contribution of carbohydrate to oxidative ATP production. Malic enzyme 1 (ME1)-mediated entry of glycolytic carbon into the TCA cycle bypasses numerous reactions that would otherwise generate reducing equivalents for ATP production (shown in red), reducing the overall ATP yield from glucose oxidation. In addition, ME1 consumes NADPH, which is used for anabolic processes such as TG synthesis as well as maintenance of cellular redox state via glutathione reduction.
Interestingly, our lab has shown that increased anaplerosis in hypertrophy can be attenuated through pharmacological activation of PDC (Pound et al., 2009). By treating intact perfused hypertrophied hearts with dichloroacetate (an inhibitor of pyruvate dehydrogenase kinase), anaplerotic flux was partially reduced, coinciding with improved LV contractility and restored TG content.

These findings of beneficial, short-term pharmacologic intervention in the hypertrophic heart provide the impetus for the current study investigating potential benefit of selective knockdown of cardiac ME1 expression in vivo in rats subjected to TAC. To accomplish this aim, we employed a technique developed in our lab to deliver adenovirus containing non-native micro RNA targeted to the ME1 gene (miR-ME1), limiting ME1 knockdown specifically to the myocardium (O’Donnell et al., 2012).

B. Methods

1. Animal model of pressure-overload hypertrophy

Cardiac hypertrophy by chronic pressure-overload was induced by constricting the transverse aorta (hemoclip) of three-week-old male Sprague Dawley rats, as previously described (Sorokina et al., 2007; O’Donnell et al., 2008a; O’Donnell et al., 2008b; Lewandowski et al., 2013). This banding procedure relies on the natural growth of the animal to produce a gradually increasing degree of aortic constriction. The rats develop a concentric hypertrophy and increased heart weight, heart weight-to-body weight ratio, and heart weight-to-tibia length ratio associated with short-term improvement in the systolic function of the heart (Vatner and Ingwall, 1986; Sack et al.,
1996; Ribeiro et al., 2003; O’Donnell et al., 2008). At 12 weeks post-banding, the animals enter a decompensated stage with depressed LVDP and rate of pressure development (dP/dt). In this model of left ventricular hypertrophy, no systemic activation of the sympathetic nervous system or of the renin-angiotensin-aldosterone system occurs (Ribeiro et al., 2003). Consequently, there are no signs of cardiac lesions, peripheral arteritis, myocardial necrosis, or extensive fibrosis. The rats progress to a dilated cardiac hypertrophy with acute end-stage heart failure at 4-6 months post-banding. The sham groups underwent similar surgery without placement of the aortic band. Rats had free access to food and water while being housed under controlled temperature and lighting. All experimental procedures were approved by the University of Illinois at Chicago Animal Care and Use Committee.

2. **Adenovirus production**

Synthesis of a non-native miR-RNA sequence was designed to target cytoplasmic rat malic enzyme 1 mRNA (O’Donnell et al, 2012). The miR-RNA sequence was cloned into pcDNA6.2-GW/miR expression vector (Invitrogen) with a CMV promoter. The miR-RNA sequence was then recombined into a pAd/CMV/V5-DEST vector (Invitrogen) and transfected into HEK 293 cells for amplification as previously described (O’Donnell and Lewandowski, 2005; O’Donnell et al, 2012). The virus was harvested and purified using cesium chloride density gradient centrifugation as previously described (O’Donnell and Lewandowski, 2005).
3. **Adenoviral delivery of micro-ribonucleic acid targeted to malic enzyme 1 to the myocardium**

Adenovirus (Adv.MiRME1) or PBS was delivered to the heart *in vivo* by isolated perfusion of the coronary arteries as previously described (O'Donnell and Lewandowski, 2005; O'Donnell et al, 2012) and summarized here. 12 weeks post-surgery, hypertrophied (TAC) or sham-operated (SHAM) rats were anesthetized and intubated. Core body temperature was monitored via rectal thermometer and lowered to 30°C using an ice pad. The chest was opened at the second or third intercostal space and all vessels leading to and from the heart were cross-clamped simultaneously. A catheter for fluid delivery was inserted into the left ventricle at the apex and advanced into the aortic root, and a second catheter was positioned in the right ventricle for fluid efflux. The coronary vessels were perfused for 7 minutes with calcium-free Tyrode solution, followed by delivery of 0.2 mL of either Adv.MiR-ME1 ($10^{13}$ viral particles units/mL in PBS) or virus-free PBS. After a 90 second incubation period, unsequestered virus was flushed from the coronary vessels with Krebs buffer containing 1.5 mmol/L calcium. The cross-clamp was removed, the chest was closed, and the rats were allowed to recover from anesthesia in an oxygen chamber.

4. **Isolated heart perfusion protocol**

6 days after delivery of either adenovirus or PBS, animals were heparinized (1000 IU, intraperitoneal injection) and anesthetized (100 mg/kg pentobarbital, intraperitoneal injection). Hearts were excised and retrogradely perfused with modified Krebs-Henseleit buffer (in mmol/L: 116 NaCl, 4 KCl, 1.5 CaCl$_2$, 1.2 MgSO$_4$...
and 1.2 NaH₂PO₄) equilibrated with 95% O₂/5% CO₂ and containing 0.4 mmol/L ¹²C palmitate complexed to bovine serum albumin in a 3:1 molar ratio, 5 mmol/L ¹²C glucose and 1 mmol/L ¹²C sodium lactate. Buffer temperature was maintained at 37°C. A water-filled latex balloon, connected to a force transducer, was fitted into the LV and set to a diastolic pressure of 5 mmHg. LVDP data was continuously acquired during perfusion with Powerlab (ADInstruments, Dunedin, New Zealand).

Isotopic enrichment was initiated by switching the perfusate supply to buffer containing 0.4 mmol/L [2,4,6,8,10,12,14,16-¹³C₈] palmitate, 5 mmol/L unlabeled glucose, and 1 mmol/L unlabeled sodium lactate. Enrichment continued for 40 minutes. At endpoint of the enrichment protocol, oxygen consumption was determined from pulmonary artery effluent with a blood gas analyzer (GEM Premier 300, Instrumentation Laboratory) prior to freeze clamping hearts with liquid N₂-cooled tongs for subsequent in vitro biochemical analysis.

5. **In vitro analysis of tissue acid extracts to determine anaplerotic flux**

Perchloric acid extracts of frozen LV tissue from perfused hearts were lyophilized and reconstituted in 0.5 mL deuterium oxide. High-resolution proton-decoupled ¹³C NMR spectra were acquired from *in vitro* samples with a 5 mm ¹³C probe (Bruker Instruments, Billerica, MA). The relative contribution of anaplerosis (y) to TCA cycle flux (ratio of anaplerotic flux to citrate synthase flux) was determined by isotopomer analysis of the glutamate 3- and 4-carbon ¹³C resonances as previously described (Malloy et al, 1988; Lewandowski et al, 1996).
6. **Reduced glutathione assay**

Reduced glutathione content was determined using a commercially available kit (Millipore). Frozen LV was homogenized, deproteinated, and centrifuged. Supernatant was incubated with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), which oxidizes GSH to form the derivative 5'-thio-2-nitrobenzoic acid (TNB), measurable at 412 nm (Rahman et al., 2006). GSH content was normalized to protein content, as determined by BCA protein assay kit (Thermo Scientific).

7. **Western blot analysis**

ME1 (Abcam) expression in perfused hearts was measured in whole-tissue lysates, with calsequestrin (CALSEQ, Thermal Scientific) as a loading control (O’Donnell et al, 2012). Western band intensity, normalized to loading control, was analyzed by NIH Image software.

8. **Statistical analysis**

Results are presented as mean ± standard error of the mean (SEM). Comparisons of two means were performed using Student’s unpaired t test. Comparisons of more than two means were performed using ANOVA and Tukey’s honestly significant difference (HSD) post hoc test. Significant differences between means were determined at the 5% probability level ($P<0.05$).
C. **Results**

1. **Adenovirus-mediated reduction in protein expression**

   Consistent with previous reports (Pound et al., 2009), TAC caused an elevation in cytosolic ME1 content ($P<0.01$) (Figure 24). Delivery of Adv.MiRME1 resulted in dramatically reduced expression of ME1 in both SHAM and TAC hearts 6 days after injection: 69% decrease in SHAM, 85% decrease in TAC ($P<0.01$).

![Figure 24. Protein levels of malic enzyme 1 (ME1).](image)

Protein expression of ME1 was increased in TAC PBS compared to SHAM PBS. Delivery of adenovirus containing miRME1 vector reduced protein expression of ME1 in both groups. ($n=3-6$ for each group). White bar, PBS injected; black bar, miRME1 injected. Error bars indicate mean ± SEM. *$P<0.01$.

2. **Reduced malic enzyme 1 expression corresponded with decreased anaplerosis and increased reduced glutathione content**

   While SHAM miRME1 hearts showed no further reduction of inherently low baseline ratios of anaplerosis to citrate synthase flux from normal hearts, miRME1 reduced anaplerosis in TAC to baseline: TAC miRME1=$0.034±0.004$; TAC
PBS=0.081±0.005 (**P<0.001) (Figure 25A). Importantly, ME1 suppression restored GSH in TAC (†P<0.05) (Figure 25B), consistent with decreased ME1-mediated carboxylation of pyruvate into malate, a reaction that consumes NADPH used for maintaining glutathione in its reduced form.

**Figure 25. Anaplerosis and reduced glutathione content (GSH).**
(A) Anaplerosis, expressed as a ratio to citrate synthase activity. In the PBS group, anaplerotic flux was increased nearly 100% with TAC. Knockdown of ME1 expression significantly reduced anaplerosis in TAC to normal levels. (B) Compared to SHAM, TAC hearts demonstrated significantly lower GSH content. Knockdown of ME1 expression restored GSH content in TAC to levels comparable to SHAM. (n=3-6 for each group). White bar, SHAM; black bar, TAC. Error bars indicate mean ± SEM. *P<0.01; **P<0.001; †P<0.05 versus SHAM PBS.

D. **Discussion**

For this mechanistic study we employed a cutting-edge *in vitro* gene transfer technique developed in our lab in order to induce acute knockdown of ME1 specifically in the heart using non-native miRNA targeted to the ME1 mRNA. This technique has been shown to affect gene expression specifically limited to the myocardium (O'Donnell
and Lewandowski, 2005), which is not trivial due to the role of ME1 in lipogenesis in liver and adipose tissue. Thus, we were able to investigate the effects of ME1 knockdown on heart metabolism without any potentially confounding changes induced by altered ME1 expression in other tissues.

The findings demonstrate the maladaptive increase in anaplerosis via ME1 with TAC can be completely attenuated by knocking down expression of the enzyme. In contrast, enzyme-substrate competition induced by activation of PDC with DCA was only able to partially reduce increased anaplerotic flux in hypertrophy (Pound et al., 2009). Maladaptive increase in anaplerosis via ME1 in TAC is associated with reduced GSH content. Suppressing increased ME1 expression in hypertrophied hearts, and thus consumption of NADPH for anaplerotic malate, produced favorable metabolic shifts, improving intracellular redox state (Figure 26).

Ongoing work will answer several remaining questions regarding the effects of ME1 knockdown in cardiac hypertrophy:

- Is glucose oxidation corrected in hypertrophied hearts in which upregulated ME1 expression is suppressed? Experiments are currently underway using $^{13}$C-enriched glucose to address the fate of carbohydrate.

- Substrate-enzyme competition induced by DCA was able to restore TG content in hypertrophied hearts (Pound et al., 2009; Sack, 2009). In a similar manner, does ME1 knockdown restore normal TG content, turnover rate, and oxidation via replenishing intracellular NADPH, which is used for TG synthesis?
• In treating hypertrophied hearts with DCA, we observed an immediate benefit on cardiac function reflected in improved LV contractility (+ and –dP/dt). Does knockdown of ME1 expression have similar beneficial effect on function?
Figure 26. Summary scheme depicting the effects of ME1 knockdown in hypertrophied hearts.

(A) Increased anaplerotic flux (bold arrow) mediated by ME1 consumes NADPH (shown in green) in order to carboxylate pyruvate into malate, which enters the second span of the TCA cycle. Depleted NADPH content. As a consequence, NADPH supply for biosynthetic reactions is limited, as reflected in decreased TG stores and GSH. (B) Knockdown of ME1 is induced by cardiac-specific delivery of adenovirus containing miRNA sequence targeted to ME1 mRNA. Anaplerosis is reduced to normal low levels, restoring TG and GSH content.
V. EXPLORING THE INFLUENCE OF ENHANCED FATTY ACID UPTAKE ON LIPID DYNAMICS IN THE FAILING HEART

Our final aim was to examine how changes in LCFA uptake and activation influence the multiple kinetic components of TG turnover in normal and hypertrophied hearts. Cardiac-specific overexpression of sarcolemmal fatty acid transport protein 1 (FATP1) served as a model to address our hypothesis that enhanced uptake and activation of exogenous LCFA would alter the rate constant of the saturable component of TG incorporation and may affect the kinetics of TG turnover due to increased LCFA content within the cell.

Our initial investigation into the effects of dietary fats palmitate and oleate on lipid dynamics in failing rat hearts (see Chapter II) expanded in scope to look at differential effects on metabolic gene transcription through nuclear receptor PPAR-α (see Chapter III). Additional experiments were performed in order to meet the standards required by peer-review for publication in the journal *Circulation* (see Figures 12-16, 21). Furthermore, we encountered unforeseen experimental difficulties with the FATP1 mouse hearts in that a significant number of transgenic hearts had dramatically dilated atria and needed to be excluded from the study. This observation was confirmed in correspondence with our collaborator Dr. Jean Schaffer at Washington University, who kindly provided the mice, and adversely impacted the success rate of mouse heart perfusion experiments following TAC or SHAM procedure.
While a mechanistic link between the FATP1 expression pattern and TG dynamics remains to be elucidated, we include data demonstrating that the two kinetic phases of LCFA incorporation into TG can be resolved by dynamic mode $^{13}$C NMR spectroscopy (Figure 27). These data reflect our group’s newfound ability to overcome the technical obstacles posed by mouse heart perfusion to resolve time points of 1 min resolution with adequate signal-to-noise ratio (Goldenberg and Lewandowski, 2014).

**Figure 27.** Exponential and linear phases of long-chain fatty acid (LCFA) incorporation into triglyceride (TG) are resolved in dynamic-mode $^{13}$C NMR of the perfused mouse heart.

A transgenic FATP1-overexpressing mouse heart was perfused with U-$^{13}$C oleate for 20 min (1 min per spectrum). Blue square data points represent the integrals of the peak at 30.5 parts per million over the course of perfusion, yielding an enrichment profile reflecting incorporation of exogenous LCFA into the TG pool. The incorporation curve displays two distinct kinetic components: an initial exponential component (red line) directly related to the process of fatty acid uptake, and a delayed linear component (black line) reflecting TG turnover. Red and black lines are determined from monoexponential and linear best fit, respectively, of the individual data points.
CITED LITERATURE


APPENDICES

APPENDIX A.

Title: Dietary Fat Supply to Failing Hearts Determines Dynamic Lipid Signaling for Nuclear Receptor Activation and Oxidation of Stored Triglyceride

Author: Ryan Lahey, Xuerong Wang, Andrew N. Carley, E. Douglas Lewandowski

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American Physician Scientists Association

**ABSTRACTS**


Lahey R, Lewandowski ED. Lipid dynamics in decompensated hearts are influenced by chain composition of major dietary fats and linked to nuclear signaling through peroxisome proliferator-activated receptor-alpha. J Mol Cell Cardiol. 2013;65:S87.


PUBLICATIONS


