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Education

University of Maryland, School of Medicine, Baltimore, MD
Doctorate of Philosophy, Candidate 2009 – 2013
Major: Molecular Medicine
Track: Physiology

St. Lawrence University, Canton, NY
Bachelors of Science, 2004 – 2008
Major: Biochemistry
Minor Concentration: Mathematics

Université Rouen, Rouen, France
Intensive study of French language, European History and culture, 2005

Honors

Dean's Merit List, St. Lawrence University, 2008
Pi Mu Epsilon, Mathematics Honorary Society, St. Lawrence University, 2008
Liberty League All-Academic Honors, Women's Volleyball Team, St. Lawrence University, 2005
1856 Scholarship Award, St. Lawrence University, 2004 – 2008

Related Experience

Graduate Research Assistant, University of Maryland, Baltimore, MD 2009 – Present

- Research the effects of dietary fatty intake on mitochondrial function in pressure overload-induced heart failure in rats
- Employ mitochondrial function assays including isolation, respiration, flow cytometry, ROS production, and calcium flux experiments
- Specialized in cardiovascular metabolism and mitochondrial physiology

Production Associate II, ThermoFisher Scientific, Inc, Lafayette, CO, April – July 2009

- Use biochemical techniques to purify synthesized RNA oligomers
- Participate in monthly department meetings
- Maintain GLP and follow SOPs

Emergency Medical Technician, New York State, Certified May 2007 – 2010

- Field experience in the ambulance and emergency room at Canton-Potsdam Hospital, NY

Fellowship, Eric M. Smith Fellowship award, St. Lawrence University, 2007 – 2008

- Molecular biology and biochemistry-focused
- Determining the mechanism of binding of Rpd3*p* to the promoter region of GAP1 in yeast upon treatment with rapamycin

Mentor, Quantitative Resource Center, St. Lawrence University, 2007 – 2008

- Assist students in mathematics and statistic-related coursework

Leadership/Activities

Vice President (2007), **Secretary** (2006) American Chemical Society, 2004-2008

- Conduct biweekly meetings and participate in ACS-sponsored events

Captain (2007), Women's Volleyball Team, 2004 – 2008

- Responsible for strategy and teamwork as well as interacting with officials on the interpretation of the rules

6th Annual Leadership Conference, St. Lawrence University, February 4, 2006

Poster Presentations/Abstracts

O'Connell KA, Cox JW, Xu W, Shekar KC, Gamble D, Woodman B, Stanley WC. Decreased Mitochondrial Respiratory Capacity does not correlate with Worsened LV Function in Heart Failure. *FASEB J.* 2013

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O'Connell, K.A., Dabkowski, E.A., Ribeiro R.F., Xu W., Galvao T., Stanley, W.C. Effects of MitoQ on Mitochondrial Function in Pressure Overload-Induced Heart Failure. *FASEB J.* 2011.

Ribeiro RF, Dabkowski ER, **O'Connell KA**, Galvao T, Jonas S, Stanley WC. Docosahexaenoic acid (DHA) Supplementation Increases Mitochondrial Membrane Fluidity and Attenuates Mitochondrial ROS Production in Hypertrophied Myocardium. *Journal of General Physiology*, 2011.

Khairallah RJ, Junhwan K, O'Shea KM, **O'Connell KA**, Polster BA, Des Rosier C, Hoppel CL, Stanley WC. Dramatic alterations in cardiolipin and phospholipid composition through dietary interventions do not affect function of cardiac mitochondria. *Journal of General Physiology*, 2011.

Hecker PA, Ribeiro RF, Brown BH, **O'Connell KA**, Stanley WC. Glucose-6-phosphate dehydrogenase deficiency promotes susceptibility to pressure overload induced left ventricular dysfunction and hypertrophy. *American Heart Association*. 2011.

Galvao TF, Brown BH, **O'Connell KA**, Hecker PA, Stanley WC. Treatment with high saturated fat diet compared with standard diet and high n-3/n-6 polyunsaturated fat diet, decreases mortality in cardiomyopathic hamsters. *FASEB J*. 2011.

Galvao TF, Hecker PA, Brown B, **O'Connell KA**, O'Shea K, Des Rosiers C, Stanley WC. High Saturated Fat Intake Improves Survival in Genetic Cardiomyopathy Compared to a High Polyunsaturated Fat Diet or a Low Fat Diet. *American Heart Association*. 2011.

Oral Presentations

Docosahexaenoic Acid Prevents Cardiac Dysfunction in Heart Failure, and Increases Mitochondrial Membrane Fluidity, Ca^{2+}Uptake, and Resistance to Permeability Transition. **Kelly A O'Connell**, Erinne R Dabkowski, Rogerio F Ribeiro, Wenhong Xu, Tatiana Galvao, William C Stanley, Univ of Maryland Baltimore, Baltimore, MD, *American Heart Association Scientific Sessions,* November 6, 2012.

Publications

O'Connell KA, Cox JW, Xu W, Shekar KC, Gamble DM, Woodman BF, Deneault C, Des Rosiers C, Stanley WC. Diets High in Monounsaturated, n6-polyunsaturated, and Saturated fats cause Profound Differences in Cardiac Phospholipids with Little Impact on Pathological Processes in Heart Failure. 2013 (*In revision*).

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Khairallah RJ, Kim J, O'Shea KM, **O'Connell KA**, Brown BH, Galvao T, Des Rosiers C, Polster BM, Hoppel CL, Stanley WC. Improved Function of Cardiac Mitochondria with a Diet Induced Increase in n3 or n6 Polyunsaturated Fatty Acids in Membrane Phospholipids. *PLoS One* 2012;7(3):e34402.

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Papanicolaou KN, Ngoh GA, Dabkowski ER, **O'Connell KA**, Ribeiro RF, Stanley WC, Walsh K. Cardiomyocyte deletion of mitofusin-1 leads to mitochondrial fragmentation and improves tolerance to ROS-induced mitochondrial dysfunction and cell death. *Am J Physiol Heart Circ Physiol*. Jan 1;302(1):H167-79, 2012.

Professional Memberships

American Heart Association
2012-Present

American Physiological Society
2011-Present

Funding/Awards

American Heart Association Mid-Atlantic Pre-Doctoral Fellowship Award
University of Maryland, Baltimore
Title: Effects of Dietary Fat Intake on Mitochondrial Function in Heart Failure
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T32 AR007592 Muscle Biology Training Grant
University of Maryland, Baltimore
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Title of Dissertation: Effects of Dietary Fat Intake on Mitochondrial and Cardiac Function in Heart Failure

Kelly A. O'Connell, Doctor of Philosophy, 2013

Dissertation Directed By: Dr. William C. Stanley

Abstract

New treatments are needed for heart failure (HF) and dietary interventions have been suggested as a potential adjunct to current therapies. Recent evidence shows that high fat diets, in the absence of obesity, can improve left ventricular (LV) dysfunction and survival in HF. Mitochondria contribute to HF pathology through impaired oxidative phosphorylation and greater susceptibility to mitochondrial permeability transition (MPT). MPT dissipates membrane potential and triggers cardiomyocyte death. Our lab found that the n3-polyunsaturated fatty acid (n3-PUFA) DHA incorporates into mitochondrial membranes and delays MPT, suggesting that dietary lipids affect mitochondrial phospholipid (PL) composition and function. Survival in HF was prolonged by a high fat diet enriched with long chain saturated fat (LCSat) and monounsaturated fat (MUFA) compared to a high fat diet comprised of a mixture of MUFA+n6-PUFA+n3-PUFA or a low fat diet. The effects of specific dietary fatty acids on the normal and failing heart are complex and poorly understood. Thus, this dissertation *1)* compares the effects of low fat/high DHA and high LCSat diets on cardiac and mitochondrial function in healthy animals, and *2)* systematically evaluates diets high in MUFA, n6-PUFA or LCSat in pressure overload-induced HF. In Aim 1, normal animals were fed diets rich in DHA or LCSat for 6 weeks. Despite dramatic changes in mitochondrial PL composition and slow mitochondrial Ca^{2+} uptake by the high LCSat diets, there were no effects on mitochondrial respiration or cardiac function. In

Aim 2, HF and sham animals were treated with various high fat diets (40% energy as fat) or a standard low fat diet (15% fat) for 15 weeks. There was no evidence of obesity or adverse effects on cardiac structure or function with the various high fat diets. The LCSat diet ameliorated the HF-induced decrease in ejection fraction compared to a standard diet (65% for LCSat vs. 48% for standard). The high MUFA diet conferred the greatest protection from MPT and better maintenance of mitochondrial phospholipid composition in sham rats. In summary, despite dramatic diet-induced differences in mitochondrial PL fatty acid composition, high fat diets had neutral effects on mitochondrial and contractile function.

Effects of Dietary Fat Intake on Mitochondrial and Cardiac Function in Heart Failure

by
KELLY A. O'CONNELL

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Dedication

To Joyce and Kevin O'Connell - my parents and my foundation

To Eric and Michael - my brothers who constantly push me out of my comfort zone

To Fraser, my best friend, my brilliant better half, and my Penguin

Words cannot express how much I love you all.

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Table of Contents

List of Tables

Chapter 1

Chapter 2

Chapter 3

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of Abbreviations

ACE, Angiotensin converting enzyme

AHA, American Heart Association

ALA, α-Linolenic acid

ANF, Atrial natriuretic factor

ANOVA, Analysis of variance

ANT, Adenine nucleotide translocase

ATP, Adenosine triphosphate

BMI, Body mass index

BSA, Bovine serum albumin

CD36, Cluster of differentiation 36, also known as FAT1

CHD, Coronary heart disease

CHO, Chinese hamster ovary

CL, Cardiolipin

COX, Cyclooxygenase

CPT-1, Carnitine palmitoyl transferase-1

CsA, Cyclosporin A

CVD, Cardiovascular disease

CypD, Cyclophilin D

DHA, Docosahexaenoic acid

DPH, 1,6-Diphenyl-1,3,5-hexatriene

EDD, End diastolic diameter

EF, Ejection Fraction

EPA, Eicosapentaenoic acid

ESD, End systolic diameter

FA, Fatty acid

FAO, Fatty acid oxidation

FAT1, Fatty acid transporter-1

FS, Fractional shortening

H_2O_2, Hydrogen peroxide

HDL, High density lipoproteins

HF, Heart Failure

HHE, 4-Hydoxy-2-hexanal

HNE, 4-Hydroxy-2-nonenal

HRP, Horseradish peroxidase

IFM, Interfibrillar mitochondria

IL, Interleukin

L_4CL, Tetralinoleoyl cardiolipin

LCAD, Long chain acyl-CoA dehydrogenase

LCHAD,

LCSat, Long chain Saturate fat

LDL, Low density lipoprotein

LV, Left Ventricle

LVH, Left ventricular hypertrophy

MCAD, Medium chain acyl-CoA dehydrogenase

MHC, Myosin heavy chain

MPT, Mitochondrial permeability transition

MUFA, Monounsaturated fatty acids

NEFA, Non-esterified fatty acids

NFκB, Nuclear factor kappa B

NYHA, New York heart association

PDH, Pyruvate dehydrogenase

PDK4, Pyruvate dehydrogenase kinase 4

PET, Positron emission tomography

PGC-1α, PPAR gamma coactivator-1α

PGE2, Prostiglandin E2

PL, Phospholipid

PPAR, Peroxisome proliferator activated receptor

Ppia, Cyclophilin A

PUFA, Polyunsaturated fatty acids

RCR, Respiratory control ratio

RNA, Ribonucleic Acid

ROS, Reactive oxygen species

SEM, Standard error

SHHF, Spontaneous Hypertensive Heart Failure

SIRT1, Sirtuin 1

TCA, Tricarboxylic Acid

TG, Triglyceride

TMA-DPH, 1-(4-Trimethylammoniumphenyl)-6-Phenyl-1,3,5-Hexatriene *p*-Toluenesulfonate

TNF-α, Tumor necrosis factor α

UCP3, Uncoupling protein 3

Chapter 1 – Introduction

Dietary lipids contribute to the regulation of cardiac function by modulating membrane phospholipids, serving as signaling molecules and ligands for nuclear receptors, and providing the primary substrate for oxidation by cardiac mitochondria. Much attention has been paid to the effects of dietary lipids on the incidence of coronary heart disease (CHD) but their effects on other types of cardiovascular diseases, specifically heart failure (HF), has been largely ignored. The American Heart Association estimated that approximately 6.6 million U.S. adults (≥18 years of age) were living with HF as of 2010[6]. Many forms of therapy exist to treat the symptoms of HF, but despite aggressive diagnosis and treatment, HF remains a major clinical problem and a huge burden on the US health care system. Current medical therapies can improve clinical symptoms and slow the progression of HF but the prognosis remains poor even for optimally-treated patients[7, 8]. Thus, there is a need for novel therapies.

Recent clinical trials and studies in small animal models of HF suggest the novel concept that a diet high in fat and low in carbohydrate prevents the development and progression of HF compared to low fat/high carbohydrate diets[4, 5, 9-15]. The idea of replacing dietary carbohydrate with fat to prevent HF may seem counterintuitive, however, it is largely in line with current thinking regarding dietary fat and prevention of CHD[16-18]. This dissertation will address the effects of dietary lipid on cardiac function and the progression of HF.

Heart Failure

Definition of Heart Failure

The American Heart Association defines heart failure as a "complex clinical syndrome that can result from any structural or functional cardiac disorder that impairs the ability of the ventricle to fill with or eject blood."[19] The two most common causes of HF are coronary heart disease or prolonged hypertension[19] which results in left ventricular hypertrophy (Figure 1.1). Similar to La Place's law describing transmural pressure differences in blood vessels, wall stress and thickness can be related according to the following expression:

$$\sigma \propto \frac{P * r}{2h}$$

where σ is wall stress, P is ventricular pressure, r is ventricular radius and h is wall thickness[20]. This equation will change slightly when considering the left ventricle as it is not perfectly spherical. By examining this equation, one can see that ventricular hypertrophy will ameliorate an increase in wall stress that occurs with hypertension. Left ventricular hypertrophy can also occur via physiological mechanisms such as chronic exercise (Figure 1.1) which does not include fibrosis or dysfunction and is colloquially known as the "athlete's heart." Myocardial infarction also can cause HF due to replacement of functioning myocardium with scar, which increases the work demand on the remaining myocardium and triggers cardiomyocyte hypertrophy (Figure 1.1).

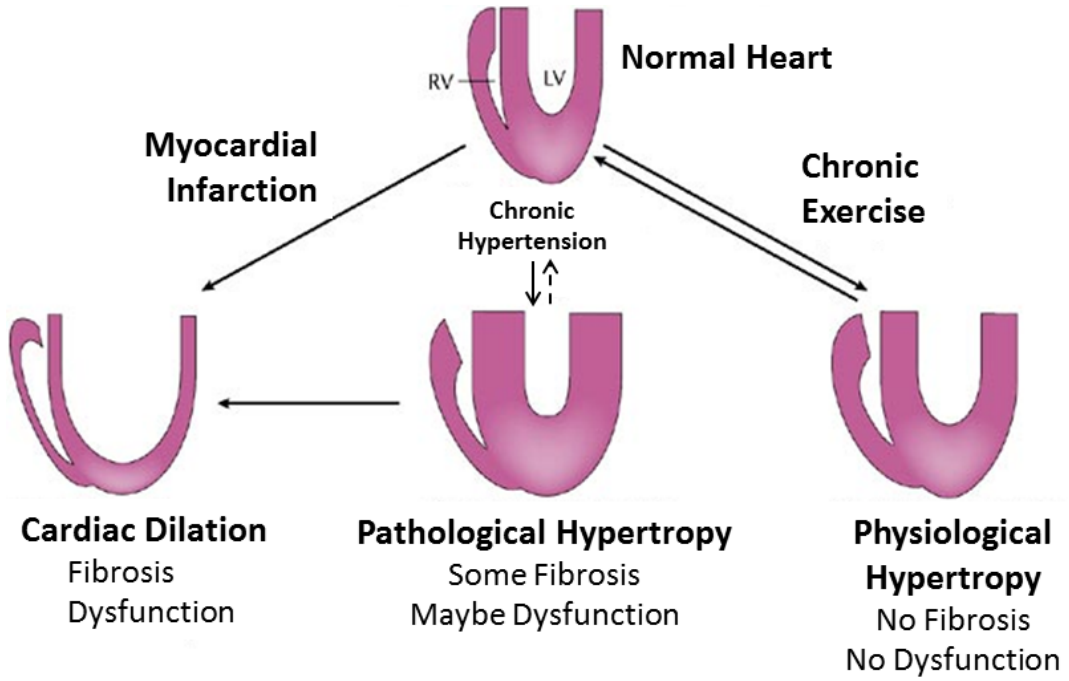

Figure 1.1. **Left ventricular hypertrophy** can arise from a multitude of situations including physiological adaptation (such as chronic exercise) and pathological hypertrophy as a consequence of chronic hypertension or myocardial infarction. (Adapted from Heineke et al. *Nature Reviews Molecular Cell Biology* 2006[21]).

The diagnosing and classification of heart failure rely heavily upon physical symptoms. According to the prospective epidemiological Framingham Heart Study, in which over 5,000 men and women were followed for over 4 decades, the criteria for diagnosing congestive HF include the concurrent presence of either 2 major criteria or 1 major and 2 minor criteria[22]. The major criteria include weight loss, dyspnea, neck vein distension, rales, pulmonary edema and radiographic cardiomegaly. Minor criteria include dyspnea on ordinary exertion, pleural effusion, tachycardia and bilateral ankle edema. Another method of classifying HF is through the use of the New York Heart Association functional classification system. This system uses four classes based on how a patient feels during physical exertion[23]. Class 1 include patients with cardiac disease but no physical limitations, Class 2 includes patients who experience fatigue upon ordinary exertion, Class 3 is characterized by marked limitations wherein less than

ordinary exertion causes fatigue, and Class 4 includes patients who experience dyspnea at rest.

Current Therapies for Heart Failure

Heart failure can often be difficult to diagnose as patients presenting to the emergency department have differential symptoms. Many patients present with a chief complaint of dyspnea (shortness of breath), related to increased left atrial pressure. Thus, approved therapies to treat patients with acute heart failure syndrome are to relieve congestion (i.e. use of diuretics to modulate fluid levels and reduce pre-load), balance hemodynamics and achieve normovolemia[24]. Inotropes (drugs which improve contractility) can also be used in the acute setting to relieve symptoms of HF, mainly pulmonary edema and dyspnea. In the chronic setting, therapies for patients with decreased left ventricular ejection fraction (EF) are through the use of ACE inhibitors or angiotensin-II receptor antagonists to reduce ventricular "remodeling," and β-adrenergic receptor antagonists (i.e. specifically selective for β1 receptors), which are negative inotropes that reduce heart rate and decrease the force of contraction thereby reducing blood pressure[25]. Though these treatments are available, the American Heart Association estimates that an additional 3 million Americans will be diagnosed with HF by 2030, a 25% increase in prevalence since 2010[6], thus more effective therapies are needed.

Current Dietary Recommendation for Heart Failure Patients

Clearly there is a need to develop recommendations for dietary macronutrients for people who are "at risk" for the development of HF (e.g. patients with CHD,

hypertension, left ventricular hypertrophy (LVH), diabetes, etc.), and for patients with established HF. There are no current recommendations for HF patients that specifically refer to dietary fat intake, and patients are encouraged to follow the current guidelines for prevention of cardiovascular diseases, CVD, mainly CHD[26-28]. Guidelines for fat intake to prevent CHD have recently changed in response to results from epidemiological studies which showed that the incidence of CVD and CHD was not related to the intake of fat[16, 17, 29], and a large interventional study that found incidence of CHD was not affected by a 25% reduction in fat intake[29]. This has prompted a major shift in the paradigm for preventing CHD, away from reducing fat intake towards the current recommendation of a diet high in unsaturated fats and low in sugars and refined carbohydrate[18] although this does not particularly address patients with HF. This concept extends to the prevention of obesity, where decreased relative intake of rapidly absorbed carbohydrate and increased consumption of unsaturated fat is associated with less weight gain[30].

Effects of Dietary Fat on Development and Progression of HF

The concept of developing optimal diets for the prevention and treatment of HF is particularly attractive as many patients are looking for treatments beyond the pharmacy. Also, any beneficial effects of dietary manipulation should be additive or synergistic with current treatments with drugs and devices. At present, there is limited information regarding the effects of dietary macronutrient composition and HF, however new research is being conducted and progress in this field is being made.

Clinical Studies on Dietary Manipulation

Based on over 30 years of data from the National Health and Nutrition Examination Surveys in 2009, Ford et al[31] describes the trends of risk for CVD since the 1970s. From the 1970s through the 1990s, the risk for CVD decreased due to decreases in smoking, hypertension, hypercholesterolemia and improvements in medical care; however there has been a reversal of risk for CVD from the late 1990s that is associated with an increased incidence of obesity and diabetes mellitus[31]. Thus, unfavorable behavior seems to have counteracted progress in medical technology and care, and suggests that a larger problem of dietary and lifestyle choices are increasing the risk of heart disease. Analysis of the dietary patterns of HF patients in the United States reveals that a generally poor diet likely has a negative impact on disease progression and the underlying pathophysiology[32].

Many initial recommendations for treating heart disease suggested that a low fat (and thus high carbohydrate) diet would be beneficial in ameliorating the increase in CVD risk. However, in a study of over 80,000 women followed for 20 years, those that ate the greatest amount of rapidly-absorbed carbohydrate had a significant increase in their risk of CHD [16, 33] whereas those who consumed the most amount of vegetable fat (olive, soybean, corn and canola oils) had a significant decrease in their risk of CHD and other cardiovascular diseases[16, 34-36]. Similarly, in a randomized controlled interventional trial of over 48,000 post-menopausal women followed for an average of 8.1 years, a 20% decrease in calories from fat did not significantly reduce the risk of CHD, stroke, or CVD[29]. These results suggest that decreased fat intake is not correlated with cardiovascular disease risk and conversely, increased fat intake may be beneficial in

reducing CVD. However, the specific quantity and type of fat that is beneficial remains uncertain. Most recently, a randomized controlled trial of 7,447 people without cardiovascular disease but considered high risk due to their risk factor profile (type 2 diabetes, or 3 or more of the following: smoking, hypertension, elevated LDL, decreased HDL, overweight or obese, or family history of CHD) were assigned to dietary treatment groups in a 1:1:1 ratio to a Mediterranean high fat diet supplemented with olive oil, a Mediterranean high fat diet supplemented with nuts, or a low fat "control" diet[15]. A Mediterranean high fat diet consists of a diet high in monounsaturated fats and low in n3-PUFAs (specifically α-linolenic acid). Patients were followed for an average of 4.8 years. There was a relative risk reduction of 30% for major CVD events (myocardial infarction, stroke, or death from cardiovascular causes) in both Mediterranean diet groups supplemented with nuts or olive oil compared to the control diet[15]. Though much of this research has been on CHD, few studies explore the effects dietary supplementation in HF.

Insights from the Animal Laboratory

Several studies from our lab and others have shown that a high fat diet, in the absence of obesity, does not adversely affect the heart[37-41] and may even prevent the development and progression of HF in response to hypertension or myocardial infarction (Table 1.1).[4, 5, 10, 11, 42, 43].

Table 1.1. **Diet Studies.** Compilation of studies assessing the effects of high fat diets in the absence of obesity or supplementation with n3-PUFA in heart failure. Studies where body mass was increased >10% were excluded.

Diet comparison	Species	Model	Duration	Primary Outcome	Ref
High Fat Diet Studies					
20% fat diets compared (high palmitate, myristate, and oleate)	Humans	Healthy	5 wks	No changes in inflammatory markers between groups	44
60% fat vs 12% fat	Rats	Salt-induced Hypertension	6 wks	High fat diet attenuated LVH and improved contractile function.	10
60% fat diet vs 10% fat diet	"	"	12 wks	High fat diet attenuated LVH and improved contractile function.	5
60% fat diet vs 10% fat diet	"	"	13 days	High fat diet prolonged survival	42
60% fat diet vs 10% fat diet	"	"	8 wks	High fat diet prolonged survival	42
35% fat diet (mainly oleate) vs 10% fat diet	"	"	15 wks	High oleate prevented LVH and attenuated increase in systolic BP	45
60% fat diet vs 14% fat diet	Rats	Coronary Artery Ligation	8 wks	High fat intake decreased LV fractional shortening, but increased peak dP/dt.	9
60% fat diet vs 10% fat diet	"	"	8 wks	High fat diet increased LV contractile function	13
60% fat diet vs 10% fat diet	"	"	8 wks	High fat diet increased LV contractile function	14
60% fat diet (mainly saturated fat) vs 10% fat diet	"	"	8 wks	High fat diet improved contractility and decreased MHCβ	46
42% fat (high linoleate or mixed fats) vs 12% fat diet	Rats	Genetic Hypertension	~6 months	Both high fat diets prevented LV dilation, and the high linoleate acid diet prolonged survival.	12
58% fat diet (mainly saturated fat) vs 10% fat diet	Rats	Aortic Constriction	8 wks	High fat diet increased contractile function.	11
60% fat diet vs 14% fat diet	"	"	8 wks	High fat diet had no effect on LV mass, function or chamber volume	47
45% fat diet (either saturated + monounsaturated fat or PUFA) vs 12% fat diet	Hamsters	δ sarcoglycan cardiomyopathy	>1 year	High saturated+monunsaturated fat diet prolonged survival compared to low fat or high PUFA diets	4
~10% n6-PUFA compared to ~13% MUFA and 13% n3-PUFA	Rabbits	Aortic valve rupture + stenosis	4 months	High MUFA and n3-PUFA prevented LVH	48

Table 1.1. Continued

60% fat diet vs 10% fat diet	Mice	Aortic Constriction	16 wks	High fat diet had no effect on LV mass, function or chamber volume	49
60% fat diet vs 10% fat diet	"	"	4 wks	High fat diet increased LVH, worsened LV dilation and contractile dysfunction	50
n-3 PUFA Studies					
Observational Epidemiological Analysis	Humans	At risk population	12 years	Intake of DHA+EPA inversely related to risk for new onset HF	51
DHA+EPA (5.4 g/d) vs. Placebo	Humans	Advanced HF patients	18 weeks	DHA+EPA decreased TNF-α	52
DHA + EPA (0.9 g/d) vs. Placebo	Humans	HF patients	3.9 years	DHA+EPA decreased risk of death and admission to hospital for cardiovascular events by 8% vs. placebo	53
DHA+EPA (1.6 g/d) vs. Placebo	Humans	Nonischemic Dilated HF	12 months	n3-PUFA increased EF by 10.4%	54
DHA+EPA (0.9 g or 3.6g/d) vs Placebo	Humans	Nonischemic dilated HF	3 months	Dose dependent increase in LV ejection fraction.	55
High α-linolenic acid/low n-6 PUFA diet vs standard low n-3 PUFA diet.	Hamsters	δ sarcoglycan cardiomyopathy	>1 year	High α-linolenic acid prolonged survival	56
DHA+EPA (2.8% of energy intake vs standard low n-3 PUFA diet.	Rats	Aortic Constriction	12 wks	DHA+EPA prevented LVH and LV dilation.	57
DHA+EPA or α-linolenic acid dose-response vs standard low n-3 PUFA diet.	"	"	12 wks	DHA+EPA prevented LV dilation and increased systemic inflammation, but α-linolenic acid did not.	58
DHA, EPA or DHA + EPA (2.3% energy intake) vs standard low n-3 PUFA diet.	"	"	17 wks	No effect on LV function or mass with moderate LVH, but increased resistance to Ca2+ -induced MPT.	59

Table 1.1. Continued

DHA+EPA (2.3% of energy intake) vs standard low n3 PUFA diet.	"	"	8 wks	DHA+EPA prevented LVH and inflammation.	47
DHA+EPA (1.5% of chow by mass) vs. standard low n-3 PUFA diet.	"	"	15 wks	DHA+EPA improved *ex vivo* cardiac mechanical function and efficiency.	60
DHA+EPA (2.3% of energy intake) vs. standard low n-3 PUFA diet.	Rats	Myocardial Infarction	12 wks	No effect on LV function or mass, but increased resistance to Ca^{2+} -induced MPT.	61
DHA + EPA (2.3% of energy intake) vs. standard low n-3 PUFA diet.	Mice	Aortic Constriction	6 wks	DHA+EPA prevented LV dilation.	62
Menhaden oil (DHA + EPA at 1% of total energy intake) vs. standard low n3-PUFA diet.	"	"	4 wks	DHA+EPA prevented contractile dysfunction and myocardial fibrosis.	63

In hypertensive rats fed a high fat diet, there was improved LV contractile function and an attenuation of hypertrophy compared to a low fat diet[5, 10, 42]. It was originally thought that a high fat diet would accelerate the pathological processes involved in the progression of HF. Early studies from our lab were performed in the Dahl salt-sensitive rat which develops hypertension upon salt feeding and displays progressive LVH, increases in LV end systolic and diastolic volumes, and recapitulates much of the pathophysiology of the clinical condition of HF with preserved ejection fraction[64]. In this model, the effects of a high saturated fat chow (60% of energy from fat, mainly from cocoa butter) to a standard low fat/high carbohydrate chow (10% of energy from fat) on the development of hypertension-induced HF were compared[5, 10]. After 12 weeks of hypertension (high salt feeding) and dietary treatment, there were no differences in body mass or the degree of hypertension[5]. In the low fat/high carbohydrate/high salt group, hypertension increased LV mass and end systolic and diastolic volume which was prevented by the low carbohydrate/high fat diet (Figure 1.2A). A possible mechanism of this beneficial effect of high fat is through the activation of PPARs (discussed below) and better maintenance of mitochondrial content and function. The cardiac activity for the mitochondrial fatty acid oxidation enzyme medium chain acyl-CoA dehydrogenase (MCAD) and Krebs cycle enzyme citrate synthase were reduced in the hypertensive rats fed a low-fat diet compared with non-hypertensive animals on low-fat chow (Figure 1.2B)[5]. The decrease in the activity and transcript levels of these enzymes was ameliorated with the high fat diet. With the low fat diet, there was a large induction of the mRNA for atrial natriuretic factor (ANF) and fetal myosin (MHCβ, a classic marker of HF) that was ameliorated with the high fat diet. Later it was

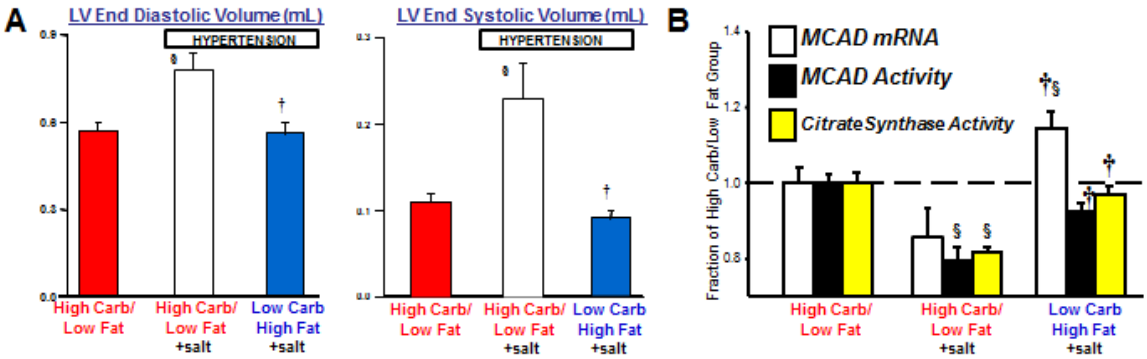

Figure. 1.2. **Effects of a high fat/low carbohydrate diet**. A high fat/low carbohydrate diet prevents expansion of the LV chamber (A) and a decrease in the mRNA and activity of the fatty acid β-oxidation enzyme medium chain acyl-CoA dehydrogenase (MCAD) and the Krebs cycle enzyme citrate synthase in Dahl salt-sensitive hypertensive rats (B). From Okere et al, Hypertension, 2006[5].

found that survival was prolonged by the high fat chow diet compared to low fat/high carbohydrate diets[42]. One must keep in mind that the Dahl rat is a model of genetic HF that involves idiopathic hypertension and a defect in the myocardial fatty acid transport protein FAT1/CD36[65], and thus likely has limited application to human HF.

Because the cardioprotective effects of a high fat diet observed in the Dahl salt sensitive rats could be unique to the Dahl rat model, similar studies were performed in a pressure overload-induced HF caused by aortic banding. In this rat model of aortic stenosis, we again found that the high fat diet significantly prevented the chamber enlargement compared to a low fat/high carbohydrate diet[11]. In mice with aortic constriction, a high fat diet did not affect LV mass or function[50] but did lead to a better maintenance of the activity of mitochondrial oxidative enzymes[49, 66], suggesting that there are species differences (Table 1.1). In rats with HF secondary to myocardial infarction, studies found a neutral effect on ventricular remodeling and function with a high fat diet, although this was confounded by the development of obesity[67].

In contrast, we and others have found different results in a genetic hamster model of dilated HF caused by a δ-sarcoglycan deficiency[4, 56]. We compared the effects of two

high fat diets (45% of energy from fat): a high n3-PUFA + n6-PUFA diet (mainly α-linolenic acid, 18:3n3, commonly found in canola and flaxseed oils and linoleate, 18:2n6), and a high long chain saturated and monounsaturated (LCSat+MUFA) diet [mainly palmitate (16:0, 11%) and stearate (18:0, 12%)]. There was only a modest ~10%

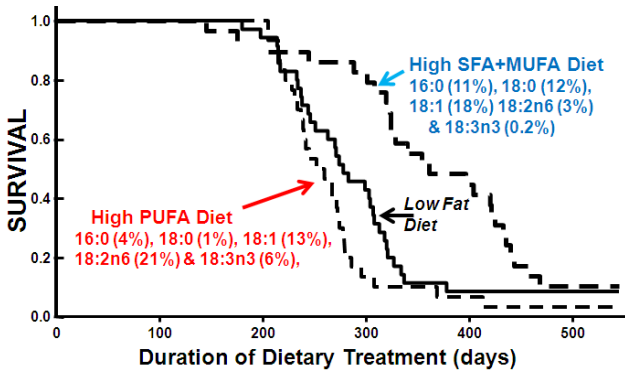

Figure. 1.3. Survival of cardiomyopathic hamsters. Median survival on treatment increased from 278 days with the low fat diet to 361 days with high LCSat+MUFA ($P<0.01$). The high PUFA group was not different from the low fat diet (260 days) but was shorter than LCSat+MUFA ($P<0.01$). The fatty acid content is expressed as per cent of total energy in the diet. Modified from Galvao et al 2006[4].

increase in body mass with the two high fat diets, but surprisingly, as shown in Figure 1.3, consumption of the high LCSat+MUFA diet prolonged life compared to either the standard low fat diet or the high n3-PUFA+n6-PUFA diet[4]. Recently, in rabbits subjected to HF induced by aortic valve rupture and subsequent stenosis leading to pressure and volume overload, supplementation with n3-PUFA or MUFA (mainly oleate) for 4 months prevented LV hypertrophy compared to a diet containing mostly linoleate (Table 1.1)[48]. Further support for a beneficial effect of a high fat diet in HF comes from studies in the spontaneously hypertensive HF (SHHF) rat, where LV chamber expansion was prevented by feeding old SHHF rats a diet high in n6-PUFA (linoleate from safflower oil) or LCSat+MUFA diet (from lard)[12]. Interestingly, only the n6-PUFA diet

prolonged survival compared to the low fat diet[12] which seems to suggest that diets high in linoleate may be beneficial for the heart in HF. However, both the cardiomyopathic hamster and SHHF models are genetic models of cardiac dysfunction so caution should be taken in extrapolating these findings to humans. At present, our understanding of the potential benefit of high fat diets in HF patients is confounded by the lack of a systematic comparison of various commonly consumed fats (LCSat, MUFA and n6-PUFA) in an established model of advanced HF. To date, this work has largely focused on prevention of the early development of HF in rodent models and has generally used a single high fat diet compared to a low fat diet.

Metabolic Effects of Substituting Fat for Carbohydrate

Replacing carbohydrate by increasing fat intake, particularly unsaturated fatty acids for sugars and rapidly absorbed starches, has beneficial effects on the plasma lipid profile, reduces insulin secretion, and lower post prandial glycaemia and lipid storage[18, 30, 68]. Historical dietary guidelines have recommended consumption of a low saturated fat/high carbohydrate diet to reduce CHD, however modern "Western" diets have adopted a greater consumption of fructose and rapidly absorbed carbohydrate and a decreased intake of complex carbohydrate and fat[69]. As detailed above, clinical studies found no evidence for beneficial effects of reducing fat intake in terms of the incidence of CHD. Low fat/high carbohydrate diets are associated with increased blood lipids, glucose and insulin, and elevated blood pressure as compared to high fat/low carbohydrate/high complex carbohydrate diets[70-72]. Clinical studies find that high plasma insulin is associated with LVH[73-77]. A high glycemic load (such as occurs with high

carbohydrate diets) stimulates insulin secretion and activation of insulin signaling pathways, enhancing protein synthesis which could induce LVH, fibrosis, reactive oxygen species and apoptosis, and contribute to the development of HF[68, 78, 79]. In addition to the effects on the myocardium, high fat/low carbohydrate diets are an effective means for reducing body weight, insulin resistance, and serum triglycerides and can increase high density lipoprotein cholesterol in humans[72, 80, 81]. In contrast, diets high in simple sugars increase LV dysfunction and mortality compared to diets high in fat or complex carbohydrate in hypertensive rats or mice with aortic constriction [42, 43, 68, 82, 83]. Taken together, evidence from epidemiologic, clinical, and mechanistic studies is consistent, showing that an increase in fat intake in exchange for carbohydrate will lower serum triglycerides and improve insulin sensitivity without adverse effects on the cholesterol profile.

Cardiac Mitochondria in Heart Failure

Energy in the form of ATP is necessary for the proper function of cardiac contraction and relaxation. Cardiac mitochondria contribute 50 - 90 % of the ATP supply[84]. Mitochondrial physiology changes with the progression of hypertrophy to HF with modifications in mtDNA, content, morphology, ETC complexes and activity and oxidative phosphorylation[85, 86]. The causative effects of mitochondrial dysfunction and cardiac disease is controversial yet amelioration of mitochondria damage remains an attractive therapeutic target for many cardiac diseases.

Mitochondrial Subpopulations

Within the cardiomyocyte, mitochondria are spatially separated into two subpopulations: subsarcolemmal mitochondria (SSM) and interfibrillar mitochondria (IFM) first observed in 1977 by Palmer et al[87]. SSM are located beneath the plasma membrane while IFM are restricted between the myofibrils. These subpopulations have distinct morphologies and functions. The maximal rate of substrate oxidation (state 3 respiration) is usually increased in IFM compared to SSM and the specific activities of mitochondrial enzymes (i.e. citrate synthase) are also increased in isolated IFM compared to SSM in normal rats[87-89] or in infarct-induced HF[39, 61]. In addition to bioenergetic differences[87], IFM are have greater resistance to mitochondrial permeability in response to apoptotic stimuli (i.e. Ca^{2+}, ROS)[90-94].

Impact of Dietary Fat on Mitochondrial Phospholipid Composition

Dietary fats can have profound effects on cardiac mitochondrial membrane phospholipids which can impact mitochondrial function and affect the progression HF. Mitochondrial membranes contain high levels of cardiolipin (CL), a signature phospholipid that accounts for 10-20% of the total mass of mitochondrial phospholipids[95-100]. CL content is higher in the inner mitochondrial membrane where it is synthesized relative to the outer membrane[101]. Tetralinoleoyl CL (L_4CL) is the most prevalent form of CL in cardiac mitochondria in humans, dogs and rats[97, 98, 100, 102]. CL is thought to contribute to oxidative phosphorylation, as it is only found in membranes that have an electrochemical gradient[101] and indeed, CL helps stabilize the assembly of respiratory complexes[101, 103, 104] and ATP synthase[105]. It is also thought to contribute to

cristae formation as cardiolipin-deficient models have abnormal inner membrane morphology[103, 105, 106] which affects mitochondrial function. This is seen in Barth syndrome which is caused by a genetic mutation in tafazzin, an essential enzyme for the synthesis of functional CL[107-109]. These patients have low systemic levels of total CL and L_4CL, resulting in dilated cardiomyopathy, skeletal muscle weakness and neutropenia[102]. Reduced L_4CL levels have been observed in genetic and pressure overload-induced HF models in rats, and in myocardium from HF patients, suggesting that loss of L_4CL might contribute to the progression of HF[12, 99, 100, 110]. However, L_4CL was increased by 60% with a high LCSat diet but was not associated with LV dysfunction or pathology in rats with aortic banding[47]. Thus, the extent that total L_4CL levels contribute to advanced HF is not clear. Linoleate, an n6-PUFA, is the primary fatty acid side chain in CL, but increases in n6-PUFA have been suggested to increase inflammation via the production of arachidonic acid (20:4n6) and inflammatory prostaglandins (discussed below). In SHHF rats, which are depleted of L_4CL, supplementation with a high linoleate increases total CL and L_4CL in myocardium and is associated with amelioration of LV dilation and extended survival compared to a low fat diet or a high fat diet comprised of LCSat+MUFA[12] suggesting that linoleate influences the stereochemical arrangement of fatty acids in CL, thereby affecting its biological function[111].

In humans, a high intake of marine n3-PUFA (between 3 and 6g/day), specifically docosahexaenoic acid (DHA; 22:6n3) and eicosahexaenoic acid (EPA; 20:5n3), results in a rapid incorporation of DHA and EPA into myocardial PL along with a proportional decrease in arachidonic acid (20:4n6)[112, 113] which plateau in effect after approximately 4 weeks[112]. DHA and EPA incorporation into membranes increases the double bond index

(equivalent to the sum of the mole percentage of unsaturated FA in the membrane multiplied by the number of double bonds in each unsaturated FA) and membrane fluidity, which can affect the ability for proteins to move and interact within the membrane. An increase in membrane fluidity can have major effects on the function of cardiac mitochondria and may increase the interaction among respiratory complexes and the capacity for oxidative phosphorylation. Indeed, a diet rich in DHA+EPA at a pharmacological dose (equivalent to >4g/day in humans) has prevented LV remodeling and contractile dysfunction in rats with aortic constriction[113]. Epidemiology studies suggest that a high intake of DHA+EPA (>0.4g/day) is strongly associated with reduced risk of HF[51, 113]. The mechanism for this effect is not clear, but may be due to improved function of mitochondria secondary to changes in membrane PL fatty acid composition.

Further, incorporation of DHA into cardiac phospholipid membranes prevented the expansion of the LV in response to aortic constriction in a dose-dependent manner[58]. This effect corresponded with a decrease in arachidonic acid in membrane phospholipids, and a decreased in systemic inflammation, evidenced by lower urine thromboxane B2 and serum TNF-α. In contrast, high intake of α-linolenic acid (18:3n3), an essential fatty acid from flaxseed oil, did not increase DHA or EPA in cardiac membranes due to its limited capacity for elongation and desaturation, and thus did not improve cardiac function or exert anti-inflammatory effects. Furthermore, an increase in n3-PUFA increases membrane fluidity and the ability for proteins to move and interact within the membrane (*Dabkowski, O'Connell et al, Unpublished*).

In the cardiomyopathic hamster model, as discussed above, the high n3-PUFA+n6-PUFA diet was compared to a high long chain saturated and monounsaturated fat diet as well as a standard low fat (12% of energy from fat)/high carbohydrate diet. Analysis of whole myocardial tissue PL fatty acid composition revealed that the combined PUFA diet increased linoleate (18:2n6) by ~70% compared to the standard low fat diet or the LCSat+MUFA diet (Figure 1.4). Linoleate can be converted to arachidonic acid which participates in the formation of pro-inflammatory mediators, thus the PUFA diet might increase inflammation. However, we observed no increase in urine thromboxane B_2 concentration suggesting this pathway was not accelerated[4]. Thus, the diet high in both α-linolenic acid and linoleate had a negative effect on survival when compared to a mixed high fat diet comprised of LCSat+MUFA. A comparison of the effects of long term treatment with diets specifically high in LCSat, MUFA, or n6-PUFA on LV remodeling, function and survival has not been performed, but are essential prior to advancing this concept into studies in HF patients.

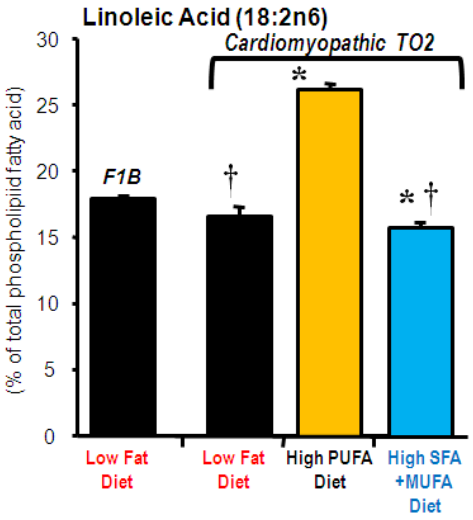

Figure 1.4. Myocardial PL content of linoleate (18:2n6) expressed as a per cent of total fatty acids after 24 weeks of dietary treatment. *P<0.05 vs. F1B; †P<0.05 vs. TO2 on the High PUFA diet. From Galvao et al 2012[4].

Mitochondrial Permeability Transition

Mitochondrial permeability transition (MPT) is a phenomenon that occurs in response to extra-mitochondrial stressors, and is characterized by the formation of a high conductance (or a low-conductance) pore that is formed across the inner mitochondrial membrane and is permeable to molecules up to 1.5 kDa. This leads to depolarization, inner membrane swelling and outer membrane rupture and release of cytochrome c which can lead to cell death[114-117]. The components of the pore are unknown, however ANT and more importantly, cyclophilin D, are thought to be regulators of this transition[118]. Most recently, new studies have suggested that Complex V of the electron transport chain, specifically subunit c of the F_0 ATP Synthase, may be a component of the permeability transition pore[119, 120]. Increased mitochondrial Ca^{2+} uptake is a known promoter of MPT. Elevated matrix Ca^{2+} increases metabolism by stimulating pyruvate dehydrogenase (PDH), isocitrate dehydrogenase and α-ketoglutarate dehydrogenase which promote increased flux through the tricarboxyilic acid (TCA) cycle (Figure 1.5). The TCA cycle produces reducing equivalents that enter the electron transport chain which can increase levels of ROS, another known promoter of MPT. Additionally, Ca^{2+} has been shown to promote the association of cyclophilin D to the pore components, facilitating MPT[121]. Addition of cyclosporin A to experimental assays can partially inhibit MPT by binding to cyclophilin D, impairing its activity and increasing the threshold for MPT formation[122, 123]. The susceptibility of isolated mitochondria to MPT can be measured using known stressors such as Ca^{2+} and ROS, and examining matrix swelling through a light scattering assay or the release of intramitochondrial Ca^{2+} into the extramitochondrial buffer[59, 61, 91, 93, 124, 125].

As discussed earlier, high intake of DHA+EPA increases the percent of these fatty acids in total cardiac and mitochondrial membranes. The increase in n3-PUFA and concomitant decrease in n6-PUFA increases membrane fluidity, which may delay the MPT[61]. Indeed, dietary supplementation with DHA+EPA attenuated Ca^{2+}-induced MPT in isolated mitochondria[61]. We also found that resistance to MPT was present only with DHA supplementation and not with EPA[59, 61, 93]. Additionally, high intake of DHA prevented cardiac dysfunction and

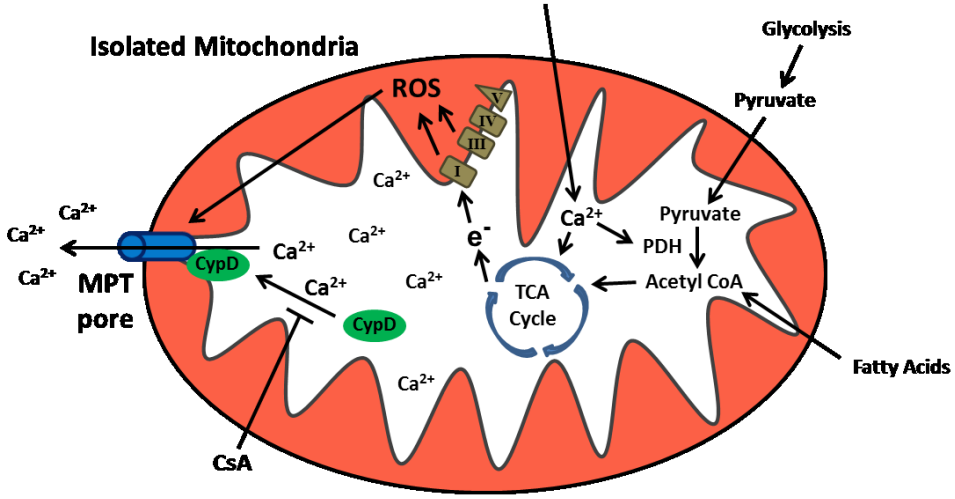

Figure 1.5. **Effects of calcium in isolated mitochondria**. A schematic representation of the experimental conditions of added extramitochondrial calcium used to promote mitochondrial permeability transition (MPT). Cyclosporin A (CsA) can also be added experimentally to partially inhibit MPT. PDH, pyruvate dehydrogenase; TCA, tricarboxylic acid cycle; CypD, cyclophilin D; ROS, reactive oxygen species; I-V, electron transport chain complexes (complex II omitted for simplicity).

cardiomyocyte apoptosis in early HF in rats[58], which could be due to less MPT caused by changes in membrane structure. Surprisingly, Ca^{2+}-induced MPT was significantly delayed in IFM with a high LCSat+MUFA diet compared to the high n6-PUFA diet in the cardiomyopathic hamster[4] which may be related to the increased linoleate in cardiac phospholipids detailed above (Figure 1.4). However, treatment with DHA ameliorated the sensitivity to Ca^{2+}-induced MPT in these hamsters but did not affect survival

suggesting that MPT may not contribute to cardiac pathology and thus is not an attractive therapeutic target.

Effects of Fat Intake on Cardiac Gene Expression of PPARs

Metabolic Substrate Utilization in HF

Under healthy conditions, catabolism of fatty acids is the predominant metabolic pathway in the heart, supplying energy for 50 to 90% of ATP production[84]. The myocardium extracts non-esterified fatty acids (NEFA) from the circulation through passive and active transport (via the fatty acid transporter FAT1/CD36). Once inside the cell, these fatty acids are re-esterified in the cytoplasm and transported into the mitochondrial matrix via the carnitine shuttle and carnitine palmitoyl transferase-1 (CPT-1). Within the matrix, β-oxidation successfully removes 2-carbon acetyl units which then enter the tricarboxylic acid cycle to generate ATP[126-130]. Other energy sources for the myocardium are glucose, lactate, ketone bodies, and amino acids[129]. In a healthy state, the heart has the ability to switch between substrates based on energy requirements and substrate availability. In HF, there is a substrate switch that occurs to favor glucose utilization over fatty acid oxidation (FAO)[126-128]. In failing human hearts (NYHA functional class III-IV, EF 10-35%), fatty acid oxidation was down regulated at both the mRNA and protein levels seen via expression of MCAD, LCAD and LCHAD enzymes involved in β-oxidation[131]. This metabolic switch has been observed by PET imaging in patients with idiopathic dilated cardiomyopathy after an IV bolus of ^{11}C-glucose for glucose utilization and ^{11}C-palmitate for myocardial fatty acid utilization and oxidation, compared to normal healthy control patients[132]. Studies with direct measurement of

substrate oxidation in HF patients using isotopic tracer observed a similar decrease in fat oxidation[133] compared to patients without heart failure This phenomenon has been also been observed in various animal models of heart failure[134, 135].

The switch in energy substrates is thought to be an initially compensatory response because the catabolism of glucose is more aerobically favorable compared to fatty acids however this shift may later become maladaptive and contribute to the progression of severe heart failure via impaired maximal capacity for energy transduction. Our lab has hypothesized that increasing FAO may delay the switch towards glucose utilization and slow the progression of heart failure.

Transcriptional Regulation of Fatty Acid Oxidation

Greater exposure of the heart to fatty acids activates peroxisome proliferator activated receptors (PPAR) in cardiomyocytes which can increase the expression of genes involved in fatty acid import and oxidation[37, 38, 127, 136, 137]. Fatty acids activate PPARs, primarily PPARα and PPARβ/δ[127, 136, 138, 139], which forms a heterodimer with retinoid X receptors and the cofactor PPARγ coactivator-1 (PGC-1α), and binds to PPAR response elements upstream of FAO genes[136]. The activity of PPARα, the predominant isoform in the heart[128], decreases in response to hypertrophy *in vitro*[134], evidenced by a decrease in the transcript of genes regulated by PPARα. *In vivo* studies showed similar effects with pressure overload-induced LVH[37, 140] and advanced HF in mouse, rat, and dog models[67, 141, 142]. Previously, Dr. Stanley's group showed that a high fat diet may prevent the down regulation of FAO and impairment in overall mitochondrial function that occurs in HF (Figure 1.1)[5, 127, 131, 142]. There is evidence that dietary MUFA, n3 and

n6-PUFA, and LCSat have distinct effects on the extent and expression of PPAR-regulated genes. High fat diets comprised of long chain saturated fatty acids (16:0 and 18:0) or n6-PUFA (18:2n6) increased the mRNA levels for known PPARα-regulated genes in the rat heart, however, there were significant differences in the expression pattern[37, 38]. In a pressure overload-induced HF model, animals fed a diet with 60% calorie intake from fat (primarily palmitate and stearate) maintained MCAD levels and prevented expansion of LV end systolic and diastolic volumes and contractile dysfunction[49, 66]. A similar effect was observed in rats with pressure overload induced by aortic constriction[11]. Work in our lab compared the effects of high fat diets comprised of LCSat to n6-PUFA in normal rats and saw that both high fat diets increased the mRNA levels of the PPARα-regulated genes, pyruvate dehydrogenase kinase (PDK-4) and uncoupling protein 3 (UCP-3) to a similar extent[37]. In cultured L6 skeletal muscle cells, exposure to high MUFA (oleate) up regulated mitochondrial biogenesis and PGC-1α, a co-activator of PPAR, which may increase mitochondrial function[143]. More controlled studies in isolated cardiomyocytes from adult rats showed that oleate (18:1) increased expression of PPARα regulated genes promoting fatty acid oxidation to a greater extent than did stearate (18:0), palmitate (16:0), or linoleate (18:2n6) (Figure 1.6)[1]. This suggests that a diet high in oleate, which is the primary fatty acid in olive oil and the Mediterranean diet, may be more effective than other fatty acids at activating PPARs and maintaining mitochondrial function in HF.

Obesity and Heart Failure

Much emphasis has recently been placed on the effect of obesity on the development and progression of HF[144, 145]. Obesity is a major risk factor for hypertension, dyslipidemia, atrial fibrillation, CHD and HF[146], and is associated with elevated cardiac triglycerides and ceramides[147] and can be accompanied by cardiac

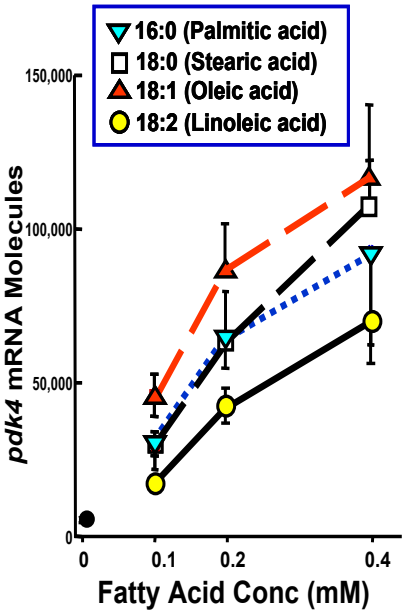

Figure 1.6. mRNA content of PDK-4, a PPARα regulated gene, in isolated adult rat cardiomyocytes. Cells were exposed to the fatty acid for 6 hrs[1-3].

insulin resistance and/or the development of diabetic cardiomyopathy[148]. The obesity epidemic appears to be mainly due to greater intake in highly processed carbohydrate and total energy, and is not the consequence of greater fat intake per se[30].

Obesity Paradox

Animal studies that investigate high fat/low carbohydrate intake should be evaluated with caution, as diet-induced obesity is frequently a confounding factor. Diet-

induced obesity in mice and rats can be generated in select strains using diets that are relatively high in fat (usually 40% to 50% of total energy intake compared to 10-15% in the typical commercial rodent chows) combined with high sugar (~20% to 30% sucrose). Obesity has complex effects on the heart largely mediated through changes in circulating hormones, impaired vascular function and altered autonomic regulation of the cardiovascular system[144, 149, 150]. Further, there exists an "obesity paradox" in HF, where overweight and obese people (overweight defined as a body mass index (BMI) of 25 to 29.9 kg/m^2 and obese as a BMI greater than 30 kg/m^2 in adults) have 2 to 3 times greater risk for the onset of HF than normal weight individuals[151], but once they are diagnosed they have slower HF progression, less hospitalization[144, 145] and lower mortality[145, 152] than non-obese patients[153].

While it is important to study the effects of obesity and HF, the experiments conducted in this project focus on the impact of dietary fat intake on HF in the absence of major diet-induced changes in body mass. Thus, we will avoid the confounding effects of elevated leptin and insulin, elevated blood pressure, and metabolic abnormalities that frequently accompany diet-induced obesity in rats. The specific effects of saturated, monounsaturated and n3 and n6-polyunsaturated fatty acids will be addressed, as well as the more general consequences of replacing carbohydrate with fat.

Effects of Specific Fatty Acids

n3 and n6 Polyunsaturated Fat

Polyunsaturated fatty acids (PUFA) are long chain fats that contain two or more doubles bonds beginning at the ω3 (n3-PUFA) or ω6 (n6-PUFA) carbon. The most

common form of daily consumption of long chain n3-PUFAs (mainly DHA and EPA) is oily fish (such as anchovy, herring and salmon) wherein most people consume about 0.1 g/day[51, 154]. Another n3-PUFA, α-linolenic acid, can be found in some plant sources, particularly flaxseed oil, though the effects of DHA/EPA compared to ALA are different[36, 58]. A large number of studies have examined the effects of n3-PUFAs and cardiac function (Table 1.1) and current guidelines now recommend at least 250 mg/day EPA+DHA or at least 2 servings of oily fish/week for the prevention of CHD[36].

On the contrary, n6-PUFA, by far the more predominant PUFA, has been implicated in adverse cardiac events and inflammation[155]. Toxic lipid products are formed when long chain PUFA undergo peroxidation via reaction with oxygen or hydroxyl radicals within mitochondria, generating highly reactive toxic aldehydes[156, 157]. The main metabolite formed upon peroxidation of n6-PUFA is 4-hydroxy-2-nonenal (HNE), while n3-PUFA generate 4-hydoxy-2-hexanal (HHE)[158]. HNE is highly reactive, and is increased in the myocardium following ischemic/reperfusion and in HF[159]. It modifies key mitochondrial and contractile proteins and is associated with impaired oxidative phosphorylation and LV function[160]. On the other hand, HHE, the more abundant metabolite, is thought to be less reactive and may not exert as toxic effects as observed with HNE[161]. This has major implications for the impact of dietary lipids, as a high intake of n3-PUFA, particularly DHA and EPA, will increase DHA and EPA in mitochondrial membranes in exchange for a decrease in n6-PUFA (arachidonic acid and linoleate). This would result in a decrease in HNE and greater HHE, and thus might be less cytotoxic for a given rate of lipid peroxidation. At present, there is no experimental

evidence for this proposed beneficial effect of dietary supplementation with DHA and/or EPA in the heart.

Supplementation with n3-PUFA has anti-inflammatory effects in both healthy people and those with chronic inflammatory conditions, including HF[52, 54]. Several studies have found that high doses of DHA and/or EPA supplementation increase adiponectin when given at pharmacological doses to mice, rats and humans[57, 162, 163]. Adiponectin is an anti-inflammatory adipokine secreted by white adipose tissue, and is negatively associated with obesity and systemic inflammation[164]. Clinical studies find that circulating adiponectin levels are increased in advanced HF and are a positive predictor of worsening HF[165-168]. On the other hand, the protective cardiovascular and anti-inflammatory effects of adiponectin in non-HF models of cardiovascular disease are well established[168 155], and the rise in adiponectin with worsening HF appears to be at least partially due to cachexia, a wasting syndrome characterized by loss of weight, muscle atrophy and fatigue. Adiponectin gene expression is regulated by PPARγ, and incubation of adipocytes with EPA and/or DHA increases adiponectin mRNA and synthesis, DHA having a greater effect than EPA[169]. Thus, the anti-inflammatory actions of n3-PUFA may also be through their actions on transcription factors to influence gene expression, or mediated through eicosanoids**.**

On a typical Western diet, arachidonic acid is the predominant PUFA in cell membrane phospholipids, comprising 15–25% of total membrane fatty acids, and is the major substrate for eicosanoid synthesis. EPA is a substrate for both cyclooxygenase and 5-lipoxygenase, generating eicosanoids with a different structure as compared to eicosanoids from arachidonic acid. In general, EPA forms weaker pro-inflammatory

eicosanoids than arachidonic acid, therefore manipulating n3 to n6-PUFA by diet alters the inflammatory environment[170]. Development and progression of HF is associated with increased levels of inflammatory mediators such as eicosanoids and cytokines. Enhanced intake of DHA and EPA decreases arachidonic acid in inflammatory cells which lowers production of prostaglandin PGE_2, thromboxane A_2, and leukotriene B_4, while the increase in EPA increases production of anti-inflammatory eicosanoids such as prostaglandin PGE_3, thromboxane A_3, and leukotriene B_5[113]. EPA and DHA also generate potent anti-inflammatory eicosanoids, resolvins, in the COX-2 pathway[171]. Supplementation with DHA+EPA blunted the increase in thromboxane B_2 and 6-keto prostaglandin F_1 in rats with early HF[47, 58], and reduced circulating levels of TNF-α, IL-1, and IL-6[52, 54, 172, 173]. Decreased cytokine levels are mostly due to the effects of n3-PUFAs on gene expression and transcription factors. Synthesis of cytokines is regulated by nuclear transcription factor kappa B (NF-kB) and other factors, which is activated in HF[174]. Taken together, high intake of DHA and EPA alters membrane phospholipids in a manner that suppresses inflammatory pathways that contribute to progression of HF.

Saturated Fat

Long chain saturated fatty acids (LCSat), encompass approximately 11-12% of total energy in the Western diet[175]. In the United States, the most common sources of saturated fat are dairy and red meat.[176] Elevated saturated fatty acids are correlated with increased ceramides[9]. Ceramides are lipid signaling sphingolipids implicated in the formation of ROS and apoptosis[177]. Long chain saturated fatty acids are the primary fatty acid moieties in cardiac ceramide, with palmitate and stearate each comprising

approximately 40% and 30%, respectively, of the total ceramide pool in the rat heart[178, 179]. *In vivo* studies show that a high fat diet with palmitate (60% of energy from fat) increased palmitoylceramide in normal rats and in animals with HF induced by myocardial infarction for eight weeks, but was not associated with impaired cardiac function or pathology[37, 39]. Palmitoylceramide levels were elevated by 40% with 20 weeks of infarct-induced HF on a low fat diet, but were not increased in rats fed a high fat/high sugar diet (45% of total energy from fat)[67]. Taken together, there is no convincing evidence to suggest elevated cardiac ceramides contribute to the development of HF or that a high fat diet triggers cardiac pathology through elevated ceramides and meta-analyses found no relationship between saturated fat intake and risk of CVD[180, 181].

Monounsaturated Fat

Monounsaturated fatty acids (MUFA) are fatty acids that contain one double bond. The most common MUFAs are palmitoleate (16:1n7) and oleate (18:1n9). Though oleate is the most predominant MUFA in the diet (found commonly in olive and canola oils), palmitoleate is the fifth most abundant fatty acid in commonly measured tissue and blood fractions[182]. Dietary agencies have varied in recommendations for total MUFAs consumed, but current guidelines recommend MUFA comprise ≤25% of total energy intake [175, 183, 184].

Benefits of high MUFA intake have been identified. In humans, several studies have shown that MUFA can preserve or even increase HDL-C[185] lower LDL-C and triacylglycerides compared to a diet high in carbohydrate,[184, 186, 187]. In type II diabetic patients, an isocaloric diet high in MUFA prevented the post-prandial decrease in

adiponectin gene expression and insulin resistance compared to a diet rich in carbohydrate[188]. A diet high in oleate decreased inflammatory markers in plasma and adipose tissue and improved insulin sensitivity in old rats fed for 16 weeks[189]. MCAD and CPT-1 mRNA expression was also increased with high MUFA diet compared to a diet high in saturated fat[189]. High oleate has also been shown to decrease total cholesterol in rats with high carbohydrate/high fat-induced metabolic syndrome[190]. LVH was ameliorated in SHHF rats fed a diet supplemented with oleate for 15 weeks[45] and in rabbits subjected to pressure and volume overload for 4 months[48].

Oleate increased CPT-1 expression and decreased ceramide content in C2C12 myotubes preincubated with palmitate[191]. In cell culture, oleate reverses palmitate-induced insulin resistance and inflammation[192, 193] and in skeletal muscle cells, treatment with oleate, but not other long chain fatty acids, enhanced the catalytic deactelyase activity of SIRT1, of which PGC1-α, a co-activator of PPARα, is a predominant downstream target[194]. Thus, oleate specifically accelerated fatty acid oxidation through a PGC1-α-dependent mechanism.

Clinical Significance

The proposed research has the potential to cause a paradigm shift in the treatment of HF. There is growing evidence that the development and progression of HF is affected by the composition of dietary fats and carbohydrate. Previous dietary recommendations for patients with coronary heart disease emphasized a low fat/high carbohydrate diet[17]. CHD guidelines have evolved and now recommend greater total fat intake, specifically unsaturated fatty acids, and reduced consumption of refined carbohydrate,[18, 80] although

this does not directly address dietary concerns of HF patients. Results from studies in rodent models of HF show that high intake of LCSat, MUFA and/or n6-PUFA can exert differential beneficial effects on LV function, remodeling and survival, and the underlying mechanisms are complex. Recent studies from our lab support the novel concept that a diet high in fat and low in carbohydrate attenuates or prevents the development and progression of HF compared to various low fat/high carbohydrate diets[5, 10, 42]. The effects of n6-PUFA, specifically linoleate, are particularly unclear, as high levels in membrane phospholipids could be detrimental due to greater HNE formation, but increased supplementation with these fats prolonged survival in a genetic model of hypertension-induced HF.

Project Aims

Rationale

Modulation of dietary fat intake has the potential to contribute to the prevention and treatment of HF. Previous dietary recommendations for patients with CVD emphasized a low fat/high carbohydrate diet[17]. Guidelines of fat/food consumption for patients with HF fall under the guidelines for treating CVD which have evolved and now recommend greater total fat intake, specifically of unsaturated fatty acids, and reduced consumption of refined carbohydrate[18, 80]. Recent studies from our lab support the novel concept that a diet high in fat and low in carbohydrate attenuates or prevents the development and progression of HF compared to various low fat/high carbohydrate diets[5, 10, 42]. There is growing evidence to support the concept that high fat intake with MUFA or mixed fatty acids, but not with very high LCSat or n6-PUFA, will improve

mitochondrial function by activating PPARs and increasing transcription of genes encoding key mitochondrial oxidative enzymes[1]. However, the optimal dietary FA composition for prevention and treatment of HF is not known. The therapeutic potential of modifying dietary lipid intake in HF patients is uncertain and clarification requires a mechanistic understanding of the specific effects of commonly consumed LCSat [palmitate (16:0) and stearate (18:0)], MUFA [oleate (18:1n9)] and n6-PUFA [linoleate (18:2n6)] on cardiac structure and function in HF.

The goal of the following research is to determine the optimal diet in terms of relative and absolute intake of saturated, monounsaturated, and n6-PUFA and the effects of these diets on cardiac structure and function in the normal and diseased heart. The criteria for defining the optimal diet will be based on beneficial changes cardiac and mitochondrial function compared among dietary treatments within a surgery group. Cardiac function will be measured by examining structural changes in the posterior and anterior wall thickness of the left ventricle and end diastolic and systolic volumes (i.e. changes in left ventricular remodeling) as measured by echocardiography. Mitochondrial functional changes will be assessed via calcium stress assays and mitochondrial oxidative capacity.

Statement of Hypothesis

The goal of this study is to determine the effects of diets high in fat on mitochondrial function and PL composition in HF, in the absence of obesity. We hypothesize that high fat intake with mixed LCSat+MUFA+n6-PUFA diet, but not LCSat, MUFA or n6-PUFA alone, will favorably change membrane PL composition,

activate PPARs, decrease sensitivity to MPT, prevent LV remodeling and dysfunction, and prolong survival in HF. These hypotheses will be tested through the following specific aims:

Aim 1. Determine the effects of a high LCSat diet, a low fat diet supplemented with DHA and a low fat/high carbohydrate diet without DHA on mitochondrial function in normal rats

Aim 2. Examine the effect of diets rich in MUFA, or n6-PUFA, LCSat, and mixed fatty acids (MIX, LCSat+MUFA+n6-PUFA) compared to a standard low fat/high carbohydrate diet on LV function in rats with advanced HF.

Aim 3. Assess the effects of these diets on mitochondrial phospholipid composition, membrane fluidity, activation of PPARα-regulated genes, and susceptibility to MPT.

I address these aims in 2 studies. The first study examines the effects of the n3-PUFA, DHA compared to a diet high in LCSat on mitochondrial and contractile function in healthy rats. The second study systematically evaluates the effects of diets high in MUFA, LCSat, n6-PUFA and a mixed diet on cardiac and mitochondrial function in the setting of pressure overload-induced heart failure.

Chapter 2 – Effects of Dietary Saturated and n3-Polyunsaturated Fat Intake on Cardiac Mitochondrial Composition in Normal Rats

Introduction

The primary sources of saturated fat in the Western diet are dairy and red meat. On average, approximately 11-12% of total energy intake is from saturated fat in the US[175]. High intake of saturated fat, particularly long chain fatty acids such as palmitate and stearate, has either neutral or adverse effects on the heart in the setting of left ventricular hypertrophy[4, 37, 195]. Epidemiological studies have found conflicting results suggesting positive correlations between saturated fat intake and coronary heart disease[195-197], a neutral effect of saturated fat on CHD[180] or, most recently, an inverse relationship between saturated fat and stroke, but positive correlation with myocardial infarction[198].

Cell culture studies, on the other hand, in Chinese hamster ovary (CHO) cells[199], cardiomyocytes[200, 201], pancreatic β-cells[202], and in mouse C2C12 cells[203] have shown that lipotoxicity occurs primarily in response to saturated fat, specifically palmitate (16:0). This could be due to reactive oxygen species (ROS)[199], ceramide synthesis[204], effects on membranes[200] and/or mitochondrial function[201]. However, in coronary artery ligation-induced heart failure in rats, a high saturated fat diet (60% total energy from fat) increased mitochondrial function (state 3 respiration) and fatty acid oxidation enzyme activity even though it did not affect overall cardiac function[9]. A high fat diet rich in saturated fat lowered plasma insulin and leptin concentrations compared to high

unsaturated fat diet. Both of these groups had high myocardial triglycerides, however, the ability to store excess fatty acids as intracellular triglycerides has been speculated to be protective from lipotoxicity[205]. Additionally, ceramides and cardiomyocyte apoptosis were higher in the saturated fat group compared to unsaturated fat, suggesting that dietary saturated fat may induce programmed cell death through elevated ceramide[37]. More recently, we found that high intake of saturated fat prolonged survival in cardiomyopathic hamsters compared to a low fat/high carbohydrate diet and a diet high in polyunsaturated fat (n6 and n3-PUFA)[4].

Polyunsaturated fat, particularly n3-PUFAs, are commonly found in oily fish (i.e. anchovy, salmon etc.). The main n3-PUFA in fish oil are docosahaexanoic acid (DHA) and eicosapentaenoic acid (EPA). The GISSI-HF trial showed a decrease in mortality in heart failure patients who consumed a low dose of fish oil[53]. Currently, DHA+EPA is approved for treating hypertriglyceridemia at a dose of 3.4g/day[206]. Our lab found that DHA readily incorporates into total cardiac and mitochondrial membranes in a dose dependent manner[58]. Elevated DHA affects mitochondrial function by reducing sensitivity to mitochondrial permeability transition (MPT)[61], a disastrous event that occurs through permeabilization of the inner mitochondrial membrane, depolarizing the mitochondria and promoting cell death. Further, we found that supplementation with DHA but not EPA has the greatest effect on reducing the occurrence of MPT in both healthy and HF rats compared to a diet low in fat and low in n3-PUFAs[59]

Taken together, it appears that high intake of saturated fatty acids have effects on the myocardium that are largely opposite those observed for n3-PUFAs, specifically

DHA. However, there has not been a direct comparison between these two extremely different dietary fatty acids, and little is known about the differences in their effects on mitochondrial phospholipid composition and cardiac function in healthy animals. Thus, before moving to pathology, we studied the effects of extreme diets either high in saturated fat or DHA on cardiac and mitochondrial function in normal healthy rats. Animals were fed for six weeks, which is sufficient duration to remodel cardiac phospholipid fatty acid composition, but avoid the complications that can occur with diet-induced obesity. We hypothesized that DHA would be beneficial or neutral on mitochondrial function (assessed by respiration and calcium uptake) and that high saturated fat would decrease cardiac and mitochondrial function. We also expected that high DHA would increase DHA in mitochondrial membranes and that a high saturated fat diet would increase the amount of palmitate and stearate and decrease monounsaturated fatty acids in membranes. Lastly, we hypothesized that both diets would not affect cardiac function or mass.

Methods

Experimental Design

The animal protocol was approved by University of Maryland, School of Medicine Institutional Animal Care and Use Committee and performed following the Guidelines for the Care and Use of Laboratory Animals. The animals were maintained on a 12-hour light/dark cycle and procedures and tissue harvest were performed within 6

hours of the start of the light cycle. Echocardiography was performed after six weeks of treatment, and animals were then euthanized.

Healthy, male Sprague-Dawley rats (Harlan, Indianapolis, IN) (350-400g) were assigned to either a standard low fat diet (n=28), a low fat diet supplemented with DHA (n=30), or a high in saturated fat diet (n=30). After 6 weeks of dietary treatment, rats were anesthetized with 5.0% isoflurane, and a thoracotomy was rapidly performed to expose the heart. Blood was drawn by cardiac puncture, and the heart was immediately harvested. The left ventricle was dissected, weighed and immediately used for mitochondrial isolation. Epididymal and retroperitoneal fat pads were dissected and weighed.

Diets

All diets were custom-manufactured by Research Diets Inc. (New Brunswick, NJ) and contained 20% of total energy content as protein from (casein + L-cystine). The standard control low fat diet contained 11% of total energy from fat (4% unsalted butter, 5% lard, 2% soybean oil) and 69% carbohydrate (56% corn starch and 12% maltodextrin). The DHA diet was the same as the standard control low fat diet except fat diet contained 2.3% DHA (as DHA ethylester, 90% pure, from KD Pharma, Bexbach, Germany), 3% unsalted butter, 4% lard, 2% soybean oil). The high saturated fat diet contained 45% of total energy from fat (43% unsalted butter and 2% soybean oil) and 35% carbohydrate (23% corn starch and 12% maltodextrin). The fatty acid composition

of the diets was analyzed by gas chromatography – mass spectroscopy as described below, and is given in Table 2.1.

Table 2.1. **Analysis of fatty acids in rodent chow** (expressed as a per cent of total fatty acids).

% kcal:	**Standard Low Fat Diet**	**DHA Low Fat Diet**	**High Saturated Fat Diet**
Protein	20	20	20
Carbohydrate	69	68	35
Fat	11	11	45
Fatty acid			
C14:0	0.1	0.1	5.1
C16:0	2.5	1.9	17.8
C16:1	0.2	0.2	0.9
C18:0	2.7	2.1	8.2
C18:1n9	3.1	2.5	11.0
C18:1n7	0.3	0.2	0.5
C18:2n6	1.6	1.4	2.2
C18:3n6	-	-	-
C18:3n3	0.1	0.1	0.1
C20:3n6	-	-	0.1
C20:3n9	-	-	-
C20:4	0.4	0.3	0.3
C20:5n3	-	0.2	0.1
C22:5n3	-	0.1	-
C22:6n3	-	2.0	-
Total	**11.0**	**11.1**	**46.3**
% Saturated	5.2	4.1	31.0
%MUFA	3.6	2.9	12.4
%n6 PUFA	2.0	1.8	2.7
%n3PUFA	0.1	2.3	0.2

Echocardiography

LV function was assessed using echocardiography (model Vevo 770, VisualSonics, Inc., Toranto, Canada) as previously described in detail[4]. Briefly, rats were

anesthetized using 2.5% isoflurane by nose cone, placed on a heated platform and 2-dimensional long and short axis images as well as guided M-mode were acquired and analyzed using software resident on the machine.

Mitochondrial Isolation

In myocardium mitochondria are located in two spatially distinct subpopulations: interfibrillar mitochondria (IFM) located between the myofibrils, and subsarcolemmal mitochondria (SSM) found in the outer region of the cell. Functional and structural differences between IFM and SSM have been described[88, 91, 94, 207], thus it is important to separately assess the two populations. The two mitochondrial subpopulations were isolated as described previously[4, 59, 61, 87, 93, 124, 208]. Briefly, freshly harvested LV tissue was minced and homogenized in 1:10 dilution with ice cold modified Chappel-Perry buffer [100 mM KCl, 50 mM MOPS, 5 mM $MgSO_4$, 1 mM EGTA, 1 mM ATP, 0.2 mg/ml bovine serum albumin (BSA)]. Homogenates were lightly centrifuged at 500 x *g* to yield subsarcolemmal mitochondria (SSM). Interfibrillar mitochondria (IFM) were extracted with a tryptic digestion (5 mg/g wet weight) for 10 min on ice, along with further purification through a series of differential centrifugation spins. Mitochondrial protein was assessed by the Lowry method using an 8-point BSA standard curve.

Mitochondrial Respiration

Oxygen consumption by SSM or IFM was measured using a Clark-type elctrode as described previously[59, 62, 93, 124, 208]. Isolated mitochondria (0.5 mg mitochondrial

protein/mL) were assayed in the same calcium-free Chappel-Perry buffer described above but containing 1 mg/ml BSA and no ATP. State 3 and 4 respiration were measured utilizing glutamate + malate (10 mM and 5 mM, respectively). Respiratory control ratio (RCR) was calculated as the ratio of State 3:State 4.

Mitochondrial Calcium Handling

Mitochondrial Ca^{2+} uptake was assessed using isolated SSM and IFM as previously described[209]. In short, Ca^{2+} uptake was measured using 1.5 mg mitochondrial protein resuspended in 1.5 mL of Ca^{2+}-free buffer containing 100 mM KCl, 50 mM MOPS, 5 mM KH_2PO_4, 5 μM EGTA, 1 mM $MgCl_2$, 10 mM glutamate, and 5 mM malate at 37˚C. The cell impermeant fluorescent calcium indicator, Ca^{2+} green-5N (Invitrogen), with an excitation and emission of 488 and 530 nm, was added to measure extra-mitochondrial Ca^{2+}. Ca^{2+} uptake was measured as the decrease in extramitochondrial Ca^{2+} following a bolus injection of 3 μL of 15 mM Ca^{2+} (30 nmol Ca^{2+}/mg mitochondrial protein).

Ca^{2+}-Induced Mitochondrial Swelling

Ca^{2+}-induced mitochondrial swelling, an established measure of MPT, was monitored in isolated cardiac mitochondria using a 96 well spectrophotometric plate reader (SpectraMax, Molecular Devices, USA) at 37°C as described previously[59]. Briefly, 50 μg of mitochondrial protein was resuspended in 200 μl of calcium free buffer (described above) and absorbance was monitored at 540 nm for 2 minutes to obtain a

baseline. Subsequently, a bolus of 100 nmol Ca^{2+}/mg mitochondrial protein was added and the absorbance recorded for 20 minutes. Samples were compared to a parallel time control group in which no Ca^{2+} bolus was added.

Membrane Microviscosity

Mitochondrial membrane microviscosity was assessed using fluorescence polarization of the membrane bound dye, 1,6-diphenyl-1,3,5-hexatriene (DPH, Invitrogen) with an excitation and emission of 360 and 430 nm, respectively. Briefly, 200 µg mitochondrial protein was incubated in 3 mL of a calcium free buffer (described above) with 10 µM DPH at 37˚C for 30 minutes. One mL of each sample was read in a cuvette using single point polarization at 650 V, at high sensitivity. The bandpass was set at 4 for excitation and 8 for emission. Anisotropy values were obtained as the inverse of membrane fluidity.

Phospholipid Analysis

Mitochondrial phospholipid fatty acid composition was analyzed by gas chromatography coupled with mass spectrometry according to a modification of the transesterification method as previously described[210].

Biochemical Parameters

Free fatty acids and triglycerides were measured in plasma by spectrophotometric enzyme assays (Wako Chemicals, USA) according to the manufacturer's instructions[211, 212].

Statistical Analyses

A 1-way ANOVA was performed between all groups with a Bonferonni post-hoc test. A 2-way repeated measures ANOVA with a Bonferonni post-hoc test was performed on the calcium uptake experiments. A Dunn's Method post-hoc test was used if data sets failed normality. SSM and IFM were not compared to each other. Mean values are presented as ± SEM with $P<0.05$ as significant.

Results

Body Mass and Cardiac Function

Initial body mass was similar among groups and increased over 6 weeks of treatments, with a 42% greater gain in body mass in the high saturated fat diet group compared to the standard low fat diet and DHA groups ($P<0.05$). (Table 2.2) This corresponded with a significantly greater epididymal and retroperitoneal fat pad mass. There were no differences in absolute LV mass or LV mass when normalized to tibia length. No changes were seen LV chamber diameter at end systole or diastole, or fractional shortening (Table 2.2). Taken together, there was no evidence of any gross cardiac dysfunction with short term diet-induced obesity with the high saturated fat diet.

Table 2.2. **Physiological parameters and echocardiography data**. EDD, end diastolic diameter; ESD, end systolic diameter; FS, fractional shortening; A, anterior wall; P, posterior wall. Data are presented as ± SEM. *P<0.05 vs. Standard Low Fat Diet, †P<0.05 vs. DHA Low Fat Diet.

	Standard Low Fat Diet	**DHA Low Fat Diet**	**High Saturated Fat Diet**
N	18	20	19
Initial Body Mass (g)	318 ± 7	323 ± 4	323 ± 5
Final Body Mass (g)	488 ± 10	494 ± 8	554 ± 11*†
Change in Body Mass (g)	170 ± 9	171 ± 6	242 ± 15*†
LV Mass (g)	0.98 ± 0.02	0.97 ± 0.02	1.02 ± 0.02
LV/tibia length	23.85 ± 0.90	22.93 ± 0.51	24.52 ± 0.68
Epididymal Fat Mass (g)	9.24 ± 0.85	7.27 ± 0.42	14.12 ± 1.00*†
Retroperitoneal Fat Mass (g)	9.66 ± 1.23	7.79 ± 0.48	14.08 ± 1.02*†
Echocardiography Data			
EDD (mm)	7.53 ± 0.26	7.33 ± 0.19	7.20 ± 0.23
ESD (mm)	3.69 ± 0.22	3.67 ± 0.17	3.59 ± 0.20
FS (%)	51.6 ± 1.6	49.8 ± 2.1	50.4 ± 1.8
Absolute Wall Thickness (mm)	4.5 ± 0.2	4.4 ± 0.2	4.3 ± 0.2
Relative Wall Thickness (mm)	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0

Plasma free fatty acids were increased in animals fed the high saturated fat diet compared to the standard low fat and DHA diets (Figure 2.1). DHA significantly decreased plasma triglycerides compared to the standard low fat diet and high saturated fat diet.

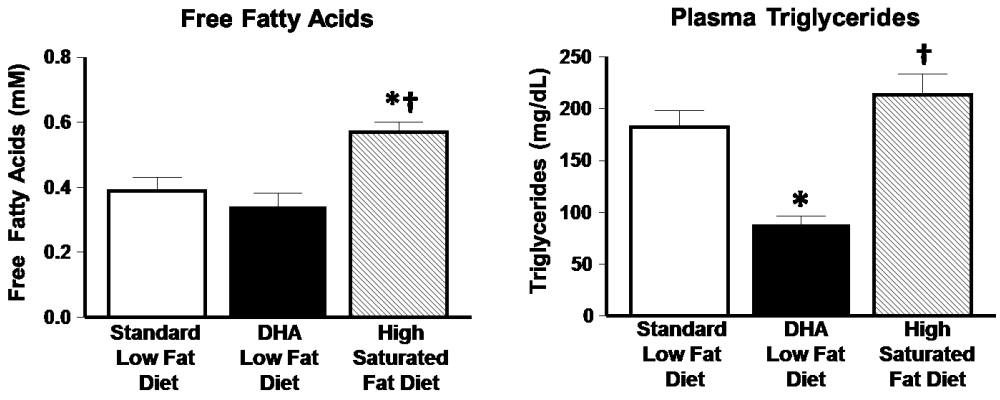

Figure 2.1. **Circulating free fatty acids and plasma triglycerides**. Data are presented as ± SEM. *P<0.05 vs. standard low fat diet, †P<0.05 vs. DHA low fat diet. The n for Standard low fat, DHA low fat and high saturated fat are as follows: n = 18, 20, 19.

Mitochondrial Yield

The yield of the two mitochondrial subpopulations, SSM and IFM, and total mitochondrial yield were not different among the three diets. Extensive assessment of mitochondrial respiration found no differences in state 3 or state 4 respiration or the respiratory control ratio (state 3:state 4) (Table 2.3).

Table 2.3. **Mitochondrial Parameters**. Mitochondrial yield, respiration and RCR for all substrates. State 3 and 4 are displayed in natoms $O*mg^{-1}*min^{-1}$. RCR, respiratory control ratio, is the ratio of State 3:State 4. Data are presented as ± SEM.

	Standard Low Fat Diet (n=20)	**DHA Low Fat Diet (n=20)**	**High Saturated Fat Diet (n=20)**
LV Mito Yield (mg protein/g wet wt)			
SSM	17.2 ± 1.3	17.3 ± 0.7	17.6 ± 0.8
IFM	11.6 ± 0.8	11.1 ± 0.5	10.7 ± 0.6
Total	28.9 ± 1.8	28.2 ± 1.0	28.3 ± 1.0

Table 2.3 Continued

	n=9	n=10	n=9
Glutamate + Malate			
SSM			
State 3	107.9 ± 9.1	105.4 6.1	119.1 ± 14.4
State 4	33.0 ± 2.9	35.8 ± 3.3	31.1 ± 3.0
RCR	3.3 ± 0.1	3.1 ± 0.3	3.9 ± 0.3
IFM			
State 3	145.6 ± 13.3	129.9 ± 6.7	140.2 ± 14.2
State 4	44.4 ± 8.3	43.1 ± 4.0	36.5 ± 4.0
RCR	3.6 ± 0.2	3.2 ± 0.3	4.0 ± 0.7
Palmitoylcarnitine			
SSM			
State 3	222.2 ± 31.5	207.1 ± 20.9	224.6 ± 14.6
State 4	61.6 ± 14.8	47.3 ± 2.7	50.3 ± 4.0
RCR	4.0 ± 0.4	4.4 ± 0.3	4.6 ± 0.4
IFM			
State 3	259.4 ± 21.9	263.9 ± 19.2	256.3 ± 15.8
State 4	57.6 ± 5.8	62.5 ± 2.5	58.2 ± 4.2
RCR	4.6 ± 0.2	4.2 ± 0.3	4.6 ± 0.4
Succinate+Rotenone			
SSM			
State 3	273.1 ± 9.7	272.7 ± 10.3	262.3 ± 7.3
State 4	89.2 ± 7.4	89.8 ± 5.7	87.1 ± 6.3
RCR	3.2 ± 0.4	3.1 ± 0.2	3.1 ± 0.2
IFM			
State 3	363.3 ± 26.9	331.1 ± 11.9	338.8 ± 15.3
State 4	111.9 ± 9.7	104.6 ± 7.9	109.3 ± 5.9
RCR	3.3 ± 0.4	3.3 ± 0.3	3.1 ± 0.1

Mitochondrial Respiration and Calcium Uptake

Previous studies have shown that supplementation with DHA delayed mitochondrial permeability transition[59, 61] and increased Ca^{2+} activation of pyruvate

dehydrogenase in the mitochondrial matrix, suggesting that DHA might be altering the ability of mitochondria to take up Ca^{2+}. Thus, we measured Ca^{2+} uptake by isolated mitochondria. Mitochondria from the rats on the high saturated fat diet had a significant decrease in the rate and total amount of Ca^{2+} uptake compared to both the standard and high DHA diets in both SSM and IFM (Figure 2.2). DHA had no effect on Ca^{2+} uptake. No changes were seen in Ca^{2+}-induced mitochondrial swelling at baseline or after the addition of Ca^{2+} (Figure 2.3).

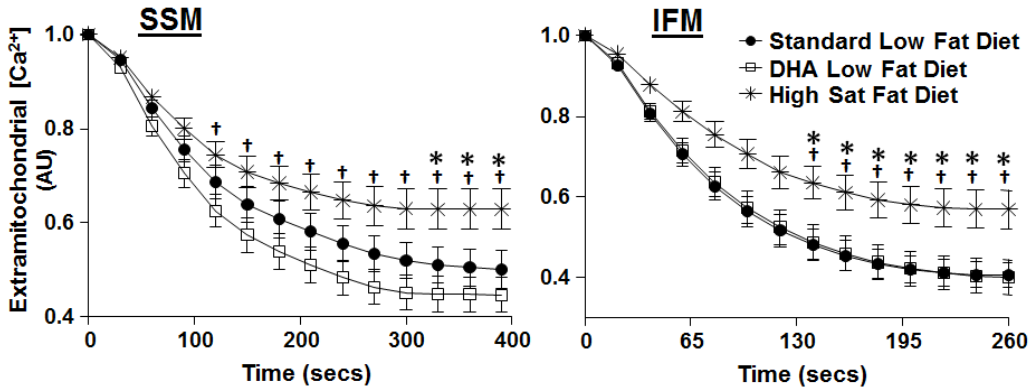

Figure 2.2. **Ca^{2+} uptake in SSM and IFM**. Data are presented as ± SEM. *P<0.05 high saturated fat vs. standard low fat, †P<0.05 high saturated fat vs. DHA low fat diet. The n for Standard low fat, DHA low fat and high saturated fat are as follows: n = 8, 8, 10 for SSM and n = 7, 9, 9 for IFM.

Membrane Fluidity

Increasing the density of long chain highly unsaturated fatty acids via high intake of n3-PUFA has the potential to affect membrane fluidity[124, 208], thus we used fluorescence polarization to measure anisotropy, the inverse of fluidity. No differences were seen in mitochondrial membrane fluidity between groups (Figure 2.4). A trend toward decreased anisotropy was observed with the high DHA diet compared to the

standard diet (P=0.07), suggesting increased fluidity of the membrane in IFM, however it was not statistically significant.

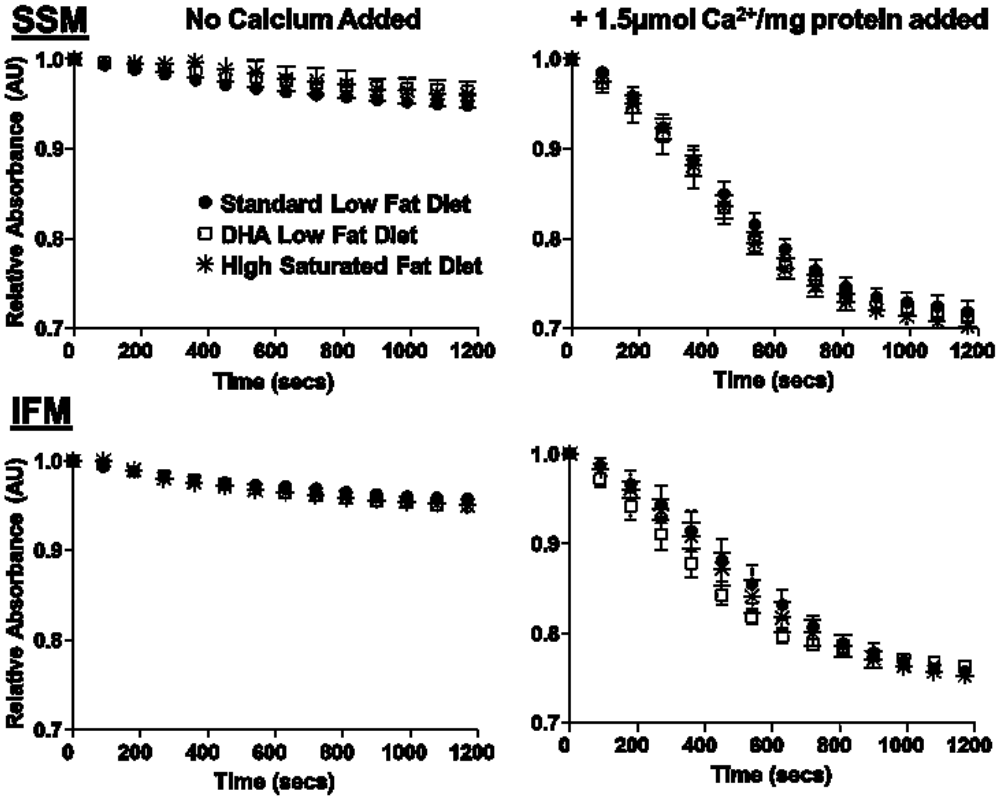

Figure 2.3. **Calcium-induced MPT** measured in isolated mitochondria using a light scattering assay in SSM (top panels) and IFM (lower panels). Data is presented as ± SEM. N = 10 for all groups.

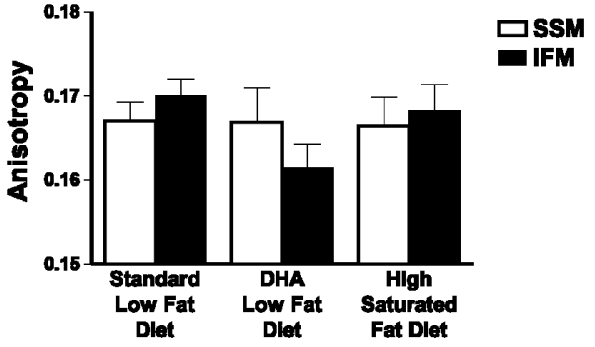

Figure 2.4. **Mitochondrial membrane microviscosity** (fluidity) measured using anisotropy of DPH. Data is presented as ± SEM. The n for Standard low fat, DHA low fat and high saturated fat are as follows: n= 8, 9, 8 for both SSM and IFM.

Mitochondrial Phospholipid Composition

Analysis of the fatty acid composition of mitochondrial phospholipids revealed dramatic differences among the three diet groups. Within SSM, the DHA low fat diet increased the amount of DHA present in mitochondrial membranes while decreasing the amount of arachidonic acid (22:4n6) (Figure 2.5, Table 2.4). The high DHA diet also increased the saturated fatty acids palmitate (16:0) and stearate (18:0) and the monounsaturated fat, oleate (18:1) while decreasing the *trans*-fatty acids, palmitoleic acid (16:1n7) and vaccenic acid (18:1n7). Thus, supplementation with DHA lowered total monounsaturated fatty acids and n6-PUFA compared to the standard low fat diet. High saturated fat significantly increased palmitate but not stearate compared to the standard low fat diet while also increasing oleate. Further, the high saturated fat diet decreased the n6-PUFA, linoleate (18:2n6) compared to both diets, and also lowered palmitoleic acid compared to the both diets and vaccenic relative to the standard low fat diet. It also raised levels of docosapentaenoic acid (DPA, 22:5n3) and there was a trend towards increased DHA, but not EPA, consistent with our previous measurements in whole myocardial extracts from rats fed a high saturated diet[47].

Phospholipid side chains were similarly changed in IFM (Figure 2.6, Table 2.4). The DHA low fat diet did not affect the saturated fatty acids in IFM but continued to decrease arachidonic acid while increasing EPA and very significantly increasing DHA. Overall, DHA supplementation decreased total monounsaturated fatty acids and n6-PUFAs while increasing n3-PUFA. The high saturated fat diet decreased both palmitoleic and vaccenic acid compared the standard low fat diet and increased oleate

relative to the DHA diet. Additionally, DPA and DHA were also increased with saturated fat intake without an increase in EPA.

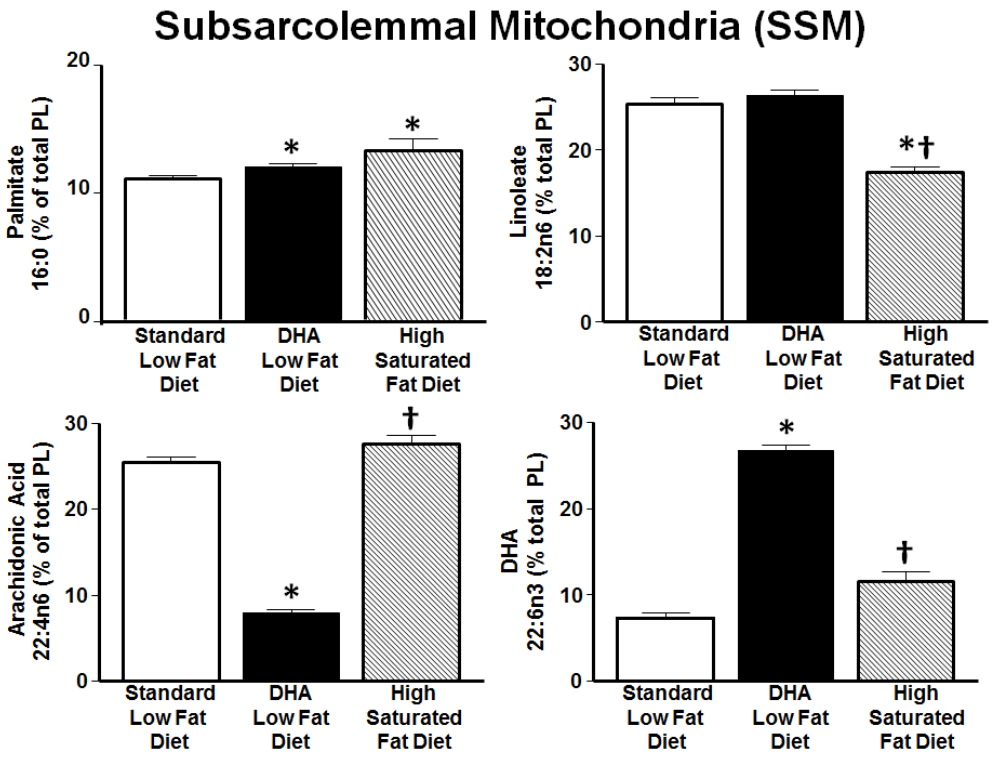

Figure 2.5. **Mitochondrial phospholipid analysis** of palmitate (16:0), linoleate (18:2n6), arachidonic acid (20:4n6) and DHA (22:6n3) in isolated SSM. Data is presented as ± SEM. *P<0.05 vs. standard low fat diet, †P<0.05 vs. DHA low fat diet. The n for Standard low fat, DHA low fat and high saturated fat are as follows: n = 9, 10, 10.

Table 2.4. **Mitochondrial phospholipid analysis**. Data are presented as ± SEM. *P<0.05 vs. Standard low fat diet, †P<0.05 vs. DHA low fat diet.

SSM

Fatty acid	**Standard Low Fat Diet (n=9)**	**DHA Low Fat Diet (n=10)**	**High Saturated Fat Diet (n=10)**
palmitic acid (16:0)	11.14 ± 0.25	12.09 ± 0.20*	13.33 ± 0.98*
palmitoleic acid (16:1n7)	0.44 ± 0.04	0.30 ± 0.02	0.21 ± 0.01*†
Stearic Acid (18:0)	23.82 ± 0.38	21.72 ± 0.28*	22.74 ± 1.08
Oleic Acid (18:1n9)	3.33 ± 0.11	2.65 ± 0.10*	3.98 ± 0.27†
Vaccenic Acid (18:1n7)	2.85 ± 0.11	1.89 ± 0.04*	1.94 ± 0.10*

Table 2.4 Continued

Linoleic Acid (18:2n6)	25.29 ± 0.91	26.30 ± 0.72	17.44 ± 0.61*†
α-Linolenic Acid (18:3n3)	0.05 ± 0.00	0.03 ± 0.00*	0.06 ± 0.00†
Arachidonic Acid (20:4n6)	25.47 ± 0.58	7.96 ± 0.36*	27.55 ± 1.06†
Eicosapentaenoic Acid (20:5n3)	0.01 ± 0.00	0.11 ± 0.01*	0.02 ± 0.00†
Docosapentasnoic Acid (22:5n3)	0.22 ± 0.01	0.14 ± 0.00*	0.84 ± 0.19*†
Docosahexaenoic Acid (22:6n3)	7.26 ± 0.65	26.71 ± 0.76*	11.56 ± 1.20†
Σ Saturated Fatty Acids	34.96 ± 0.57	33.80 ± 0.44	36.08 ± 1.30
Σ Monounsaturated Fatty Acids	6.61 ± 0.15	4.83 ± 0.14*	6.11 ± 0.36†
Σ n-3 PUFA	7.53 ± 0.65	24.99 ± 0.76*	12.47 ± 1.36†
Σ n-6 PUFA	50.76 ± 0.69	34.26 ± 0.49*	44.98 ± 1.58†

IFM

Fatty acid	**Standard Low Fat Diet (n=9)**	**DHA Low Fat Diet (n=10)**	**High Saturated Fat Diet (n=8)**
palmitic acid (16:0)	12.10 ± 1.26	15.17 ± 2.10	13.62 ± 1.28
palmitoleic acid (16:1n7)	0.41 ± 0.03	0.30 ± 0.02	0.21 ± 0.02*
Stearic Acid (18:0)	24.83 ± 1.17	24.38 ± 1.38	24.30 ± 0.87
Oleic Acid (18:1n9)	3.47 ± 0.17	2.82 ± 0.24	3.88 ± 0.41†
Vaccenic Acid (18:1n7)	2.80 ± 0.11	1.85 ± 0.06*	1.91 ± 0.09*
Linoleic Acid (18:2n6)	25.30 ± 1.53	25.27 ± 1.33	17.98 ± 0.75*†
α-Linolenic Acid (18:3n3)	0.05 ± 0.00	0.04 ± 0.01	0.07 ± 0.01†
Arachidonic Acid (20:4n6)	24.10 ± 0.99	7.01 ± 0.67*	25.64 ± 1.96†
Eicosapentaenoic Acid (20:5n3)	0.01 ± 0.00	0.11 ± 0.01*	0.02 ± 0.00
Docosapentasnoic Acid (22:5n3)	0.21 ± 0.01	0.13 ± 0.01*	0.83 ± 0.19†
Docosahexaenoic Acid (22:6n3)	6.57 ± 0.61	22.78 ± 2.19*	11.18 ± 1.32
Σ Saturated Fatty Acids	36.93 ± 2.41	39.55 ± 3.47	37.92 ± 1.87

Table 2.4. Continued

Σ Monounsaturated Fatty Acids	6.69 ± 0.26	4.97 ± 0.30*	6.00 ± 0.49
Σ n-3 PUFA	6.84 ± 0.62	23.06 ± 2.19*	12.09 ± 1.42
Σ n-6 PUFA	49.40 ± 2.29	32.28 ± 1.73*	43.63 ± 2.43

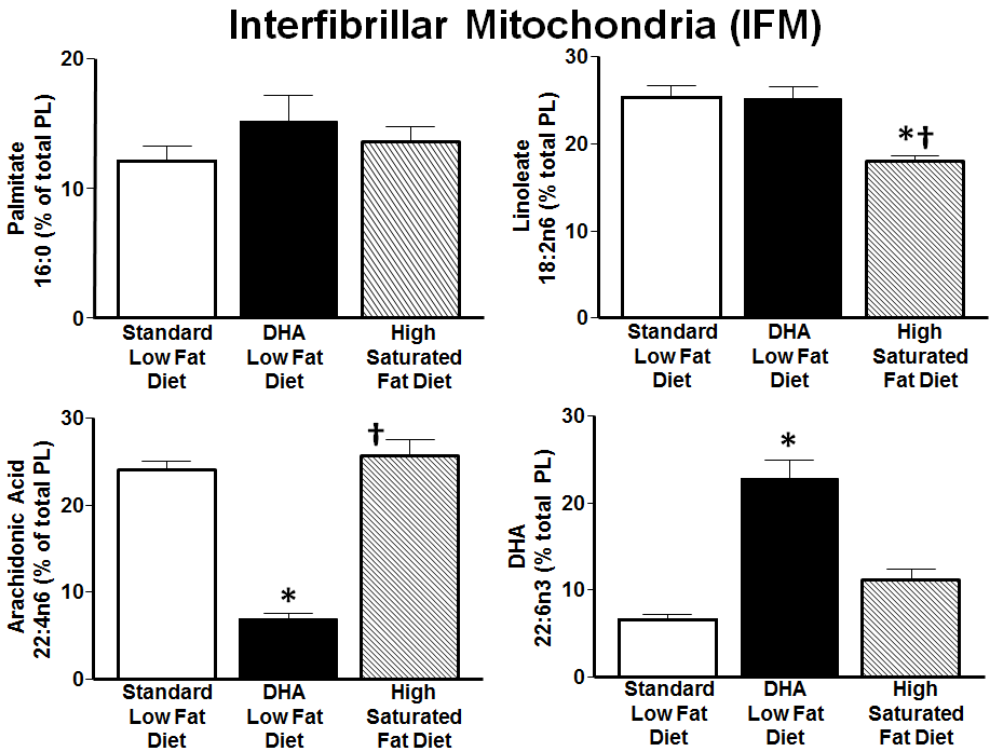

Figure 2.6. **Mitochondrial phospholipid analysis** of palmitate (16:0), linoleate (18:2n6), arachidonic acid (20:4n6) and DHA (22:6n3) in isolated IFM. Data is presented as ± SEM. *P<0.05 vs. standard low fat diet, †P<0.05 vs. DHA low fat diet. The n for Standard low fat, DHA low fat and high saturated fat are as follows: n = 9, 10, 8.

Discussion

This study compared the effects of extreme diets, specifically one high in the very long chain n3-PUFA, DHA, to a high long chain saturated fat diet in healthy animals. Mitochondrial phospholipid side chains were dramatically altered with both treatments compared to a standard low fat diet, however there was surprisingly no difference in mitochondrial respiration, Ca^{2+}-induced mitochondrial swelling, or cardiac mass, LV chamber size or systolic shortening. Taken together, these findings illustrate the adaptability of cardiac mitochondria to extreme dietary lipid intakes, and resultant

changes in mitochondrial phospholipid fatty acid composition without a major change in cardiac function or structure.

Supplementation with DHA has previously been shown by our lab to readily incorporate into mitochondrial membranes in a dose-dependent manner[58]. In this study, we see a dramatic increase of almost 3-fold of DHA into mitochondrial membranes with ~2-fold decrease in arachidonic acid relative to the standard low fat diet. We have observed a similar effect of DHA replacing arachidonic acid in mitochondrial membranes which correlated with decreased MPT sensitivity in both normal and hypertrophied hearts after feeding for 17 weeks[93, 208]. However, the present study did not find evidence of an association between increased DHA and significant changes in Ca^{2+}-induced MPT as assessed by the Ca^{2+}-induced swelling assay, or by Ca^{2+} uptake, suggesting that short-term DHA supplementation is not sufficient to affect MPT. We also saw increased palmitate and stearate in SSM but not IFM with DHA supplementation as well as decreases in the *trans*-fatty acid palmitoleic acid (16:1n7) in both subpopulations and both palmitoleic acid and vaccenic acid (18:1n7) in IFM. Trans-fatty acids are associated with systemic inflammation in both healthy and heart failure patients[213, 214]. Thus, decreased *trans*-fatty acids with DHA supplementation may be partially responsible for the anti-inflammatory effects of fish oil.

High intake of long chain saturated fatty acids increased body weight in these animals with dramatic increases in fat pad mass. Though circulating free fatty acids and triglycerides were increased with saturated fat, this did not worsen LV function. High saturated fat modestly affected mitochondrial phospholipid composition but significantly

decreased mitochondrial Ca^{2+} uptake capacity, though this did not affect Ca^{2+}-induced MPT, as assessed by the swelling assay, providing further evidence for a disassociation between phospholipid changes and mitochondrial function in healthy rats. Further, the high saturated fat diet increased DHA by ~60% in SSM membranes and 70% in IFM compared to a standard low fat diet, as well as a dramatic significant increase in the DHA-precursor, docosapentaenoic acid (DPA). This supports previous data that showed saturated fat intake increases DHA[4, 47] suggesting that excess saturated fat may contribute to the elongation of fatty acids to DHA. Increased DHA with saturated fat may explain the opposing conclusions of epidemiological data showing inverse associations between saturated fat intake and disease[198] and cell culture and animals studies showing increased cell death with high saturated fat[37, 200, 201].

In conclusion, despite dramatic changes in mitochondrial phospholipid fatty acid side chain compositions and differential effects on mitochondrial Ca^{2+} uptake capacity, low fat diets with and without DHA as well as a high saturated fat diet do not affect contractile function in healthy rats. Additionally, DHA is increased with high saturated fat intake which may account for the neutral or beneficial effects of high saturated fat in some models[47, 58, 61, 62, 93].

Chapter 3 – Impact of a High Fat Diet on the Development of Heart Failure: Differential Effects of Monounsaturated, n6 Polyunsaturated and Saturated Fats

Introduction

As covered in the introduction, cardiovascular diseases, including heart failure (HF), are the leading cause of death in the US. Though many effective drugs are available to treat heart failure[25], the AHA estimates an increase of 25% in the prevalence of HF by 2030. Thus, dietary interventions have the potential to be effective as an adjunct to currently used drugs and devices, and thus are an attractive goal[6]. Mitochondrial dysfunction contributes to the pathology of HF through impaired oxidative ATP production and greater susceptibility to mitochondrial permeability transition (MPT). MPT is a catastrophic event that occurs when a large pore forms across the inner mitochondrial membrane, dissipating the membrane potential and triggering cardiomyocyte death[215]. Recent evidence shows that specific high fat diets, in the absence of obesity, can prevent mitochondrial and left ventricular (LV) dysfunction and/or improve survival in HF induced by infarction[39, 67], hypertension[5, 49, 66], or genetic cardiomyopathy[56, 216]. This suggests greater relative intake of select lipids may improve clinical outcome in HF patients[217, 218].

The mechanisms responsible for the beneficial effect of high fat diets in HF are not clear, and the optimal dietary fatty acid composition is not known. The fatty acid composition of dietary lipid affects mitochondrial phospholipid (PL) composition, which impacts mitochondrial structure and function[61, 93, 219]. Monounsaturated fatty acids

(MUFA), n6-polyunsaturated fatty acids (n6-PUFA), and long chain saturated fatty acids (LCSat) have different effects on cardiac mitochondria and LV function in normal and HF animals[37, 38, 58, 113, 216], however the specific effects are complex and poorly understood, and a direct comparison has not been performed.

Recent studies in our lab suggest that dietary MUFA, n6-PUFA and LCSat affect the progression of HF through differential regulation of fatty acid oxidative (FAO) genes[37, 38, 57]. Long chain fatty acids are the endogenous activating ligand for peroxisome proliferator activating receptors (PPAR), specifically PPARα[37], which activates the expression of genes that encode keys proteins involved in the uptake and oxidation of fatty acids. Exposure of isolated cardiomyocytes to various fatty acids increases the mRNA levels of PPARα target genes, with oleate having a greater effect than other long chain fatty acids[1]. We recently found prolonged survival in HF with a high fat diet (45% energy from fat) enriched with LCSat and MUFA compared to a high fat diet comprised of a mixture of MUFA, n6-PUFA and n3-PUFA or a standard low fat diet[216]. Dietary lipid composition also effects mitochondrial membrane PL composition which may affect susceptibility to MPT. The optimal fatty acid composition to elicit a beneficial effect in HF is not known, and a systematic evaluation of long-term treatment with LCSat, MUFA and n6-PUFA on LV function and survival in HF is needed

Therefore, the goal of this present study was to systematically compare the effects of diets high in MUFA, n6-PUFA, LCSat, and a MIX diet (high in each these fatty acids) on cardiac and mitochondrial function in the setting of pressure overload-induced HF. We hypothesized that a high MUFA diet, similar to a Mediterranean diet, would

ameliorate the decrease in contractile function and preserve mitochondrial function in HF. HF was generated in rats by subjecting them to aortic pressure overload caused by transverse aortic constriction. HF rats and sham operated animals were placed on a purified low fat/high carbohydrate diet for 10 weeks, and then either left on this diet or given one of four different high fat diets (40% total energy from fat) for an additional 15 weeks. Myocardial function was assessed by echocardiography and mitochondrial function was examined using respiration, membrane PL side chain composition and fluidity, and Ca^{2+}-induced MPT.

Methods

Experimental Design

Experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the University of Maryland Institutional Animal Care and Use Committee. Investigators were blinded to the treatment groups when experiments were performed. All mitochondrial assays were performed within 4 hours of isolation.

Surgery

Transverse aortic constriction surgery was performed in 6-7 week old (70-90g) male Sprague Dawley rats (Harlan, Indianapolis, IN) to induce cardiac hypertrophy and heart failure. Rats were placed in a chamber with 5% isoflurane to induce anesthesia, and then intubated, mechanically ventilated and maintained on isoflurane (1.5 - 2.5% to

effect). The aortic arch was exposed and a tantalum clip (0.50 mm internal diameter; Pilling-Weck, Germany) was positioned around the transverse aorta between the brachiocephalic trunk and the left common carotid artery, as described previously[220-222]. Sham animals underwent the same procedure but without clip application. The incision was sutured closed and the animal was maintained for 25 weeks.

Diets

Ten weeks following surgery, rats were assigned to one of five custom manufactured diets made with purified ingredients (Research Diets, New Brunswick, NJ, USA) maintaining no differences in end diastolic and systolic diameter and volume, ejection fraction and body weight (Table 3.1). The standard (STD) diet contained 15% energy intake from fat (mainly soybean oil, lard and high oleic sunflower oil) with 67% energy from carbohydrate (maltodextrin, 12% of total energy and corn starch, 55%) (Table 3.2). Four high fat diets contained 40% of total energy intake from fat and 40% energy from carbohydrate (maltodextrin, 11% of total energy and corn starch, 29%). These diets consisted of a Mediterranean-like high monounsaturated fatty acid diet [MUFA, predominately from high oleic sunflower oil and soybean oil consisting of oleate (18:1, 25.2% of total energy intake), linoleate (18:2n6, 8.2%), palmitate (16:0, 3.0%) α-linolenic acid (18:3n3, 1.7%) and stearate (16:0, 1.2%)], n6-polyunsaturated fatty acid [n6-PUFA mainly from safflower and soybean oil containing linoleate (32.7%), oleate (3.8%), palmitate (2.6%) and α-linolenic acid (0.1%)], long chain saturated fatty acid [LCSat from cocoa butter and lard containing stearate (19.1%), palmitate (9.8%),

oleate (9.0%), linoleate (1.6%) and α-linolenic acid (0.1%)], and a mixed high fat diet [MIX from peanut oil, lard, cocoa butter, and soybean oil consisting of oleate (14.6%), palmitate (9.1%), linoleate (7.9%), stearate (7.5%) and α-linolenic acid (0.1%)]. Each diet contained 18% of energy from protein (casein + L-cystine). Animals were housed on a 12 hour light-dark cycle and fed *ad libitum*, with food intake monitored weekly.

Table 3.1. Echocardiography data at 10 weeks post-surgery. Animals were grouped into dietary groups maintaining no differences in the following parameters.

			Sham		
	STD	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
Ejection Fraction (%)	83.34±2.32	81.27±2.02	80.78±2.93	79.98±1.92	82.53±1.95
End Diastolic Volume (mL)	0.21±0.15	0.23±0.13	0.21±0.15	0.23±0.22	0.21±0.19
End Diastolic Diameter (mm)	6.40±.20	6.60±0.16	6.34±0.22	6.54±0.32	6.38±0.25
End Systolic Volume (mL)	0.38±0.07	0.44±0.06	0.41±0.08	0.49±0.08	0.39±0.07
End Systolic Diameter (mm)	2.95±0.23	3.20±0.22	3.08±0.24	3.30±0.26	3.02±0.23
Body Weight (g)	368±6	383±6	371±6	379±5	374±10

			HF		
	STD	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
Ejection Fraction (%)	60.58±4.42	60.79±2.76	60.99±3.12	60.65±4.60	62.31±5.03
End Diastolic Volume (mL)	0.28±0.27	0.28±0.14	0.26±0.19	0.25±0.18	0.25±0.20
End Diastolic Diameter (mm)	7.23±0.33	7.25±0.16	6.94±0.23	6.88±0.23	6.88±0.25
End Systolic Volume (mL)	1.26±0.24	1.11±0.11	1.05±0.14	1.08±0.16	1.08±0.20
End Systolic Diameter (mm)	4.83±0.42	4.99±0.27	4.60±0.27	4.55±0.35	4.47±0.41
Body Weight (g)	341±11	357±7	352±13	342±8	355±9

Table 3.2. Dietary fatty acid composition of purified diets (expressed as per cent of total dietary fatty acids as well as per cent of energy intake). The high fat diets contained 40% of total energy from fat while the STD low fat/high carbohydrate diet contained 15% from fat.

Fatty Acid	% of Total Dietary Fatty Acids				
	STD	MUFA	n6-PUFA	LCSat	MIX
Myristic acid (14:0)	0.58	0.00	0.00	0.41	0.61
Palmitic acid (16:0)	20.04	7.39	6.38	24.50	22.73
Palmitoleic acid (16:1n7)	0.45	0.17	0.00	0.36	0.51
Stearic Acid (18:0n1)	6.20	3.03	1.71	47.66	18.77
Oleic Acid (18:1n9)	32.67	62.98	9.61	22.42	36.57
Vaccenic Acid (18:1n7)	0.82	1.62	0.24	0.40	0.67
Linoleic Acid (18:2n6)	38.43	20.45	81.85	4.03	19.81
α-Linolenic Acid (18:3n3)	0.80	4.35	0.21	0.17	0.26
Arachidonic Acid (20:4n6)	-	-	-	-	-
Eicosapentaenoic Acid (20:5n3)	-	-	-	-	-
Docosahexaenoic Acid (22:6n3)	-	-	-	-	-
Σ Saturated Fatty Acids	26.83	10.43	8.09	72.56	42.11
Σ Monounsaturated Fatty Acids	33.94	64.77	9.85	23.18	37.76
Σ n-3 PUFA	0.80	4.35	0.21	0.17	0.26
Σ n-6 PUFA	38.43	20.45	81.85	4.03	19.81
Total	100	100	100	100	100

Table 3.2. Continued

Fatty Acid	% of Energy Intake				
	STD	MUFA	n6-PUFA	LCSat	MIX
Myristic acid (14:0)	0.1	-	-	0.2	0.2
Palmitic acid (16:0)	3.0	3.0	2.6	9.8	9.1
Palmitoleic acid (16:1n7)	0.1	0.1	0.0	0.1	0.2
Stearic Acid (18:0n1)	0.9	1.2	0.7	19.1	7.5
Oleic Acid (18:1n9)	4.9	25.2	3.8	9.0	14.6
Vaccenic Acid (18:1n7)	0.1	0.6	0.1	0.2	0.3
Linoleic Acid (18:2n6)	5.8	8.2	32.7	1.6	7.9
α-Linolenic Acid (18:3n3)	0.12	1.7	0.08	0.07	0.104
Arachidonic Acid (20:4n6)	-	-	-	-	-
Eicosapentaenoic Acid (20:5n3)	-	-	-	-	-
Docosahexaenoic Acid (22:6n3)	-	-	-	-	-
Σ Saturated Fatty Acids	4.0	4.2	3.2	29.0	16.8
Σ Monounsaturated Fatty Acids	5.1	25.9	3.9	9.3	15.1
Σ n-3 PUFA	0.12	1.74	0.08	0.07	0.10
Σ n-6 PUFA	5.8	8.2	32.7	1.6	7.9
Total	15.0	40.0	40.0	40.0	40.0

Echocardiography

Echocardiography was used to evaluate LV function. A MS250 probe (20 MHz) on a high-resolution small animal imaging system was used (Vevo 2100 High-Resolution Imaging System using a MS200 9-18 MHz probe, VisualSonics Inc., Toronto, Canada). Rats were anesthetized with isoflurane (1.5%) by mask, the chest shaved, and the animal situated in the supine position on a warming platform (37˚C). M-mode and two-dimensional echocardiographic studies were performed from the short and long axis views as previously described[58]. Analysis was performed separately using VisualSonics software by two independent readers and averaged.

Left Ventricular Pressure Measurements and Tissue Harvest

The terminal procedure was performed between 1 and 3 hours after the onset of the light phase. Rats were anesthetized with 5% isoflurane by mask for 5 minutes. Isoflurane was reduced to 2.0% and rats were intubated via tracheotomy. Pressure measurements were taken directly from the LV by inserting a 1.4-Fr Millar pressure catheter (Model SPR671 with a MPVS-400 signal processor, Millar Instruments, Houston, TX) through the apex of the heart after thoracotomy. After 10 seconds of pressure stabilization, LV pressure was recorded for 30 seconds[14]. Immediately after pressure measurements were taken, plasma and serum were collected by cardiac puncture, and organs were immediately harvested, weighed, frozen in liquid nitrogen, and stored at −80°C for later analysis. Mitochondria were freshly isolated from the LV.

Mitochondria Isolation

Mitochondrial isolation was performed according to the methods described in Chapter 2.

Mitochondrial Respiration

Mitochondrial respiration was performed according to the methods described in Chapter 2. State 4 was also calculated using the complex V (ATP synthase) inhibitor, oligomycin, to determine proton leak across the inner mitochondrial membrane. The respiratory control ratio (RCR) was calculated as state 3:state 4 without oligomycin.

Membrane Microviscosity

Membrane fluidity was assessed in isolated mitochondria using the fluorescent membrane staining dye 1-(4-Trimethylammoniumphenyl)-6-Phenyl-1,3,5-Hexatriene *p*-Toluenesulfonate (TMA-DPH) dye with an excitation and emission of 350 and 420 nm,, respectively. Briefly, 200 μg mitochondrial protein was incubated in 3 mL of a calcium free buffer (described above) with 5 μM TMA-DPH at 37˚C for 30 minutes. 200 uL of each sample was plated on a black opaque 96-well plate (Nunc) and read on a spectrophotometric plate reader (FlexStation 3, Molecular Devices) using fluorescence polarization. Samples were run in triplicate and anisotropy values, the inverse of membrane fluidity, were averaged.

MPT Assessment from Mitochondrial Ca^{2+} Uptake

The ability of mitochondria to take up progressive additions of Ca^{2+} was used as a measure of Ca^{2+}-induced MPT (FlexStation 3, Molecular Devices, Sunnyvale, CA) at 37˚C. Briefly, 0.25 mg/mL mitochondrial protein was suspended in Ca^{2+}-free buffer containing 100 mM KCl, 50 mM MOPS, 5 mM KH_2PO_4, 5 μM EGTA, 1 mM $MgCl_2$, 5 mM glutamate, and 5 mM malate. Fluorescent measurements were taken every 2 seconds from the fluorescence of Ca^{2+} using 750 nM of an extra-mitochondrial Ca^{2+} indicator, Calcium Green-5N (Invitrogen) with an excitation and emission of 488 and 530 nm, respectively. Injections of 10 μL of a 70 μM Ca^{2+} solution (14 nmol Ca^{2+}/mg mitochondrial protein) were made every 3 minutes, which was sufficient time for the extra-mitochondrial calcium fluorescence to return to baseline after each bolus addition.

A total of 12 injections were made, data was analyzed by averaging the stable baseline of Ca^{2+} fluorescence before each successive Ca^{2+} injection and plotting against total amount of Ca^{2+} added. The occurrence of MPT was defined by a sharp increase in slope which suggests that the mitochondria are no longer able to buffer the increased Ca^{2+}.

Ca^{2+}-Induced Mitochondrial Swelling

Assessment of mitochondrial swelling was performed according to the methods described in Chapter 2.

Mitochondrial Hydrogen Peroxide Production

Hydrogen peroxide production by isolated mitochondria was determined from the oxidation of the fluorogenic indicator amplex red in the presence of horseradish peroxidase (HRP)[223]. The concentrations of HRP and amplex red in the incubation were 0.1 unit/mL and 50 μM, respectively, and detection of fluorescence was assessed (Flex Station 3, Molecular Devices) with excitation and emission wavelengths of 530 nm and 590 nm, respectively. H_2O_2 was quantified using a standard curve which was obtained by adding known amounts of H_2O_2 to the assay medium in the presence of amplex red and HRP. H_2O_2 production was supported in mitochondria using glutamate+malate or succinate in the presence of rotenone as substrates.

Metabolic and Enzymatic Measurements

Plasma was analyzed for leptin (Alpco Diagnostics), free fatty acids and triglycerides (Wako Diagnostics). Serum was analyzed for glucose (Wako), insulin and TNF-α (Alpco). Urine was analyzed for thromboxane B_2 which was normalized to creatinine concentration (Caymen Chemicals). Frozen LV tissue was also analyzed for enzymatic analysis of activities of citrate synthase, aconitase, and medium- and long-chain acyl coenzyme A dehydrogenase (MCAD and LCAD) using spectrophotometric assays as previously described[61, 83]. Isolated mitochondrial subpopulations were also analyzed for enzymatic activity of citrate synthase, MCAD, and LCAD.

Gene Expression

Assessment of mRNA was performed on frozen LV tissue. Briefly, LV tissue was homogenized using a bullet blender and mRNA isolated using RNeasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer's instructions. Reverse transcription was performed using Superscript RTII on a Step One Plus® (Applied Biosystems) machine. Quantitative RT-PCR was performed using the following Taqman gene expression assays: Nppa (ANF), acyl-CoA dehydrogenase (Acadm), CPT-1b (muscle specific isoform), MHCα and β (Myh6 and 7, respectively), PDK4, UCP3, Aconitase (Aco), and PPARα (Applied Biosystems, Foster City, CA) normalized to Ppia (cyclophilin A) using Applied Biosystems StepOne Plus.

Phospholipid Analysis

Mitochondrial phospholipid composition was analyzed according the methods described in Chapter 2.

Statistical Analysis

The sham and HF groups were compared within the STD diet using a Student's t-test. The effects of diet was assessed within each surgery group (sham or HF) using a 1-way ANOVA, with a Bonferroni post hoc test. A 2-way repeated measures ANOVA, with a Bonferonni post hoc test, was used to analyze Ca^{2+}-induced mitochondrial swelling and permeability transition. A Dunn's Method post-hoc test was used if data sets failed normality. Data are reported as mean ± SEM, and a $P<0.05$ was considered significant.

Results

Body and Cardiac Mass

Transverse aortic constriction caused clear cardiac hypertrophy in all dietary groups. In the STD diet, there were increases of 44%, 130% and 326% in the LV, RV and atrial masses in the HF animals compared to shams ($P<0.001$, Figure 3.1A and B, Table 3.3). Diet had no effect on cardiac mass in either the sham or HF groups. There was no difference in mortality in any of the groups (31% in the STD group, 24% in MUFA, 35% n6-PUFA, 29% LCSat, and 38% MIX) though there was a trend towards increased survival with the high fat diets (Figure 3.2)

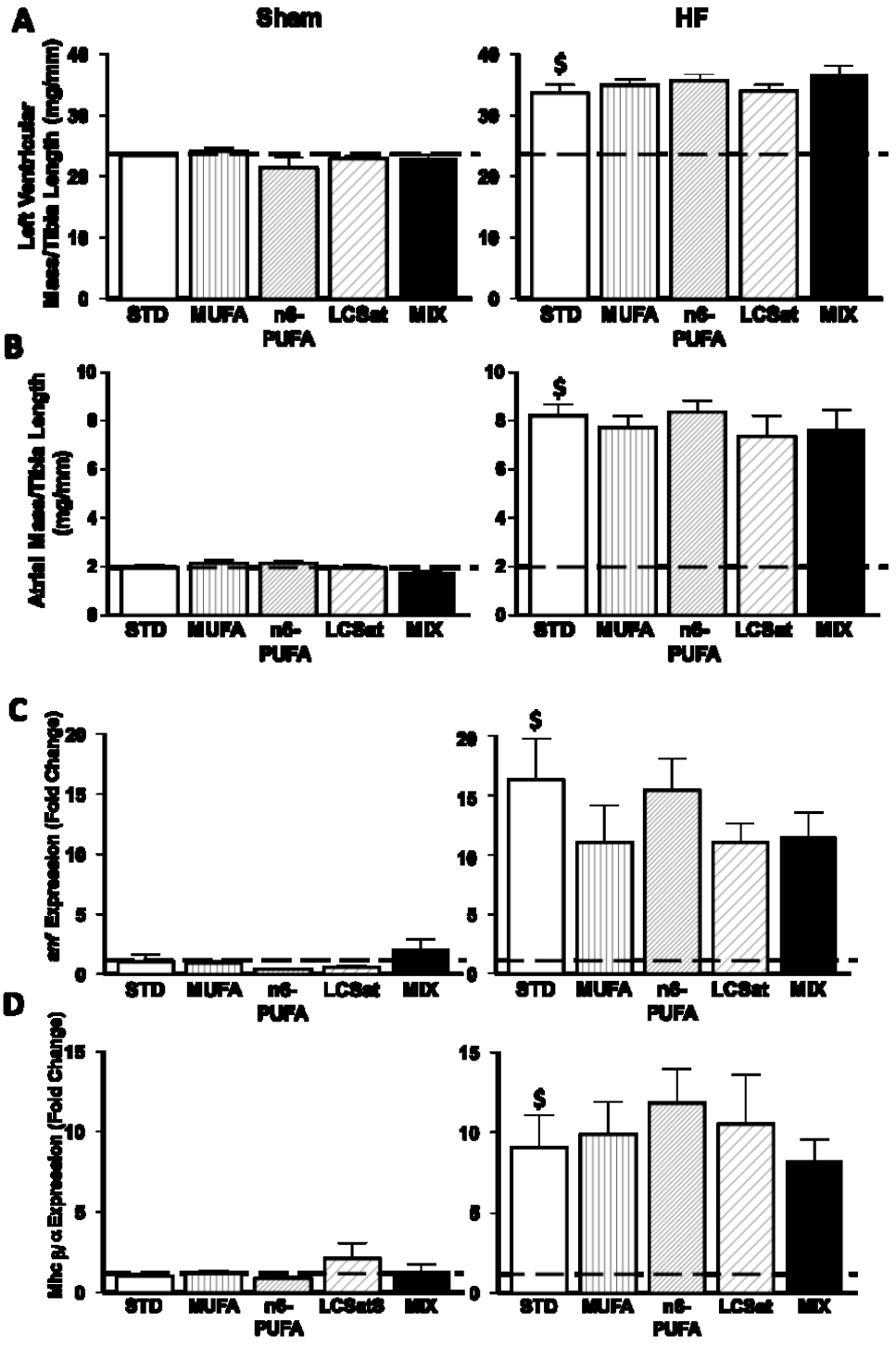

Figure 3.1. **Changes in heart mass and mRNA**. Left ventricle (A) and atrial masses (B) normalized to tibia length. mRNA expression (as fold change) of ANF (C) and myosin heavy chain ratio (D). Data are presented as mean ±SEM. $P<0.05 STD Sham vs. HF.

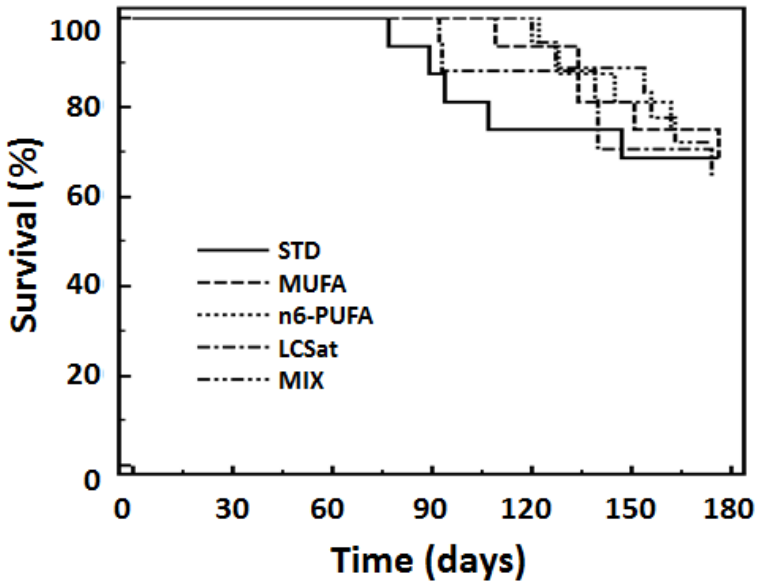

Figure 3.2. Kaplan-Meier **survival curve of HF animals** from surgery (day 0) to termination (an average of day 176). Deaths were recorded after assignment to dietary group at day 70.

Table 3.3. **Body parameters**. LV, left ventricle; RV, right ventricle; TL, tibia length; HW, heart weight. *P<0.05 vs. Respective STD, $P<0.05 STD Sham vs. STD HF.

			SHAM		
	STD	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
N	13	14	13	14	13
LV weight (g)	1.01±0.03	1.01±0.02	0.97±0.03	0.99±0.02	1.00±0.03
RV weight (g)	0.24±0.01	0.25±0.01	0.24±0.08	0.25±0.01	0.25±0.01
Atria (g)	0.08±0.01	0.09±0.01	0.09±0.01	0.08±0.01	0.08±0.01
Total Heart Weight (g)	1.33±0.04	1.36±0.02	1.31±0.03	1.32±0.03	1.33±0.04
Epididymal Fat (g)	3.95±0.15	6.07±0.46*	4.99±0.33	4.80±0.31	5.63±0.65*
Retroperitineal Fat (g)	3.95±0.25	6.26±0.32*	5.06±0.40	4.75±0.30	5.36±0.88
Total Fat Pad (g)	7.90±0.36	12.33±0.69*	10.05±0.69	9.54±0.56	10.99±1.51
LV/TL (mg/mm)	23.5±0.6	24.0±0.6	21.4±0.2	22.9±0.4	22.9±0.6
Atria/TL (mg/mm)	1.9±0.6	2.1±0.1	2.1±0.1	1.9±0.1	1.7±0.1
HW/TL (mg/mm)	30.9±0.8	32.1±0.7	30.9±0.7	30.5±0.5	30.4±0.7

Table 3.3 Continued

			HF		
	STD	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
N	11	11	12	11	13
LV weight (g)	1.41±0.06$	1.52±0.05	1.54±0.05	1.42±0.05	1.59±0.07
RV weight (g)	0.52±0.02$	0.51±0.03	0.53±0.02	0.44±0.04	0.50±0.03
Atria (g)	0.35±0.02$	0.33±0.02	0.36±0.02	0.31±0.03	0.33±0.04
Total Heart Weight (g)	2.28±0.08$	2.35±0.08	2.43±0.07	2.17±0.10	2.43±0.09
Epididymal Fat (g)	3.42±0.39	4.28±0.54	4.20±0.39	3.92±0.35	3.73±0.43
Retroperitineal Fat (g)	2.78±0.37$	3.78±0.51	3.29±0.29	3.63±0.53	3.12±0.42
Total Fat Pad (g)	6.19±0.72$	8.06±1.02	7.49±0.62	7.56±0.82	6.85±0.84
LV/TL (mg/mm)	33.0±1.5$	35.6±1.4	35.6±1.2	33.3±1.1	36.7±1.4
Atria/TL (mg/mm)	8.3±0.5$	7.6±0.5	8.3±0.5	7.2±0.8	7.7±0.8
HW/TL (mg/mm)	53.6±1.7$	55.1±1.9	56.1±1.6	50.9±2.4	56.0±1.9

The high fat diets did not result in obesity and there were no differences in body weight gain between any of the diets in either sham or HF groups. There was also no difference between STD Sham and HF. Fat pad mass (epididymal and retroperitoneal fat) was similar among all diets in the HF group, however in the sham groups the high MUFA group had a modest but significant increase in fat pad mass compared to the other diets (Table 3.3). This was not accompanied by an increase in circulating leptin (Table 3.10), consistent with the absence of obesity. Food consumption varied among groups. Among sham animals, the high saturated fat group consumed significantly more energy per day than the other groups (Table 3.4). Similarly, HF animals in the high saturated fat group consumed more energy than the STD, MUFA, and MIX diet groups.

Table 3.4. Food consumption in kcals/rat/day. *P<0.05 vs. STD, #P<0.05 vs. MUFA, ^P<0.05 vs. n6-PUFA, †P<0.05 vs. LCSat, $P<0.05 STD Sham vs. STD HF. The n for STD, MUFA, n6-PUFA, LCSat and MIX are as follows: n = 95, 100, 96, 96, 98 for Sham and n = 113, 124, 127, 140, 147 for HF.

	Sham	HF
STD	62.0 ± 0.9	58.2 ± 0.9$
MUFA	68.5 ± 0.6*^	63.0 ± 0.7*^
n6-PUFA	63.7 ± 0.7	65.5 ± 0.8*
LCSat	71.5 ± 0.7*#^	66.3 ± 0.8*#†
MIX	68.9 ± 0.8*^†	63.1 ± 0.8*

Cardiac Dimensions and Performance

Heart failure decreased ejection fraction by 33% (83.11±1.18% for STD Sham compared to 47.93±4.21% for STD HF, Figure 3.3A) and increased end diastolic and systolic volumes by 55% and 350%, respectively (from an EDV of 0.43±0.03 in the STD Sham to 0.68±0.05 in HF and from an ESV of 0.07±0.01 in the STD Sham to 0.36±.05 in HF, P<0.0004), providing evidence for heart failure (Table 3.5). The high fat diets did not affect LV volumes and function in the sham groups (Table 3.5). Among the HF groups the LCSat HF animals had a significantly greater ejection fraction (65.16±4.97%) compared to rats on the STD diet (47.93±4.21%).

HF increased peak LV pressure due to aortic constriction in the STD diet, but did not cause changes in dP/dt max, a measure of contractility (Table 3.5). HF caused LV diastolic dysfunction as seen in a 13-fold increase in end diastolic pressure and lower dP/dt min, a measure of lusitropy, compared with HF in the STD diet group (Figure 3.3B). However, there were no differences in these dP/dt max or min, end diastolic pressure, or maximum LV pressure among diet in either the sham or HF groups (Table 3.5).

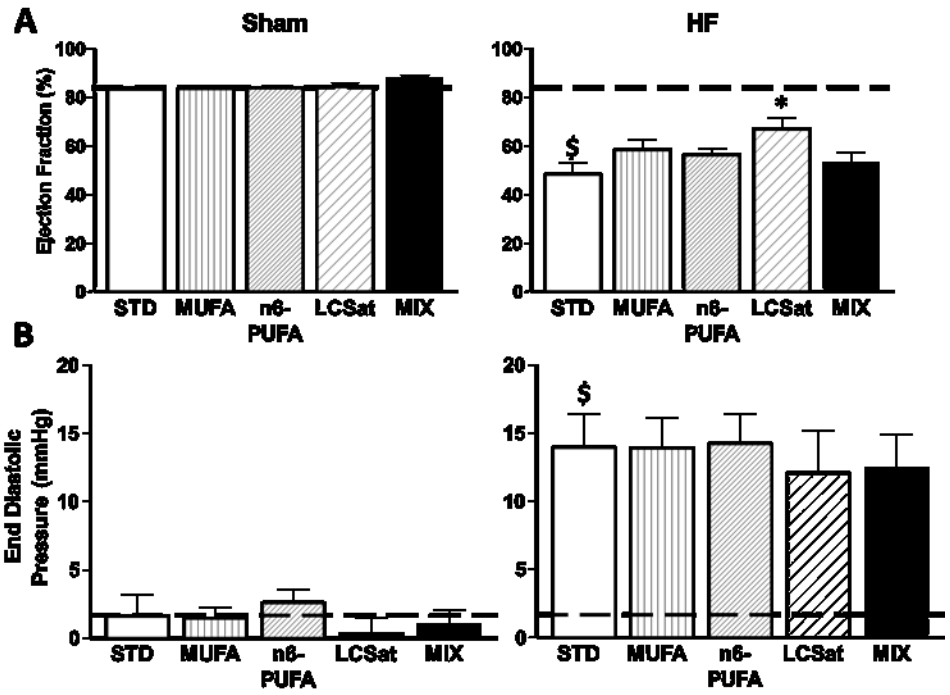

Figure 3.3. **Ejection fraction (A) and end diastolic pressure (B).** Data are presented as ± SEM. $P<0.05 STD Sham vs. HF, #P<0.05 vs. STD TAC.

Mitochondrial Phospholipid Fatty Acid Analysis

Previous studies from our lab have shown that dietary intake can modulate mitochondrial phospholipid composition[59, 61, 93, 124, 208]. Changes in phospholipid composition can affect mitochondrial membrane fluidity[100, 224, 225] and function[208, 226]. In general, HF has a greater effect on IFM than SSM[59, 91] so we analyzed phospholipids from isolated IFM.

In rats fed the STD diet, HF increased saturated fatty acids while decreasing n6-PUFAs. Myristate (14:0) was increased by 64%, palmitate (16:0) by 26%, stearate (18:0) by 11% (Table 3.6). HF also increased the *trans*-fatty acids, palmitoleic acid (16:1n7) by

Table 3.5. **Echocardiography and LV pressure measurements at 25 weeks post-surgery**. Data are presented as mean ± SEM. $P<0.05 STD Sham vs. HF. *P<0.05 vs. respective STD. LVP, left ventricular pressure; EDP, end diastolic pressure.

Sham	STD	MUFA	n6-PUFA	LCSat	MIX
N	13	14	13	14	13
End Diastolic Area (mm^2)	44.64±3.12	40.57±1.46	41.74±1.99	43.68±2.55	40.68±3.11
End Systolic Area (mm^2)	16.08±1.44	13.77±0.86	15.39±0.96	14.71±0.97	15.27±1.99
End Diastolic Diameter (mm)	7.36±0.18	7.47±0.13	7.40±0.15	7.26±0.18	7.04±0.18
End Systolic Diameter (mm)	4.06±0.17	4.06±0.12	3.97±0.13	3.94±0.19	3.49±0.17
End Diastolic Volume (mL)	0.43±0.03	0.44±0.02	0.43±0.03	0.41±0.03	0.37±0.03
End Systolic Volume (mL)	0.07±0.01	0.07±0.01	0.07±0.01	0.07±0.01	0.05±0.01
Stroke Volume (mL)	0.35±0.02	0.37±0.02	0.36±0.03	0.34±0.02	0.33±0.02
Cardiac Output	127.98±8.09	135.81±7.39	128.75±9.81	124.89±8.06	117.61±7.39
Relative Wall Thickness (mm)	0.66±0.03	0.62±0.03	0.62±0.02	0.64±0.03	0.70±0.04
Absolute Wall Thickness (mm/mm)	4.82±0.14	4.60±0.13	4.58±0.11	4.61±0.17	4.86±0.18
Heart Rate	364±4	367±6	355±8	367±6	365±8
Ejection Fraction (%)	83.11±1.18	83.60±1.22	83.90±1.43	83.68±1.52	87.44±1.26
Fractional Shortening	0.45±0.01	0.46±0.01	0.46±0.02	0.49±0.02	0.51±0.02
N	13	13	13	14	13
dP/dt Max (mmHg/s)	4977.99±523.68	5607.98±288.57	5173.01±425.49	5273.82±425.73	5157.95±402.94
dP/dt Min (mmHg/s)	-5992.44±716.20	-7328.71±522.66	-6130.68±585.72	-6862.65±539.89	-5630.93±541.04
LVP Max (mmHg)	83.00±5.24	93.92±3.44	87.96±4.36	88.93±5.09	90.17±4.45
LVP Min (mmHg)	-5.85±0.61	-5.52±0.55	-4.49±0.72	-6.09±1.36	-4.98±0.71
EDP (mmHg)	1.60±1.68	1.44±0.82	2.58±1.02	0.32±1.18	1.04±1.08

Table 3.5. Continued

HF	STD	MUFA	n6-PUFA	LCSat	MIX
N	11	12	12	12	13
End Diastolic Area (mm^2)	64.38±2.07$	63.00±3.08	66.00±4.42	58.46±4.77	60.75±3.01
End Systolic Area (mm^2)	46.61±2.14$	37.88±2.98	43.88±4.18	36.93±5.37	38.74±3.16
End Diastolic Diameter (mm)	8.62±0.23$	8.52±0.32	8.57±0.39	8.10±0.37	8.42±0.20
End Systolic Diameter (mm)	6.89±0.32$	6.36±0.33	6.61±0.38	5.66±0.51	6.45±0.33
End Diastolic Volume (mL)	0.68±0.05$	0.68±0.08	0.70±0.10	0.59±0.08	0.64±0.04
End Systolic Volume (mL)	0.36±0.05$	0.29±0.05	0.33±0.06	0.238±0.06	0.31±0.04
Stroke Volume (mL)	0.32±.03$	0.38±0.05	0.37±0.05	0.35±0.03	0.33±0.03
Cardiac Output	104.30±9.95$	123.53±16.80	119.92±15.09	118.38±11.23	109.43±8.17
Relative Wall Thickness (mm)	0.69±0.04	0.75±0.05	0.76±0.07	0.77±0.06	0.73±0.04
Absolute Wall Thickness (mm/mm)	5.96±0.32$	6.21±0.23	6.31±0.35	6.05±0.25	6.07±0.26
Heart Rate	327±8$	319±10	332±8	333±10	333±7
Ejection Fraction (%)	47.93±4.21$	57.38±3.78	53.71±3.23	65.16±4.97*	53.76±4.59
Fractional Shortening	0.20±0.02$	0.25±0.02	0.23±0.02	0.31±0.03	0.24±0.03
N	11	11	11	10	13
dP/dt Max (mmHg/s)	3990.64±278.32	4082.81±427.80	3849.26±413.27	4589.12±404.67	4714.28±382.19
dP/dt Min (mmHg/s)	-3863.34±327.67$	-4188.49±414.76	-3911.06±630.47	-5163.20±806.27	-4314.55±426.55
LVP Max (mmHg)	114.07±5.73$	117.18±10.57	110.10±9.58	123.11±11.11	124.29±7.97
LVP Min (mmHg)	5.39±1.71$	4.90±1.72	5.44±1.19	2.77±2.13	2.30±1.39
EDP (mmHg)	13.92±2.60$	13.91±2.30	14.22±2.32	12.08±3.28	12.45±2.59

67% and vaccenic acid (18:1n7) by 62%. There was a dramatic 48% HF-induced decrease in the n6-PUFA linoleate (18:2n6), but no change in arachidonic acid (20:4n6).

The various high fat diets profoundly altered phospholipid side chain fatty acid composition within the sham and HF groups (Table 3.6, Figure 3.4). In sham animals, the high MUFA diet increased oleate and DHA while decreasing linoleate. Further, high MUFA increased the Σn3-PUFA, decreased Σn6-PUFA, and increased the Σn3/n6-PUFA ratio (Table 3.6). The high n6-PUFA group increased the n6/n3-PUFA ratio by increasing linoleate and decreasing DHA, and dramatically reduced oleate content. The n6-PUFA diet differentially affected saturated fatty acids by increasing stearate while not significantly affecting palmitate. The high LCSat diet significantly increased stearate, palmitate, and arachidonic acid. Lastly, the MIX diet had intermediate effects by increasing stearate, arachidonic acid and DHA and decreasing linoleate.

Among the HF animals, the high MUFA diet increased oleate and decreased linoleate, and decreased the sum of n3-PUFA. The n6-PUFA diet decreased oleate, DHA, and the sum of n3-PUFA, but did not raise arachidonic acid as would be expected if elongation and desaturation of linoleate were accelerated by greater supply. LCSat, however, significantly increased arachidonic acid but this was not associated with decreased contractile function.

Mitochondrial Respiration and Yield

Overall, HF significantly decreased mitochondrial yield and maximal respiration on the standard diet, with little effect of the various high fat diets.

Mitochondrial yield was significantly decreased with HF for SSM, IFM and for total yield (SSM+IFM) compared to sham, but was not affected by diet in either sham of HF groups (Table 3.7).

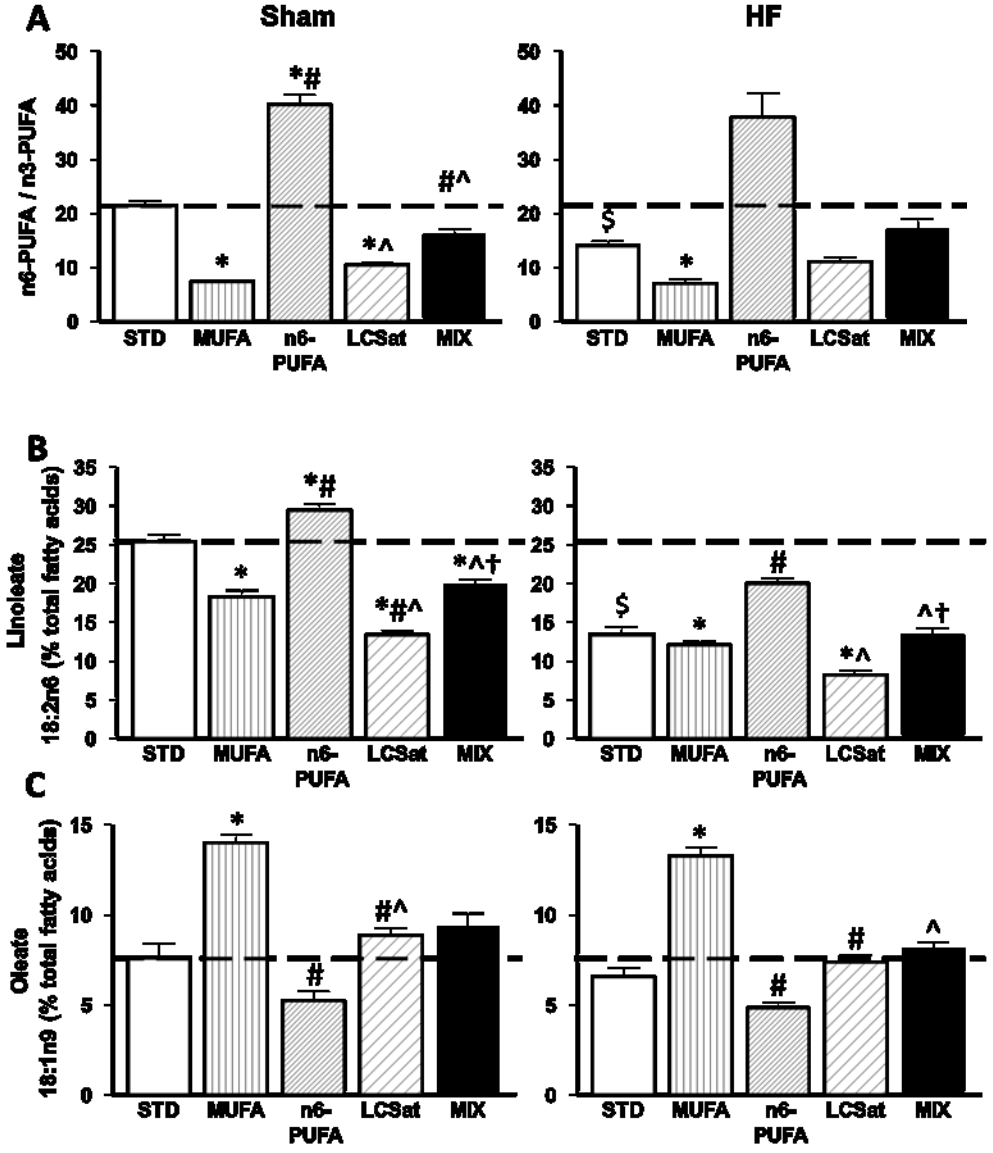

Figure 3.4. Mitochondrial phospholipid composition (IFM) displaying n6/n3-PUFA ratio (A), linoleate (B) and oleate (C) expressed as a per cent of total fatty acids. Data are presented as ± SEM. *P<0.05 vs. respective STD, $P<0.05 STD sham vs. HF, ^P<0.05 vs, n6-PUFA, †P<0.05 vs. LCSat.

Table 3.6. **Mitochondrial phospholipid analysis in isolated IFM**. *P<0.05 vs. STD, $P<0.05 STD Sham vs. HF, #P<0.05 vs. MUFA, ^P<0.05 vs. n6-PUFA,†P<0.05 vs. LCSat

	Sham				
	STD	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
N	12	12	13	12	10
Myristic acid (14:0)	0.11±0.02	0.11±0.03	0.12±0.02	0.14±0.01	0.11±0.02
Palmitic acid (16:0)	23.14±0.97	23.47±1.34	20.29±0.44	25.20±1.12^	21.09±.82
Palmitoleic acid (16:1n7)	0.18±0.03	0.06±0.04	0.03±0.01*	0.02±0.02	0.06±0.02
Stearic Acid (18:0)	27.97±0.58	28.83±0.70	31.89±0.85*#	34.05±0.49*#	32.37±0.64*#
Oleic Acid (18:1n9)	7.58±0.80	13.99±0.45*	5.23±0.51#	8.87±0.38#^	9.36±0.70
Vaccenic Acid (18:1n7)	3.39±0.15	3.36±0.09	1.89±0.07*#	2.54±0.08*#^	2.05±0.09*#†
Linoleic Acid (18:2n6)	25.37±0.91	18.28±0.82*	29.42±0.77*#	13.35±0.60*#^	19.85±0.72*^†
α-Linolenic Acid (18:3n3)	0.02±0.02	0.09±0.04	0.03±0.03	0	0.04±.04
Arachidonic Acid (20:4n6)	10.36±0.47	8.09±0.45	9.90±0.55	13.02±0.76*#^	12.84±0.93#^
Docosapentaenoic Acid (22:5n3)	0.15±0.02	0.34±0.02*	0.07±.01*#	0.23±0.02*#^	0.19±0.01#^
Docosahexaenoic Acid (22:6n3)	1.56±0.07	3.24±0.14*	0.90±0.04#	2.41±0.17^	1.87±0.13#^
Σ Saturated Fatty Acids	51.37±1.25	52.5±1.40	52.51±0.81	59.46±1.06*#^	53.70±0.82†
Σ Monounsaturated Fatty Acids	11.14±0.79	17.41±0.51*	7.15±0.49#	11.47±0.44#^	11.47±0.66#
Σ n-3 PUFA	1.73±0.06	3.67±0.16*	0.99±0.04#	2.64±0.18^	2.10±0.11#^
Σ n-6 PUFA	35.76±1.11	26.41±1.22*	39.35±0.72#	26.42±1.17*^	32.73±0.79#^†
Σ n-6 PUFA / Σ n-3 PUFA	20.99±1.00	7.26±0.31*	40.32±1.76#	10.39±0.57*^	16.06±1.13#^

Table 3.6. Continued

			HF		
	STD	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
N	**11**	**10**	11	**8**	**11**
Myristic acid (14:0)	0.18±0.01$	0.14±0.02	0.14±0.02	0.09±0.03	0.12±0.02
Palmitic acid (16:0)	28.92±1.11$	25.21±1.34	25.24±1.12	26.31±1.75	26.16±1.38
Palmitoleic acid (16:1n7)	0.30±0.02$	0.08±0.02*	0.05±0.02*	0.07±0.02*	0.06±0.01*
Stearic Acid (18:0)	31.15±1.00$	29.64±0.88	32.75±1.02	34.10±1.72	33.86±1.33
Oleic Acid (18:1n9)	6.57±0.51	13.27±0.47*	4.84±0.28#	7.37±0.39#	8.15±0.33^
Vaccenic Acid (18:1n7)	5.51±0.28$	5.98±0.15	3.83±0.14*#	4.58±0.20	3.56±0.11*#
Linoleic Acid (18:2n6)	13.37±1.03$	12.10±0.41*	20.00±0.64#	8.19±0.40*^	13.42±0.86^†
α-Linolenic Acid (18:3n3)	0.01±0.01	0.12±0.04	0	0.01±0.01	0
Arachidonic Acid (20:4n6)	11.88±1.05	10.05±0.78	12.00±1.18	16.75±1.43#	12.55±1.39
Docosapentaenoic Acid (22:5n3)	0.18±0.02	0.31±0.03	0.13±0.03#	0.18±0.02	0.17±0.02#
Docosahexaenoic Acid (22:6n3)	1.70±0.12	2.86±0.19*	0.81±0.09*#	2.19±0.21#^	1.50±0.15#^†
Σ Saturated Fatty Acids	60.45±1.37$	55.17±0.88	58.31±1.53	60.60±1.67	60.55±1.69
Σ Monounsaturated Fatty Acids	12.38±0.39	19.33±0.48*^	8.73±0.31*#	12.02±0.28#^	11.77±0.36#^
Σ n-3 PUFA	1.90±0.13	3.31±0.22*	0.95±0.10*#	2.38±0.22#^	1.67±0.17#^
Σ n-6 PUFA	25.28±1.35$	22.19±0.90	32.20±1.71*#	24.98±1.64^	26.01±1.83
Σ n-6 PUFA / Σ n-3 PUFA	13.68±0.87$	7.00±0.58*	37.77±4.61*#	11.02±0.98^	17.11±1.90#

Table 3.7. **Mitochondrial respiration and yield of SSM and IFM**. *P<0.05 vs. respective STD, $P<0.05 STD Sham vs. STD HF. Mitochondrial yield contains the same n as respective respiration parameters. Data are presented as mean ± SEM.

Mitochondrial Yield		STD	MUFA	n6-PUFA	LCSat	MIX
Sham	SSM	15.6±0.6	15.7±0.4	15.6±1.0	14.3±0.5	15.5±0.5
	IFM	12.0±0.6	12.1±0.4	11.1±0.8	11.5±0.4	11.5±0.6
	Total	27.6±0.9	27.8±0.5	26.7±1.2	25.8±0.7	26.9±0.9
HF	SSM	12.0±0.7$	13.5±0.9	12.6±0.7	12.0±0.5	13.1±0.7
	IFM	6.5±0.5$	7.3±0.3	6.5±0.4	7.4±0.7	8.1±0.5
	Total	18.5±0.8$	20.8±0.9	19.1±0.9	19.4±1.0	21.1±0.9
SSM Sham		STD	MUFA	n6-PUFA	LCSat	MIX
	N	13	14	13	13	12
Glutamate+Malate	State 3	303.4±15.8	277.0±28.8	320.7±24.5	265.0±12.2	289.6±16.0
	State 4	76.0±4.8	81.7±6.5	85.1±10.5	73.1±4.3	75.8±6.9
	State4+Oligomycin	28.5±3.3	26.5±3.5	29.4±6.2	22.6±2.2	26.6±3.7
	RCR	4.2±0.3	3.6±0.4	4.0±0.3	3.7±0.2	3.9±0.2
	ADP:O	2.3±0.1	2.1±0.1	2.2±0.1	2.9±0.3	2.4±0.3
Rotenone+Succinate	State 3	288.8±8.0	304.7±14.4	303.3±17.3	285.9±16.3	291.7±15.3
	State 4	134.7±5.5	140.0±5.5	141.9±10.9	128.4±9.7	133.6±6.3
	State4+Oligomycin	82.2±5.2	90.7±3.1	84.5±9.6	78.4±6.8	77.2±5.9
	RCR	2.2±0.1	2.2±0.1	2.2±0.1	2.3±0.1	2.2±0.1
	ADP:O	1.7±0.1	1.9±0.1	1.6±0.2	1.8±0.1	1.7±0.2
Palmitoyl Carnitine	State 3	193.5±23.1	199.9±15.9	215.3±20.6	179.5±16.7	201.3±17.3
	State 4	60.3±4.6	74.7±8.7	72.2±6.2	65.4±4.5	70.6±7.3
	State4+Oligomycin	19.9±3.7	25.6±4.2	28.2±4.6	18.7±2.0	24.7±3.8
	RCR	3.2±0.3	2.8±0.2	3.1±0.3	2.8±0.2	2.9±0.2
	ADP:O	2.2±0.1	2.3±0.3	2.3±0.1	2.4±0.2	2.3±0.2

Table 3.7. Continued

IFM Sham		STD	MUFA	n6-PUFA	LCSat	MIX
	N	13	14	13	13	12
Glutamate+Malate	State 3	335.2±22.3	299.5±20.8	292.0±14.1	280.5±24.8	299.3±22.7
	State 4	89.2±5.0	95.2±7.9	90.3±7.1	84.2±4.9	84.6±5.5
	State4+Oligomycin	33.3±2.7	30.4±4.0	29.8±3.2	30.9±1.7	29.8±2.9
	RCR	3.8±0.3	3.2±0.2	3.4±0.2	3.3±0.3	3.6±0.2
	ADP:O	2.7±0.2	2.9±0.3	2.9±0.1	3.3±0.3	2.8±0.2
Rotenone+Succinate	State 3	389.6±25.9	387.1±17.2	399.4±12.6	369.1±24.6	390.8±19.6
	State 4	189.3±13.2	179.1±10.5	177.3±11.1	153.8±7.8	177.2±10.6
	State4+Oligomycin	127.8±10.7	121.7±11.7	117.7±9.0	104.6±5.8	120.4±7.4
	RCR	2.1±0.1	2.2±.1	2.3±0.1	2.4±0.1	2.2±0.1
	ADP:O	1.6±0.1	1.7±0.1	1.6±0.1	1.7±0.2	1.8±0.1
Palmitoyl Carnitine	State 3	196.0±20.6	201.7±14.2	229.8±14.8	192.4±18.6	189.6±15.0
	State 4	76.7±7.7	71.6±5.8	85.1±8.2	71.0±4.9	74.9±6.4
	State4+Oligomycin	34.3±8.0	29.7±3.5	38.0±6.7	23.6±2.5	33.2±5.9
	RCR	2.6±0.2	2.8±0.1	2.8±0.2	2.7±0.1	2.6±0.2
	ADP:O	2.5±0.2	2.6±0.1	3.0±0.2	3.1±0.8	2.6±0.1
SSM HF		**STD**	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
	N	11	11	12	11	12
Glutamate+Malate	State 3	248.0±22.7$	267.6±23.9	270.6±27.7	259.8±26.6	257.2±19.8
	State 4	83.6±9.8	89.7±6.7	84.5±9.8	79.8±4.8	84.1±6.9
	State4+Oligomycin	25.0±2.8	31.1±3.4	26.9±3.3	26.9±2.0	28.6±3.7
	RCR	3.2±0.4$	2.7±0.2	3.4±0.4	3.5±0.5	3.1±0.2
	ADP:O	1.8±0.2	2.1±0.3	2.2±0.3	2.2±0.3	2.3±0.2
Rotenone+Succinate	State 3	233.4±20.8$	266.0±19.8	293.4±18.7	254.1±25.0	266.0±13.5
	State 4	127.7±6.2	143.0±13.8	165.7±16.7	139.2±9.0	145.2±6.6
	State4+Oligomycin	76.7±6.4	85.6±9.5	96.5±13.9	74.7±6.1	86.5±7.4

Table 3.7. Continued

	RCR	1.8±0.1	1.9±0.2	1.9±0.1	1.8±0.1	1.9±0.1
	ADP:O	1.5±0.2	1.6±0.1	1.4±0.2	1.2±0.1	1.5±0.1
Palmitoyl Carnitine	State 3	113.2±12.8$	168.8±18.9	116.0±19.0	141.9±19.3	149.3±9.2
	State 4	58.3±6.4	71.3±7.5	55.1±4.7	62.5±6.3	67.8±5.3
	State4+Oligomycin	15.5±2.2	21.9±3.7	14.9±2.5	18.5±4.9	18.0±1.8
	RCR	2.0±0.2$	2.4±0.3	2.0±0.2	2.3±0.4	2.3±0.2
	ADP:O	2.6±0.6	2.3±0.2	2.5±0.4	2.5±0.3	2.7±0.2
IFM HF		**STD**	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
	N	11	11	12	11	12
Glutamate+Malate	State 3	242.3±27.8$	256.7±30.0	245.5±34.5	257.0±26.5	217.6±26.1
	State 4	105.6±6.8$	96.0±6.3	100.7±9.8	98.7±8.3	89.2±6.9
	State4+Oligomycin	45.4±4.4	35.9±6.5	44.1±8.0	33.2±4.2	32.9±3.5
	RCR	2.3±0.2$	2.7±0.2	2.5±0.3	2.7±0.3	2.4±0.2
	ADP:O	2.0±0.3	2.3±0.3	2.3±0.2	2.4±0.3	2.5±0.2
Rotenone+Succinate	State 3	327.6±31.2	306.4±21.0	363.7±35.8	332.6±28.4	309.1±25.6
	State 4	196.6±21.8	159.0±10.6	196.4±15.2	183.9±19.2	167.7±13.2
	State4+Oligomycin	109.7±15.2	95.8±6.9	117.6±11.1	113.8±13.5	102.7±8.6
	RCR	1.7±0.1$	2.0±0.1	1.9±0.1	1.9±0.2	1.9±0.1
	ADP:O	1.2±0.1$	1.4±0.1	1.5±0.2	1.4±0.1	1.4±0.1
Palmitoyl Carnitine	State 3	117.6±16.4$	145.4±15.6	128.5±14.2	149.3±23.3	120.7±13.4
	State 4	71.6±4.6	75.6±5.9	72.5±3.9	72.4±6.5	64.5±4.4
	State4+Oligomycin	20.6±3.3	28.9±4.8	23.9±3.4	20.4±3.0	25.9±2.9
	RCR	1.6±0.2$	1.9±0.2	1.8±0.2	2.0±0.3	1.9±0.2
	ADP:O	2.1±0.3	2.5±0.3	2.4±0.3	2.5±0.3	2.5±0.2

In the STD diet groups HF decreased state 3 respiration with all substrates tested: glutamate+malate, succinate with rotenone, and palmitoylcarnitine. Diet did not affect mitochondrial respiration in sham or HF groups with any substrate. There was a trend toward increased in State 3 respiration by SSM HF with palmitoylcarnitine as substrate in the MUFA diet, although this was not statistically significant (Figure 3.5).

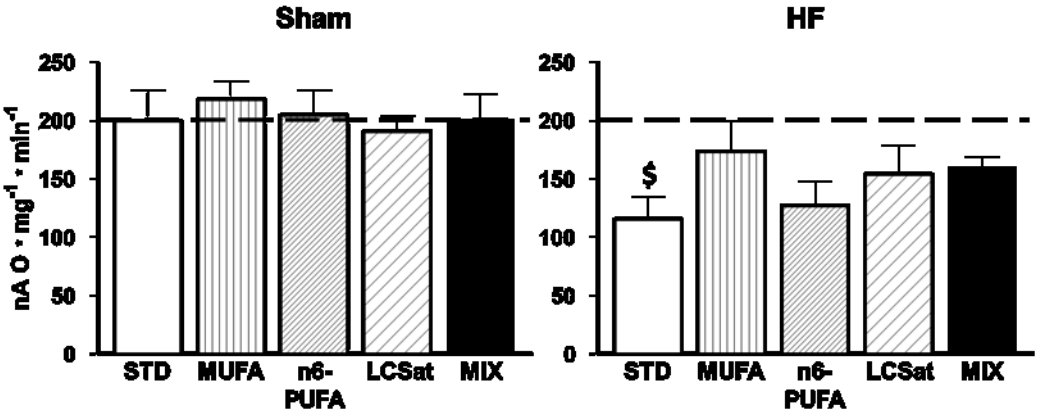

Figure 3.5. **Mitochondrial respiration with palmitoylcarnitine**. Data are presented as mean ± SEM. $P<0.05 vs. STD Sham.

Reactive Oxygen Species Production

Production of reactive oxygen species by isolated mitochondria was measured using Amplex Red with either glutamate+malate or succinate in the presence of rotenone as substrates. Heart failure significantly increased H_2O_2 production with glutamate+malate compared to sham in both subpopulations in the STD diet (Table 3.8). Diet did not effect H_2O_2 production in either the sham or HF groups, though there was a statistically insignificant trend towards increased H_2O_2 with n6-PUFA.

Table 3.8. **Hydrogen peroxide production by isolated mitochondria over 30 mins**. Data are presented as ± SEM with units of nmol/mg/min. $P<0.05 STD Sham vs. HF. The n is included for each substrate and surgery group with a number in parentheses if the n between subpopulations is different, SSM (IFM).

Sham		**STD**	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
N		12(11)	14	13	12(11)	13
Glutamate+Malate	**SSM**	204.1±92.0	129.7±16.3	234.8±83.7	136.1±30.1	121.0±23.0
	IFM	74.3±20.2	92.5±21.0	164.4±88.3	106.5±33.2	81.3±11.9
N		12	12	13	12	12
Succinate+Rotenone	**SSM**	228.9±15.3	244.4±14.2	228.4±23.4	221.3±19.2	217.6±16.2
	IFM	238.519.8	258.3±15.2	246.9±27.2	221.9±24.7	231.2±15.8
HF		**STD**	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
N		11	11	12	11	13
Glutamate+Malate	**SSM**	337.5±39.2$	334.3±52.0	294.0±46.8	355.5±52.1	373.1±44.3
	IFM	388.0±42.3$	345.56±59.0	333.5±50.2	401.5±65.6	334.8±41.0
N		8	9	9	10	11
Succinate+Rotenone	**SSM**	210.0±19.7	216.0±15.4	215.9±22.9	193.0±13.6	220.5±28.0
	IFM	270.7±25.0	253.6±18.7	337.3±83.6	257.4±27.1	269.6±32.4

Membrane Microviscosity

Fluorescence polarization was used to measure anisotropy, which decreases with an increase in membrane fluidity or a fall in membrane microviscosity. Heart failure significantly decreased anisotropy (corresponding to increased membrane fluidity) in SSM but not IFM of the STD diet (Figure 3.6). Among the sham animals the MUFA group had decreased fluidity compared to LCSat in SSM, and in IFM the MUFA group had decreased fluidity compared to the STD, LCSat and n6-PUFA groups (Figure 3.6).

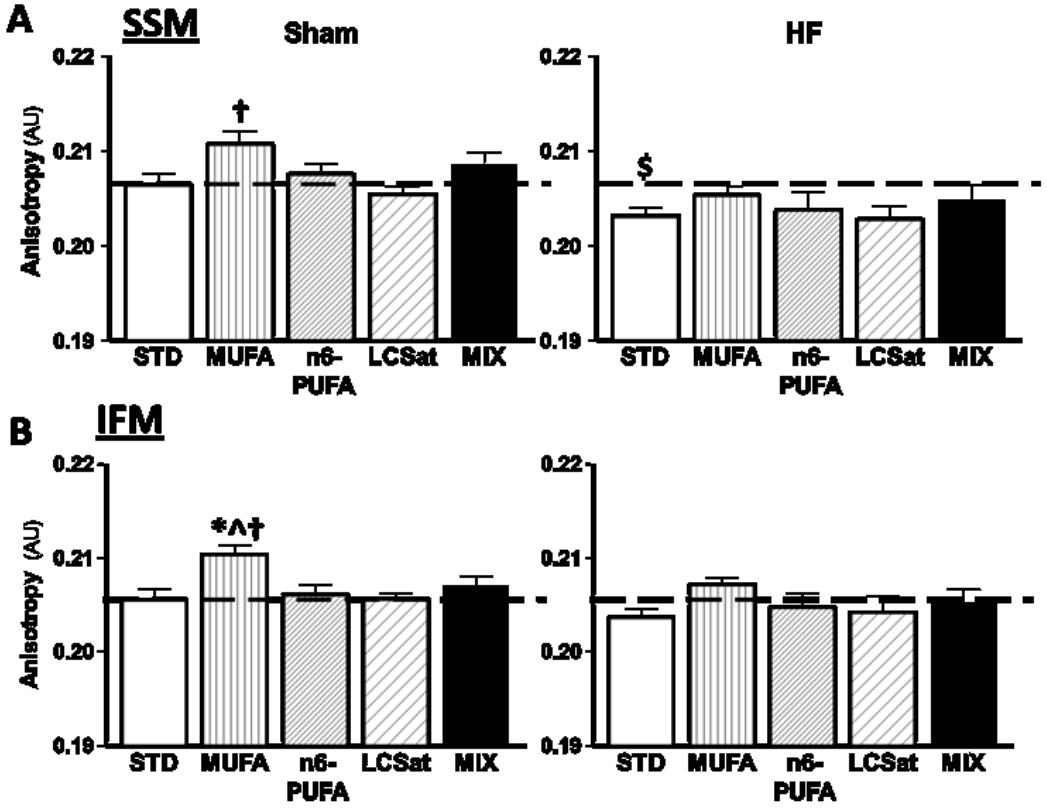

Figure 3.6. Mitochondrial membrane fluidity measured using TMA-DPH in SSM (A) and IFM (B). Data are presented as ± SEM. *P<0.05 vs. respective STD, $P<0.05 STD Sham vs. HF, ^P<0.05 vs. n6-PUFA, †P<0.05 vs. LCSat. The n for STD, MUFA, n6-PUFA, LCSat and MIX are as follows: n = 13, 14, 13, 13, 13 for SSM sham, n = 11, 11, 12, 11, 13 for SSM HF, n = 13, 14, 12, 13, 13 for IFM sham, and n = 11, 11, 12, 11, 13 for IFM HF.

Ca^{2+}-Induced Light Scattering Assay for MPT

A Ca^{2+}-induced light scattering assay was employed wherein decreased absorbance over time is indicative of mitochondrial swelling. There was no effect of HF on the rate of swelling however the final plateau of absorbance was greater with HF SSM compared to sham in the STD diet. Within SSM, MUFA was more resistant to swelling under Ca^{2+}-free conditions compared to all other diets and was also resistant when a bolus of Ca^{2+} was added (100 nmol Ca^{2+}/mg mitochondrial protein) compared to the STD and LCSat diets (Figure 3.7). With no Ca^{2+} added, LCSat SSM swelled significantly

more compared to STD and MIX diets but not to n6-PUFA. No differences were seen among diets in the sham group under Ca^{2+}-free conditions in IFM however HF increased the rate of swelling in STD IFM compared to sham. Similar to SSM, MUFA sham IFM displayed decreased swelling compared to LCSat upon the addition of Ca^{2+} (data not shown). The subtle changes with MUFA were not due to changes in the initial baseline absorbance readings as it was unchanged among groups (Figure 3.8). No differences were observed among diets within the HF surgery group of either subpopulation of mitochondria.

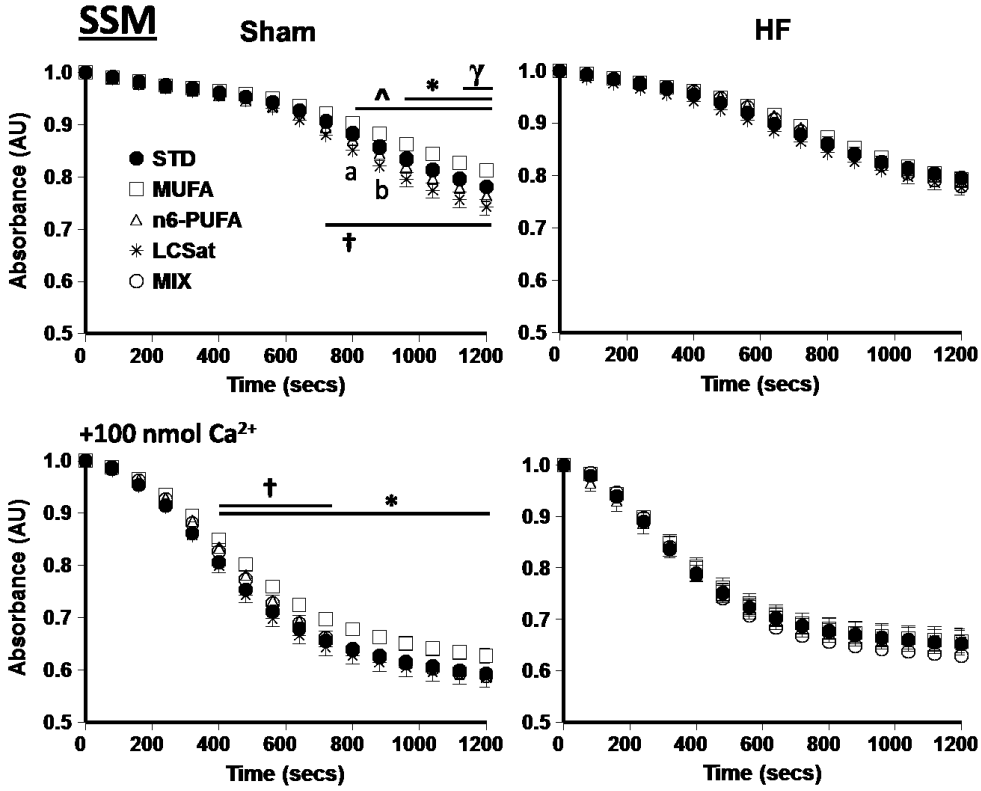

Figure 3.7. **Ca^{2+}-induced mitochondrial swelling in SSM**, as measured by a decrease in absorbance over time. *P<0.05 MUFA vs. STD, †P<0.05 MUFA vs. LCSat. Data are presented as ± SEM. The N for STD, MUFA, n6-PUFA, LCSat and MIX are as follows: n = 13, 14, 13, 13, 13 for sham and n = 11, 11, 12, 11, 13 for HF.

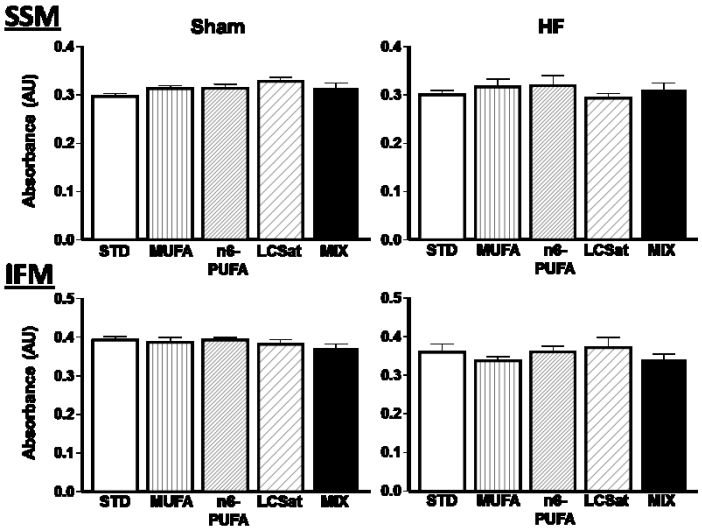

Figure 3.8. **Baseline absorbance values for isolated SSM and IFM** at 540 nm. Data are presented as ± SEM. The n for STD, MUFA, n6-PUFA, LCSat and MIX are as follows: n = 13, 14, 13, 13, 13 for SSM sham, n = 11, 11, 11, 11, 13 for SSM HF, n = 13, 12, 12, 12, 13 for IFM sham, and n = 10, 11, 12, 10, 13 for IFM HF.

Ca^{2+}-Induced Mitochondrial Permeability Transition

Extramitochondrial Ca^{2+} green 5N fluorescence was increased in HF compared to sham IFM upon the addition of Ca^{2+}, indicating decreased Ca^{2+} buffering capacity (Figure 3.9). No differences were observed in SSM but IFM within the n6-PUFA diet displayed decreased Ca^{2+} handling compared to the STD and MIX diets.

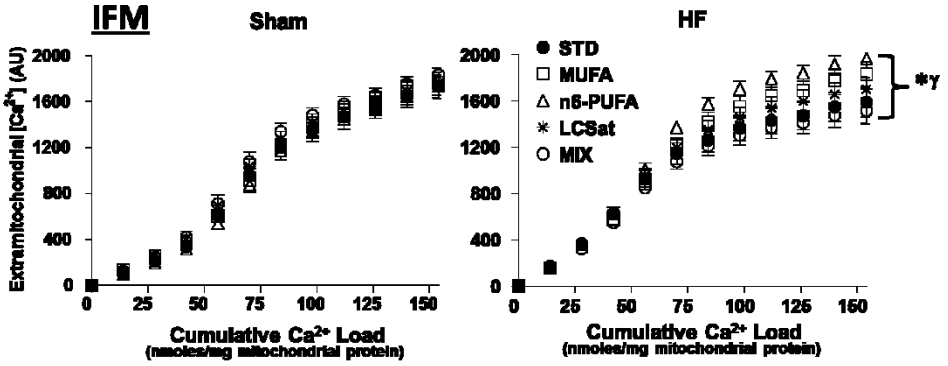

Figure 3.9. **Ca^{2+}-induced MPT measured in isolated IFM** as an increase in extramitochondrial Ca^{2+} with increased Ca^{2+} load. No differences reported in SSM. Data are presented as ± SEM. *P<0.05 vs. respective STD, γP<0.05 vs. MIX. The n for STD, MUFA, n6-PUFA, LCSat and MIX are as follows: n = 11, 13, 12, 12, 12 for Sham and n = 11, 10, 10, 10, 12 for HF.

Fatty Acid Oxidation Enzymatic Activity

Whole LV Tissue

The activities of citrate synthase, aconitase, MCAD and LCAD, expressed per gram of wet tissue, were unaffected by HF and there were no differences among diets within the HF group. Within Sham, MUFA had greater aconitase and LCAD activity compared to STD (Table 3.9).

Isolated Mitochondria

No changes in citrate synthase, MCAD or LCAD in SSM with HF or between diets. HF did not have an effect on mitochondrial enzyme activity in IFM, however high MUFA decreased MCAD activity relative to STD (Table 3.9).

Biochemical Parameters

Circulating free fatty acids, glucose, insulin, and leptin were not different among diets within each surgery group, and with the standard diet HF was not different from sham (Table 3.10). Plasma triglycerides were lower in the HF group compared to sham on the STD diet. Within sham groups MUFA and LCSat had elevated plasma triglycerides compared to STD and n6-PUFA. No differences in triglycerides were seen with HF. Diet had no effect on markers of inflammation, as the urine thromboxane/creatinine ratio and serum TNF-α were unaffected by diet.

Table 3.9. Enzymatic analysis of LV tissue and isolated mitochondrial subpopulations. CS, citrate synthase; MCAD, medium chain acyl-dehydroganse; LCAD, long chain acyl-dehydrogenase. *P<0.05 vs. STD Sham.

Whole Tissue			Sham		
	STD	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
N	13	14	13	14	13
Aconitase					
mmol*gww-1*min-1	0.77±0.13	1.68±0.16*	1.06±0.16	1.46±0.21	1.06±0.20
CS					
µmol*gww-1*min-1	105.33±15.23	157.12±14.28	107.27±8.87	129.68±11.40	116.96±15.70
MCAD					
µmol*gww-1*min-1	3.32±0.50	4.10±0.50	2.92±0.33	4.33±0.41	3.37±0.44
LCAD					
µmol*gww-1*min-1	5.09±0.52	7.49±0.64*	5.80±0.68	7.08±0.59	5.42±0.60
			HF		
	STD	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
N	11	11	12	11	13
Aconitase					
mmol*gww-1*min-1	1.00±0.16	0.86±0.15	0.82±0.16	0.80±0.19	0.72±0.14
CS					
µmol*gww-1*min-1	104.34±15.92	115.77±22.68	91.40±12.42	95.30±21.70	88.31±11.64
MCAD					
µmol*gww-1*min-1	2.43±0.41	2.91±0.42	2.39±0.27	2.93±0.71	2.16±0.27
LCAD					
µmol*gww-1*min-1	4.49±0.71	4.32±0.65	3.85±0.53	3.73±0.85	3.00±0.37

Table 3.9. Continued

SSM	STD	MUFA	Sham n6-PUFA	LCSat	MIX
N	13	14	12	13	11
CS µmol*gww-1*min-1	1.69±0.12	1.79±0.12	1.85±0.12	1.74±0.11	2.10±0.26
MCAD µmol*gww-1*min-1	0.08±0.01	0.08±0.01	0.07±0.01	0.13±0.05	0.08±0.00
LCAD µmol*gww-1*min-1	0.04±0.00	0.04±0.00	0.04±0.00	0.04±0.00	0.04±.00
	STD	**MUFA**	**HF n6-PUFA**	**LCSat**	**MIX**
N	10	10	12	11	13
CS µmol*gww-1*min-1	2.25±0.60	1.75±0.18	1.73±0.11	2.17±0.52	1.80±0.21
MCAD µmol*gww-1*min-1	0.08±0.01	0.07±0.01	0.07±0.01	0.07±.00	0.07±0.01
LCAD µmol*gww-1*min-1	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00	0.03±0.00

IFM	STD	MUFA	Sham n6-PUFA	LCSat	MIX
N	13	14	13	13	13
CS µmol*gww-1*min-1	2.13±0.06	2.26±0.13	2.22±0.10	2.21±0.09	2.11±0.13
MCAD µmol*gww-1*min-1	0.12±0.01	0.09±0.01	0.09±0.00	0.10±0.01	0.10±0.01
LCAD µmol*gww-1*min-1	0.07±0.00	0.06±0.00	0.06±0.00	0.05±0.00	0.06±0.01
	STD	**MUFA**	**HF n6-PUFA**	**LCSat**	**MIX**
N	11	11	12	11	13
CS µmol*gww-1*min-1	2.29±0.11	2.06±0.09	2.22±0.14	2.31±0.14	2.14±0.14
MCAD µmol*gww-1*min-1	0.10±0.01	0.08±.01	0.08±0.00	0.09±0.01	0.08±0.01
LCAD µmol*gww-1*min-1	0.06±0.01	0.05±0.00	0.06±0.00	0.05±0.01	0.05±0.00

Table 3.10. **Metabolic Parameters**. *P<0.05 vs. STD, $P<0.05 STD Sham vs. HF, #P<0.05 vs. MUFA, ^P<0.05 vs. n6-PUFA,†P<0.05 vs. LCSat

Metabolite or Hormone	Sham					HF				
	STD	MUFA	n6-PUFA	LCSat	MIX	STD	MUFA	n6-PUFA	LCSat	MIX
N	11	13	10	13	12	11	10	11	10	12
Serum Glucose, mM	18.39±1.33	17.38±0.80	17.32±1.28	19.34±1.17	18.86±0.88	15.29±0.78	14.89±1.01	15.14±0.96	16.81±1.81	15.44±1.22
Plasma Leptin, pg/mL	807.5±105.3	988.6±132.2	1014.3±157.9	716.0±83.5	934.9±113.6	591.2±78.3	541.1±101.2	615.1±96.7	745.2±138.7	799.8±101.4
Serum Insulin, ng/mL	0.582±0.083	0.629±0.056	0.762±0.095	0.945±0.196	0.844±0.073	0.621±0.070	0.615±0.119	0.729±0.129	0.633±0.107	0.856±0.193
Plasma Triglycerides, mg/dL	95.8±9.8	143.4±9.7*^	88.6±9.4	137.7±12.8*^	120.9±11.8	66.2±7.9$	80.3±18.8	83.0±14.2	108.3±10.5	97.2±9.8
Plasma Free Fatty Acids, mM	0.298±0.056	0.420±0.023	0.371±0.027	0.331±0.033	0.384±0.026	0.300±0.050	0.331±0.039	0.397±0.056	0.366±0.031	0.372±0.030
Serum TNF-Alpha, pg/ml	4.643±2.173	6.190±1.815	5.858±1.959	6.540±1.907	5.283±1.693	9.984±2.228$	10.797±2.177	8.411±2.395	12.741±2.520	9.795±2.080
Thromboxane/Creatinine, pg/umol	50.3±5.4	52.3±6.4	46.8±5.2	32.5±5.4	48.2±6.9	99.9±28.6	48.2±17.4	73.4±22.4	49.0±10.4	78.7±31.9

mRNA Expression

The classic mRNA markers of HF, ANF and MHCβ/α, were significantly elevated compared to sham (Figure 3.1C and D), but there were no changes among diets within HF or sham groups. Many of the PPARα-regulated genes were down regulated with HF in the STD diet groups, specifically PDK4, UCP3 and MCAD, but there were no differences between diets within each surgery group (Table 3.11).

Table 3.11. **mRNA expression** of fatty acid oxidation genes from frozen LV tissue. CPT-1, carnitine palmitoyl transferase-1; UCP3, uncoupling protein 3; PDK4, pyruvate dehydrogenase kinase 4; Acadm, medium chain acyl dehydrogenase; Nppa, atrial natriuretic factor; Aco, aconitase; MHC, myosin heavy chain. Data are presented as mean ± SEM. $P<0.05 STD Sham vs. HF.

			Sham		
	STD	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
N	13	12	13	14	12
CPT-1	1.00±0.19	1.19±0.24	0.88±0.18	1.18±0.13	1.05±0.19
UCP3	1.00±0.21	1.44±0.34	1.22±0.33	0.84±0.11	1.30±0.29
PDK4	1.00±0.19	2.28±0.46	1.57±0.38	1.57±0.19	1.62±0.34
Acadm	1.00±0.20	1.26±0.16	1.02±0.20	1.16±0.12	1.30±0.20
Aco	1.00±0.19	0.99±0.14	0.88±0.18	1.24±0.16	1.07±0.17
PPARα	1.00±0.25	1.08±0.22	0.88±0.26	1.06±0.19	0.95±.21
MHCα	1.00±0.22	1.06±0.17	0.89±0.23	1.10±0.24	1.05±0.21
MHCβ	1.00±0.45	0.62±0.12	0.34±0.07	0.49±0.07	0.54±0.11
			HF		
	STD	**MUFA**	**n6-PUFA**	**LCSat**	**MIX**
N	11	11	12	10	12
CPT-1	0.66±0.10	0.86±0.20	0.82±0.10	0.82±0.17	0.72±0.13
UCP3	0.23±0.04$	0.93±0.35	0.41±0.10	0.51±0.16	0.61±0.11
PDK4	0.57±0.08$	1.22±0.37	0.81±0.13	1.20±0.25	0.99±0.11
Acadm	0.43±0.04$	0.68±0.14	0.74±0.08	0.67±0.15	0.56±0.08
Aco	0.76±0.10	0.73±0.15	0.82±0.08	0.70±0.15	0.80±0.14
PPARα	0.51±.11	0.54±0.15	0.53±0.07	0.73±0.23	0.65±0.15
MHCα	0.24±0.05	0.24±0.06	0.24±0.04	0.31±0.09	0.22±0.04
MHCβ	0.98±0.15	1.05±0.18	1.23±0.13	1.02±0.18	0.91±0.14

Discussion

The novel findings of this study are that *1)* a Mediterranean-like high MUFA diet dramatically altered mitochondrial phospholipid composition by decreasing the n6/n3-PUFA ratio by 35% compared to the STD diet in healthy rats and by 50% in HF, as well as decreasing membrane fluidity which was associated with increased resistance to calcium-induced swelling and maintenance of fatty acid oxidation in sham-operated animals, *2)* a diet high in n6-PUFA dramatically increased the n6/n3-PUFA ratio in sham and HF compared to the STD diet which was associated with increased susceptibility to calcium-induced MPT (or decreased calcium buffering capacity) and a trend towards increased ROS production, and *3)* a diet high in LCSat increased the levels of saturated fat and arachidonic acid in mitochondrial membranes but was not associated with decreased mitochondrial function and in fact ameliorated the decreased in ejection fraction compared to the STD diet. Taken together, our results show that increased intake of diverse long chain saturated or unsaturated fatty acids is well tolerated in severe pressure overload heart failure. Further, they suggest that a Mediterranean diet high in MUFA with a moderate level of linoleate decreases MPT sensitivity and increases fatty acid oxidation compared to other high fat diets, but is not effective for treating advanced HF in this model.

Effects of Diet in Healthy Rats

In healthy rats, a high MUFA diet increased oleate at the expense of the n6-PUFA, linoleate, with no effect on saturated fat. The MUFA diet also increased DHA, most likely due to the elongation of α-linolenic acid, which accounted for 4.35% of the

total fatty acids in the chow (1.7% of energy intake). The n6/n3-PUFA ratio was the lowest in the high MUFA diet which may account for the increased conversion of α-linolenic acid to DHA[227]. MUFA displayed resistance to Ca^{2+}-induced light scattering, a measure of MPT, compared to LCSat which was not due to inherent absorbance of mitochondria. This finding is consistent with previous studies highlighting the protective effect of oleate over palmitate against cell death and MPT activation in cell culture studies[191-193, 228, 229].

Mitochondrial membrane fluidity, assessed using fluorescence anisotropy, was decreased with MUFA compared to LCSat. The correlation between decreased membrane fluidity and increased resistance to Ca^{2+}-induced MPT was surprising as earlier studies found that dietary supplementation with long chain n3-PUFA conferred resistance to MPT[59, 61, 93] and increased membrane fluidity in both healthy and hypertrophied myocardium (*Dabkowski, O'Connell et al, unpublished*). Conversely, early studies on membrane fluidity suggested that increased lipid peroxidation, as occurs with increased polyunsaturated fatty acid intake, contributes to increased membrane fluidity via opening channels in the OMM which increases in membrane permeability thus a loss in membrane integrity[224]. Our results are similar to *in vitro* studies in isolated cardiomyocytes from animals with streptozotocin-induced diabetes, where increased fluidity in mitochondrial membranes in diabetes correlate with increased expulsion of fluid into the extra-mitochondrial space, interpreted as MPT-induced membrane rupture[232, 233].

The high n6-PUFA diet dramatically increased the n6/n3-PUFA ratio by elevating linoleate and decreasing DHA without a change in arachidonic acid. This diet also

significantly increased stearate while decreasing palmitate. The high LCSat diet also increased stearate and decreased the n6-PUFA linoleate while increasing arachidonic acid. Although arachidonic acid is thought to be pro-inflammatory[161], cardiac function in the hearts of the LCSat group was greater compared to the STD group. The MIX diet was intermediate. Thus, it seems that linoleate is a flexible phospholipid that can be substituted for oleate or stearate depending on the type of fatty acid supplementation.

Effects of Heart Failure and Diet on PL Fatty Acid Composition

In this study, HF increased the percent of saturated fatty acids and arachidonic acid in mitochondrial membranes at the expense of MUFA and the n6-PUFA, linoleate in rats fed the STD diet (Table 3.6). Similar changes in saturated and monounsaturated fatty acids in cardiac dysfunction have been observed in rat SSM 12 weeks after myocardial infarction[61], rat IFM 25 weeks after abdominal aortic banding[59], and in a cardiomyopathic hamster model, described previously[4].

In this study, the high MUFA diet had the highest levels of oleate and lowest linoleate among the HF groups. The MUFA diet lowered palmitate and stearate, preventing the HF-induced increase in these fatty acids observed with the STD diet. This diet also ameliorated the decreased in DHA that occurs with HF. In a previous study using cardiomyopathic hamsters fed a diet high in MUFA and LCSat (18% energy from oleate, 20% from palmitate+stearate), there were mixed effects on individual saturated fatty acids but overall long chain saturated fats were increased, while MUFAs were decreased despite comprising almost 50% of total energy from fat intake. The present

study found differential mRNA expression of PPAR-regulated genes with a trend toward maintenance of expression with MUFA in HF (P=0.047 for main effect of diet).

The n6-PUFA group maintained the increase in linoleate and surprisingly did not increase arachidonic acid. This diet, however, decreased DHA and oleate in HF which may have contributed to the trend toward increased ROS production in n6-PUFA. Also similar to the sham group, the LCSat group decreased linoleate and raised arachidonic acid but this did not affect cardiac function as the EF in this group was greater compared to the STD group. Previous studies in the hypertrophied myocardium show a high fat/low carbohydrate diet (60% total energy intake, mainly from saturated fat) ameliorated LVH and altered gene expression by increasing mRNA and activity of MCAD, a PPARα-regulated gene[5]. However, when a high unsaturated fat (60% total energy with 34% from MUFA+PUFA and 26% from palmitate+stearate) was compared to a high saturated fat diet (56% palmitate+stearate) in healthy rats for 2 months, cardiac function was unaffected but apoptosis and ceramide content was increased with high LCSat compared to unsaturated fat. Both high fat diets upregulated PPARα-regulated genes[37]. Although these various high fat diets have either neutral or beneficial effects on cardiac function, phospholipid composition is dramatically altered which may account for the differential effects of the high fat diet combinations.

Conclusion

In summary, the diets used in this study had differential effects on mitochondrial phospholipids, fluidity, and Ca^{2+}-induced MPT in healthy animals, and had generally neutral effects in HF. Overall, the high MUFA diet, similar to a high Mediterranean diet,

had the greatest protection from MPT and better maintenance of mitochondrial phospholipid composition.

Chapter 4 – Summary, Conclusions, and Future Directions

Summary

This dissertation examined the effects of diets high in specific fatty acids on mitochondrial and contractile function in healthy and diseased hearts. First, our initial study demonstrated that intake of diets high in saturated fat dramatically alters mitochondrial membrane phospholipid composition which slowed mitochondrial Ca^{2+} uptake, but did not alter cardiac function in healthy animals. In TAC-induced HF, 15 weeks of high fat diet feeding was either neutral or modestly beneficial in many of the parameters measured compared to feeding a standard low fat diet. However, we found a disassociation between resistance to MPT and contractile function, suggesting that inhibition of MPT may not be an effective therapeutic target for slowing the progression of HF. Specifically, a diet high in MUFA, similar to a Mediterranean diet, increased the amount of oleate in mitochondrial membranes, decreased membrane fluidity, and conferred resistance to Ca^{2+}-induced MPT, but did not affect cardiac function. We found that a high saturated fat diet had differential effects in the healthy and diseased heart. It decreased Ca^{2+} uptake by isolated mitochondria in the healthy heart but ameliorated the HF-induced decrease in EF. Lastly, a diet high in n6-PUFA decreased the Ca^{2+} buffering capacity of mitochondria but did not affect contractile function or mitochondrial respiration.

Considerations and Limitations

One of the limitations in our studies is the mitochondrial isolation procedure. As described in the methods, we used a standard method with polytron homogenization,

trypsin digestion, and differential centrifugation to separate out the mitochondrial subpopulations. In failing myocardium, this procedure likely favors the isolation of the healthiest mitochondria, with the loss of fragmented and damaged mitochondria during the isolation process[234]. Increased time and intensity of homogenization can damage initially released mitochondria[87]. Thus, an artificially segregated healthy population of mitochondria may account for the similarity in mitochondrial respiration among groups, particularly in the HF study. Evidence for this comes from the decrease in mitochondrial yield with HF despite no decrease in the activity of mitochondrial marker enzymes in whole tissue or isolated mitochondria.

Additionally, there is a limitation in our approach to assessing MPT, as we used only Ca^{2+} stress, and did not evaluate other triggers of MPT, specifically ROS. Further, we did not assess the effects of cyclosporine A, an inhibitor of cyclophilin D that delays stress-induced MPT. Earlier studies from our lab used cyclosporine A to delay the opening of the pore and establish that MPT was causing the release of Ca^{2+}. Thus, in the current studies it is possible that Ca^{2+} was being released through a mechanism other than MPT. We initially examined whether Ca^{2+}-induced MPT, assessed using successive injections of Ca^{2+} released total matrix Ca^{2+} which would indicate membrane rupture (see Chapter 3 for detailed methods). Prior to conducting the studies presented in Chapter 3, I performed a preliminary experiment using an addition of alamethicin (20 ug/mL; Sigma Aldrich), a membrane channel-forming peptide, and observed no additional increase in extramitochondrial Ca^{2+} when applied after 15 injections of 14 nmol Ca^{2+} (Figure 4.1), confirming that all mitochondrial Ca^{2+} was released. We did not add alamethicin at the end of our measurement of Ca^{2+}-induced MPT in our experiments. However, since the

response to added Ca^{2+} was similar we assume that all Ca^{2+} was released (compare Figure 3.5 to Figure 4.1).

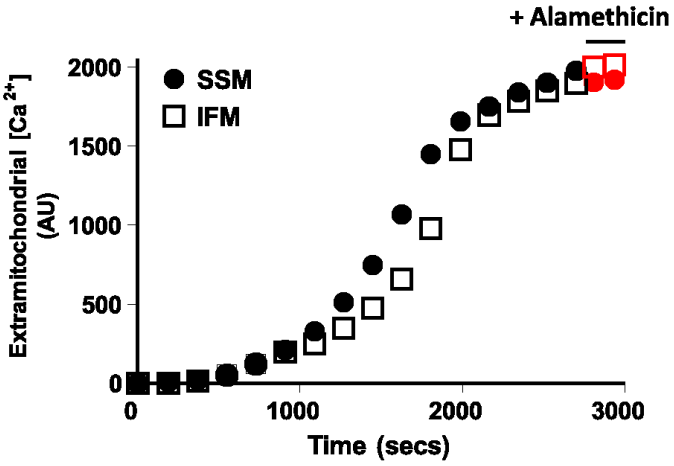

Figure 4.1. Ca^{2+}-induced MPT followed by the addition of 20 µg/mL alamethicin (highlighted in red).

Future Directions

The results presented in this dissertation generate new questions about the association between mitochondrial respiration, MPT and contractile function in HF, and on the overall impact of fat intake on the failing heart. Further, these results question the therapeutic potential of examining susceptibility to MPT as a clinically relevant end point in the treatment for heart failure.

Altering Membrane Composition

The studies presented in Chapters 2 and 3 explore the impact of changes in mitochondrial phospholipid fatty acid side chain composition on the function of isolated cardiac mitochondria. We found dramatic changes in fatty acyl side chain composition based on the intake of specific fatty acids. In some cases, membrane fluidity was affected (as with the decrease in fluidity observed with high intake of MUFA in Chapter

3) which was associated with enhanced resistance to Ca^{2+}-induced light scattering but not to amelioration of contractile dysfunction induced by TAC. In other cases, despite changes in phospholipid composition, membrane fluidity remained unaffected while Ca^{2+} uptake was altered (Chapter 2). Future studies should examine the importance of fluidity on the biophysical properties of mitochondrial membranes. Additionally, the use of dyes that bind to various sites within the phospholipid membrane (i.e. polar head groups vs. hydrophobic core)[235] could be used to compare the time course of fluidity changes in response to known changes in fluidity (i.e. temperature).

It is important to note that the analysis of PL fatty acid composition performed here did not separate out individual mitochondrial PL classes [phosphotidylcholine, phosphotidylethanolamine, phosphatidylinositol, sphingomyelin, lyso phosphotidylcholine, and cardiolipin (CL)], nor did we assess the effects of diet and HF on the relative amount of each PL class. The molecular composition of CL, which is largely comprised of linoleate side chains, was not analyzed in these studies. Deficiencies in CL are linked to cardiomyopathy as evidenced by Barth Syndrome, caused by a genetic mutation in tafazzin, an essential enzyme for the synthesis of functional CL[107, 108]. Previous studies from our lab have shown that arachidonic acid supplementation decreases tetralinolenyl CL (L_4CL) by ~80% through the substitution of arachidonic acid side chains, yet mitochondrial respiration was unaffected and MPT was significantly desensitized compared to animals on a standard control diet[208]. Similarly, a high saturated fat diet fed to banded rats also depleted L_4CL without any adverse effect on cardiac function[47]. These data suggest that L_4CL is not essential for normal mitochondrial function.

It has been proposed that heart failure caused depletion of total CL content and L_4CL[100], however this was not observed in HF patients[102] or rats with HF[61]. Similarly, dogs with HF secondary to myocardial ischemic damage have severe mitochondrial dysfunction but normal L_4CL[95, 236]. Thus, in the present investigation we did not measure CL because there is little no evidence that CL changes with HF, and the literature does not support a role for depletion of CL in impaired mitochondrial function in the failing heart[226].

Prevention of HF

The results from Chapter 2 suggest that dramatic dietary-induced changes in phospholipid composition are not associated with altered cardiac function in healthy rats, while Chapter 3 showed that treating rats that have TAC-induced HF with a high fat diet also was not strongly associated with improved contractile function compared to the standard low fat diet. However the LCSat group had significantly increased ejection fraction in HF compared to the low fat/high carbohydrate diet. Previous studies showed a 60% high fat diet (mainly saturated fat) ameliorated hypertension-induced LVH, decreased markers of HF, and prolonged survival[5, 42] in Dahl salt-sensitive rats. An earlier study using the same diet provided further evidence for the beneficial effects of high saturated fat on LVH, showing improvement in contractile performance[10].

Transitioning to the Clinic

The studies described in this dissertation take a reductionist approach to defining the effects of high dietary fat in the healthy and diseased heart. Though knowledge of the

mechanisms of specific fatty acids is important, translating studies about dietary fat to the clinic will require research on the combinations of ingredients within specific foods. Thus, studies on whole foods which contain a variety of compounds (i.e. fat, cholesterol, endogenous antioxidants) are essential to advising patients on dietary changes in the face of disease risk.

Our data support the concept that a diet high in MUFA with moderately elevated levels of n3-PUFA would benefit patients at risk for HF. Recently, a Mediterranean diet was found to reduce the incidence of myocardial infarction, stroke or death from cardiovascular causes in people at high risk for cardiovascular events (i.e. had type 2 diabetes or at least 3 of the following: smoking, hypertension, increased LDL, low HDL, overweight or obese, or had a family history of CHD)[15]. In this primary prevention study, patients were assigned to a Mediterranean diet supplemented with either olive oil or mixed nuts (walnuts, almonds and hazelnuts) high in oleate and α-linolenic acid. The use of olive oil and nuts not only increases monounsaturated fat and plant based n3-PUFA in the diet but also provides polyphenol antioxidants. Also, by comparing consumption of a similar background diet but supplemented with either olive oil (rich in monounsaturated fat) and nuts (high polyunsaturated fats like linoleate and α-linolenic acid) the authors could potentially separate the differential effects of MUFA and PUFA. However, this study did not control for total fat intake but biomarker analysis on urinary hydroxytyrosol (the main phenolic compound in extra virgin olive oil) and plasma α-linoleate (the main fatty acid from walnuts) indicated dietary compliance[15]. This study provides an excellent step towards transitioning a whole foods preventative approach to patients.

To further this concept in patients, long-term trials of a controlled Mediterranean diet with known concentrations of MUFA, n3 and n6-PUFA from pre-tested food items should be given to patients that are healthy, at risk for HF and to those diagnosed with HF. This will help examine whether this diet is effective at preventing cardiac disease even in healthy individuals and whether supplementation with MUFA + n3 and n6-PUFA has synergistic effects with current HF therapies.

Overall conclusion

In summary, high fat diets, in the setting of heart failure, do not adversely affect the heart. Increased resistance to Ca^{2+}-induced MPT imply that high MUFA diets may be the most beneficial in preserving mitochondrial function. On the other hand, decreased Ca^{2+} buffering capacity of mitochondria from animals fed a high n6-PUFA diet suggests that n6-PUFA may adversely affect mitochondrial function, although this did not translate to changes in contractile function.

Lastly, several substitutions in the fatty acid side chains of mitochondrial phospholipids were identified in this dissertation and indicate that a complex interplay exists among long chain fatty acids (Figure 4.2). We have previously described the known interactions between various fatty acids (indicated by the solid lines in Figure 4.2) in Chapter 1 and elsewhere[237]. Briefly, previous studies in our lab have shown that n3-PUFA, specifically DHA and, to a smaller extent, EPA, can delay MPT[59, 93], decreases inflammation[58, 238] and replaces arachidonic acid in membranes[93]. In the presented studies, we found that DHA also increases long chain saturated fatty acids in SSM but not IFM (indicated by light blue dashed lines, Figure 4.2). Early studies on saturated fat have

shown that palmitate has a greater effect on inducing lipotoxicity than stearate[199-203]. However, epidemiological studies have been conflicting and inconclusive. We have found that a high saturated fat diet increases arachidonic acid but also DHA. The increase of both pro and anti-inflammatory mediators may account for the mixed or neutral effects of saturated fat. Several cell cultures studies have suggested that oleate may prevent the adverse effects of palmitate[191-193, 228, 229]. In our HF study, we have found that high oleate also increases DHA while concomitantly decreasing linoleate in both healthy and sick animals which may partially account for the beneficial effects of monounsaturated fats. Lastly, n6-PUFA, specifically linoleate, can elongate to form arachidonic acid, which participates in inflammation, though we did not observe this in our study (represented by a thinner line in Figure 4.2). Linoleate is also required for the formation of L_4CL, an essential fatty acid in inner mitochondrial membranes[101] which may improve mitochondrial function[101, 103, 104]. We have found that supplementation with high linoleate increases saturated fat in membranes (specifically stearate) while decreasing oleate in healthy but not HF animals (Chapter 3). Thus, the notion of one specific fatty acid exerting effects in a linear fashion is largely outdated. Rather, new research should focus on combinations of fatty acids found in common foods, assisting the translation of this work into patients.

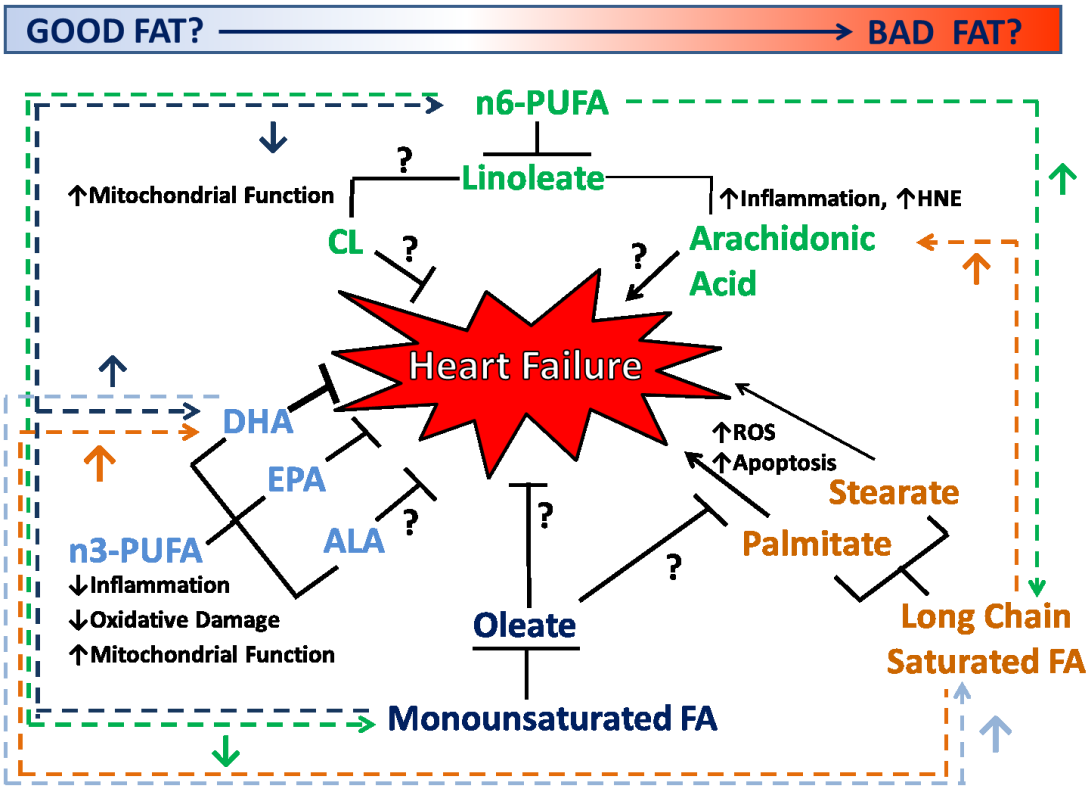

Figure 4.2. Summary of Increased Complexity of the Effects of Fatty Acids. Schematic depicting the complex interplay among common long chain fatty acids. Solid lines indicate current known and questionable interactions. Thinner lines (i.e. from linoleate to arachidonic acid and stearate to HF) indicate decreased flux. Dashed lines indicate relationships identified in this dissertation. ALA, α-linoleic acid; CL, cardiolipin.

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