EXPLORING DIFFERENCES IN INDIVIDUAL SUSCEPTIBILITY TO LIPOTOXICITY AND THE UNDERLYING MECHANISMS IN THE MITOCHONDRIA OF PRIMARY PREADIPOCYTES

By

Jared M. Shine

April, 2018

Director of Dissertation: Dr. Jacques Robidoux

Department of Pharmacology and Toxicology, Brody School of Medicine, East Carolina University

ABSTRACT

Prolonged exposure to fatty acids (FAs), at levels normally seen during fasting, can be cytotoxic in vulnerable cells including preadipocytes. Prior work from our laboratory has shown that FAs cause mitochondrial inner membrane (MIM) permeabilization due to the formation of the permeability transition pore (PTP) and ultimately cell death. Cyclophilin D (CypD) is well known, and the F_1F_0 ATP synthase has emerged, to be necessary for PTP. However, the role of the Bcl-2 (b-cell lymphoma 2) family members in the formation of the PTP is less clear. The

initial hypothesis was that the cytotoxicity of FAs in primary human preadipocytes correlates with the binding of CypD to, and the release of Bcl-x$_L$ (b-cell lymphoma extra-large) from, the F_1F_0 ATP synthase complex.

Our results showed a wide range of FA sensitivity in primary human preadipocytes as the number of cells undergoing MIM permeabilization and cell death was 2.3-fold and 3.4-fold higher, respectively, in groups labeled FA-sensitive (FAS) compared to the groups labeled FA-resistant (FAR) quintile. The purpose of selecting these groups was to examine differences in mitochondrial handling of elevated FAs and how this led to differences in preadipocyte cell death. Under thought of the adipose expandability hypothesis, we hypothesize that decreased preadipocyte number (indicative of our FAS group) during times of elevated FAs could play a role in adipose tissue dysfunction and the resulting metabolic abnormalities known as the metabolic syndrome. Furthermore, MIM permeabilization and cell death were prevented by incubating preadipocytes with mitochondrion-selective superoxide and lipid peroxide targeting antioxidants MitoTempo and MitoQ, as well as L-carnitine.

Co-immunoprecipitation showed CypD was already present in the F_0F_1 ATP synthase complex and not further incorporated following FA exposure. Cyclosporine A (CsA), which induced the dissociation of CypD from the F_0F_1 ATP synthase, prevented FA-induced cytotoxicity. We confirmed that Bcl-$_{xL}$ binds to the F_0F_1 ATP synthase and that FAs induced the release of Bcl-$_{xL}$. ABT-737, which inhibits Bcl-$_{xL}$ protein to protein interactions, exacerbated FA-induced ATP depletion and eliminated mitochondrial respiratory reserve capacity in the FAR preadipocytes only. However, this only marginally affected cell viability. Interestingly, FA exposure induced the expression of the carnitine acetyltransferase and sirtuin 3 (SIRT3) genes in

the FAR preadipocytes only. We propose that SIRT3 induction decreased acetylation of the F_1F_o ATP synthase.

Altogether the results of our first study illustrated that FAs cause a CypD-mediated MIM permeabilization that is responsible for cell death. FAs also lead to ATP depletion, but it is not the cause of cell death. Additionally, FAs increase F_1F_o ATP synthase acetylation, but it does not explain the full extent of cell death or FA sensitivity.

In our second study we furthered the aforementioned work by hypothesizing that improving preadipocytes FA sensitivity will correlate with improvements in metabolic health following 12 weeks of exercise training. As observed in our older cohort, the FA sensitivity of preadipocytes ranged several folds. Interestingly, FA sensitivity correlated with lower high-density lipoprotein-cholesterol (HDL-c) levels. Following the training program, the individuals that improved their FA sensitivity also decreased their low-density lipoprotein-cholesterol (LDL-c) levels. One goal of the exercise study was to assess whether fatty acid sensitivity testing prior to exercise could predict the effectiveness of exercise on metabolic health. Though no significance was found, there was a trend for pre-exercise fatty acid sensitivity to correlate with the changes in triglycerides and HDL-c after exercise.

The work done in this dissertation provides a deeper understanding of how chronically elevated levels of FA can cause mitochondrial dysfunction and preadipocyte death. The variance in this sensitivity to the toxic effects of FA need further elucidation yet could possibly be at play in determining metabolic health of obese individuals.

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A Dissertation

Presented To

The Faculty of the Department of Pharmacology and Toxicology

The Brody School of Medicine at East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Pharmacology and Toxicology

By

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Jared M. Shine

APPROVED BY:

DIRECTOR OF
DISSERTATION:_____
(Jacques Robidoux, PhD)

COMMITTEE MEMBER: _____
(Kymberly Gowdy, PhD)

COMMITTEE MEMBER: _____
(Ethan Anderson, PhD)

COMMITTEE MEMBER: _____
(Joe Houmard, PhD)

COMMITTEE MEMBER, CHAIR OF
THE DEPARTMENT
OF (Pharmacology and Toxicology): _____
(David Taylor, PhD)

DEAN OF THE

GRADUATE SCHOOL: _____

Paul J. Gemperline, PhD

Acknowledgements

Aim for the sky, but move slowly, enjoying every step along the way. It is all those little steps that make the journey complete. - Chanda Kochhar

First off, I have to thank God for the life and opportunities that I've been provided and blessed with, and for those watching over me above. I'd like to thank my mom Cheryl Shine for everything she has done. Being raised in a single parent household, I had no want or need that wasn't met; didn't miss out at all in life; and that's solely due to you working hard day in and day out to provide for your family. I would not be where I am today if it wasn't for the morals, work ethic, life lessons that you instilled in me, as well as the trust for me to move 600 miles away from home to pursue an education and a life.

I'm eternally gracious to have met my wife Sherri Moore Shine while in graduate school. I'd like to thank you for accompanying me through the ride of obtaining a PhD, whilst obtaining yours as well. Your love and support are immeasurable and can't be said enough how grateful I am to have had you by my side.

I wouldn't be obtaining my PhD. today without the lessons and guidance from Dr. Jacques Robidoux. You patiently taught me the correct way, not the easy way, to becoming a scientist and independent thinker. I can't thank you enough for everything you've done for me along the way and believing in me.

Finally, the microcosm of people that I have had the opportunity to develop relationships along the way, each in their own way owing in part to my arrival towards my dissertation and growth as a person; Barbara Davis, Jackie Masterson, Drs. Domico, Anderson, Taylor, and Ladin.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADP	Adenosine Diphosphate
ALDH	aldehyde dehydrogenase
ANOVA	Analysis of variance
ARE	antioxidant/anti-inflammatory response elements
ATP	adenosine triphosphate
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-$_{xL}$	B-cell lymphoma extra large
BF%	Body fat percentage
BMI	Body mass index
BSA	Bovine Serum Albumin
CABG	Coronary artery bypass graft
CRAT	Carnitine o-acetyltransferase
CDC	Center for Disease Control and Prevention
cDNA	Complementary DNA
CoA	Coenzyme A
CPT	Carnitine palmitoyltransferase
CsA	Cyclosporin A

CVD	Cardiovascular Disease
CypD	Cyclophilin D
DMEM	Dulbecco's modified eagle media
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
ETC	Electron transport chain
FA	Fatty Acid
FADH2	Dihydroflavin adenine dinucleotide
FAR	Fatty acid resistant
FAS	Fatty acid sensitive
FCCP	Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone
FFA	Free fatty acid
FOXO	Forkhead box protein 0
GCL	Glutamate cysteine ligase
GPx	Glutathione peroxidase
GSH	Glutathione
GSR	Glutathione reductase
GST	Glutathione s-transferase
H_2O_2	Hydrogen peroxide
HDL-c	High density lipoprotein-cholesterol
HFD	High fat diet

HIIT	High intensity interval training
HO-1	Heme oxygenase
HOMA-IR	Homeostatic model assessment of Insulin Resistance
HR_{max}	Maximum heart rate
IP	Immunoprecipitation
IR	Insulin Resistance
I/R	Ischemia/Reperfusion
KO	Knockout
LDL-c	Low density lipoprotein- cholesterol
MHO	Metabolically healthy obese
MIM	Mitochondrial inner membrane
MOM	Mitochondrial outer membrane
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
MUO	Metabolically Unhealthy obese
NAD^+ / NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAM	Nicotinamide
NMN	Nicotinamide mononucleotide
NOX4	NADPH Oxidase 4
NQO1	NADPH dehydrogenase quinone 1

NFE2L2	Nuclear factor erythroid 2 related factor 2
OCR	Oxygen consumption rate
OSCP	Oligomycin sensitivity conferring protein
PBS	Phosphate buffered saline
PPARγ	Peroxisome proliferator-activated receptor gamma
Prx	Peroxiredoxin
PTP	Permeability transition pore
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SDS- PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SIRT	NAD-dependent deacetylase sirtuin
SOD	Superoxide dismutase
T2D	Type 2 diabetes
TG	Triglyceride
TPx	Thioredoxin peroxidase
TRx	Thioredoxin reductase
VDAC	Voltage dependent anion channel

CHAPTER 1 INTRODUCTION

Obesity and the Metabolic Syndrome

The CDC estimates that over one-third of the U.S adult population (116 million) have obesity, which is defined as a grossly overweight state of BMI>30. Though obesity may not be considered by some to be a disease on its own, it is among a group of conditions known as the metabolic syndrome that are highly associated with diabetes, cardiovascular disease (CVD), and other diseases [1] [2] [3]. Other risk factors include hyperglycemia; high blood pressure; high cholesterol and triglyceride (TG) levels; elevated low-density lipoprotein-cholesterol (LDL-c); and decreased high density lipoprotein cholesterol (HDL-c) levels; and insulin resistance. Furthermore, the risk of having these risk factors is closely linked to obesity and the lack of physical activity. Metabolic syndrome is becoming more common with the rise in obesity rates and may overtake smoking as the leading risk factor for heart disease. Due to the close relationship of obesity, diabetes, and cardiovascular disease, and the alarming mortality rates associated with these diseases, determining the mechanisms at the origin of obesity are key in prevention of these diseases in the future.

Adipose Tissue

Adipose tissue is the primary organ of which energy, in the form of fat, is stored. Adipocytes make up the primary cell type in adipose tissue and are responsible for storing energy in times of excess and releasing energy in times of need. This ability to store fat without lipotoxicity that is exhibited in mostly every other organ makes adipose tissue highly unique and a vital aspect in whole body metabolism. Aside from fat storage, adipose tissue has other

functions including acting as a secretory organ, secreting hormones, proteins, and other factors that contribute to energy homeostasis, inflammation, angiogenesis, as well as immunity [4]. Other cell types in adipose tissue include adipocyte precursor cells, preadipocytes, fibroblasts, vascular endothelial cells, and a variety of immune cells. However, multitudes of evidence show that the normal functions of adipose tissue can become compromised with obesity, and this loss of function, aka adipocyte dysfunction, is the basis for much of pathophysiology corresponding with the metabolic syndrome [4].

Mechanisms of Obesity

Mechanisms of the onset of obesity are not fully known, but it is believed to be a result of a chronic positive energy imbalance, being more energy consumed than physiologically needed. Upon an increase in energy, adipose tissue must respond by increasing fat storage. Adipose tissue can increase fat storage by increasing fatty acid (FA) uptake from the serum, and esterifying FA to store them as TG in lipid droplets within the adipocyte. Chronically however, this increase in fat storage is primarily achieved by either increasing adipocyte size (hypertrophy), or adipocyte number (hyperplasia). Adipocyte hyperplasia occurs at early stages in adipose tissue development, whereas hypertrophy occurs prior to hyperplasia to meet increased fat storage needs [5] [6]. However adipose tissue seems to have a limit to its hypertrophic and hyperplastic abilities.

In obesity, hypertrophic adipocytes reach a point that the excess fat storage is detrimental to the adipose tissue as a whole, leading to adipocyte dysfunction. During this phase, the adipocytes grow at a rate that exceeds angiogenesis rates, or the creation of new blood vessels to supply oxygen and nutrients to the cells. With a lack of oxygen and nutrients, these cells can

become hypoxic, leading to the activation of hypoxia-inducible factor 1α, a transcription factor that serves as an oxygen sensor in the cell [7]. Hypoxia is a condition that is toxic to cells and leads to a pro-inflammatory response, which may impair insulin sensitivity [8]. This pro-inflammatory state of adipose tissue during excess energy imbalance is a hallmark of adipose tissue dysfunction and obesity, and is characterized by an increase in pro-inflammatory cytokine release and macrophage infiltration. Furthermore, adipose tissue dysfunction leads to excess FA release systemically to other organs such as the liver, pancreas, skeletal muscle, and the heart, which are not equipped to handle elevated FA loads. Indeed, recent evidence suggests that once adipocyte functional capacity has been exceeded, metabolic diseases occur [9] [10]. Interestingly, decreasing this elevated FA load in the serum improves insulin sensitivity in obese and T2D individuals [11] [12].

Evidence suggests that adipocytes may become dysfunctional due to an inability to increase fat mass, and this highly correlates, and possibly precedes, development of the metabolic syndrome. This phenomenon has given rise to the adipose tissue expandability hypothesis, which puts this dysfunction of adipogenic turnover as a key factor in linking energy imbalance and the metabolic syndrome, rather than obesity [13]. The hypothesis states that once adipose tissue loses its ability to store fat, then net lipid flux to non-adipose tissues increases and lipids begin to be deposited ectopically. This ectopic deposition in organs not meant to store lipids leads to insulin resistance and apoptosis, and further progression to the metabolic syndrome [10] [13] [14]. This dissertation is in agreement with the adipose tissue expandability hypothesis, and experiments within sought to answer questions as to how fat becomes lipotoxic to adipose tissue, specifically in the adipocyte precursor cells, preadipocytes.

Metabolically Healthy Obese vs. Unhealthy Obesity

If there was a simple correlation between insulin resistance and adipose tissue mass, then all individuals with similar amounts of adipose tissue would be diabetic, which is not true. Despite the significant correlations between obesity and metabolic diseases, there exists a subpopulation of obese individuals that do not display metabolic abnormalities, "metabolically healthy obese" individuals (MHO) [15]. This subset of the obese population is still unclear but is estimated to occur in about 15% of the total obese population. As mentioned before, metabolically unhealthy obese (MUO) individuals have a reduced number of adipocytes that are larger in size and exist in a chronic pro-inflammatory state including a M1 polarized macrophage phenotype [15] [16] [17]. On the other hand, MHO individuals display a greater number of adipocytes that are smaller in size, and display an anti-inflammatory profile, with more M2 polarized macrophages [15] [18] [19]. Additionally, these MHO individuals have a greater adipogenic potential, which is the ability of adipose tissue to differentiate adipocyte precursor cells, preadipocytes, into mature adult adipocytes. This phenomenon is in support of the adipose tissue expandability hypothesis and provides rationale to further investigate obesity-induced changes within the adipogenic environment. Further elucidating the mechanisms that determine MHO vs. MUO may provide an effective target in treating obesity or preventing the progression towards metabolic diseases.

Preadipocytes Roles in Disease

It used to be common thought that adipose tissue is resistant to the toxic effects of FAs, however recent literature suggests that adipose tissue is one of the first tissues to experience lipotoxicity in obese models [20] [21]. In order for adipose tissue to maintain its storage function

and capacity, hypertrophic adipocytes during a chronic energy imbalance phase must be replaced by new, healthy adipocytes. This is achieved by the differentiation of preadipocytes into smaller, healthy adipocytes. However with adipose dysfunction in the obese state, preadipocyte turnover has been found to be decreased, halting the ability of adipose tissue to perform their FA storage function [21] [14] [22]. How this decrease in turnover comes about is still not known.

Preadipocyte differentiation is under control of a host of transcription factors such as ccaat-enhancer-binding proteins and peroxisome proliferator-activated receptor γ (PPARγ). Studies have suggested that in the obese state, PPARγ expression is reduced, as well as preadipocyte number, which may be the result of increased FA exposure [14] [23]. PPARγ deficiency is also correlated with severe insulin resistance, shown by multiple genetic and rodent models [24],

As mentioned before, with chronic positive energy imbalance and the development of adipocyte dysfunction, there's a resulting excess pool of FAs. FAs are normally bound by two fates in adipose tissue; 1) esterification and storage; or 2) used by the mitochondria for energy production via β-oxidation. Preadipocytes have a low FA esterification rate, causing a reductive pressure upon nutrient overload. This pressure to handle the increased FA load is represented as an increase in NADH and FADH2 greater than the levels needed to regenerate ATP [25] [26]. As a result, mitochondrial ROS production is increased, and chronically elevated ROS levels may lead to mitochondrial dysfunction, a hallmark of obesity. Previous work done in immortalized preadipocytes by Rogers *et al*, has shown that upon FA exposure, representative of nutrient overload, a sequence of events occurs within the mitochondria which leads to it becoming dysfunctional [27]. Initially FA cause an increase in ROS formation. This ROS may "prime" the preadipocytes for a sequence of events later on that ultimately leads to a cellular energetic crisis

and cell death. Among these events are nicotinamide adenine dinucleotide (NAD^+) depletion,

increase in mitochondrial inner membrane (MIM) permeability, and ATP depletion [27]. Rogers

also found for this FA-toxicity to be dependent on formation of the permeability transition pore

(PTP). Work in this dissertation investigated the sequence of these events leading to FA-induced

preadipocyte death, as well as the mechanism and structure of permeability transition pore (PTP)

in preadipocytes.

Mitochondrial Permeability Transition Pore

Dysfunctional mitochondria are at the center of the pathophysiology for a number of

diseases including; neurodegenerative diseases such as Alzheimer's, Huntington's, and

Parkinson's disease; multiple sclerosis; Friederich's ataxia; fibromyalgia; autistic spectrum

disorder; cancer; as well as CVD and diabetes. Though exact mechanisms vary, mitochondrial

dysfunction is characterized by a loss in the maintenance of the electrochemical gradient of the

MIM needed for ATP regeneration; alterations of the functions of the electron transport chain

(ETC); or a decrease in the transport of vital metabolites into the mitochondria. Among the

possible pitfalls that may cause mitochondrial dysfunction, the PTP is believed to be at the center

of a number of the aforementioned diseases [28] [29] [30]. PTP is described as a large

conductance pore that causes the collapse of the chemiosmotic gradient across the MIM,

effectively halting ATP regeneration, and through prolonged opening, promoting cell death.

Though its role in disease has been extensively studied in the past decade, the components that

make up the PTP are still up for debate.

PTP was first discovered in 1975 by Haworth and Hunter, who found for Ca^{2+} addition to

cause an increase in mitochondrial permeability, an uncoupling from oxidative phosphorylation,

and mitochondrial swelling [31]. Since then it has been found for PTP to be modulated by a number of different factors; pore opening is increased by Ca^{2+} addition, inorganic phosphate (Pi), reactive oxygen species generation (ROS), and mitochondrial depolarization; and pore opening is decreased by divalent cations such as Mg^{2+}, increase in $\Delta\Psi$, and alterations of pH above/below 7.3 [31] [32]. However, modulation of PTP seems to be clearer than its actual composition.

Initial studies suggested for the PTP to be a multi-component pore spanning the MIM and mitochondrial outer membrane (MOM); and involving adenine nucleotide translocase (ANT), and the voltage-dependent anion channel (VDAC) [33] [34] [35] [36]. However genetic studies were able to disprove both of these potential PTP components, as pore opening still occurred despite their genetic deletion or knockdown [37] [38]. Recently the consensus appears to include the F_1F_0 ATP synthase as being a central component of PTP, despite its characterization and suggestion to compose the core back in 1975 [31]. The F_1F_0 ATP synthase, otherwise known as Complex V of the ETC, is the enzyme responsible for creating energy in the cell in the form of ATP. Overall, the enzyme utilizes ADP, P_i, and H^+, to create ATP and H_2O, which is achieved by utilizing the electrochemical gradient created by the ETC.

The F_1F_0 ATP synthase is a large multi-component complex embedded in the MIM. It consists of two main subunits, F_0 and F_1, along with oligomycin sensitivity conferring protein (OSCP), which are connected by a central stalk and work together in a rotational engine mechanism to regenerate ATP. The F_1F_0 ATP synthase is commonly isolated as a functional monomer; however, electron microscopy studies have revealed that it exists physiologically as dimers, forming long rows of oligomers [39]. The F_0 portion forms a transmembrane ring composed of a, b, and c subunits, and pumps protons back into the mitochondrial matrix. Proton

binding causes a rotation in the F_0 subunit, which is coupled to F_1 rotation by the γ stalk. The F_1 portion is composed of alpha, beta, gamma, delta, and epsilon subunits which undergo conformational changes (closed, open states) to bind ADP and Pi, leading to ATP regeneration and release. This rotational "coupling" between the subunits is vital for proper mitochondrial functioning and ATP regeneration and is disrupted by elevated Ca^{2+} levels and ROS, leading to PTP formation.

Cyclophilin D role in PTP

One of the only universally accepted components of the PTP is cyclophilin D (CypD). Consensus includes CypD as being a major component of PTP as numerous genetic and functional studies have detailed its role [40] [41] [42] [43]. CypD is a mitochondrial matrix protein and a member of the peptidylprolyl isomerase (PPIase) class of enzymes that regulate PTP and cell death [40]. It has been shown to bind to the lateral stalk, as well as OSCP of the F_1F_0ATP synthase regulating F_1F_0 ATP synthase activity, causing Ca^{2+} sensitization, decreased ATP synthesis, and PTP formation [41] [44] [45]. Cellular stressors, such as oxidative stress, cause CypD translocation from the mitochondrial matrix to the MIM, allowing it to bind to the F_1F_0 ATP synthase [46] [47].

Genetic studies have focused on the role of CypD in PTP and disease. Stemming from these studies is the founding of cyclosporine A (CsA), a potent immunosuppressive agent, as being a CypD inhibitor and effectively inhibiting PTP [43]. Used acutely, CsA was found to be therapeutically beneficial in cases of I/R (ischemia/reperfusion) injury, and myocardial infarction [48]. In the context of obesity, CypD -/- mice exhibit an increase of white adipose tissue and develop early onset obesity [49]. Though CsA use as an immunosuppressive agent may limit its

8

long term use in cases of obesity and the metabolic syndrome, promising new inhibitors of CypD are being developed in hopes of bypassing these additional effects of CsA [50] [51].

Consequences of PTP opening vary on the amount of time in the open state, as well as the number of these "pores" in the mitochondrial population at one given time. Normal PTP opening is observed physiologically and is described to occur in a transient manner and play a role in cellular health by regulating ionic homeostasis, as well as small solute exchange between the cytosol and mitochondria [30] [52]. However, if this opening persists, then a sequence of events can occur ultimately leading to cell death. Prolonged PTP opening leads to an increase in ionic and small solute flux into the mitochondria, ultimately causing mitochondrial swelling [53]. This increase in ionic flux also dissipates the proton gradient necessary for ATP regeneration, causing a collapse in $\Delta\Psi$ of the MIM, decreased ATP regeneration, and increase in ROS production. Furthermore, mitochondrial swelling can rupture the outer mitochondrial membrane causing cytochrome C release from the mitochondria, which is a vital component of the ETC, and subsequent apoptotic pathway activation [54] [55].

B-cell Lymphoma Extra-Large (Bcl-$_{xL}$)

Another possible component, or at the least, modulator of PTP has been suggested to be B-cell lymphoma extra-large (Bcl-$_{xL}$). Bcl-$_{xL}$ is a transmembrane mitochondrial molecule that is among the Bcl-2-related family of anti-apoptotic proteins. Bcl-$_{xL}$ is long known for its anti-apoptotic effects thought to be due to MOM action on inhibiting the pro-apoptotic Bak/Bax [56] [57]. Upon apoptotic stimuli, Bax translocates to the mitochondria and promotes Cytochrome C release, caspase activation, and apoptosis induction [57]. Bcl-$_{xL}$ has been thought to inhibit Bax-

mediated apoptosis through direct binding and prevention of Bax translocation to the mitochondria.

Besides Bax inhibitory actions, Bcl-$_{xL}$ more recently has been suggested to modulate energy homeostasis. These actions include stabilizing MIM potential, and increasing metabolic efficiency, again conferring cellular protection [58] [59]. Recent evidence suggests these metabolic and protective actions may be due to Bcl-$_{xL}$ binding to the β-subunit of the F_1F_0 ATP synthase [59] [60]. However, the interaction between Bcl-$_{xL}$ and positive chronic energy imbalance, obesity, and adipocyte dysfunction have not been studied. Furthermore, most studies to date on PTP structure and composition have focused on remodeling/reconstituting model mitochondrial membranes, and few have studied the structure in vitro or in human models.

Mitochondrial Acetyl-Coenzyme A Levels and Protein Acetylation

As mentioned previously, post-translational modifications such as acetylation play an important role in oxidative stress balance, as well as bioenergetics, mitochondrial health, and cellular survival. Protein acetylation, a reversible modification, is affected by changes in nutrient availability and cellular energy status. Acetylation in the mitochondria can occur non-enzymatically in conditions of high acetyl-coenzyme A levels [61], which is a molecule that participates in many biochemical reactions including protein, carbohydrate, and lipid metabolism. Being at the center of nutrient metabolism, acetylation thus is sensitive to metabolic changes. Acetylation is increased during times of fasting, and paradoxically, during times of high fat diet HFD [62].

FAs enter the mitochondria through the L-carnitine shuttle (Figure 2). L-carnitine is an essential nutrient that serves as a substrate for a family of acetyltransferases, allowing for

membrane permeable transferring of fatty acyl-CoAs (FA-CoA). The shuttle consists of three enzymes, carnitine palmitoyltransferase 1 (CPT1), carnitine palmitoyltransferase 2 (CPT2), carnitine 0-acetyltransferase (CRAT), as well as the small soluble molecule central to the shuttle, L-carnitine. Before FAs can be transported into the mitochondria they are esterified with CoA to form FA-CoA. This molecule is then transported into the mitochondria via CPT1, exchanging L-carnitine for the attached CoA, and into the matrix via CPT2 which exchanges CoA for L-carnitine to produce FA-CoA once again, which is then ready to enter the β-oxidation process, with the free L-carnitine ready to transfer out to the cytosol and resupply the cytoplasmic pool of L-carnitine for further shuttling. Furthermore, β-oxidation results in acetyl CoA resupply within the mitochondria which can be utilized in the tricarboxylic acid (TCA) cycle for energy production, or cause acetylation of proteins. Additionally, acetyl-CoA can also be transported back out into the cytosol via CRAT shuttling with L-carnitine.

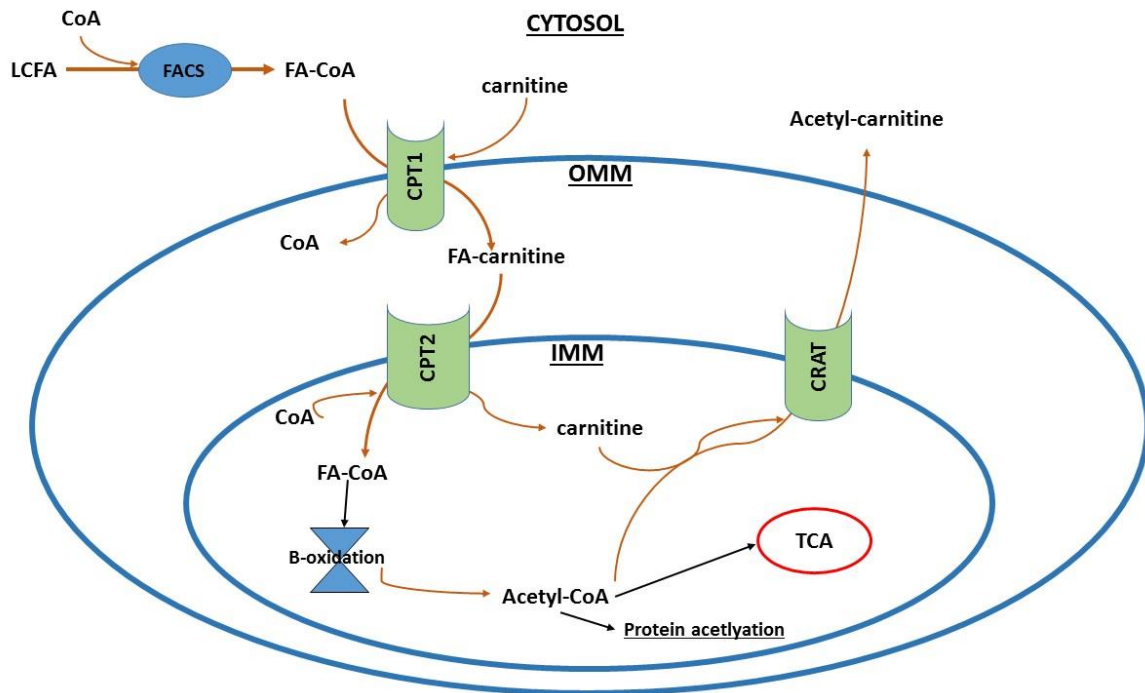**Figure 1.1. Fatty acid and acetyl CoA transport into the mitochondria via L-carnitine shuttle**. FAs must be transported into the mitochondria via CPT1/2 for energy production. During β-oxidation, acetyl CoAs are generated which can further lead to energy production via the TCA cycle, cause non-enzymatic protein acetylation, or can be transported back out into the cytosol with L-carnitine via CrAT.

Evidence suggests perturbations in FA and acetyl CoA shuttling in/out of the mitochondria have an impact on mitochondrial, cellular, and organ health. CRAT, which converts acetyl CoA to the membrane permeant acetylcarnitine, has been shown to be a key regulator of substrate switching and glucose tolerance. In obesity-associated FA accumulation, there is a decrease in free L-carnitine and CRAT activity which lead to incomplete β-oxidation, and supplementing human subjects with L-carnitine enhances acetyl-carnitine urinary excretion, whole body glucose tolerance, and metabolic flexibility [63] [64]. Furthermore, CPT1 and CRAT expression has also been found to be decreased in adipose tissue in humans with obesity and T2D which correlated with lipid accumulation and decreased preadipocyte differentiation [65] [66]. However there exists a system to balance acetylation status of the mitochondria.

The acetylation status of proteins is regulated by NAD^+-dependent protein deacetylators called sirtuins. There are 7 isoforms of SIRTs, however SIRT1 (cytosolic/nuclear) and SIRT3 (mitochondrial) seem to be the most important cellular deacetylators. SIRT activity is dependent on NAD^+ levels, suggesting their activity is directly linked to the energy status of the cell, either via NAD^+/NADH ratio, or absolute levels of NAD^+, NADH, or the intermediate, NAM (nicotinamide) [67]. Preliminary data in our lab have shown for 1000μM FA to cause a decrease in NAD^+ levels, correlating with PTP and cell death. (Figure 1.2)

Interestingly, studies have found that HFD causes decreased SIRT3 expression and activity, leading to mitochondrial hyperacetylation and obesity [62]. Studies utilizing SIRT3 KO mice demonstrate in parallel to increased mitochondrial acetylation, glucose intolerance and increased insulin resistance (IR) [62]. Furthermore, SIRT3 activity has also been shown to play a role in energy metabolism due its deacetylating actions on several mitochondrial proteins involved in energy pathways [68]. Since HFD/obesity both modulate acetylation and sirtuin

expression and activity, manipulating this relationship may provide an attractive therapeutic

target for many mitochondrial-related diseases.
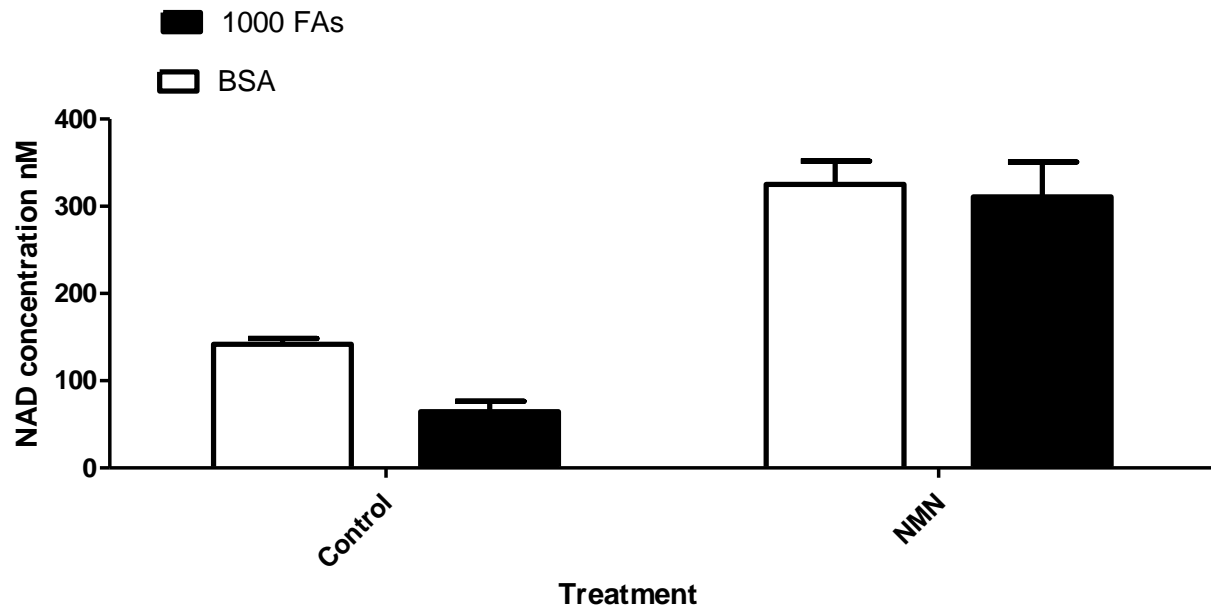
Figure 1.2. **Fatty acids cause a decrease in NAD$^+$ levels**. Immortalized preadipocytes were treated with 1000μM FA and NAD$^+$ levels were assessed after 24 hours. NMN co-treatment with 1000μM FA replenishes NAD$^+$ pool.

Mitochondrial Oxidative Stress

One of the hallmarks of obesity is increased oxidative stress in adipose tissue, which is an early instigator of the metabolic syndrome [69]. Oxidative stress impairs glucose uptake in adipose tissue and decreases insulin secretion from the pancreas [70] [71]. Furthermore, HFD and hyperglycemia increase ROS production in adipose in obese individuals [69]. At a cellular level, ROS reduces oxygen consumption in adipocytes, and blocks FA oxidation, exacerbating already increased circulating FA levels [72]. The importance of ROS in obesity and the metabolic syndrome is highlighted by the finding that IR is attenuated through the use of selective mitochondrial antioxidants [73].

The mitochondria is one of the main sources of cellular ROS production. There exists a delicate balance between ROS production and antioxidant systems within the mitochondria, but high concentrations of ROS may cause lipid peroxidation, carbonylation of proteins, formation of the PTP, and mitochondrial DNA (mtDNA) oxidative damage [74]. During oxidative phosphorylation, electrons are transferred down the ETC to oxygen, the final electron acceptor. As mentioned before, it is this passing of electrons down the ETC that creates the proton gradient necessary for ATP regeneration. ROS is generated at several different sites along the ETC, but the main sites seem to be Complex I and III, where electron passing may leak to oxygen (O^2) to form a superoxide anion (O^{2-}), which is the initial form of ROS in the mitochondria [74] (Schematic image in Figure 1). Superoxide is relatively unreactive due to its negative charge and poor permeability; however, it can rapidly react with nitric oxide (NO) to form the highly reactive oxidant peroxynitrite ($ONOO^-$). Most superoxide is converted to hydrogen peroxide (H_2O_2) through dismutation reactions via superoxide dismutase (SOD), Hydrogen peroxide is a stable, membrane permeable free radical that plays a role in several different cellular processes

including acting as an oxidant or a signaling molecule. In fact, H_2O_2 is described as playing a vital role in cellular processes and health [75] [76] [77]. Hydrogen peroxide can be neutralized and converted into water (H_2O) by several different antioxidants including catalase, glutathione peroxidase (GPx), and thioredoxin reductase (TPx). If not detoxified, it can further produce the highly reactive hydroxyl radical. Hydroxyl radicals are powerful oxidants that can indiscriminately damage several types of macromolecules including lipids causing lipid peroxidation.

As mentioned before, the mitochondria contain a well-balanced antioxidant defense system in order to control ROS production (Figure 1.3). Antioxidant enzymes include SOD, catalase, GPx, and TPx. The thioredoxin system is a vital for the protection against peroxides. The system is composed of TPx, thioredoxin, and thioredoxin reductase, which keeps thioredoxin reduced in order to act as an electron donor for TPx neutralization of H_2O_2. HFD has been shown to reduce TPx and play a role in IR [78].

SOD2 is a highly abundant mitochondrial antioxidant that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. Previous studies have shown differential effects of FAs on SOD2 levels and activity, as well as its expression/activity in T2D and obesity. In one of those studies, FA induced oxidative stress leading to SOD2 induction, which is suggested to protect against the toxic effects of FAs [79]. However, FAs, which are elevated in T2D, also increased lipid peroxidation, which was shown to lower SOD2 activity and protein levels, and ultimately lead to a pro-oxidant state [79] [80].

Another key component of mitochondrial antioxidant defense is the glutathione system, which essentially converts hydrogen peroxide into water. The system includes GPx, glutathione (GSH), which acts as the electron donor, and glutathione reductase (GSR) which reduces

oxidized GSH to be used again. Numerous studies have shown for greater levels of GPX4 to be cytoprotective in cases of elevated FA, as well as in obesity and IR [81] [82], however some studies include both GPX1 and GPX4, or total GPX measurements which does not discern between the different isozymes. This is important because there has been an opposite trend for reduced GPX1 activity and expression to be cytoprotective as well as insulin sensitizing [76] [83] [84]. GPX4 is composed of hydrophobic amino acids, allowing it to exist and interact with complex lipids in membrane, and is believed to be the primary defense against lipid peroxidation [85] [86], which may contribute to its FA buffering benefits. The study by Katunga *et al,* showed for GPX4 haploinsufficiency to cause increased lipid peroxidation in mice on a high fat/high sucrose diet, and for patients with diabetes to exhibit reduced GPX4 levels compared to insulin sensitive counterparts [87]. On the other hand, GPX1 can be said to be a general hydrogen peroxide scavenger and may decrease H_2O_2 levels to a level detrimental to the cell [75] [76]. In fact, it has been shown by multiple studies for increased GSH levels to actually reduce insulin sensitivity [81] [88].
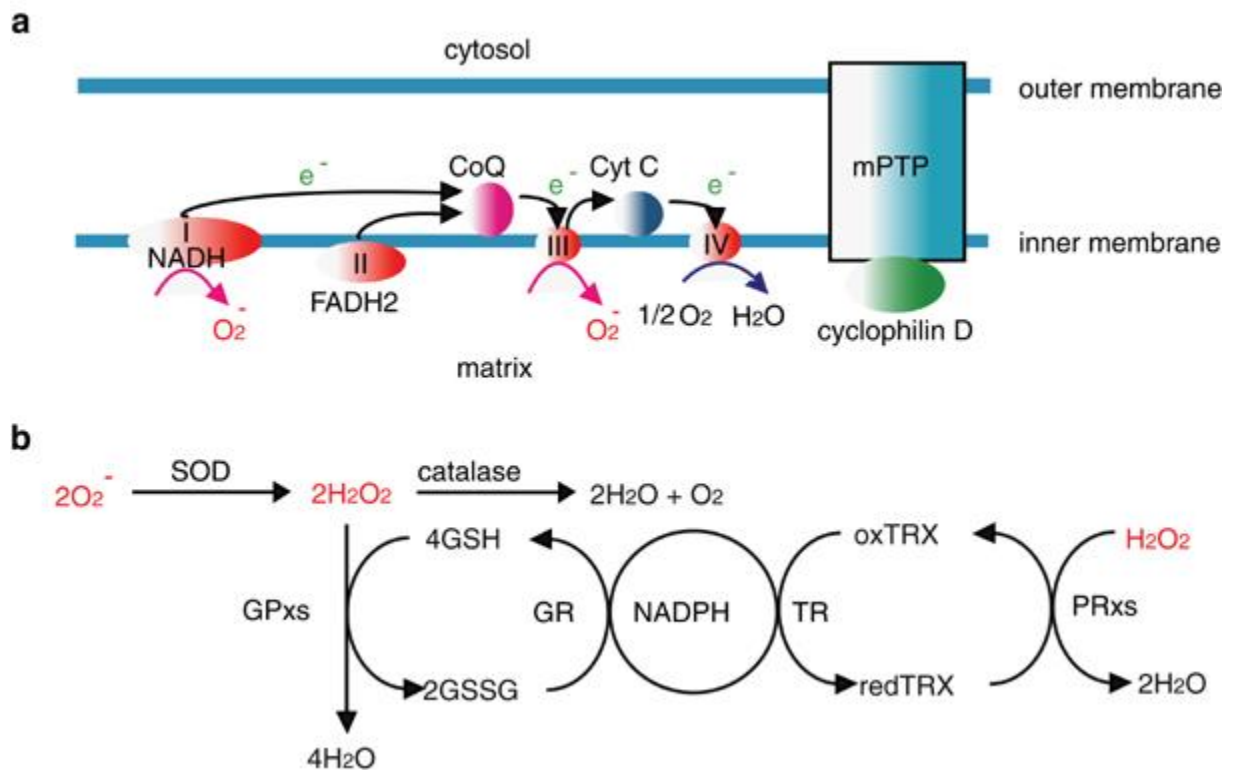
Figure 1.3: **Generation of ROS in the mitochondria and antioxidant elimination of ROS** [89]**.** *(A)* The electron transport chain consists of 5 complexes of which electrons are transferred to molecular oxygen. Electrons may leak during transport down the ETC, leading to superoxide production. *(B)* Mitochondrial antioxidant system elimination of superoxide and self-maintenance.

Antioxidant Response Regulation

As mentioned earlier, ROS serves as important signaling mediator at physiologic and pathological levels. Cellular antioxidant response is regulated by several different transcription factors including nuclear factor erythroid 2-like 2 (NFE2L2) and the forkhead box, class O (FOXO) family of factors. Transcription factors of the FOXO family allow for the production of antioxidant proteins in different cellular compartments including the mitochondria. However, FOXOs are also regulated and affected by ROS and other stressors such as fasting which can modulate FOXO activity [90]. Some of the FOXO-targeted genes include SOD2, catalase, Prx3/Prx5, Trx2, and TrxR2, which were mentioned earlier to play a vital part in mitochondrial ROS protection. The activity of FOXOs is regulated by several means of post-translational modifications, including acetylation [91]. Under conditions of oxidative stress, FOXOs can become acetylated which modulates its promoter selectivity. Acetylated FOXOs can interact with SIRT1, which has been shown to increase antioxidant response abilities of FOXOs [91] [92], yet decrease another one of FOXOs abilities, cell death. This modulation of FOXO activity has been suggested to allow cells to survive during elevated levels of oxidative stress. The importance of FOXOs has been further supported by studies showing its role in SIRT1-dependent β-cell protection and insulin synthesis/secretion preservation [93], as well as oxidative stress protection in heart failure models [94]. In relation to adipose tissue, SIRT-dependent deacetylation of FOXOs, mainly FOXO1 in adipose tissue, has been shown to display an inhibitory effect on preadipocyte differentiation, which would ultimately affect adipose tissue mass and health as mentioned previously [95]. Furthermore, hyperactivation of FOXOs in obesity and diabetes has been associated with hyperglycemia, hyperlipidemia, and insulin

resistance [96]. It stands to reason that the interaction between FOXOs and oxidative stress play an important role in the development of adipocyte dysfunction, obesity, and metabolic syndrome.

Normally Nrf-2 is sequestered in the cytoplasm until increases in oxidative stress trigger its translocation to the nucleus. Here it binds and activates the ARE (antioxidant response element) pathway, inducing transcription of antioxidant genes including heme oxygenase-1 (HO-1), thioredoxin reductase, PRDX1, GPX, glutathione-S-transferase (GST), glutathione-S-reductase (GSR), glutamate-cysteine ligase (GCL) NAD(P)H: quinone oxidoreductase-1 (NQO1) [97]. In relation to adipose tissue, there's conflicting evidence as to NFE2l2's effects on adipogenesis being inhibitory or stimulatory [98] [99], however these studies utilized different in vitro models which may owe to opposing observations. IN HFD-mouse models, NFE2l2 does seem to play an inhibitory role on adipogenesis, as NFE2l2 KO mice displayed less fat mass, and smaller adipocytes that were also greater in number, suggesting a beneficial effect of adipogenesis here in the absence of NFE2l2 [98] [100]. However, NFE2l2 has also shown a vital role in maintaining mitochondrial health as it has been shown to enhance mitochondrial biogenesis [101]. More studies are needed to elucidate the dual effects of NFE2l2 on mitochondrial health and adipogenesis in the context of obesity and the metabolic syndrome.

Exercise Effects on Obesity, Metabolic Health, and the Mitochondria

Obese, metabolically unhealthy individuals display a loss of metabolic fitness which can manifest as reduced HDL-c, raised triglycerides, cholesterol, blood pressure, fasting plasma glucose, and a large waist circumference [2] [1]. Furthermore, loss of metabolic fitness has been found to be a strong predictor for cardiovascular disease (CVD), especially low HDL-c concentrations and elevated LDL-c concentrations [102] [103] [104]. One of the first approaches

to managing obesity, CVD and many diseases of the metabolic syndrome is diet and exercise, the latter of which has been shown in a number of studies to decrease weight and improve metabolic health [102] [105] [106] [107]. Some of these improvements include preventing and treating the aforementioned cardiovascular risk factors [108] [109] [110] [111] [112].

The exact mechanisms owing to the beneficial effects of exercise are still being elucidated, however much evidence points to improvements in mitochondrial health. Some of these exercise-related benefits include increased mitochondrial biogenesis, decreased PTP susceptibility, and an increase in Bcl-2 family of proteins, ultimately attenuating cell death [113] [114] [115] [116].

Conclusion

Despite the numerous studies linking mitochondrial dysfunction, adipocyte dysfunction, obesity, and the metabolic syndrome, few have examined the role of FA, which are elevated in adipocyte dysfunction, on preadipocytes. Furthermore, the lipotoxic mechanisms by which FAs cause mitochondrial dysfunction may be a linking event to the aforementioned events and need to be elucidated. This dissertation will seek to address this deficiency in knowledge by utilizing human primary preadipocytes and examining their mitochondrial response to elevated physiologic FA exposure. I aim to further elucidate the mitochondrial mechanism of FA-induced toxicity, and further the understanding of the structure of the PTP. These findings may have over-reaching impacts on not only adipose health and the development of metabolic syndrome, but also on other energy imbalance-related diseases.

CHAPTER TWO:

Individuals Vary in their Susceptibility to Fatty Acid Toxicity Mediated by ROS-Sensitive, and Cyclophilin D-Dependent Formation of the Mitochondrial Permeability Transition Pore

Jared Shine[12], Barbara Davis[1], Alan Kypson[3], Ethan Anderson[12], Jacques Robidoux[124]

[1]Department of Pharmacology and Toxicology, [2]The East Carolina Diabetes and Obesity Institute, [3]Department of Cardiovascular Sciences and East Carolina Heart Institute, East Carolina University, Greenville, North Carolina

ABSTRACT

Mitochondrial inner membrane (MIM) permeability transition is critical for the loss of bioenergetic efficiency in preadipocytes exposed to elevated fatty acid (FA) levels. Recently, it has been shown that the permeability transition pore (PTP) forms from within the F_1F_0ATP synthase complex under conditions of oxidative stress. It has been suggested that this transition involves F_1F_0ATP synthase acetylation, cyclophilin D (CypD) binding, and the release of the prosurvival Bcl-2 family members Bcl-2 and Bcl-$_{XL}$. We report that primary human preadipocytes display a range of sensitivity to these events induced by FA. MIM permeabilization and ATP-depletion were prevented similarly by mitochondrion-selective antioxidants, as well as cyclosporin A, which uncouples CypD from the PTP, in both FA-resistant (FAR) and FA-sensitive (FAS) preadipocytes. Although ABT-737, which prevents Bcl-2 and Bcl-x_L to interact with other proteins, brought down ATP levels of the FAR preadipocytes to the levels of FAS preadipocytes, it only marginally affected MIM permeabilization and cell viability. Mitochondrial acetyl-CoA accumulation was higher in FA-exposed FAS preadipocytes. Finally, sufficient F_1F_0ATP synthase acetylation leads to similar MIM permeabilization in the FAS cells and can only be reached in the FAR preadipocytes in the presence of mildronate, a gamma-butyrobetaine hydroxylase and carnitine acetyltransferase (CRAT) inhibitor. However,

totally preventing F_1F_0ATP synthase acetylation with L-carnitine was insufficient in totally preventing MIM permeabilization and cell death. Finally, FA exposure led to an increase in the expression of CRAT and sirtuin 3 (SIRT3). Altogether, these results presented suggest that FA sensitivity is partly regulated at the level of steady state levels of acetyl-CoA or deacetylation of the F_1F_0ATP synthase complex.

Introduction

Adipose tissues are endocrine and metabolic organs essential to systemic metabolic health, and considerable evidence suggests that adiposopathy, or moribund adipose tissue, is central to the pathogenesis of type 2 diabetes [117] [118] [119] [120]. In that respect, failure of adipose tissue to accommodate for chronic caloric excess through preadipocyte proliferation and/or differentiation has been hypothesized to have negative systemic metabolic consequences [120] [121] [13]. In support of this hypothesis are many studies showing evidence of decreased adipogenesis in patients that are insulin resistant [27] [122] [123]. Also supporting this hypothesis are studies showing decreased preadipocyte number in adipose tissue of subjects with obesity, which is further decreased in individuals that are prediabetics or diabetics [124] [125]. Notwithstanding that the underlying mechanisms for a correlation between reduced ability of preadipocytes to differentiate and a loss of metabolic health are still not fully understood, to understand the consequences of positive energy imbalances in preadipocytes is of importance.

The range of fatty acid (FA) concentration to which preadipocytes are exposed daily is somewhat impossible to determine. However venous plasma nonesterified FA concentrations raise from nadir to more than 1000μM during the post-absorptive to postprandial transition and is maintained at least that high during fasting in subjects with or without type 2 diabetes [126]. Therefore, it is reasonable to speculate similar or higher adipose tissue interstitial FA levels during postprandial and fasting conditions. Palmitate-induced injury in human preadipocytes has been reported [127]. More recently, a study using prolonged exposure to a physiologically relevant mixture of FAs identified mitochondrial inner membrane (MIM) permeabilization and ATP depletion as critical steps to the cytotoxic effects of FAs [27].

MIM permeability transition is defined as an increase in MIM permeability to ions and solutes of small molecular weight that dissipate the MIM potential and slowdown ATP

25

regeneration. MIM permeability transition is due to the formation of the mitochondrial permeability transition pore (PTP), which can be triggered by FAs, thiol oxidants, or elevated mitochondrial matrix calcium [31] [128]. One of the best characterized components of the PTP is cyclophilin D (CypD), which is involved in oxidative stress-triggered and calcium dependent MIM permeabilization [47] [41]. More recently the F_1F_0ATP synthase has emerged as the core component of a calcium-activated channel indistinguishable from the PTP [45] [129]. Interestingly, CypD was previously found to interact with the oligomycin sensitivity conferral protein (OSCP) subunit of F_1F_0ATP synthase and to decrease its ATP synthetic efficiency [44]. This interaction, which can be disrupted with cyclosporine A (CsA) or the OSCP-interacting drug Bz-423, was found to be responsible for F_1F_0ATP synthase transition to its PTP mode [45].

Acetylation of CypD at lysine 166 has been shown to regulate CypD-dependent PTP occurrence, and mitochondrial dysfunction [130] [131]. Reciprocally, acetylation of OSCP lysine 70 confers CypD sensitivity to the PTP [132]. Finally, another F_1F_0ATP synthase interacting protein is b-cell lymphoma extra-large (Bcl-$_{xL}$) [59]. Bcl-$_{xL}$ binds the β subunit of the F_1F_0ATP synthase which confers ATP synthetic efficiency and conversely reduces CypD- and calcium-dependent PTP formation [59] [129].

The mechanisms responsible for the decreased number of preadipocytes and/or reduced adipogenic potential in adipose tissue of patients with systemic insulin-resistance are unknown. In this study we investigated the role of CypD, Bcl-$_{xL}$, and mitochondrial acetylation levels in FA-induced loss in ATP synthetic efficiency and MIM permeabilization.

Materials and Methods

CABG Patient Enrollment

Approval for this study was granted by the Institutional Review Board of the Brody School of Medicine at East Carolina (UMCIRB 09-0669). A total of 16 patients undergoing coronary artery bypass graft between November 2014 and January 2016 were enrolled.

Primary Preadipocyte Isolation

Following sternotomy, a small sample of subcutaneous adipose tissue was collected and immediately minced in Dulbecco's modified Eagle's medium (DMEM):F12 (1:1; Hyclone, Thermo Scientific, Logan, UT) containing 10mM glucose, 1mM pyruvate, 33µM Biotin, 17µM D-Pantothenate (all from Sigma Aldrich St. Louis, MO). Thereafter this medium will be referred to as the base medium. Upon arrival in the laboratory, the tissue was transferred to a tube containing the base medium completed with 1% fatty acid (FA)-free bovine serum albumin (BSA; Gemini Bio-Products) and 1 mg/mL of type 1 collagenase (Worthington, Lakewood, NJ). After being further minced the tissue was digested for 60-75 min at 37˚C and agitated at 120 rpm. The larger debris were trapped via filtration through a 420 µm filter. This filtrate was centrifuged at 300g for 10 min, the mature fat cells aspirated, and the pellet suspended in 10mL of an erythrocyte lysis buffer composed of 154mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM EDTA, which eliminated most erythrocytes within 3 to 6 min. This incubation was stopped by adding 40mL of base cell culture medium, filtered through a 100µm filter and centrifuged at 300g for 10 min. The resulting pellet was suspended in 2mL of base cell culture medium containing 10% fetal bovine serum, 1µM epidermal growth factor, 1µM basic fibroblast growth factor, 100nM insulin, and 10nM hydrocortisone (growth factors and serum were from Gemini Bio-Products, West Sacramento, CA; insulin was from Sigma-Aldrich, St-Louis, MO). This was considered passage zero (0) and cells were cultured for four to eight passages before their use.

Most experiments were performed at passage 3 to 6. None were done pass passage 8, as preliminary experiments performed on successive passages established that passage 8 is when markers of senescence are significantly upregulated. Between 1 to 3 hr prior to the experiments, the medium was replaced by DMEM containing 5% of calf serum, 25mM Hepes, 10mM glucose, reduced level of pyruvate (0.5mM), and 600μM FA-free BSA. We will thereafter refer to this as the experimental medium.

Preparation of Fatty Acids

All 50mM FA stock solutions (all FAs were from Sigma Aldrich) were prepared as previously described [27] [132]. These individual stocks were used to prepare the 2000μM (2X) working mixture prepared in the experimental medium. The FA proportion of this mixture was oleic acid (40%), palmitic acid (25%), linoleic acid (20%) and stearic acid (15%). One vol. of this 2X mixture was added to cells already in 1 vol of the experimental medium containing, or not, the appropriate inhibitors at the onset of the incubations. The final FA to BSA ratio was 1.67:1. Based on the binding characteristics of the various fatty acids with BSA, the total free FA concentrations of our mixture was ~11nM with no individual fatty acid reaching concentrations above 5nM [133].

Determination of FA-Induced Cell Death

Cells were maintained in the experimental medium until 30 min prior to the addition of FAs, which is the time at which they received either the appropriate inhibitor which included 5nM ABT-737, 500μM L-carnitine, 10μM Cyclosporine A (CsA), 100μM mildronate, 1nM MitoQ, 10μM MitoTempo or the solvents (control). All these reagents were from the best commercial source except MitoQ, which was a gift from Michael P. Murphy (MRC Mitochondrial Biology

Unit, Cambridge, UK). These preincubations were followed by a 24 hr incubation with either the experimental medium (BSA) or 1000μM of the FA mixture described above. At the end of the 24 hr incubation period, cells were collected with enzyme-free cell dissociation solution (Millipore Sigma) and incubated for 15 min at room temperature in Dulbecco's phosphate-buffered saline (DPBS) containing calcein AM and ethidium homodimer 1 at a final concentration of 0.1μM [134].

The number of live and dead cells was evaluated by measuring calcein AM and ethidium homodimer-1 fluorescence (Thermo Fisher Scientific, Pittsburgh, PA) by flow cytometry (BD Accuri Cytometers, Ann Arbor, MI, USA). Calcein AM is a live cell dye that is non-fluorescent and accumulates in intact healthy cells, where it acquires a strong green fluorescence upon cleavage by intracellular esterases [134]. Because intracellular esterase activity is lost as cells die, little gain in fluorescence occurs in dying or dead cells. To detect calcein AM we used at an excitation wavelength of 488 and a 530 ± 30nm emission filter. For ethidium homodimer 1 we used an excitation wavelength of 488 and a 675 ± 25nm emission filter. Ethidium homodimer-1 is a dye that enters damaged plasma membranes and interacts with nucleic acids [134]. As this interaction increases, an increase in fluorescence indicates dying or dead cells. In each experiment 100 % cell death was established by treating the cells with 100 % methanol and FA-induced cell death was obtained by subtracting the values obtained with BSA alone. Each of the measurement was performed in triplicate.

Measurement of Mitochondrial Inner Membrane Permeabilization

Cells were treated as described above. At the end of the 24 hr incubation period, the cells were lifted from the dish with the enzyme-free cell dissociation solution described above and incubated at 37°C with 0.1μM calcein AM in DPBS for 30 min, followed by 200μM $CoCl_2$ for 15

min. Mitochondrial inner membrane (MIM) permeabilization was evaluated by measuring cobalt-quenched calcein fluorescence by flow cytometry [135]. This is because calcein AM has access to most cellular compartments including the mitochondria. However, cobalt ($CoCl_2$) which quenches calcein fluorescence does not freely enter mitochondria unless rendered permeable due to permeability transition [135]. Calcein AM fluorescence was measured at an excitation wavelength of 488nm and emission through a 535 ± 30nm filter. The percent of cells experiencing quenching of calcein fluorescence was calculated by subtracting the histogram plots in the presence of calcein AM from the one obtained in its absence. Each of the measurement was performed in triplicate.

Measurement of Cellular Respiration

7,200 preadipocytes were seeded to each well of Seahorse XF96 plates (Seahorse Bioscience, Agilent Technology, Wilmington, DE) in basal cell culture medium without growth factor and grown at 37°C for 24 hr. 1 to 3 hr before the experiments, the medium was changed to the experimental medium. Cells were maintained in the experimental medium until 30 min prior to the addition of FAs, which is the time at which they received either the appropriate inhibitor or the solvents. These preincubations were followed by a 24 hr incubation with either BSA or 1000µM FAs. At the end of the 24 hr incubation period, cells were washed two times with 250µL phosphate-buffered saline containing magnesium and calcium. Finally, 175µl of Seahorse assay media containing 10mM glucose, 2mM glutamine, 1mM pyruvate was added to the cells about 1 to 2 hr before the XF96 assay was run (Seahorse Bioscience, Agilent Technology, Wilmington, DE). The measurement cycle consisted of 1 minute mix; 1 minute wait; and three minute measurements. Three basal cycles were performed, followed by 3 cycles after 1µM of oligomycin was injected, 3 after 750nM FCCP, and finally 3 cycles after 0.5µM rotenone.

To calculate ATP regeneration-driven respiration, oligomycin-resistant respiration was subtracted to basal respiration. To calculate uncoupled respiration, rotenone-resistant respiration was subtracted to oligomycin-resistant respiration. To calculate the respiratory reserve capacity basal respiration was subtracted to FCCP-elicited respiration. To account for the decrease in cell density we multiplied these oxygen consumption rates by the ratio of live cells (live cells in the control conditions/live cells in the presence of FA). Each of the measurement was performed in quadruplicate.

Determination of ATP Concentration

Cells were treated as described in the determination of FA-induced cell death section. At the end of the 24 hr incubation period, cells were washed twice with PBS and ATP was extracted by adding 200μL boiling water [123]. Following extraction, ATP levels were determined using a bioluminescence ATP determination kit (Life technologies) as described by the supplier. An Infinite 200 Pro Series plate reader was used to quantify the luminescence (Tecan, Morrisville, NC).

Measurement of Mitochondrion-specific steady state levels of ROS

Cells were treated as described in the determination of FA-induced cell death section. At the end of the 24 hr incubation period, cells were loaded with 4μM MitoSox (Invitrogen, Carlsbad, CA) for 30 min at 37°C. After loading, the cells were washed in twice in DPBS and collected with enzyme-free cell dissociation solution before being suspended in DPBS. MitoSox Red fluorescence was measured by flow cytometry using an Accuri C6 at an excitation wavelength of 488nm and a 585 ± 40nm emission filter., a hydroethidine-derived fluorescent probe coupled to

the mitochondria-derived hexyl triphenylphosphonium cation that detects mitochondrial matrix ROS, essentially superoxide [136].

Mitochondrial Isolation from Preadipocytes

Cells were grown in 500cm^2 dishes in order to yield a sufficient number of cells for mitochondrial isolation. Cells were treated as described in the determination of FA-induced cell death section. At the end of the 24 hr incubation period, the following was performed in a 4°C cold room, or on ice. Media was aspirated from the plates and plates were washed 2x with 25mL ice-cold PBS. 15mL of PBS+10mM nicotinamide (Sigma Aldrich) was added and the cells were scraped from the plates and transferred to a 50mL microcentrifuge tube. The plates were scraped a second time after the addition of 10mL of PBS+nicotinamide and transferred to the same tube. Tubes were centrifuged at 1000g for 15 min and supernatant was discarded. Lysates were resuspended in 20mL of an ice-cold hypotonic buffer which consisted of PBS, 10mM NaCl, 1.5mM MgCl$_2$, 10mM TrisHCl, 500nM trichostatin A (TSA), 10mM nicotinamide, and a 100x protease inhibitor cocktail. Nicotinamide and TSA are deacetylase inhibitors and were added to prevent the deacetylation of mitochondrial proteins which was later assessed. Working concentrations of our protease inhibitor cocktail included 400μM aprotinin, 50μM bestatin, 10μM E64, 50μM leupeptin, with 2000x pepstatin and 100xPMSF being added to the cocktail the day of experiments.

The resuspended cells sat on ice for 5 minutes and were then homogenized 10 times via a Dounce homogenizer, checking for complete homogenization. 1/3 volume of mitochondrial homogenization buffer was added which consisted of PBS, 500nM TSA, 10mM nicotinamide, 100x protease inhibitor cocktail, and 50mM sucrose. Samples were then centrifuged at 800g for

10 min which yielded a nuclear pellet and the supernatant containing mitochondria. The supernatant was transferred to an ice-cold microcentrifuge tube and centrifuged at 17,000g for 15 min, yielding the mitochondrial pellet. The supernatant was carefully aspirated to not disturb the pellet, and the pellet was resuspended in 250μL of isolation buffer which consisted of PBS, 1% dodecyl maltoside, .5mM EDTA, 150mM TrisHCl, 150mM NaCl, 500nM TSA, 10mM nicotinamide, and 100x protease inhibitor cocktail. Protein concentration was measured via a BCA Protein Assay Kit (Thermo Fisher Scientific) and samples frozen at -80˚C until further use.

Measurement of Mitochondrial Acetyl CoA

Cells were treated as described in the determination of FA-induced cell death section. At the end of the 24 hr incubation period, mitochondria were isolated from primary preadipocytes as previously described. Samples were deproteinated through a 10 kDa spin column and centrifuged at 4˚C at 10,000g for 10 min. Supernatant was collected and acetyl CoA levels were measured according to PicoProb Acetyl CoA Flurometric Assay kit and protocol (BioVision). Fluorescence was assessed by an Infinite 200 Pro Series plate reader (Tecan, Morrisville, NC).

Evaluation of F_1F_0 ATP synthase acetylation

Cells were treated as described in the determination of FA-induced cell death section. At the end of the 24 hr incubation period, cells were washed twice with DPBS and lysed in non-denaturing condition. The lysing buffer was DPBS-based and contained 1% of Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific), 0.5% IGEPAL (Sigma Aldrich), and 10mM nicotinamide. The protein concentration was quantified using a BCA Protein Assay Kit (Thermo Fisher Scientific) and standardized to 1 mg/mL. The F_1F_0 ATP synthase complex was purified by

incubating this lysate overnight on a Rotator at 4°C with 20µL of an ATP Synthase-Agarose Beads Immunocapture Antibody (Abcam, Cambridge, MA). The next morning the samples were centrifuged at 500g for 1 min, and supernatant discarded. The pelleted beads were washed three times with 1mL of the immunoprecipitation buffer. The pulled down F_1F_0 ATP synthase complex was eluted from the beads by adding 60µL of 1.5X Laemmli loading buffer containing 1.5% sodium dodecyl sulfate (both from Biorad Life Sciences Research, Hercules, CA), mixing gently for 10 min, and filtering through Ultrafree-MC-SV centrifugal filter units (Millipore Sigma) by spinning the units at 500g for 3 min. The samples from this step were loaded on Mini-protean TGX 4%-20% gels (Biorad Life Sciences), transferred to nitrocellulose membranes (GE Healthcare Bio-Sciences, Pittsburg, PA), and immunodetected using antibodies specific for acetyl-lysine (Sigma Aldrich), Cyclophilin D (Thermo Fisher Scientific), Bcl-$_{xL}$ (Cell Signaling), and ATPB5 (Sigma Aldrich).

Gene Expression Analysis

Cells were treated as described in the determination of FA-induced cell death section. At the end of the 24 hr incubation period, cells were washed twice with DPBS and lysed in 1mL TRI-Reagent (Sigma Aldrich) and frozen at -80°C until RNA isolation. On the day of isolation 1 vol of ethanol was added to the samples and they were loaded to Zymo-Spin IIC columns, washed, and eluted as described in the Direct-Zol RNA MiniPrep Isolation kit (Zymo Research, Irvine, CA). cDNA was synthesized from 2µg total RNA according to the High Capacity cDNA Reverse Transcription kit protocol (Applied Biosystems, Forest City, CA). Relative real-time TaqMan PCR was performed with primers and 6-carboxyfluorescein dye-labeled TaqMan probes (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as endogenous

control. This control was validated in a comparison with 18S, HPRT, and POLII as it was found to be the most stable among the samples. Each reaction contained 5ng cDNA from total RNA, in a total reaction volume of 10µl. ΔΔCt values were obtained by normalizing to GAPDH in all experiments.

Results

16 subjects, 10 males and 6 females were enrolled in this study whom underwent a coronary artery bypass graft surgery and consented to a small (less than a gram) subcutaneous fat biopsy. They were all middle aged (49 to 69 years old), half were African American, and half were European American, 9 were obese and 7 were diabetic. This study was about the molecular mechanisms of preadipocyte sensitivity to fatty acids (FAs) and no attempt were made to reach clinical correlates.

Preadipocytes Isolated from Sixteen Individuals Differ in Their Susceptibility to the Lipotoxic Effects of Prolonged Fatty Acid (FA) Exposure.

We first set out to determine if preadipocytes isolated from the upper abdominal subcutaneous adipose tissue of 16 individuals differed in their sensitivity to a 1000 µM mixture of FAs composed of oleic acid (40%), palmitic acid (25%), linoleic acid (20%) and stearic acid (15%) for a duration of 24 h. This FA mixture roughly mimics physiological FA composition while the concentration of 1000 µM is in the range of concentrations seen in adipose tissue during postprandial and fasting conditions [126]. In addition, this concentration and incubation time was chosen from the results of our previous study, which established that mitochondrial inner membrane (MIM) permeabilization start to occur following 12 h FA treatment, while cell death

takes place between 18 to 24 h of FA exposure [27]. As shown in Fig. 1*A*, the mean percent of FA-induced cell death $\pm$ SD was 8.1 $\pm$ 1.9 for the lowest, or FA-resistant quintile (FAR), and 27.8 $\pm$ 3.2 for the highest, or FA-sensitive (FAS), quintile ($P \leq 0.0001$). As shown in Fig. 1*B*, the preadipocytes from the FAR group also displayed less MIM permeabilization as 13.3 $\pm$ 0.2 percent did compared to 30.1 $\pm$ 2.0 in the FAS group.

Intramitochondrial Superoxide or Lipid Peroxides are Responsible for FA-Induced ATP Depletion, MIM Permeabilization and Cell Death.

A transient increase in the steady state levels of superoxide and/or lipid peroxides was shown in a preadipocyte cell line to be required for FA-induced MIM permeabilization, ATP-depletion, a second and more substantial wave of ROS production that spills out of the mitochondria, and eventually cell death [27]. We first evaluated the impact of prolonged FAs exposure in both FAS and FAR primary preadipocytes. As shown in Fig. 2*A,* despite being significant in both groups, FA-induced a 4.5-fold higher ($P \leq 0.001$) increase in mitochondrial ROS in the FAS group (90 vs 20 %). FAs also led to more severe ($P \leq 0.05$) ATP depletion in FAS (64%) than FAR (36%) preadipocytes (Fig. 2*B).* Next, we assessed the role mitochondrial superoxide and/or lipid peroxides in FA-induced ATP depletion, MIM permeabilization, and cell death. To this end, we incubated the cells with MitoQ or MitoTempo, two mitochondrion-targeted superoxide and lipid peroxide scavengers. First, as shown in Fig. 2*C,* FA-induced mitochondrial ROS production was almost totally (78-93%) prevented by the two scavengers. Surprisingly, FA-induced ATP-depletion was not only prevented but reached levels 40 to 104% above control conditions (Fig. 2*D*). MitoQ and MitoTempo also prevented 75-84% of FA-induced MIM

permeabilization (Fig 2*E*) and 87-91% of FA-induced cell death (Fig 2*F*) in both FAS and FAR without distinction. Suggesting similar mechanisms of FA-induced cytotoxicity in both groups.

Cyclophilin D (CypD) Interaction with the Mitochondrial Permeability Transition Pore (PTP) is Implicated in FA-Induced Cytotoxicity in Preadipocytes.

Cyclosporin A (CsA), a well know PTP inhibitor, has been shown to prevent the interaction between CypD and F_1F_0ATP synthase, preventing the formation of the PTP [132]. Confirming this finding in our preliminary experiment showed that 10 μM CsA, a concentration we established to have no impact on basal MIM permeability (Suppl. Fig. 1*A*), prevented FA-induced MIM permeabilization by about 75%, and displaced CypD from F_1F_0ATP synthase (Suppl. Fig. 1*B*). Therefore, preadipocytes were preincubated with or without 10 μM CsA for 30 min and co-incubated 24 h with FAs. As shown in Fig. 3*A*. CsA decreased FA-induced ROS production by 64% in the FAR and 96% in the FAS group. Similarly, to MitoQ and MitoTempo, CsA not only prevented ATP depletion, but increased ATP concentration above (57-66%) control levels (Fig. 2*B*). CsA prevented FA-induced MIM permeabilization by 42% in FAR preadipocytes and 72% in FAS preadipocytes (Fig. 3*C*). CsA also prevented FA-induced cell death by 41% in FAR preadipocytes and 69% in FAS preadipocyte (Fig 3*D*).

Inhibition of Bcl-2 and/or Bcl-xL Exacerbates FA-induced Toxicity in FA-Resistant Preadipocytes

ABT-737 is potent inhibitor of Bcl-2, Bcl-xL and Bcl-w [137]. These Bcl-2 family members are well known for their role as regulators of the mitochondrial pathways of various types of regulated cell death. In addition, Bcl-2 was shown to directly interact with the respiratory

complex IV, which was shown to reduce superoxide production [138]. Bcl-xL, on the other hand, was shown to interact with the F_1F_0 ATP synthase complex, reducing proton leak and increasing ATP regeneration efficiency [59]. Our preliminary experiments confirmed the presence of Bcl-$_{xL}$ in the F_1F_0 ATP synthase complex and showed that this interaction is decreased by 85% after 24 h of exposure to FAs (Suppl. Fig. 2A). Therefore, preadipocytes were preincubated with or without 5 nM ABT-737, a concentration that did not affect basal steady state levels of ATP (Suppl. Fig. 2B), but exacerbated FA-induced increases in the steady state levels of mitochondrial ROS by 20% in the FAS and by 138% in the FAR group (Fig. 4A). This increase in mitochondrial ROS in the FAR group was 50% of what was seen in the FAS group ($P \leq 0.001$). Despite this, ABT-737 exacerbated the FA-induced ATP depletion in the FAR group only as the depletion became undistinguishable between the two groups (Fig. 4B). ABT-737, exacerbated FA-induced MIM permeabilization by 16% in the FAS and 55% in the FAR group (Fig. 4C). Finally, ABT-737 increased FA-induced cells death in FAR, but not FAS, preadipocytes (Fig. 4D).

The Respiratory Reserve Capacity of Preadipocytes is Higher and FAs Induce Uncoupling in FAR Preadipocytes Only.

We next assessed the impact of ABT-737 or CsA treatment on the effects of 24 h of FA exposure on the mitochondrial health of preadipocytes. To this end, preadipocytes were preincubated with or without 5 nM ABT-737 or 10 µM CsA for 30 min and incubated 24 h with FAs. As shown in Fig. 5A, incubating the cells with FAs add no effect on basal respiration. However, FAs induced an increase in respiration in the presence of 1 µM oligomycin, meaning that it increased oxygen consumption while uncoupled to ATP regeneration (Fig. 5B). This increase in uncoupling was totally prevented by ABT-737 (Fig 5B). Interestingly, the respiratory

reserve capacity, which indicates how close the cells are to their maximal capacity, was 3 times greater ($P \leq 0.01$) in FAR compared to FAS preadipocytes (Fig. 5*C*). In the presence of FAs and ABT-737 this difference between the two groups was gone (Fig. 5*C*).

FAs Cause a Further Increase in Acetyl-CoA and F_1F_0 ATP Synthase Acetylation in FAS Preadipocytes and Increase the Expression of L-Carnitine Acetyltransferase (CRAT) and sirtuin 3 (SIRT3) in FAS Preadipocytes Only.

The role of mitochondrial protein acetylation was shown to influence mitochondrial functioning and SIRT3 ablation to cause protein hyperacetylation and metabolic abnormalities [62]. Moreover, mitochondrial protein acetylation was shown to be driven by FA oxidation-derived acetyl-CoA [139]. Since the effects of acetyl-CoA are concentration dependent and regulated by CRAT [140], we tested the effects of two drugs with opposite effects on acetyl-CoA levels. First, we treated the cells with 500 µM L-carnitine, which should reduce the pool of activated acetyl groups by promoting acetylcarnitine efflux [63]. Second, we treated the cells with 100 µM mildronate, an inhibitor of both gamma-butyrobetaine hydroxylase, a key enzyme in L-carnitine synthesis and CRAT, which should increase acetyl-CoA levels [141] [142].

As shown in Fig. 6*A*, mitochondrial acetyl-CoA levels were increased in both FAS and FAR preadipocytes, although the increase in the FAR group was 20% smaller ($P \leq 0.001$). Moreover, although L-carnitine significantly prevented the increase in acetyl-CoA in both groups, it only was prevented fully in the FAR group (Fig. 6*A*). On the other hand, mildronate led to a further increase in acetyl-CoA in the FAR only (Fig. 6*A*). Similarly, there was increased acetylation of many proteins within the F_0F_1 ATP synthase complex (Fig. 6*B*). However, although it did not fully prevent the increase in acetyl-CoA, L-carnitine totally prevented the increase in

acetylation of the F_0F_1 ATP synthase complex (Fig. 6*B*). FA-induced MIM permeabilization, in the same conditions, was decreased by 71% in the FAR and 60% in the FAS group (Fig. 6*C*), and cell death by 60% in both groups (Fig. 6*D*). However, despite similar relative deceases in MIM permeabilization and cell death, the remaining MIM permeabilization and cell death in the FAS group was still significantly above the control basal BSA condition ($P \le 0.001$) for both endpoints.

Finally, we evaluated the effect of 24 h FA exposure on the expression of SIRT1, SIRT3 and CRAT. As sown in Fig. 6*E*, FA exposure had opposite effects on the expression of SIRT3 and CRAT, increasing the levels of their mRNA in FAR by 56 and 146% respectively, but decreasing by 30 and 65% respectively in FAS preadipocytes.

DISCUSSION

This study was performed to investigate the role of CypD, BCl-2 or Bcl-$_{xL}$, and mitochondrial acetyl-CoA levels in FA-induced loss of ATP synthetic efficiency, MIM permeabilization, and cell death. First, we found a 3.5-fold range of FA-induced MIM permeabilization and cell death in primary culture of human preadipocytes. However, it was beyond the scope of this study to establish clinical correlates from these observations as this study would be severely underpowered for such type of analysis. However, we systematically compared the FAR and FAS preadipocytes as it had the potential to suggest the molecular mechanism underlying this difference in sensitivity. Our main conclusion is that mitochondrion-generated ROS and CypD are central to FA-induced MIM permeabilization, loss in ATP regeneration capacity, and cell death. This mechanism appears to be shared in FAR and FAS sensitive preadipocytes as MitoQ and MitoTempo treatments totally eliminate MIM permeabilization, ROS production and cell death.

One interesting finding was the increase, rather than the normalization of the steady state levels of ATP after a pretreatment with MitoQ and MitoTempo as well as CsA. Recycling ATP from $ADP \cdot Mg^{2+}$ and P_i is the day job of the mitochondrial F_1F_0 ATP synthase. Cellular levels of ATP are very stable, even in tissues with high fluctuations in ATP demand, which is the main driver for regeneration [143]. There are other factors that optimize electron and proton flow, which ultimately power the F_1F_0 ATP synthase, but none are more influential than the mitochondrial redox environment [144]. When FA levels exceed the reducing equivalent needed to maintain ATP turnover, the cells are under reductive pressure, a state in which ROS are produced at a rate somewhat superior to the buffering potential of the antioxidant system [145]. One possibility is that the shift in intramitochondrial redox environment caused by reductive pressure favors electron leak from complex I and III, and that the presence of MitoQ and MitoTempo prevent this redox break to occur.

Among the plethora of components that have been proposed to be part of the PTP, CypD is the only one recognized as necessary [40]. CsA, which prevents CypD to bind to F_1F_0 ATP synthase [45], has also been shown to cause an increase in ATP levels. CypD binding to PTP was shown to be involved in both the reversible and short-lasting flicker-like opening, and the irreversible large conductance opening of the PTP [146]. This may explain what we observed as the flickering mode of the PTP reduces ROS production by serving as a valve that prevents the back pressure that opposes proton transfer to the intermembrane space and the buildup of the proton motive force.

Contrary to what we observed in immortalized XA15A1 human preadipocytes [27], FAs did not reduce basal respiration driven by glucose, pyruvate, and glutamine. We can only speculate that the immortalized cells relied more on these substrates to maintain ATP regeneration during

proliferation [147]. However, FAs caused an increase in respiration uncoupled from the need for ATP regeneration in the FAR preadipocytes only. In a state of reductive pressure, uncoupling may be a beneficial adaptation [145], reducing ROS production. This is in line with our mitochondrial ROS measurement as FAs only increased MitoSox Red fluorescence by 20-23% in FAR, as opposed to 90-96% in the FAS preadipocytes. The absence of an inhibitory effect of CsA on uncoupling suggests that this adaptive uncoupling process is not mediated by CypD and the PTP and could be possibly through more traditional uncoupling mechanisms involving uncoupling proteins [148]. However, the prevention of uncoupling by ABT-737 suggests that Bcl2, bcl-xL or even Bcl-w, can participate in this uncoupling. This would be consistent with the suggested role for Bcl-2 family members in FA-induced uncoupling in human adipocytes [149]. Also supporting a role for Bcl-2 family members in uncoupling is the exacerbation of FA-induced ATP depletion in ABT-737 treated FAR preadipocytes. FAR resistant preadipocytes also had a much higher reserve capacity, which was decreased by FA, and further exacerbated by ABT-737. Both of these effects of ABT-737 were accompanied by a parallel increase in cell death in FAR cells, suggesting that ATP depletion may partly cause cell death, and that the higher reserve capacity may be explaining the resistance to FA-induced cell death.

We found that FAs caused an accumulation of acetyl-CoA in the mitochondria of both FAR and FAS preadipocytes, but F_0F_1 ATP synthase acetylation in FAR cells was 50% of what was measured in the FAS preadipocytes. This could be due the induction of SIRT3 in the FAR, but not FAS preadipocytes, as SIRT3 has been shown to deacetylate both the F_0F_1 ATP synthase [132] [150], and CypD [130] [131], to protect against nutrient-induced stress. However, total elimination of FA-induced F_0F_1ATP synthase acetylation with L-carnitine pretreatment prevented cell death by 60% in both FAR and FAS preadipocytes, suggesting that although CypD is

responsible for cell death, the difference in acetylation levels is not the sole reason for the difference in FA sensitivity.

Altogether this study characterized the mechanism of FA-induced cell death in primary human preadipocytes. The mechanism being an increase in mitochondrial ROS production that leads to a CypD-dependent MIM permeabilization. PTP formation, the cause of MIM permeabilization and ATP depletion, is occurring at a higher rate in FAS than FAR preadipocytes. Although F_0F_1 ATP synthase acetylation is reduced in FAR preadipocytes, it only partly explains their sensitivity. Additionally, although FAS experience much more profound ATP-depletion, the latter is not the cause of cell death. It is likely that alternative ROS-dependent mechanisms are involved in FA-induced cells death.

Sex		Age (years)	Race		BMI	A1C
M	F		White	African American		
10	6	60.31 +/- 5.71	8	8	29.25 +/- 5.28	6.88 +/- 2.68

Table 1. Patient anthropometric data. N=16. Data are presented as mean +/- SD.

Fatty Acid Resistant (FAR) group			Fatty Acid Sensitive (FAS) group		
Age (years)	BMI (average)	A1C (average)	Age (years)	BMI (average)	A1C (average)
57.67 +/- 4.73	**26.09** +/- 4.82	**5.77** +/- .21	**55.33** +/- 5.51	**35.64** +/- 4.93 p=.08	**6.2** +/- .9

Table 2. Average anthropometric data of FAR and FAS groups. Data shown as mean +/- SD.

Figure 1.

A.

p<0.0001

FA-induced Cell Death
(% of cells)

40

30

20

10

0

First (FAS)   Second   Third   Fourth   Fifth (FAR)

Quintiles

B.

p<0.0001

FA-induced MIM Permeabilization
(% of cells)

40

30

20

10

0

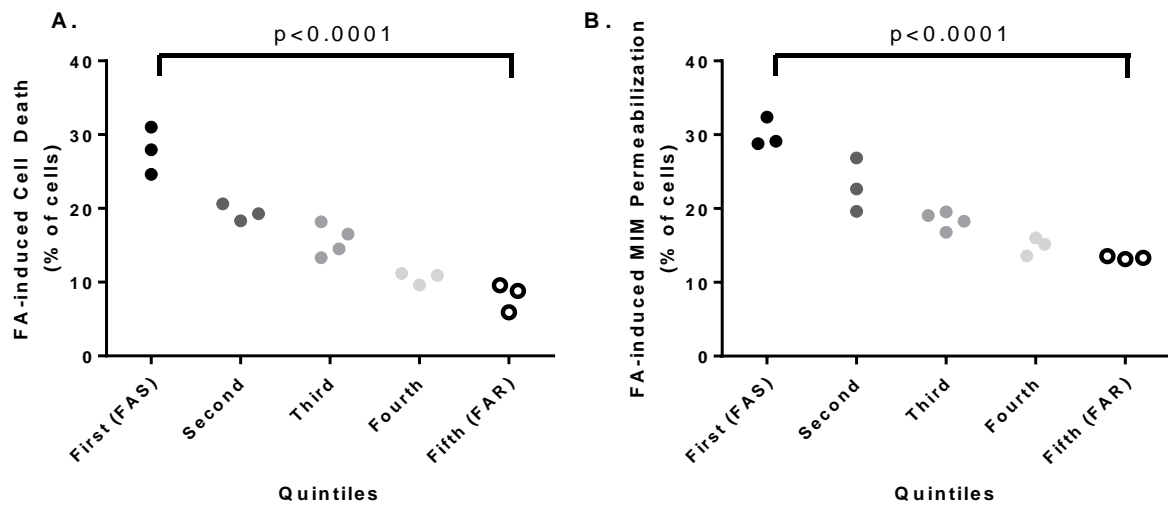First (FAS)   Second   Third   Fourth   Fifth (FAR)

Quintiles

Figure 1. Preadipocytes isolated from the upper abdominal subcutaneous adipose tissue of 16 subjects differ in their susceptibility to cytotoxic effects of fatty acids. Preadipocytes were incubated for 24 hr with 1000μM of fatty acids (FAs) and (*A*) the percent of dead and (*B*) percent of cell experiencing mitochondrial inner membrane (MIM) permeabilization were evaluated as described in the Materials and Methods section. The 1st and 5th quintiles were selected and termed fatty acid sensitive (FAS) and fatty acid resistant (FAR). The results were analyzed by one-way ANOVA followed with Dunnett's as post hoc test. The first and last quintile was significantly significant at $P \leq 0.0001$ for both variables.
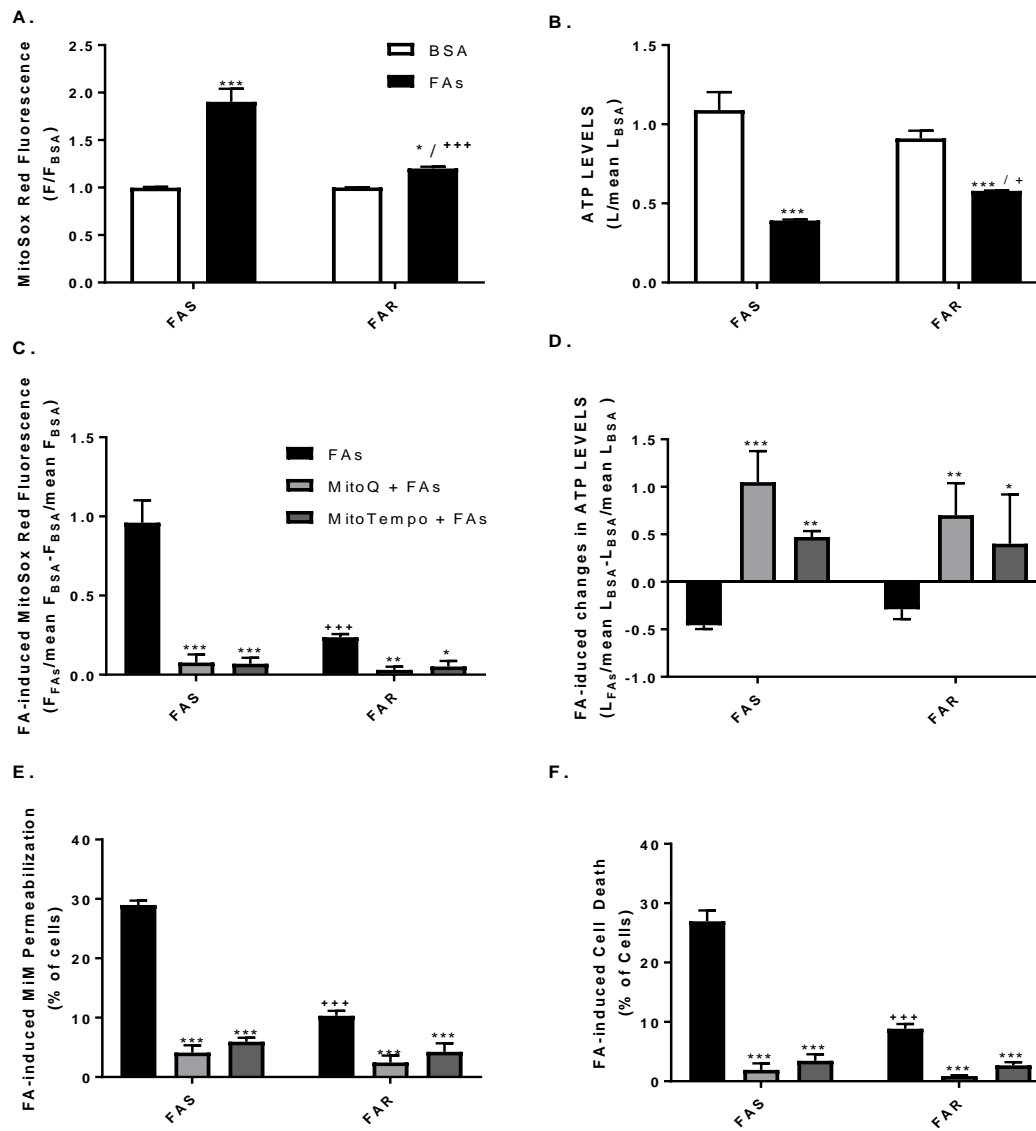
Figure 2.

Figure 2. FA-induced mitochondrial reactive oxygen species (ROS) and ATP depletion are induced to a greater extent in FAS compared to FAR preadipocytes. Effect of the mitochondrion-targeted antioxidants MitoQ and MitoTempo on FA-induced mitochondrial ROS, ATP depletion, MIM permeabilization and cell death. Preadipocytes from the FAS and FAR groups were incubated for 24 hr with 1000 µM of FAs and (*A*) MitoSox Red fluorescence and (*B*) ATP levels were measured as described in Materials and Methods section. In (*C* to *F*) Cells were preincubated 10 min with or without 1 nM MitoQ or 10 µM MitoTempo for 10 min prior to 24 h with 1000 µM FAs and (*C*) MitoSox Red fluorescence, (*D*) ATP levels, (*E*) MIM permeabilization, and (*F*) cell death were measured. Results were expressed as the mean ±SD and analyzed by two-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ compared to BSA (*A*, *B*) or FAs (*C* to *F*); [++] $P \leq 0.01$, [+++] $P \leq 0.001$ comparing the FAS and FAR groups.
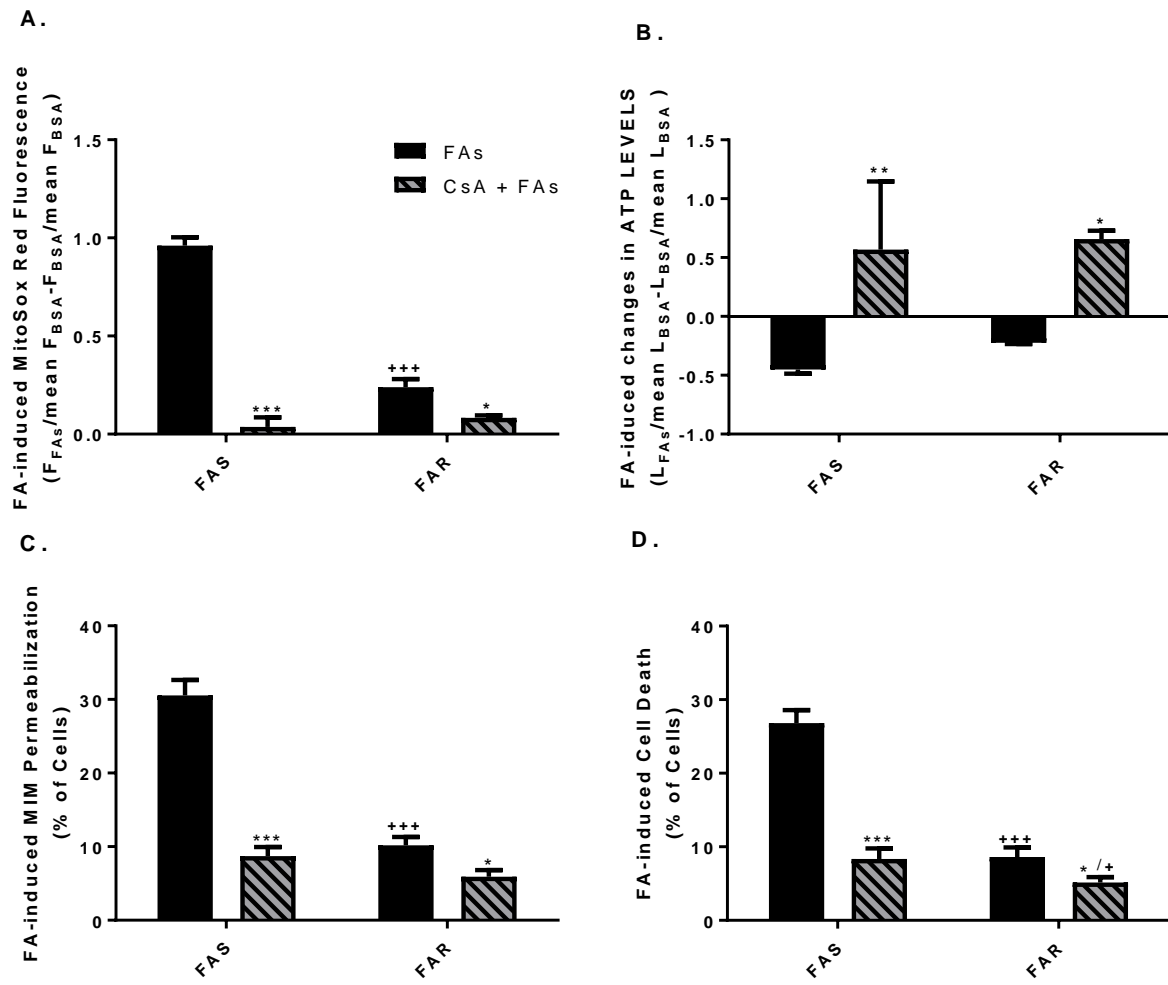
Figure 3.

Figure 3. Inhibiting cyclophilin D (CypD) binding to the F_1F_0ATP synthase with cyclosporin A (CsA) attenuates FA-induced toxicity in preadipocytes. Preadipocytes from the FAS and FAR groups were preincubated with or without 10µM CsA for 10 min prior to 24 hr with 1000µM FAs and (*A*) MitoSox Red fluorescence, (*B*) ATP levels, (*C*) MIM permeabilization, and (*D*) cell death were measured. Results are expressed as the mean ±SD and analyzed by two-way ANOVA followed by the Sidak pairwise multiple-comparison test. * $P{\leq}0.05$, ** $P{\leq}0.01$, *** $P{\leq}0.001$ compared to FAs; [+++] $P{\leq}0.001$ comparing the FAS and FAR groups.
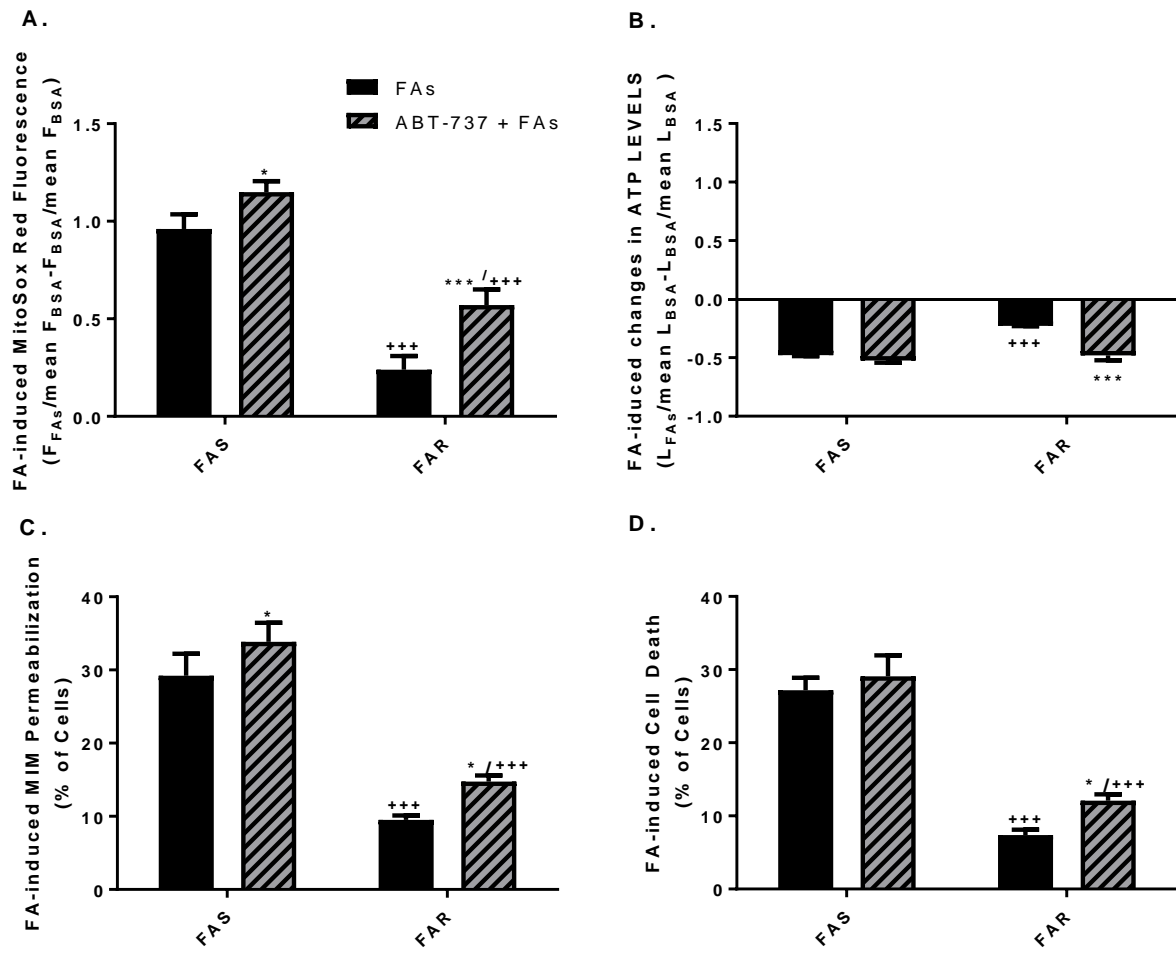
Figure 4.

Figure 4. Inhibiting b-cell lymphoma extra-large (Bcl-$_{xL}$) binding to the F$_1$F$_0$ATP synthase with ABT-737 exacerbates FA-induced ROS production and ATP depletion in FAR preadipocytes, while marginally affecting MIM permeabilization and cell death. Preadipocytes from the FAS and FAR groups were preincubated with or without 5nM ABT-737 for 10 min prior to 24 hr with 1000 µM FAs and *(A)* MitoSox Red fluorescence, *(B)* ATP levels, *(C)* MIM permeabilization, and *(D)* cell death were measured. Results are expressed as the mean ±SD and analyzed by two-way ANOVA followed by the Sidak pairwise multiple-comparison test. * *P≤0.05*, *** $P≤0.001$ compared to FAs; +*P≤0.05*, +++ $P≤0.001$ comparing the FAS and FAR groups.
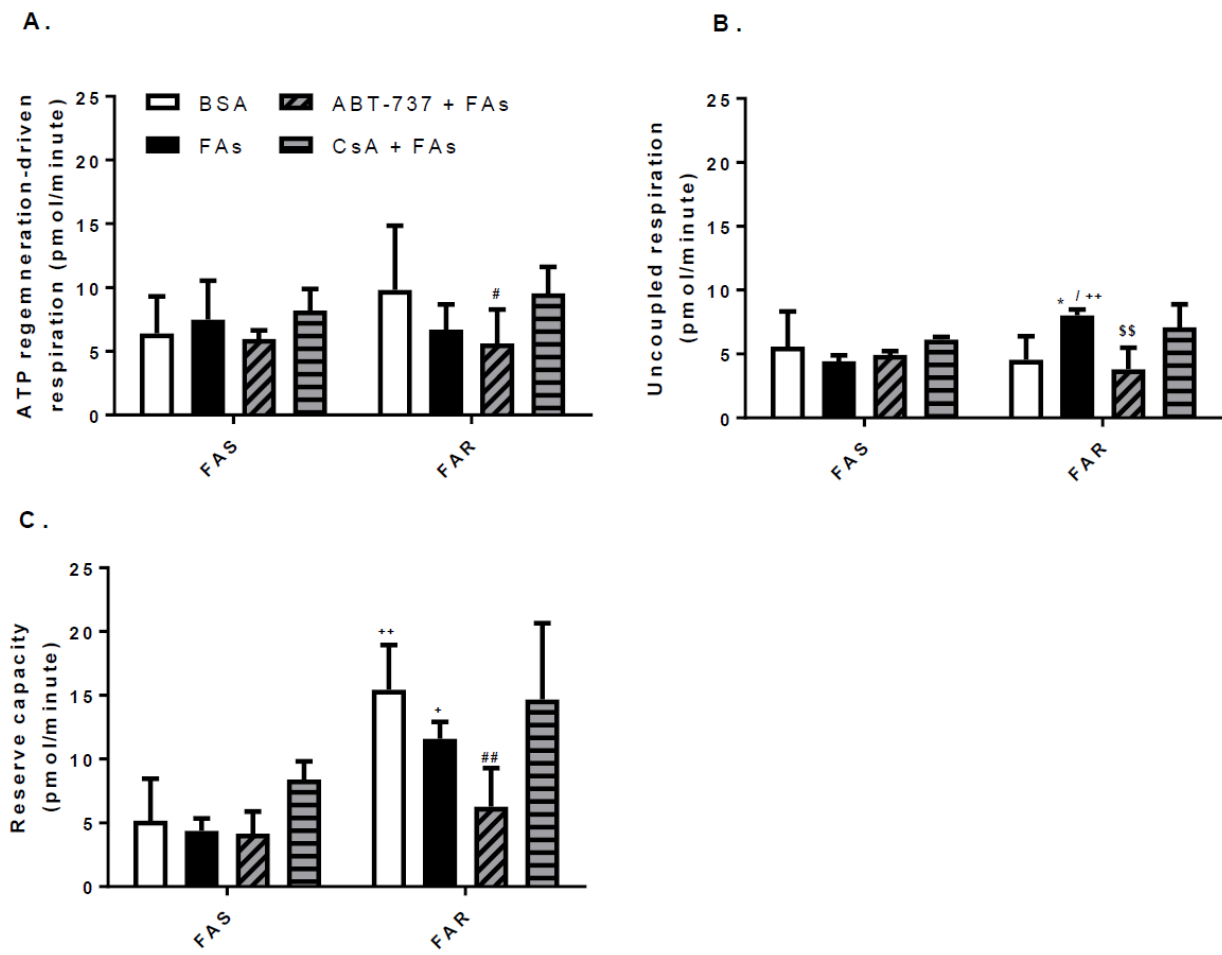
Figure 5.

Figure 5. FA-induced mitochondrial dysfunction in FAR and FAS cells. Inhibiting Bcl-xL exacerbated FA-induced dysfunction, whereas CypD inhibition attenuated FA-mediated changes in mitochondrial respiration. *(A-C)* Preadipocytes from the FAS and FAR groups were preincubated with or without 5nM ABT-737, or 10µM CsA for 10 min prior to 24 hr with 1000µM FAs and mitochondrial respiration was measured via respirometry**.** At the end of the 24 hour incubation basal, oligomycin-sensitive, FCCP-elicited and rotenone-sensitive respiration were measured. *(A-C)* ATP-regeneration driven, uncoupled, and reserve capacity were calculated according to the Materials and Methods section. Results presented in *A-C* were analyzed by two-way ANOVA on repeated measures with Boneferroni's as post hoc test. Results are expressed as the mean ±SD. * *P≤0.05* compared FAs to BSA*,* \$\$ *P≤0.05* compared to FA, ## *P≤0.05* compared to BSA, and ++ *P≤0.05* compared FAR and FAS groups.
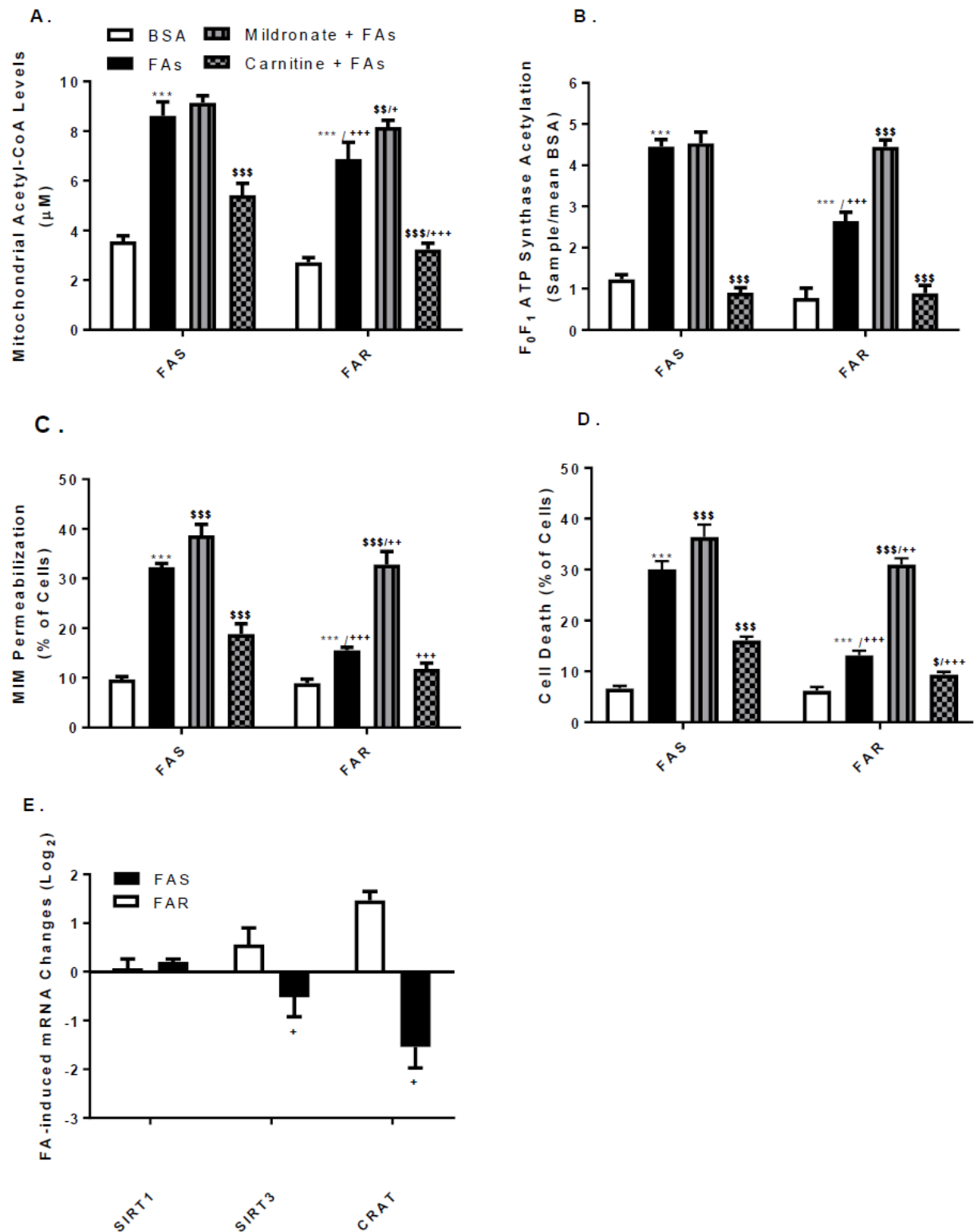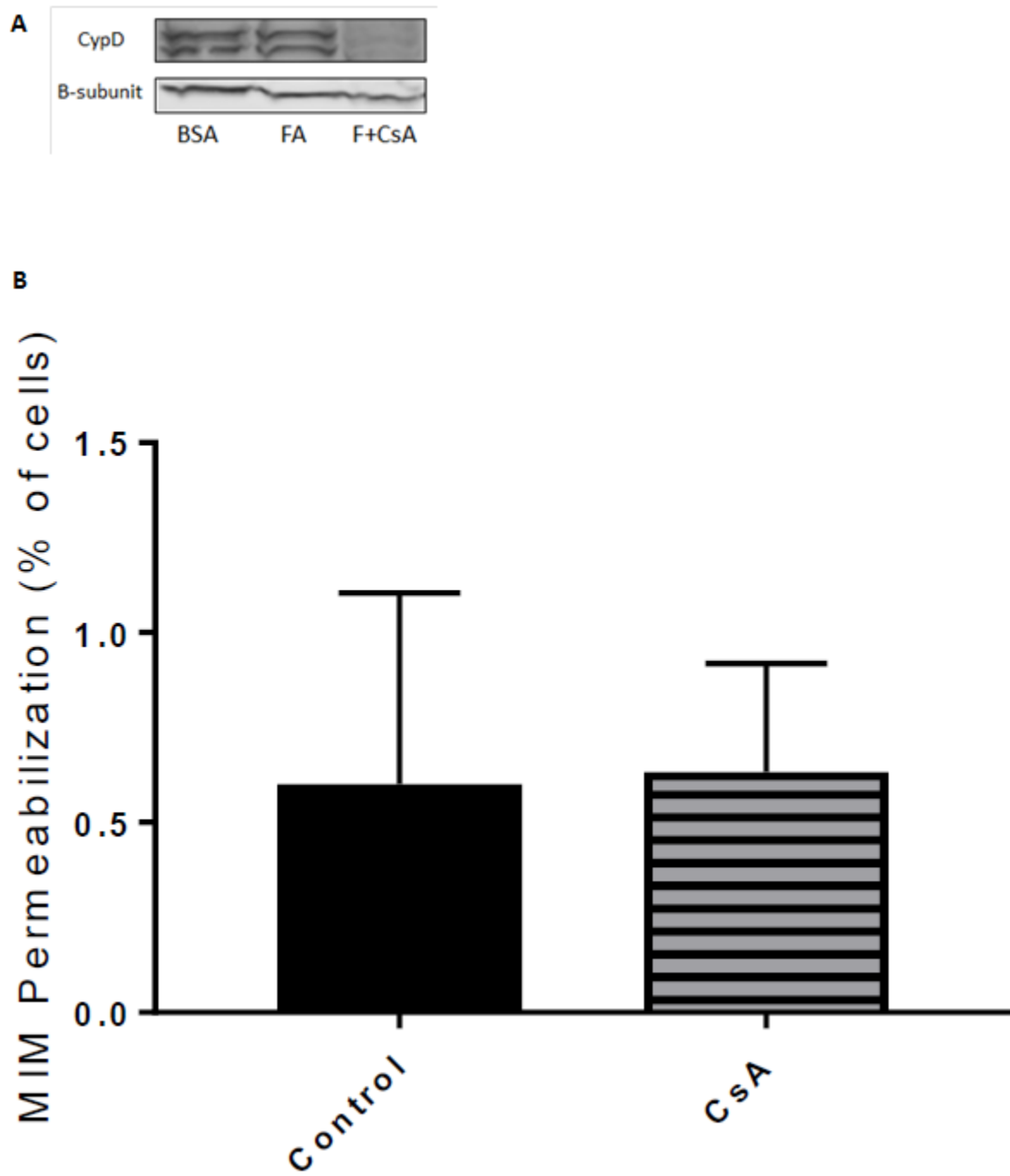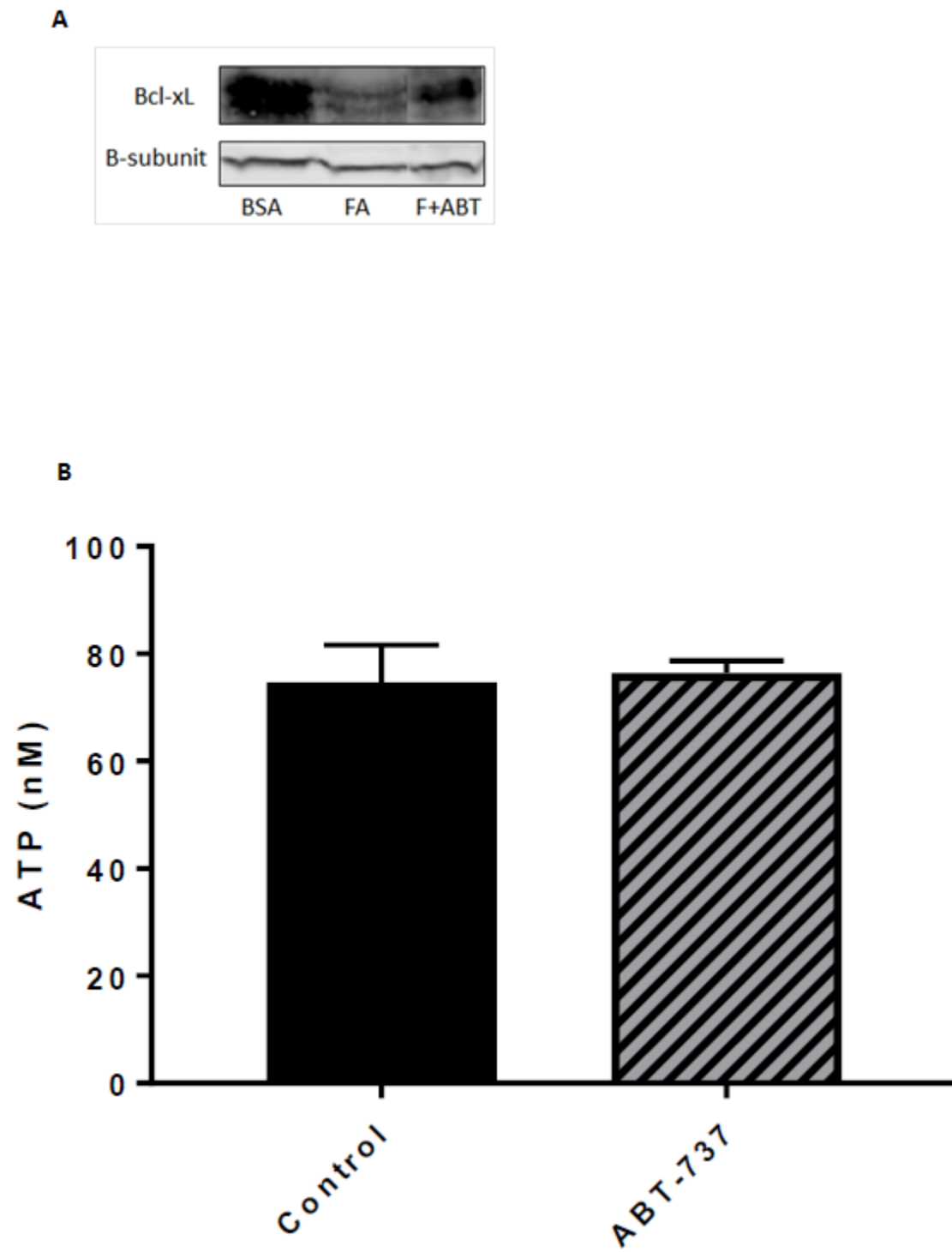
Figure 6.

Figure 6. FAs induce an increase in the steady state levels of acetyl CoA, F_1F_0 ATP synthase hyperacetylation, and increase in MIM permeabilization. These effects being more pronounced in the FAS compared to the FAR preadipocytes. Preadipocytes from the FAS and FAR groups were preincubated for 10 min with or without 500 μM L-carnitine or 100 μM mildronate prior to 24 hr with or without 1000 μM FAs. (*A*) Acetyl CoA levels were measured in isolated mitochondria as described in the Materials and Methods section. (*B*) F_1F_0 ATP synthase was immunoprecipitated and acetyl-lysine levels was evaluated by western blot as described in the Materials and Methods section. (*C*) MIM permeabilization and (*D*) cell death were measured as previously described**.** *(E)* mRNA expression of sirtuin 1 and 3 (SIRT1 and SIRT3) and L-carnitine O-acetyltransferase (CRAT) was measured via real-time PCR as described in Materials and Methods section. Results are expressed as the mean ±SD and analyzed via two-way ANOVA with followed by the Sidak pairwise multiple-comparison test. ****P≤0.001* comparing BSA to FAs; $^+$*P≤0.05,* $^{++}$ *P≤0.01,* $^{+++}$ *P≤*0.001 comparing the FAS and FAR groups. $^{\$\$}$ *P≤*0.01, $^{\$\$\$}$*P≤0.001* comparing the FAs to Drug + FAs. In (*E*) the results were analyzed with a paired t-test. *005* compared to BSA control; *** P≤0.001* compared to FAs; $^+$ *P≤*0.05 comparing the FAS and FAR groups.

Supplemental Figure 1

Supplemental Figure 1. CsA completely prevented CypD binding to the F_1F_0 ATP synthase, and had no impact on basal MIM permeability. Preadipocytes from the FAS and FAR groups were preincubated with or without 10µM CsA for 10 min prior to 24 h with 1000 µM FAs. *(A)* The F_1F_0 ATP Synthase was immunoprecipitated as described in Materials and Methods and western blots measured CypD binding to the F_1F_0 ATP synthase. (B) Preadipocytes were preincubated with or without 10 µM CsA, a concentration that did not affect basal MIM permeability.

Supplemental Figure 2

A

B

Supplemental Figure 1. FA displace Bcl-$_{xL}$ binding from the F$_1$F$_0$ ATP synthase, however 5 nM ABT-737 doesn't affect control steady state levels of ATP. Preadipocytes from the FAS and FAR groups were preincubated with or without 5nM ABT-737 for 10 min prior to 24 h with 1000 µM FAs. *(A)* The F$_1$F$_0$ ATP Synthase was immunoprecipitated as described in Materials and Methods and western blots measured Bcl-xL binding to the F$_1$F$_0$ ATP synthase. (B) Preadipocytes were preincubated with or without 5 nM ABT-737, a concentration that did not affect basal steady state levels of ATP.

CHAPTER THREE

Twelve-Weeks of Aerobic Interval Training Increases Subcutaneous Adipose Tissue Preadipocytes Fatty Acid Resistance and Decreases Cardiometabolic Risk Factors

Jared Shine[1][2], Joseph White[2][3], Timothy Heden[2][4], Barbara Davis[1], Robert Hickner[2][4], Jacques Robidoux[1][2][3]

[1]Pharmacology and Toxicology, East Carolina University, Greenville, NC, [2]East Carolina Diabetes and Obesity Institute, [3]Kinesiology, East Carolina University

ABSTRACT

Prolonged exposure of preadipocytes to concentrations of fatty acids (FAs) seen during prolonged fasting has been shown to be cytotoxic through mechanisms involving mitochondrial inner membrane (MIM) permeabilization and ATP depletion. Moribund preadipocytes have been suggested to be part of the pathogenesis of dysfunctional adipose tissue, which in turn, precipitates the appearance of cardiometabolic risk factors. Exercise training has been shown to impact metabolic organs including subcutaneous adipose tissue (SAT). To determine the effects of exercise training on the FA sensitivity of preadipocytes isolated from SAT we enrolled 35 subjects (10 males/25 females) with obesity but normoglycemic in a 12-week aerobic interval training (AIT) exercise program and obtained abdominal and gluteal subcutaneous adipose tissue (SAT) biopsies at baseline and after the intervention. At baseline, FA sensitivity evaluated by measuring FA-induced MIM permeabilization and cell death spanned 4-folds and indiscriminately between the two tissues. Of the 28 subjects completing the training 22 improved their aerobic capacity (VO_{2peak}), indicating that the training was efficient, and 25 improved their FA sensitivity. Training was also efficient at improving insulin sensitivity as well as decreasing total and low-density lipoprotein (LDL)-cholesterol.

We tested three hypotheses: 1) baseline FA sensitivity predicts who will benefit from AIT training; 2) AIT training improves preadipocyte FA sensitivity; 3) The improvement in FA sensitivity correlates with the decrease in cardiometabolic risk factors. We found that: 1) there is a trend for baseline FA sensitivity to correlate with the changes in triglycerides as well as HDL-c after training, despite the fact that the FA resistant subjects had a significant higher baseline HDL-c levels; 2) AIT improves preadipocytes FA sensitivity; 3) improvement in FA sensitivity correlates with reduction in total cholesterol and LDL-c. In conclusion, AIT improves cardiometabolic risk factors, and also improves the FA sensitivity of preadipocytes, but the usefulness of measuring FA-sensitivity as a predictor of who would benefit the most from AIT training seems minimal especially considering the invasiveness of the procedure.

Introduction

Obesity is considered to be caused by a combination of a high caloric diet and a lack of physical activity, resulting in a positive energy imbalance. If this imbalance exists chronically, it may also result in a loss of metabolic fitness which can manifest as reduced HDL-c, raised triglycerides, cholesterol, blood pressure, fasting plasma glucose, and a large waist circumference [2] [1]. Furthermore, loss of metabolic fitness has been found to be a strong predictor for cardiovascular disease (CVD), especially low HDL-c concentrations and elevated LDL-c concentrations [102] [103] [104]. One of the first approaches to managing CVD and many diseases of the metabolic syndrome is diet and exercise, the latter of which has been shown in a number of studies to decrease weight and improve metabolic health [102] [105] [106] [107]. Some of these improvements include preventing and treating the aforementioned cardiovascular risk factors [108] [109] [110] [111] [112].

The exact mechanisms of the beneficial effects of exercise are still being elucidated, however much evidence points to improvements in mitochondrial health [113] [114] [115]. The mitochondria have become a popular therapeutic target recently for an array of diseases, with the mitochondrial permeability transition pore (PTP) being at the center of a number of different pathological disorders. PTP is described as a large conductance pore that causes the collapse of the chemiosmotic gradient across the mitochondrial membrane (MIM), effectively halting ATP synthesis, and through prolonged opening, promoting cell death.

Our previous findings showed that inhibiting PTP afforded cellular protection and preservation of mitochondrial health in human primary preadipocytes [27] [151]. Chronic positive energy imbalance and adipose tissue dysfunction, both hallmarks of obesity, result in elevations of FFA in adipose tissue and serum [9] [27] [151]. Previous work in our lab has

64

found elevated FFA to cause an increase in the steady state levels of mitochondrial reactive oxygen species and lipid peroxides, which, in turn, cause delayed PTP, ATP depletion, and cell death in preadipocytes [27]. Additionally, we found for preadipocytes isolated from older patients undergoing CABG surgery to differ in their susceptibility to FA-induced toxicity [151].

In the present study we isolated primary subcutaneous preadipocytes from overweight/obese subjects participating in a 12-week exercise training regimen. Preadipocytes isolated prior to exercise were treated with a physiologically relevant FA cocktail to determine pre-exercise sensitivity to fatty acids, and their correlation to metabolic health markers. We hypothesized that 1) Subject FA-sensitivity (FAS) would correlate with pre-exercise metabolic health markers, being the higher the FA-induced toxicity, the worse the subject's metabolic profile; 2) 12-week exercise training would decrease FAS and improve metabolic health; 3) Pre-exercise FAS determination could be a predicative tool for the efficacy of exercise in managing obesity and cardiovascular risk factors. Our major findings were that subject pre-exercise FAS varied among young subjects, and this FAS correlated with decreased subject metabolic health; and 12-week exercise training improved this FA-sensitivity, along with lowering cardiovascular risk factors total cholesterol, and LDL-c.

Materials and Methods

Participants.

Normoglycemic, obese (BMI > 30 kg/m^2) men and women (ages 25-47) were recruited to participate in this study. Exclusion criteria were set as previously described [152]. All procedures were approved by the University and Medical Center Institutional Review Board of East Carolina University (UMCIRB 11-0262).

Anthropometrics, Assessment of Aerobic Capacity, and Blood Analyses

Height was measured with a stadiometer to the nearest 0.1 cm; body mass was measured with a digital electronic scale to the nearest 0.05 kg. Body fat percentage was determined using dual-energy x-ray absorptiometry (DXA; GE Lunar Prodigy Advance, Madison, WI, USA) as previously described [153]. At baseline and after exercise training subjects performed a standardized maximal exercise test to assess peak aerobic capacity (VO_{2peak}). VO_{2peak} was assessed via open circuit spirometry (TrueMax 2400; Parvomedics; Salt Lake City, UT, USA) using the Storie protocol, a treadmill ramp protocol where the speed or incline is increased every 2 min until volitional fatigue is reached. Heart rate (HR) was recorded every minute throughout the test and immediately upon fatigue to determine the maximum heart rate (HR_{max}). Before and after the training intervention a fasted blood draw was performed where blood was collected from the antecubital vein. Blood samples were allowed to clot and were then centrifuged at 3300 rpm for 10 min. Serum glucose, insulin, triglycerides, total-cholesterol, and high-density lipoprotein cholesterol were assessed by a commercial laboratory (Laboratory Corporation of America). Low-density lipoprotein cholesterol was calculated by the Friedewald formula [154] and the homeostatic model of insulin resistance (HOMA-IR) was calculated as previously described [155].

Adipose Tissue Biopsies

Abdominal and gluteal SAT biopsies were performed as previously described [156] after an overnight fast ($\geq$10 h) at baseline and after 3 months of exercise training. SAT samples were immediately rinsed with Krebs Ringer Bicarbonate buffer, blood clots and blood vessels were

then removed. The biopsy sample was then minced and transported for cell isolation in DMEM-F12.

Aerobic Interval Training

Participants performed an aerobic interval exercise training intervention 3d/wk for 12 wks. Exercise consisted of walking/running on a motor-driven treadmill. During each exercise session participants performed a 10-min warm-up at ~70% HR_{max}, followed by four 4-min intervals at 88-92% HR_{max} interspersed by 3-min active recovery periods at ~70% HR_{max}; intervals were followed by a 4-min cool down at ~60% HR_{max}, amounting to a total exercise time of 42 min. HR was monitored throughout each training session, and intensity (speed or incline) was increased when HR failed to reach at least 88% HR_{max} during the high-intensity interval. The fasting blood draw and SAT biopsies were repeated 2d following the final exercise session to assess the impact of the exercise intervention and to avoid the acute effects of the last exercise bout on experiments. VO_{2peak} and anthropometric tests were repeated within 1-2d of the blood draw and adipose biopsies.

Primary Preadipocyte Isolation

Following tissue biopsy, a small sample of subcutaneous adipose tissue was collected and immediately minced in Dulbecco's modified Eagle's medium (DMEM):F12 (1:1; Hyclone, Thermo Scientific, Logan, UT) containing 10mM glucose, 1mM pyruvate, 33μM Biotin, 17μM D-Pantothenate (all from Sigma Aldrich St. Louis, MO). Thereafter this medium will be referred to as the base medium. Upon arrival in the laboratory, the tissue was transferred to a tube containing the base medium completed with 1% fatty acid (FA)-free bovine serum albumin (BSA;

Gemini Bio-Products) and 1 mg/mL of type 1 collagenase (Worthington, Lakewood, NJ). After being further minced the tissue was digested for 60-75 min at 37˚C and agitated at 120 rpm. The larger debris were trapped via filtration through a 420 µm filter. This filtrate was centrifuged at 300g for 10 min, the mature fat cells aspirated, and the pellet suspended in 10mL of an erythrocyte lysis buffer composed of 154mM NH_4Cl, 10mM $KHCO_3$ and 0.1mM EDTA, which eliminated most erythrocytes within 3 to 6 min. This incubation was stopped by adding 40mL of base cell culture medium, filtered through a 100µm filter and centrifuged at 300g for 10 min. The resulting pellet was suspended in 2mL of base cell culture medium containing 10% fetal bovine serum, 1µM epidermal growth factor, 1µM basic fibroblast growth factor, 100nM insulin, and 10nM hydrocortisone (growth factors and serum were from Gemini Bio-Products, West Sacramento, CA; insulin was from Sigma-Aldrich, St-Louis, MO). This was considered passage zero (0) and cells were cultured for four to eight passages before their use.

Most experiments were performed at passage 3 to 6. None were done pass passage 8, as preliminary experiments performed on successive passages established that passage 8 is when markers of senescence are significantly upregulated. Between 1 to 3 hr prior to the experiments, the medium was replaced by DMEM containing 5% of calf serum, 25mM Hepes, 10mM glucose, reduced level of pyruvate (0.5mM), and 600µM FA-free BSA. We will thereafter refer to this as the experimental medium.

Preparation of Fatty Acids

All 50 mM FA stock solutions were prepared in 100mM NaOH and stored at −20°C. On the day of experiments, these stocks were used to prepare various 2X working solutions in the appropriate medium (DMEM containing 5% of calf serum, 10mM glucose, 25mM Hepes,

0.5mM pyruvate, and 600μM FA-free BSA). The solutions were mixed on an orbital shaker for 45 min at 37°C. The FA proportion of these mixes was oleic acid (40%), palmitic acid (25%), linoleic acid (20%) and stearic acid (15%). The final FA to BSA ratio was 1.67:1. Based on the binding characteristics of the various fatty acids with BSA, the total free FA concentrations of our mixture was ~11nM with no individual fatty acid reaching concentrations above 5nM.

Cell Death Determination

Cells were maintained in the experimental medium until 30 min prior to the addition of FAs. At the end of the 24 hr incubation period, cells were collected with enzyme-free cell dissociation solution (Millipore Sigma) and incubated for 15 min at room temperature in Dulbecco's phosphate-buffered saline (DPBS) containing calcein AM and ethidium homodimer 1 at a final concentration of 0.1μM [134]. The number of live and dead cells was evaluated by measuring calcein AM and ethidium homodimer-1 fluorescence (Thermo Fisher Scientific, Pittsburgh, PA) by flow cytometry (BD Accuri Cytometers, Ann Arbor, MI, USA). Calcein AM is a live cell dye that is non-fluorescent and accumulates in intact healthy cells, where it acquires a strong green fluorescence upon cleavage by intracellular esterases [134]. Because intracellular esterase activity is lost as cells die, little gain in fluorescence occurs in dying or dead cells. To detect calcein AM we used at an excitation wavelength of 488 and a 530 ± 30nm emission filter. For ethidium homodimer 1 we used an excitation wavelength of 488 and a 675 ± 25nm emission filter. Ethidium homodimer-1 is a dye that enters damaged plasma membranes and interacts with nucleic acids [134]. As this interaction increases, an increase in fluorescence indicates dying or dead cells. In each experiment 100 % cell death was established by treating the cells with 100 %

methanol and FA-induced cell death was obtained by subtracting the values obtained with BSA alone. Each of the measurement was performed in triplicate.

Measurement of Mitochondrial inner membrane Permeabilization

Cells were treated as described above. At the end of the 24 hr incubation period, the cells were lifted from the dish with the enzyme-free cell dissociation solution described above and incubated at 37°C with 0.1μM calcein AM in DPBS for 30 min, followed by 200μM $CoCl_2$ for 15 min. Mitochondrial inner membrane (MIM) permeabilization was evaluated by measuring cobalt-quenched calcein fluorescence by flow cytometry [135]. This is because calcein AM has access to most cellular compartments including the mitochondria. However, cobalt ($CoCl_2$) which quenches calcein fluorescence does not freely enter mitochondria unless rendered permeable due to permeability transition [135]. Calcein AM fluorescence was measured at an excitation wavelength of 488nm and emission through a 535 ± 30nm filter. The percent of cells experiencing quenching of calcein fluorescence was calculated by subtracting the histogram plots in the presence of calcein AM from the one obtained in its absence. Each of the measurement was performed in triplicate.

RESULTS

Pre-Exercise Fatty Acid Sensitivity did not differ between depots; GSQ FAS correlated with metabolic health markers

In our previous study analyzing FAS in primary subcutaneous preadipocytes from an older population of patients undergoing CABG surgery found for individuals to vary in their susceptibility to FA-induced cell death and PTP [151]. We wanted to determine if this variability in individual FAS was also present in a younger population of healthy individuals. Primary

preadipocytes isolated from these young subjects also varied in their FAS (Fig. 1A-B). There were no differences between gluteal depots (GSQ) and abdominal depots (ASQ), thus GSQ results was used for the remainder of the study.

One goal of this study was to assess the relationship between FAS and cardiovascular risk factors, including elevated cholesterol, LDL-c, TG, and diminished HDL-c levels. Our leading hypothesis was that FAS is a measure of adipocyte health, as the more sensitive the precursor cells are to physiologic levels of FA, the less healthy the adipose tissue is as a whole. Pre-exercise levels of cholesterol, TG, and LDL-c did not show any association with FAS; however, these was a significant negative correlation with HDL-c (Fig.1C-F). This is not unexpected as lower HDL-c levels are considered to be disadvantageous.

12-week exercise training improved FAS and lowered cardiovascular risk factors

After the 12-week exercise training regimen, adipose biopsies were again collected in abdominal and gluteal depots, of which primary preadipocytes were isolated. During the course of the study, there were 7 subjects that did not complete the full 12 weeks of training (Table 2). FAS was decreased in all but 2 of the gluteal samples (only 1 of the abdominal samples didn't reduce FAS after training) (Fig.2). Exercise training is a recommended first line approach for lowering cardiovascular risk factors prior to pharmacologic interventions. After 12 weeks of exercise training, subject's blood was again collected, and metabolic markers were measured (Table 2). Exercise training decreased cardiovascular risk factors cholesterol, and LDL-c, however had no significant effect on TG and HDL-c levels (Fig.3A-D).

We next tested whether the preadipocytes with the highest FAS post-exercise had any relationship with the changes in metabolic markers with exercise. We hypothesized that the

higher the FAS post-exercise, the less metabolic improvement exercise conferred on the subjects. However, it appears that higher post-exercise FAS actually associated with more significant metabolic improvements (Fig.4A-D). The greater the subjects improved/lowered their cholesterol, TG, and LDL-c, the higher their post-exercise FAS (Fig.4A-D). This inverse relationship of what we expected may be due to the fact that subjects with higher post-exercise FAS, also exhibited higher levels of these metabolic markers pre-exercise. It stands to reason that the higher the levels of these metabolic markers pre-exercise, the more there is to improve with exercise. So, we looked at whether these pre-exercise markers held any relationship with post-exercise FAS. Indeed, higher levels of these markers were associated with higher post-exercise FAS (Fig.4E-H). Interestingly, of the subjects who lowered their cholesterol and TG with exercise (positive responders), they also had greater improvements in their FAS as well (Supplemental Figure).

Comparing pre-exercise FAS measurements as a predicative tool for the effectiveness of exercise training on lowering cardiovascular risk factors

One aim of the study was to determine if pre-exercise FAS testing could be used as a predictive tool for metabolic improvements with exercise. Though there weren't any significant correlations for this case, there were negative trends for changes in cholesterol, TG and HDL-c levels (Fig.5A-D). The higher the subjects pre-exercise FAS, the greater the improvements (or lowering) of cholesterol and TGs with exercise. Conversely, a decrease in HDL-c concentrations is not beneficial, but the low-mid FAS readouts increased their HDL-c, which can be seen as an improvement.

One established tool for evaluating cardiovascular and metabolic health is fitness testing, or VO2 max testing. Many studies results have suggested for fitness testing to display a positive relationship with cardiovascular health and increasing the level of fitness matches with improvements in health. Our study corroborated this relationship between improvements in fitness with improvements in cardiovascular health (Supplemental Figure). However, in comparison to pre-exercise FAS measurements, fitness testing was not a predicative tool for the efficacy of exercise in lowering these cardiovascular health markers (Fig 5.E-H). Interestingly, fitness testing could be a valid tool in predicting the efficacy of improving insulin sensitivity with exercise, as subject glucose and insulin levels, as well as HOMA score, displayed negative trends with pre-exercise fitness levels (Fig.4I-K).

DISCUSSION

In this present study we evaluated whether primary subcutaneous preadipocytes from younger individuals differed in their susceptibility to lipotoxicity from a physiologically relevant FA cocktail mix. Indeed, we did corroborate previous findings of FAS differences in older populations [151]; younger individuals also varied in their FAS. Furthermore, exercise improved this FAS, along with decreasing some of the cardiovascular risk factors. We aimed to further elucidate the relationship between FAS and these cardiovascular risk factors through correlational analyses, in which we found multiple associations between the two variables before and after exercise. This study provided evidence of a relationship between our measure of FAS and cardiovascular risk factors.

After 12-weeks of exercise training, FAS in gluteal and abdominal depots was lowered in 92% (22/24), and 95% (19/20) subjects. Though molecular changes with exercise in this study

have not been elucidated, further studies to determine how this FAS is affected by exercise are in store. Some possible explanations could be in part due to exercise-induced changes at the mitochondrial level. Exercise has been suggested to decrease mitochondrial susceptibility to undergo PTP [113] [115], though there aren't any studies presently looking at this effect in preadipocytes. We have previously found for PTP to be a vital part of the mechanism of FA toxicity [27] [151], so it stands to reason that exercise-induced changes in either the molecular composition of the PTP, or causative agents (mtROS, calcium handling, etc.) could account for the improvements in FAS. Furthermore, others have found for exercise training to increase protein expression of Bcl-$_{xL}$ [157] which we have found to be a critical component of the F_1F_0 ATP Synthase and PTP [151].

One of the main goals of this study was to establish the relationship of an individual's FAS and their metabolic health. Through correlational analyses, we did find for FAS to associate with these markers of cardiovascular health. Along with pre-exercise FAS correlations with HDL-c (Fig.1F), post-exercise FAS also associated with post-exercise cholesterol, TG, and LDL-c (Supplemental figure). Additionally, post-exercise FAS negatively correlated with changes in these metabolic markers (Fig.4). This was not an expected result, however may be simply explained by the fact that these subjects had higher levels of these markers to begin with. Indeed, the higher the levels of these markers prior to exercise, the higher the post-exercise FAS. It stands to reason then that this relationship would explain why higher post-exercise FAS would correlate with greater changes in these markers: there was more to lose.

A correlation between the changes in FAS and changes in these markers with exercise would be a more beneficial finding in support of our proposed FAS-metabolic health relationship. We did not find any significance correlations between the two, however LDL and

74

HDL levels held strong trends with changes in FAS (Supplemental Figure). Furthermore, of the subjects who responded positively to exercise by lowering cholesterol and TG, they also trended to lower their FAS as well (Supplemental). This study utilized a relatively small number of subjects for analysis (pre-exercise n=35, post-exercise n=28) so additional subjects could give the statistical power to see more significant relationships.

In terms of metabolic health and the relationship of these metabolic markers, adipose tissue and the liver would appear to be a linking factor. Speculatively, the regulation of cholesterol, TG, and fatty acid metabolism by the liver and adipose tissue could be a source of this FAS and cardiovascular risk factor relationship. Dysfunctional adipose tissue, as indicated by our FAS measurements, could have cause increased serum FFA levels, and an adverse lipid profile [158]. Furthermore, this increase in serum FFA, which has been found to have detrimental effects in multiple tissues, can exacerbate this adverse lipid problem in the liver [159]. Some effect of exercise training could act on both the liver, being decreased cholesterol synthesis, increased LDL-c uptake, and also the adipose tissue: increased cholesterol, TG, and FFA storage. Further work would need to be performed to evaluate these changes with exercise.

Lastly, we aimed to determine if pre-exercise FAS could be a predicative tool for the efficacy of exercise in lowering cardiovascular risk factors. Again, we found trends supporting this relationship with cholesterol, TG, and HDL-c. However, our sample size may be limiting the power to see statistical significance. We decided to compare these results with a commonly used tool in fitness testing (VO$_2$ max). Interestingly, pre-exercise fitness testing did not show the relationships with these cardiovascular risk factors that the FAS testing did. This is supported by previous work which displayed that though weak, there were associations between blood lipids and body fatness, which may be related to FAS, and no associations between aerobic fitness and

blood lipids [110] [112]. Whereas fitness testing was not a predicative tool for cardiovascular improvements with exercise, it could be a valuable tool in predicting the improvements in insulin sensitivity with exercise. Altogether, results indicate that these two tools, FAS and fitness testing, both correlate with metabolic health and may be valid tools in determining metabolic improvements with exercise.

The magnitude of the effect of exercise is influenced by characteristics of the study design; type of exercise (chronic vs acute, endurance vs. HIIT); changes in body weight; control of diet, other physical activity, etc. In this study, we utilized a form of HIIT over 12 weeks, with an emphasis on limiting the weight loss variable. 12 weeks exercise decreased subject cholesterol and LDL-c levels. The effect of exercise on cardiovascular risk factors, which was evident in this study, can be large in some individuals, and lesser in others. Compared to current pharmacologic treatments for cardiovascular risk factors, the effect of exercise on these factors is not as significant [160], although exercise interventions have many positive components compared to pharmacological therapies including the lack of side effects. Additionally, the effects of exercise and its possible relation to FAS can be increased by other lifestyle changes such as changes in diet, and weight loss.

Pre-Exercise n=37	Mean. (+/-SD)	Post-Exercise n=31	Mean (+/-SD)	Change	p-value
Age (years)	34 (6.35)	**Age** (years)	34	-	.
BMI	35 (5.14)	**BMI**	35 (5.17)	0	.22
BF%	42 (5.53)	**BF%**	42 (5.61)	-1	.31
VO2 max (mL/kg)	3 (.75)	**VO2 max** (mL/kg)	25 (5.45)	+22	P<.0001
Glucose (mg/dL)	91 (7.78)	**Glucose** (mg/dL)	91 (8.12)	-1	.67
Insulin (uIU/mL)	16 (8.15)	**Insulin** (uIU/mL)	14 (8.51)	-2	.04
HOMA	4	**HOMA**	3 (2.08)	-1	.06
Total Cholesterol (mg/dL)	183 (30.99)	**Total Cholesterol** (mg/dL)	172 (23.76)	-11	.04
TGs (mg/dL)	111 (49.94)	**TGs** (mg/dL)	108 (47.30)	-3	.59
LDL-c	114 (26.83)	**LDL-c**	104 (23.11)	-10	.03
HDL-c	47 (12.28)	**HDL-c**	47 (11.59)	-1	.52

Table 1. Pre and.Post-Exercise Subject Anthropometric data.
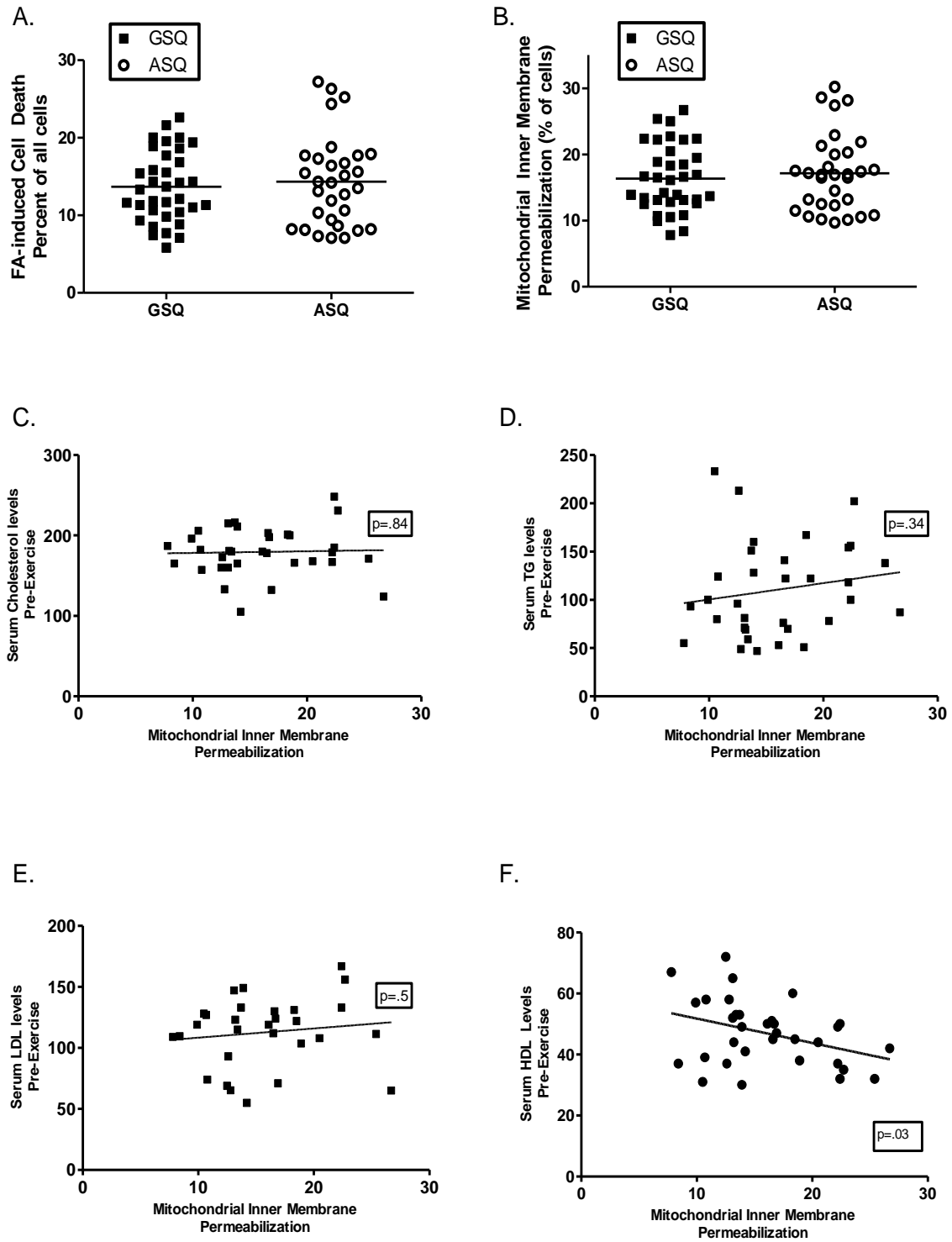
Figure 1.

Figure 1. Pre-exercise FA sensitivity (FAS) of primary preadipocytes isolated from abdominal and gluteal subcutaneous adipose depots. Pre-exercise FAS negatively correlates with HDL-c levels. Preadipocytes were treating for 24 hours with 1000µM and *(A)* FA-induced cell death, and *(B)* Mitochondrial inner membrane (MIM) permeabilization was measured after 24 hours in both preadipocytes from gluteal (GSQ, n=35) subcutaneous and abdominal (ASQ, n=30) subcutaneous depots. *(C-F)* Regression analysis was performed using the GSQ MIM permeabilization results with pre-exercise levels of serum markers; total cholesterol, triglycerides (TG), LDL-c, and HDL-c.

Figure 2.

A.

B.

C..

D.
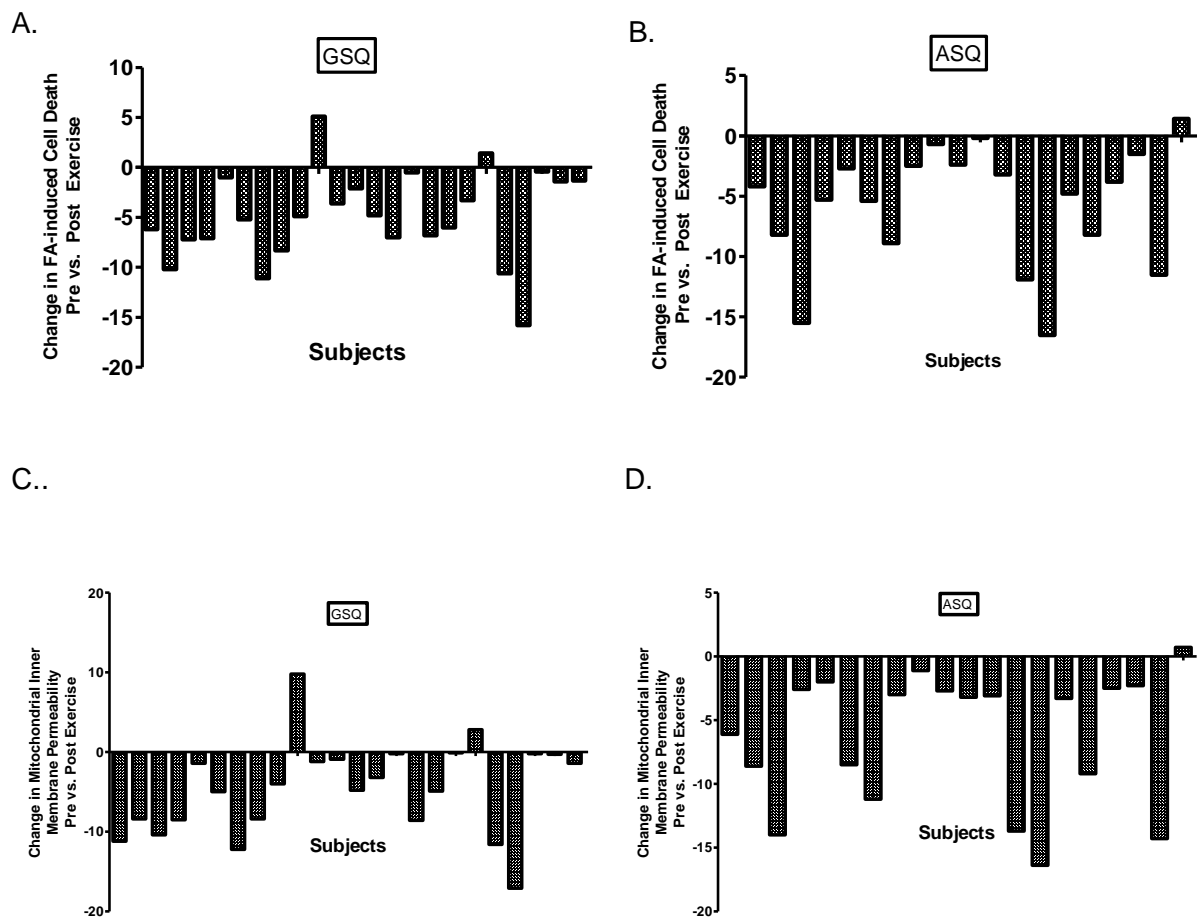
Figure 2. **Fatty acid sensitivity is decreased following 12-week exercise training**. After 12-week exercising training, primary preadipocytes were isolated from the same subjects in GSQ and ASQ depots, treated with 1000μM FA cocktail for 24 hours and measured *(A)* the change in FA-induced preadipocyte cell death (pre-exercise FA-induced cell death – post-exercise cell death) in GSQ primary preadipocytes (n=24), *(B)* ASQ primary preadipocytes (n=20), *(C)* the change in FA-induced PTP (pre-exercise FA-induced PTP– post-exercise PTP) in GSQ primary preadipocytes, and *(D)* ASQ primary preadipocytes.

Figure 3.

A.

B.

C..

D.
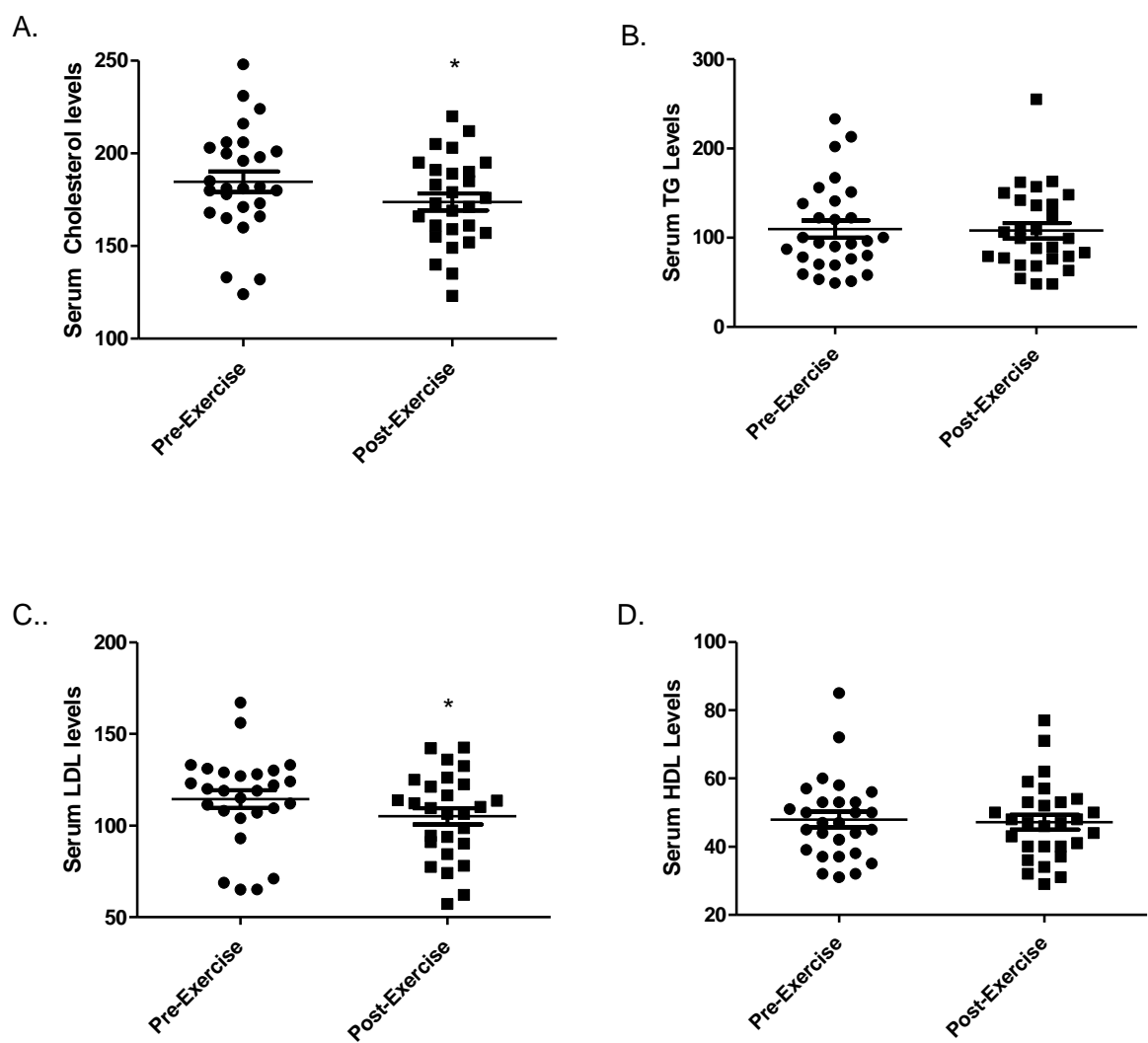
Figure 3. 12-week exercise training decreased cardiovascular risk factors. Serum markers of

(A-D) cholesterol, TG, LDL-c, and HDL-c were collected and analyzed pre vs. post exercise

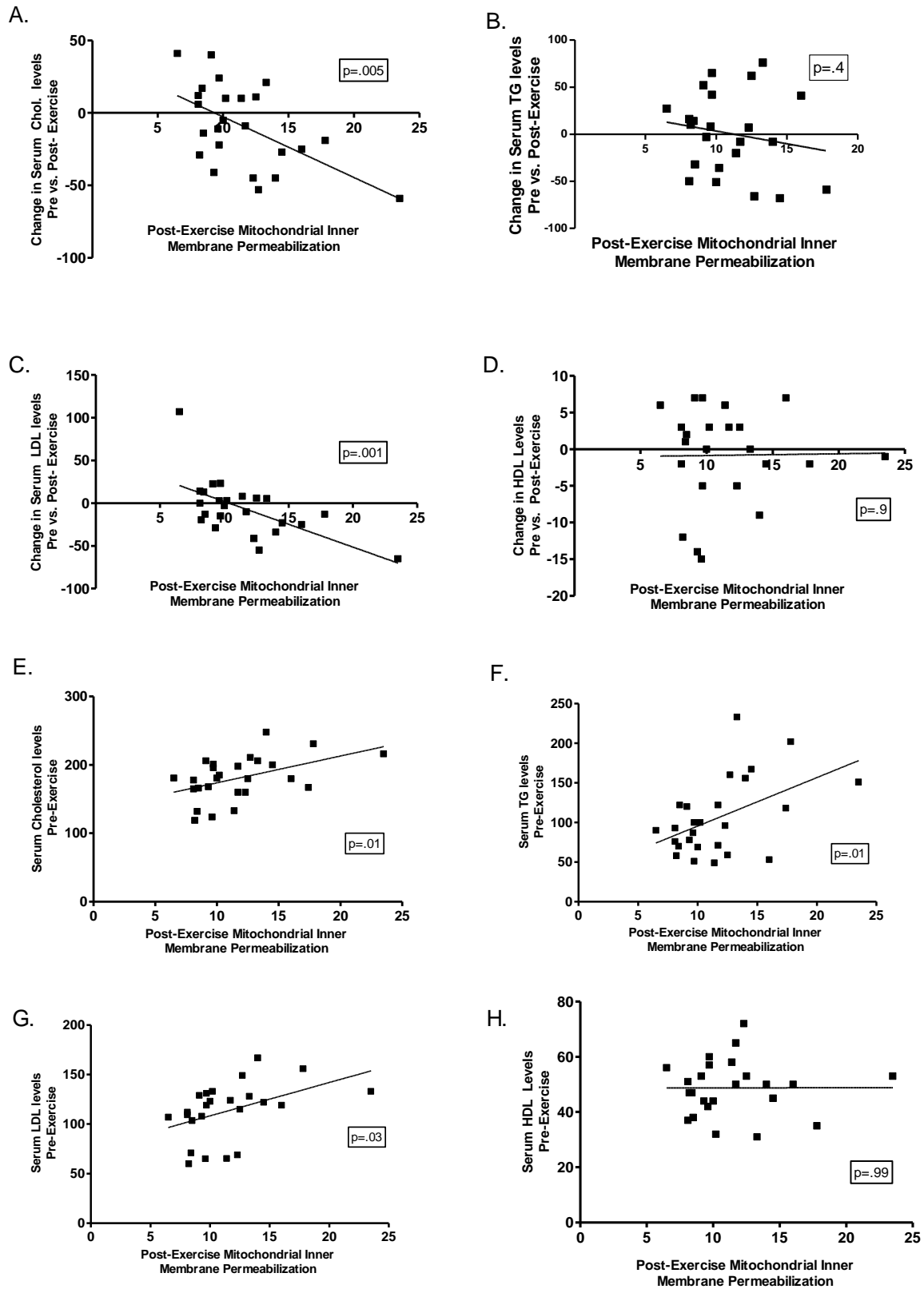(n=28). Results were analyzed via paired t-tests. ** P<.05.*
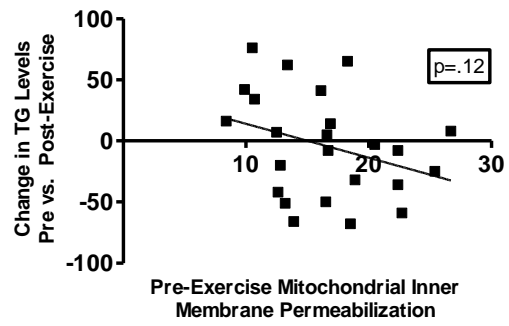
Figure 4.

Figure 4. Post-exercise FAS correlates with changes in serum markers of cholesterol and LDL-c with exercise. Preadipocytes were isolated after exercise from patient subcutaneous adipose tissue, were treated with 1000µM FA for 24 hours and MIM permeabilization was measured. *(A-D)* Post-exercise FAS correlations with the change in serum levels cholesterol, TG, LDL-c, and HDL-c (post-exercise – pre-exercise) (n=23). Pre-exercise serum levels of cholesterol, TG, and LDL-c correlate with post-exercise FAS *(E-H)* Post-exercise FAS correlations with pre-exercise serum levels of cholesterol, TG< LDL-c, and HDL-c (n=25). All results were analyzed via regression analysis.
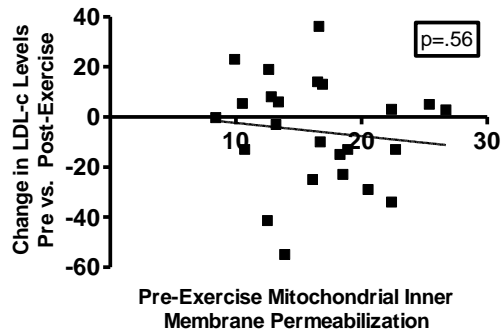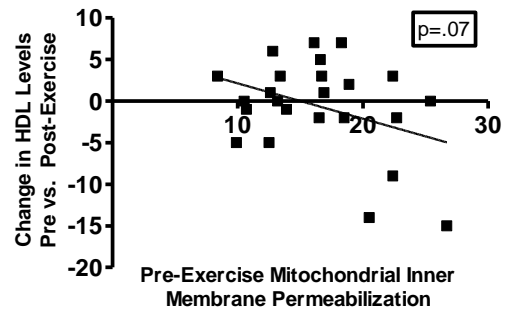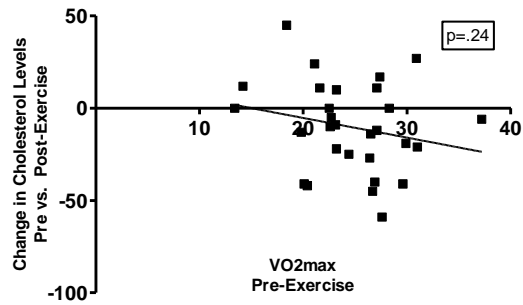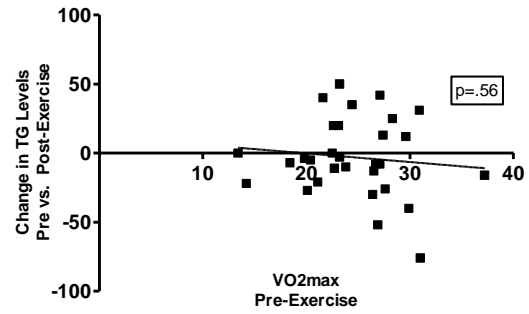
Figure 5.

E.

F.

G.

H.

I.

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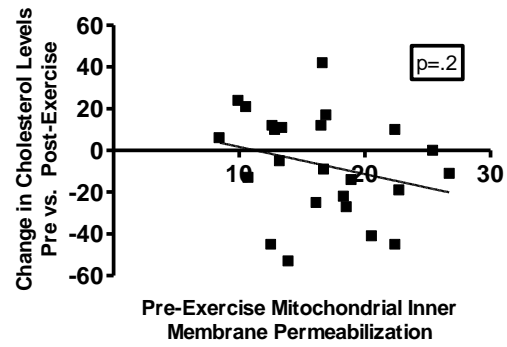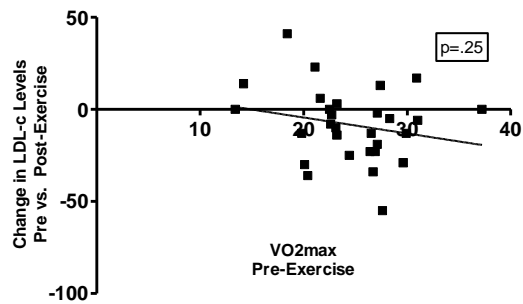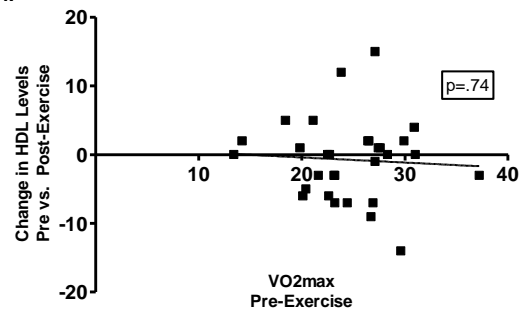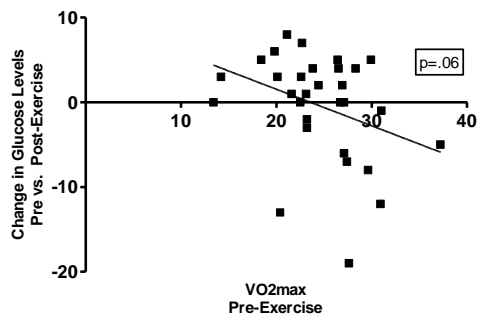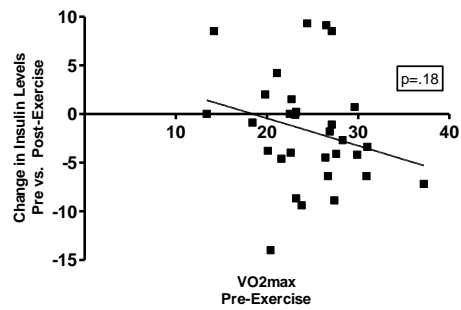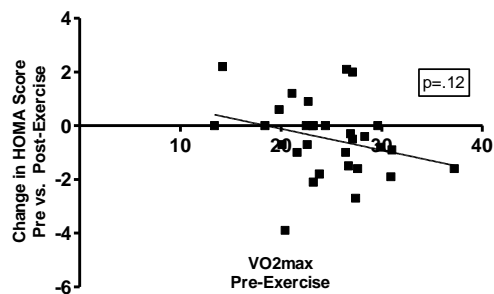
Figure 5. Pre-exercise FAS relationship with changes in metabolic markers with exercise.

(A-D) Pre-exercise FAS relationship with the change in serum levels cholesterol, TG, LDL-c, and HDL-c (post-exercise – pre-exercise) (n=23). *(E-H)* Pre-exercise VO_2 max (mL/kg) relationship with the change in serum levels cholesterol, TG, LDL-c, and HDL-c (post-exercise – pre-exercise) (n=30). *(I-K)* Pre-exercise VO_2 max correlations with the change in subject glucose levels, insulin levels, and HOMA score (post-exercise – pre-exercise) (n=30). All results were analyzed via regression analysis.

SUPPLEMENTAL FIGURES

A.
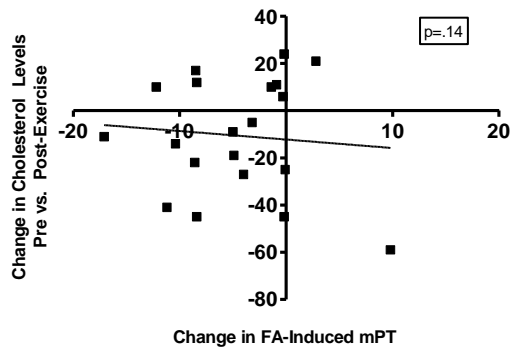
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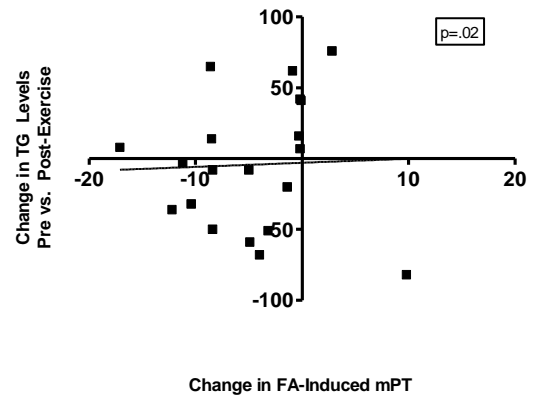
C.
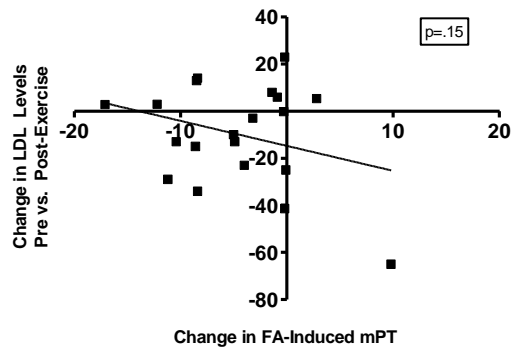
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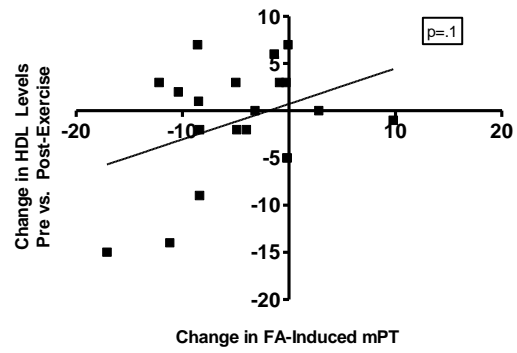
Supplemental Figure 1. Relationship between change in FAS and change in metabolic markers after exercise. Post-exercise MIM permeabilization minus pre-exercise MIM permeabilization was calculated and analyzed vs the change in serum markers of *(A-D)* cholesterol, TG, LDL-c, and HDL-c (post-exercise levels minus pre-exercise levels). All results were analyzed via regression analysis.

A.

Cholesterol Non-responders

Change in Mitochondrial Inner Membrane Permeability Pre vs. Post Exercise

+ chol   - chol

B.

TG non-responders

Change in Mitochondrial Inner Membrane Permeability Pre vs. Post Exercise

+TG   -TG
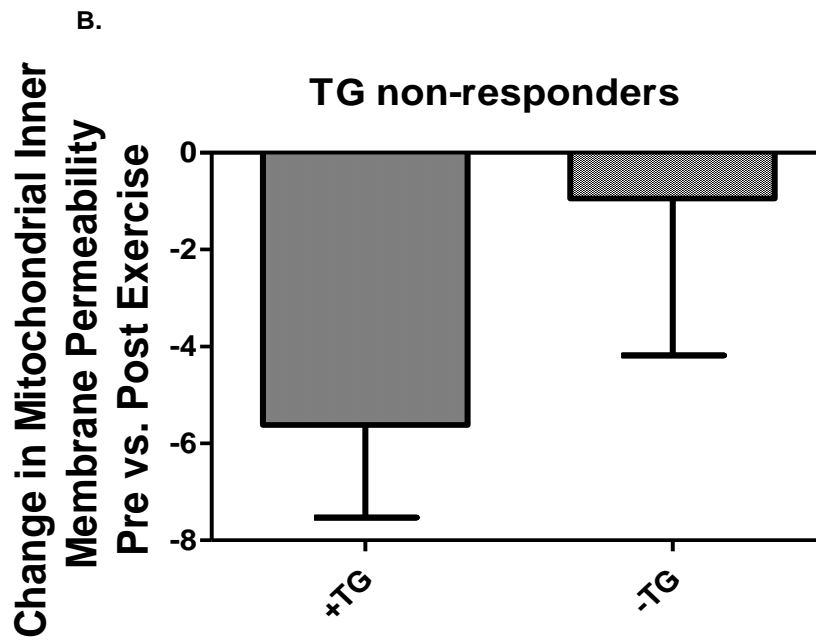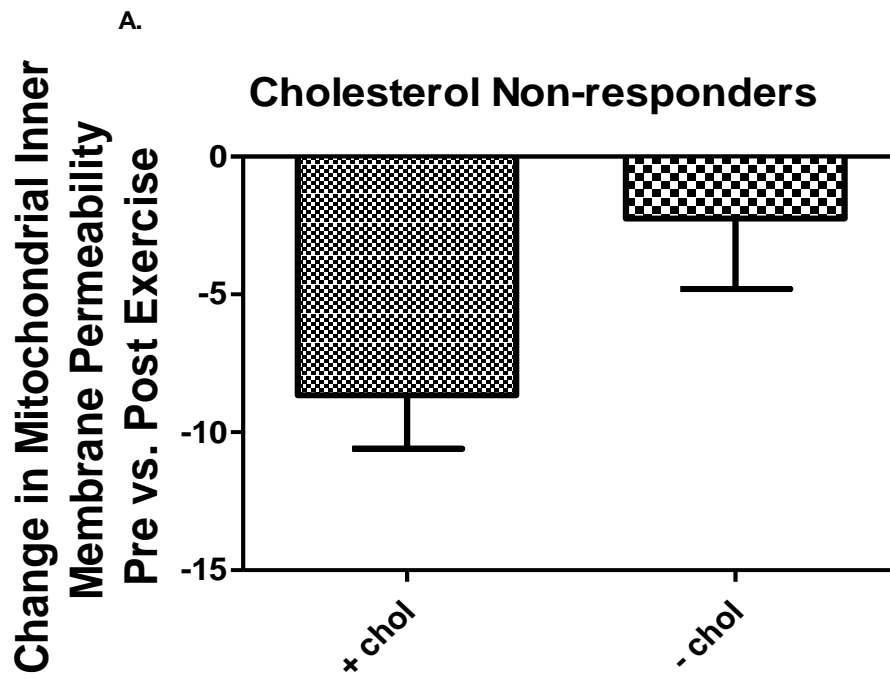
Supplemental Figure 2. Relationship of Positive/Negative Responders to Exercise vs. Changes in FAS with exercise. MIM permeabilization changes post-exercise – pre-exercise was calculated in individuals who *(A)* lowered cholesterol (positive (+ chol) responder, or increased cholesterol post-exercise (negative (- chol) responder) n=7 in each group, and *(B)* who lowered TG (positive (+ TG) responder, or increased TG (negative (- TG) responder post-exercise) n=6 in each group.

FINAL DISCUSSION

The prevalence of obesity and related diseases of the metabolic syndrome is increasing exponentially, creating a public health and clinical need to improve understanding of the onset of obesity, and progression to metabolic diseases. It is known that FA levels rise during times of chronic positive energy imbalance and obesity, however how these elevated FA levels may cause detriment is still under investigation. Collectively, this dissertation sought to elucidate this gap in knowledge. The overall objective of this dissertation was to further determine the mechanism of FA-induced mitochondrial dysfunction and lipotoxicity in human primary preadipocytes, and if/how individuals differ in susceptibility to this FA toxicity.

In the first study, subcutaneous adipose tissue biopsied from human patients undergoing CABG surgery was used to investigate how primary preadipocytes from different individuals respond to prolonged FA exposure, and also to further FA-induced mitochondrial dysfunction knowledge by focusing on the PTP. The findings of this study support that preadipocytes from different individuals indeed do respond differently to FA exposure, which is hypothesized to play a role in metabolic health determination (MHO vs. MUO). Preadipocytes that were more sensitive to FA (FAS) were isolated from individuals that trended to be more obese compared to FAR counterparts, however there wasn't a significant increase in BMI (p=.08) or A1C. However, the goal of this study was to study the mechanistic differences of FA sensitivity, not the clinical correlations. These findings do call for future studies examining aspects of adipocyte turnover and its role in FA sensitivity, and any clinical correlations. The first step to address this, on top of increasing the number of patients in the study, is to measure differentiation ability of the preadipocytes between these FAS and FAR subjects.

Furthermore, we provide evidence of a snapshot of the components of the PTP, as MIM permeabilization was CypD-dependent, and Bcl-$_{xL}$ sensitive. Though a few studies have studied Bcl-$_{xL}$ and its relationship to the F_1F_0 ATP synthase in neurons, we are the first to confirm that Bcl-$_{xL}$ is bound to the F_1F_0 ATP synthase in preadipocytes, and in some part modulates PTP activity, expanding on its prior known anti-apoptotic roles.

Together the first study furthered data mechanistically on FA-induced mitochondrial dysfunction. Prior work in our lab started to elucidate this mechanism in an immortalized preadipocyte cell line [27], in which we confirmed in primary human preadipocytes. Prolonged FA exposure did increase mitochondrial ROS and preventing this oxidative stress through mitochondrial-specific antioxidants MitoQ and MitoTempo resulted in attenuated FA cytotoxicity. What is striking about these findings is the degree to which PTP and cell death was prevented, nearly 100%, which was the greatest mito/cytoprotective effect observed out of all our interventions, aside from CsA. Interestingly, MitoQ and MitoTempo each increased ATP regeneration at levels greater than baseline. Other studies have suggested for MitoQ to cause an uncoupling effect on respiration, which lowers ATP synthesis in comparison [161]. In this dissertation however, we did not measure the effect of these antioxidants on mitochondrial respiration. Further work examining this observation might add to the benefits of these antioxidants, and the push for the use of these supplements in self-treatment.

Modulating FA/acetyl-CoA shuttling in/out of the mitochondria appears to play a significant role in FA-induced mitochondrial dysfunction. FA caused mitochondrial hyperacetylation, and acetylation of the F_1F_0 ATP synthase. Previous work has shown for SIRT3-mediated deacetylation of CypD to induce dissociation of CypD binding within the mitochondria however we did not measure specific CypD acetylation [130] [162]. Additionally, Bcl-$_{xL}$ has

94

been shown to modulate acetyl-CoA levels further preventing cell death, possibly through its metabolic effects [163]. Therefore, assessing the interplay between CypD-Bcl-$_{xL}$ and acetylation-specific effects in the future will be of value to further elucidate the mechanism of PTP induction.

FAR preadipocytes displayed greater SIRT3 mRNA, which may account for reduced mitochondrial acetylation, and possibly reduced PTP and cell death. Measuring SIRT3 activity, as well as NAD$^+$ levels in these groups would provide valuable insight into SIRT3's role in protecting against conditions featuring elevated FA.

L-carnitine, a substrate for CPT1-induced FA/acetyl-CoA transport in/out of the mitochondria, alleviated mitochondrial hyperacetylation, and resulted in PTP prevention, as well as preadipocyte cell death. Furthermore, trapping acetyl-CoA inside the mitochondria via Mildronate further exacerbated mitochondrial hyperacetylation and FA-induced mitochondrial dysfunction. Together the first study provides some evidence for the further exploration of therapeutic use of L-carnitine, targeting CRAT, and/or mitochondrial-specific antioxidants, in alleviating conditions resulting from elevated FAs, PTP, and/or mitochondrial oxidative stress. Whether L-carnitine's protective effects were due to increased shuttling into the mitochondria for energy use or shuttling excess acetyl-CoA out of the mitochondria to reduce mitochondrial acetylation, is unclear at this time.

One drawback of the first study was that in theory, all the participants can be considered to be ultimately metabolically unhealthy as they were undergoing open heart surgery, discrediting any relationship between FA-sensitivity and metabolic health. So we next furthered our findings on individual differences in FA-sensitivity in a younger, healthier population.

In the second study, we aimed to confirm our previous findings in the older patients, with younger patients undergoing 12-week exercise training. We also aimed to determine if exercise affects fatty acid sensitivity, and if there's a correlation with the metabolic health of these participants, pre and post-exercise. Indeed, we did confirm our earlier findings for preadipocytes isolated from 35 different individuals to vary in their sensitivity to FA-induced cell death, though there were not any differences among abdominal (ASQ) and gluteal (GSQ) subcutaneous depots. FA-sensitivity negatively correlated with pre-exercise subject HDL-c levels, however didn't show any significant relationships with cholesterol, LDLc, or TG prior to exercise.

After 12 weeks of exercise training, 92% - 95% of subjects displayed improved FA-sensitivity in their isolated preadipocytes. This effect could be due to a number of reasons including exercise-induced increases in FA-utilization, or greater sirtuin deacetylase activity in the mitochondria [164] [165]. Exercise training also significantly lowered subject cholesterol and LDL-c levels. Unexpectedly, these improvements were correlated with higher post-exercise FA-sensitivity. We hypothesized FA-sensitivity would be driving metabolic health, however it is possible that pre-exercise levels of lipids could be determining FA-sensitivity. This was seen in a correlation of subjects with higher pre-exercise levels of these lipid markers and higher post-exercise FA-sensitivity. In the future this relationship could be further investigated substituting cholesterol-lowering agents in place of exercise to determine their effect on FA-sensitivity.

A goal of the study was to determine if pre-exercise FA-sensitivity could be used as a predicative tool in determining the efficacy of exercise training on metabolic health. There were trends for pre-exercise FA-sensitivity to correlate with greater improvements in cholesterol and TG, however we did not reach the power to see significance. All of the subjects in the exercise study were considered metabolically healthy, so in the future it would be interesting to determine

the effects of exercise training, and relationship of FA-sensitivity in subjects with some metabolic disturbance (insulin resistance, hyperlipidemia, etc.). Also, it would be worthwhile to determine the role of Bcl-$_{xL}$, CypD, and the FA-acetyl-CoA transport system serves in the determination of FA-sensitivity in these younger individuals. There's plenty of evidence of age-related decline in NAD$^+$ and sirtuin activity, making it interesting to determine if mitochondrial acetylation which we observed in the first study, could be playing as much of a role in these younger individuals.

Altogether, this dissertation furthered knowledge of the mechanism of FA-induced mitochondrial dysfunction and lipotoxicity in human primary preadipocytes. However, it is important to note that whether this the observed cell death in an in-vivo atmosphere is good, or bad, has not been elucidated. It's known that cell death can serve a physiologic role and can be beneficial for a system as a whole. Our hypothesis was that observed cell death is a negative consequence of elevated FA exposure. However, it stands to reason that this cell death could possibly be a protective feature of the adipose atmosphere; ridding adipose tissue of these unhealthy precursors, possible to make way for new healthy preadipocytes and concurrent adipogenesis. However, it is the belief of this dissertation that the chronic "killing off" of a high number of preadipocytes decreases the adipogenic potential of the adipose organ system, and hence leads to metabolic detriment

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