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Lipotoxicity in diabetic cardiomyopathy

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Résumé

Le diabète est en croissance à un rythme alarmant. Sa fatalité la plus importante provient de ses effets sur le système cardiovasculaire. Effectivement, le diabète est un facteur de risque majeur pour la maladie coronarienne et l'hypertension artérielle. En plus, les diabétiques courent le risque de développer une cardiomyopathie diabétique (DCM) qui est présente indépendamment de l'athérosclérose et de l'hypertension. Les causes exactes de cette maladie cardiaque ne sont pas encore définies complètement mais une anomalie du métabolisme lipidique a émergé comme étant un facteur contributeur clé. Il est intéressant de noter qu'une caractéristique commune de la DCM est l'accumulation des lipides intracellulaires ce qui est connu comme la stéatose cardiaque. Cette dernière est probablement causée par un déséquilibre entre l'absorption et la clairance cellulaires des lipides. En tant que tel, nous nous sommes concentré nos études sur l'élucidation des mécanismes de lipotoxicité dans le contexte de la DCM. Nous avons donc investigué le rôle du métabolisme lipidique sur des voies de signalisation reliées à la DCM telles que le stress du réticulum endoplasmique (ERS), l'activité du Récepteur activé par les proliférateurs de peroxysomes (PPAR), l'inflammation et la dysfonction mitochondriale avec un intérêt spécifique sur l'oxydation des acides gras.

Nous avons caractérisé l'ERS et l'apoptose induits par les lipides; nous avons confirmé que le palmitate, un acide gras lipotoxique, induit l'ERS et la mort des cardiomyocytes primaires. Ensuite, on a démontré que la lipotoxicité médiée par cet acide gras est associée à un degré d'inflammation significatif qui peut être dû à une régulation à la baisse des récepteurs de PPAR.

La stéatose cardiaque et la lipotoxicité peuvent altérer des voies de signalisation ce qui conduit à la dysfonction mitochondriale. On a découvert que la toxicité du palmitate est associée à une déficience de l'oxydation complète des acides gras (FAO). Spécifiquement, le palmitate atténue la β-oxydation et le cycle de l'acide citrique sans toucher à l'activité de Cpt1b qui est l'étape cinétiquement déterminante dans la FAO. Il est intéressant de noter que l'augmentation de la FAO atténue les effets toxiques du palmitate, alors que l'atténuation de la FAO de l'oléate, qui est normalement un acide gras non toxique, induit la mort cellulaire. Ces résultats suggèrent que l'augmentation de la FAO peut avoir des effets cliniques utiles comme être une cible pour le traitement de la DCM.

Nous avons également cherché à savoir si la déficience en FAO contribue à la DCM *in vivo*. Des études précédentes ont démontré que la FAO augmente chez les souris diabétiques. Cependant, les souris diabétiques montrent une absorption élevée des acides gras ce qui pourrait contribuer significativement à l'augmentation de la FAO dans leurs tissus cardiaques. Par la suite, nous avons tenté d'évaluer la FAO directement des mitochondries au lieu des tissus cardiaques complets afin d'éviter le facteur de confusion, qui est l'augmentation de l'absorption des acides gras. Nous avons démontré alors que les souris atteintes de diabète chronique présentent une déficience en FAO accompagnée d'une dysfonction cardiaque. En revanche, les souris atteintes de diabète aigu possédaient des fonctions cardiaques normales associées de taux normaux de FAO.

Ensemble, ces études ont amélioré notre compréhension des mécanismes de lipotoxicité associée à la DCM et ils soulignent l'importance de l'ERS, de l'inflammation et de la dysfonction mitochondriale comme des facteurs clefs dans la promotion de la lipotoxicité dans la DCM.

Mots-clés : acides gras, cardiomyopathie diabétique, lipotoxicité, oxydation des acides gras, stress du réticulum endoplasmique, diabète type 2, stéatose, cardiomyocytes.

Abstract

Diabetes is growing at an alarming rate in North America. The biggest killer of patients with diabetes is heart disease. Indeed, diabetes is a major risk factor for both coronary artery disease and hypertension. However, patients with diabetes are also at risk for developing diabetic cardiomyopathy (DCM), which is characterized as heart disease in the absence of atherosclerosis and hypertension. The exact causes of this diabetic heart disease has not been completely elucidated but abnormal lipid metabolism has emerged as a key contributing factor. Interestingly, a common characteristic of DCM is the accumulation of intra cellular lipids otherwise known as cardiac steatosis. Cardiac steatosis is likely caused by a mismatch between lipid uptake and lipid clearance from the cells. As such, we focused on elucidating the mechanisms of lipotoxicity in the context of diabetic cardiomyopathy. To this end, we investigated the role of lipid metabolism on key pathways related to diabetic cardiomyopathy including Endoplasmic Reticulum (ER) stress, Peroxisome proliferator-activated receptors (PPARs) activity, inflammation, and mitochondrial dysfunction with a specific focus on fatty acid oxidation.

We characterized lipid induced ER stress and apoptosis *in vitro*, using primary neonatal cardiomyocytes. We were able to confirm that palmitate, a lipotoxic fatty acid, induces ER stress and cell death in primary cardiomyocytes. We also demonstrated that palmitate mediated lipotoxicity is associated with a significant degree of inflammation which may be due to down-regulation of PPAR receptors.

Cardiac steatosis and lipotoxicity can alter metabolic signaling pathways leading to mitochondrial dysfunction. We discovered that palmitate toxicity is associated with an impairment of complete fatty acid oxidation (FAO). Specifically, palmitate impairs β-oxidation and citric acid cycle simultaneously with no effect on Cpt1b activity, the rate limiting step in FAO. Interestingly enhancing FAO attenuated the toxic effects of palmitate while inhibiting FAO caused oleate, which is normally non-toxic, to induce cell death. These results suggest that enhancing FAO might have some clinical utility as a therapeutic target for the treatment of diabetic cardiomyopathy.

As such, we have investigated whether impaired fatty acid oxidation might contribute to DCM pathology *in vivo*. Previous studies have shown that FAO is actually increased in diabetic mice. However, diabetic mice also exhibit elevated fatty acid uptake which could significantly contribute to the elevated FAO rates in these hearts. Therefore, we aimed to assess FAO rates directly from isolated mitochondria instead of whole hearts to exclude the potentially confounding factor of enhanced fatty acid uptake. We found that older (chronic) diabetic mice exhibited impaired fatty acid oxidation rates and this was associated with ER stress and impaired cardiac function. In contrast, acutely diabetic mice had normal cardiac functions which was associated with normal FAO rates.

Taken together, these studies have furthered our understanding of lipotoxic mechanisms in the context of diabetic cardiomyopathy and they accentuated the importance of ER stress,

inflammation, and impaired mitochondrial function as key factors promoting lipotoxicity in diabetic cardiomyopathy.

Key words: Fatty acids, Diabetic cardiomyopathy, Lipotoxicity, fatty acids oxidation, Endoplasmic reticulum stress, type 2 diabetes, steatosis, cardiomyocytes.

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List of abbreviations and acronyms:

SA	Sinoatrial Node
AV	Atrioventricular Node
FAO	Fatty acid oxidation
PDH	Pyruvate dehydrogenase
AMPK	AMP-activated protein kinase
ACC	Acetyl-CoA carboxylase
RXR	Retinoid x receptor
PPRE	PPAR response elements
ALA	Alpha linolenic acid
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acids
LPL	Lipoprotein lipase
CACT	carnitine/acylcarnitine translocase
ACAD	Acyl CoA dehydrogenase
NADH	Nicotinamide adenine dinucleotide
FADH2	Flavin adenine dinucleotide
CAC	Citric acid cycle
DAG	Diacylglycerol
LD	Lipid droplets
IP3	Inositol trisphosphate

PIP2	Phosphatidyl inositol bisphosphate
OGTT	Oral Glucose Tolerance Test
T1DM	Type 1 diabetes
T2DM	Type 2 diabetes
MODY	Maturity Onset Diabetes of the Young
NDM	Neonatal Diabetes Mellitus
SNP	Single nucleotide polymorphism
CAPN10	Calpain-10
TCF7L2	Transcription factor 7-like 2
GLUT4	Glucose transporter type 4
IRS	Insulin receptor substrate
NEFA	Non-esterified fatty acids
PKC	Protein kinase C
JNK	c-Jun N-terminal Kinase
PTEN	Phosphatase and TENsin homolog deleted on chromosome ten
SHIP	Src Homology 2 domain-containing Inositol 5-Phosphatase
AGE	Glycation end-products
RAGE	Receptor for advanced glycation end products
ROS	Reactive oxygen species
DCM	Diabetic cardiomyopathy
SERCA	sarco/endoplasmic reticulum Ca2+-ATPase
NCX	Sodium-Calcium exchanger
RyR2	Ryanodine Receptor 2

CaMK	Ca2+/calmodulin-dependent protein kinase
PLB	Phospholamban
PKA	Protein kinase A
HSL	Hormone sensitive lipase
apoB	apolipoprotein B
VLDL	Very low-density lipoproteins
LDL	Low-density lipoprotein
CD36	Cluster of Differentiation 36
FATP1/6	Fatty acid transport protein 1/6
FABPpm	membrane associated fatty acid binding protein
GPI	Glycosylphosphatidylinositol
TZD	Thiazolidinediones
STZ	Streptozotocin
MAPK	Mitogen-activated protein kinase
NF-κB	Nuclear factor kappa B
PI3K	Phosphoinositide 3-kinase
RNS	Reactive nitrogen species
GFAT	glutamine:fructose-6-phosphate amidotransferase
ER	Endoplasmic reticulum
GRP78	Glucose-related peptide 78
UPR	Unfolded Protein Response
PERK	Protein kinase R (PKR)-like endoplasmic reticulum kinase
ATF6	Activating Transcription Factor 6

IRE1	Inositol Requiring Enzyme 1
XBP-1	X-box binding protein 1
elF2-α	Eukaryotic Initiation Factor 2 alpha
ATF4	Activating Transcription Factor 4
UTR	Open Reading Frame (ORF) in their 5' untranslated region
CHOP	CAAT/Enhancer binding protein (C/EBP) homologous protein
S1P	site 1 protease
S2P	site 2 protease
CREB	cAMP response element binding protein
CRE	cAMP response element
UPRE	Unfolded protein response element
ERSE	ER stress response element
ERAD	Endoplasmic-reticulum-associated protein degradation
SR	Sarcoplasmic reticulum
PP1	Protein Phosphatase 1
p38 MAPK	p38 mitogen-activated protein kinases
BI-1	Bax Inhibitor-1
IKK	IκB kinase
TLR-4	Toll Like Receptor 4
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
LCAD	Long-chain acyl-CoA dehydrogenase
CD95	Cluster of Differentiation 95
TRAIL	TNF-related apoptosis-inducing ligand

TL1A	TNF-like ligand 1A
TNFR1	TNF-receptor 1
DR-3	Death receptor 3
FADD	Fas associated death domain
TRADD	TNFR-associated death domain

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Chapter 1: Introduction and literature review

1.1 The heart

1.1.1 Heart anatomy

The heart is a vital organ in the human body. This hollow intrathoracic muscular organ has the role of pumping blood to the blood vessels of the body through its rhythmic contractions. The heart is made up of left and right sectors, each of which consists of two cavities: an atrium and a ventricle. The four cavities thus distinguish a "right heart" and a "left heart," which normally do not communicate with each other (Figure 1.1). The role of the atria is to receive the blood and then redistribute it to the ventricles, while the role of the ventricles is to provide the force necessary to propel the blood towards the organs. The left and right ventricles are separated by a septum called the interventricular septum (1).

The heart has four valves, which are non-muscular elastic membranes. Two separate each atrium from the adjacent ventricle and are called atrioventricular valves. A third valve called the aortic valve separates the left ventricle from the aorta, while the fourth valve, the pulmonary valve, is located between the right ventricle and the pulmonary artery. These valves prevent the blood from going backward and thus preventing backflow (2).

The heart wall is made of three different layers: 1) endocardium, 2) myocardium, and 3) pericardium. The endocardium is a sheet of epithelial tissue found in the inner layer, which is in direct contact with the blood thus preventing it from clotting. The myocardium is the heart muscle and the thickest component of the heart wall. It is the only part of the heart wall that contracts, and it is made mainly from cardiac muscle cells. The myocardium is composed of identifiable thick and thin filaments organized in sarcomeres that give it striated appearance like skeletal muscle (3). Finally, the pericardium is a double-walled sack that contains the pericardial fluid whose role is to prevent friction during the heartbeat, while giving the heart sufficient freedom of movement to achieve rapid and vigorous contractions. Additionally, the heart is shaped by connective tissue made from collagen, among other cells. Since it does not conduct electric signals, it provides a boundary for the heart's electrical conduction system.

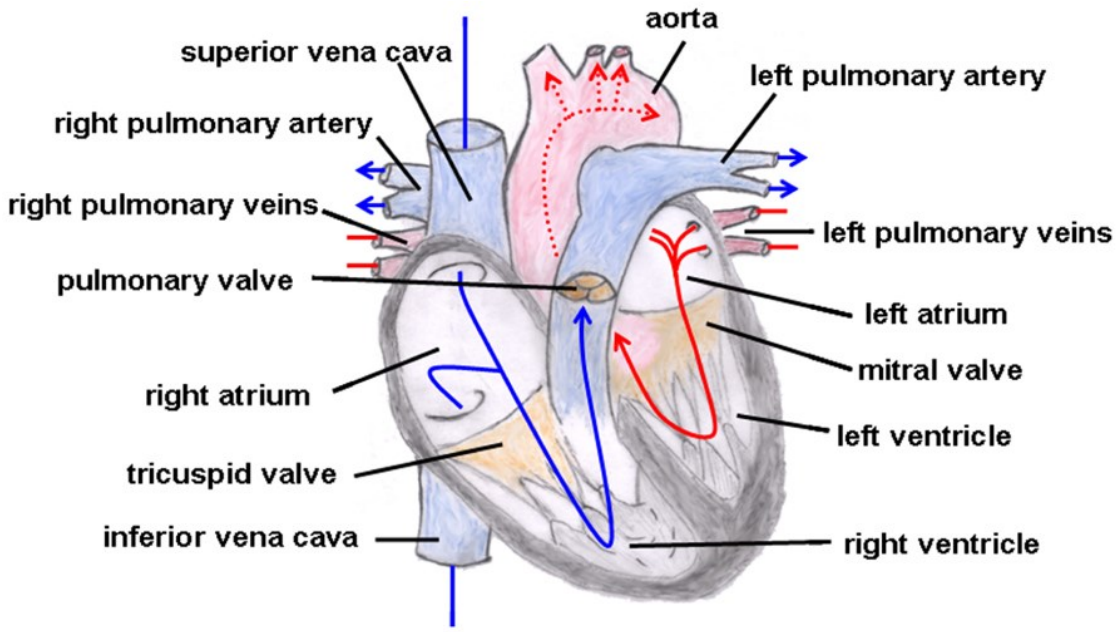

Figure 1.1: Heart anatomy. The arrows indicate the direction of blood flow in the heart (4).

Cardiomyocytes and fibroblasts represent the majority of heart cells (5). Endothelial cells, vascular smooth muscle cells, and different stem cells account for the rest. In adult rat hearts, cardiomyocytes represent around 25% of total cells and almost 80% of total heart volume (5). Cardiomyocytes contract simultaneously because, unlike other muscle cells, they are connected to each other by intercalated discs. The electrical resistance between adjacent myocytes is low because of gap junctions within the intercalated disks. These gap junctions allow ions to move freely between two cardiac cells, enabling the cells to contract simultaneously, since the electrical signal can reach all cells in few milliseconds.

1.1.2 Heart physiology

The heart pumps blood across the body through two circulations called the systemic circulation and the pulmonary circulation. The systemic circulation consists of transporting blood full of oxygen and nutrients to the peripheries before transporting blood full of CO_2 and waste out of the peripheral organs (6). The pulmonary circulation consists of transporting blood between the lungs and the heart.

Blood transport function in a cycle where the heart is at its center. The cycle starts with venous, deoxygenated blood returning to the right atrium via the superior and inferior vena cava. The blood continues to the right ventricle followed by the lungs via the pulmonary valve. The pulmonary valve is connected to the pulmonary trunk, which splits into the right and left pulmonary arteries. These two arteries transport deoxygenated blood to the right and left lungs

respectively. It is important to note that the term “arteries” indicates the blood vessels coming out of the heart independently of whether the blood is oxygenated or deoxygenated. The oxygenated blood returns to the heart via the left and right pulmonary veins and fills the left atrium. Once the left atrium is filled, the blood passes to the left ventricle via the mitral valve, which then ejects it into the systemic circulation through the aorta. Interestingly, heart cells are not supplied with oxygen and nutrients by either of the two circulations but by a circulation specific to them: the coronary circulation. This circulation, which is irrigated during the diastole, is formed of two coronary arteries (right and left) that are derived from the aortic artery and divide into a network bringing nutrition to the cardiac cells (7). Each day, the heart pumps the equivalent of 8,000 liters of blood, the equivalent of 100,000 heartbeats.

Each heartbeat consists of three major movements: atrial systole, ventricular systole (contraction) and ventricular diastole (relaxation). During the atrial systole, the atria pump blood to the ventricles (though almost 70% of the blood flows passively). During the ventricular systole, which is the strong contraction, the ventricles contract and eject the blood accumulated from atrial systole to different organs and peripheries through the arteries. This step is followed by a rest phase known as diastole where the pressure in the ventricles decreases, allowing the filling of the atria. The rhythm, frequency, and rate of this cycle are regulated by two nervous systems known as the sympathetic and parasympathetic nervous system. These two systems influence the conduction system of the heart.

The conduction system, which generates and distributes electrical impulses, is composed of specialized cardiac muscle cells found in the heart wall. It consists of: the sinoatrial node (SA),

the atrioventricular node (AV), the bundle of His, the bundle branches, and the Purkinje fibers. The SA node, or sinus node is where the heartbeat generates; it is considered the heart's natural pacemaker (3). The cells of the SA node can spontaneously depolarize and generate an electrical impulse that reaches the left and right atria. The AV node receives the electrical signal from the SA node, then transmits the signal to the ventricles. The AV node is the only pathway by which the depolarization can pass from the atria to the ventricles. The conduction of the electrical impulse through the AV node is slow enough to prevent the atria and ventricles from contracting and filling up with blood simultaneously. The His bundle, which is an extension of the atrioventricular node, is divided into two branches, each of which is divided into a network of fibers called the Purkinje fibers. The Purkinje cells are myocytes, not nerve cells, that conduct the depolarization to all part of the ventricles. The sinus node, the bundle of His and the Purkinje network are the centers of the autorhythmicity of the cardiac fiber, each having their own intrinsic rhythm (3). They can generate their own potential action and dictate the heart rhythm. The one with the fastest rhythm dictates the rhythm of the others, since their slower rhythms cannot be imposed. The SA node is the primary center and controls the heart rate in normal conditions. The bundle of His and the Purkinje network are secondary centers that can only impose their rhythm if the SA node is failing.

Propagation of the electrical impulse from the sinus node to cardiomyocytes induces their contraction. This contraction, which is well paced and synchronized, is partially modulated by the sympathetic and parasympathetic nervous systems. The sympathetic system increases the heart rate, while the parasympathetic system decreases it.

Cardiac cells possess one or several electrophysiological properties, including automaticity, excitability, conductivity, contractility and refractoriness.

Automaticity is controlled by the conduction system which represents less than 1% of heart cells. However, all myocardial cells can conduct an electrical impulse. Excitability is defined by the cells ability to react to an electrical, mechanical, or chemical stimulus and convert it into a mechanical function such as contraction (3). Excitability depends on the refractory period, which is defined as the inability of cardiomyocytes to respond to any stimulus, cancelling its excitability. The refractory period in cardiomyocytes spans the depolarization phase, the plateau phase, and most of the repolarization phase (about 250 ms). Due to the refractory period, cardiac cells cannot contract until the end of systole. This protects the heart from arrhythmias and helps coordinate the heart contractions (8).

1.2 Cardiomyocytes

1.2.1 Description, histology and contraction

Cardiomyocytes are rod-shaped cells responsible for cardiac contraction. They are about 80-140μm long and 15-25μm in diameter, and usually have a single nucleus located in the center. In human hearts, binucleated, trinucleated, and tetranucleated cardiomyocytes can also be found (9). Like other striated muscle cells, cardiomyocytes consist mainly of myofibrils, wherein the contractile units of the cell are found: the sarcomeres. Myofibrils are made up of mainly three proteins: 1) actin, 2) myosin, and 3) titin. These proteins are organized into two types of

filaments: 1) thick and 2) thin filaments. Multiple myosin molecules are clustered together to form each thick filament while thin filaments are made from actin and other proteins. The striated appearance of cardiac muscle is caused by overlapping arrays of thick and thin filaments, which appears under a microscope as a sequence of dark and light bands. Dark bands, named A Bands, are found in the center of sarcomeres. They are flanked by two light bands named I bands. While I bands contain only thin filaments, A bands contain both thin and thick filaments. Dark Z-lines separate two adjacent sarcomeres and serve as an anchoring point for actin filaments.

Even though thin and thick filaments are juxtaposed, they can only interact in the presence of calcium. Calcium released from the sarcoplasmic reticulum binds to troponin, causing tropomyosin to modify the actin structure. As a result, modified actin molecules can interreact with myosin, causing thin filaments to slide along thick filaments. This contraction needs adenosine triphosphate (ATP) and thus depends on the activity of myosin molecules and the ability of myosin to convert chemical energy (ATP) into motion. This huge demand for ATP caused by the continuous activity of cardiomyocytes is explained by the high density of mitochondria in these cells (around 40% of cytoplasm).

In order to contract simultaneously, gap junctions within intercalated discs connect cardiomyocytes together to form one functional syncytium. This allows the wave of excitation to reach all cardiomyocytes. When an action potential reaches the cell, a depolarization occurs, followed by the entry of calcium into the cells through voltage-gated calcium channels. These channels are present on cell membranes and transport the “trigger calcium” into the cell. Trigger

calcium binds to ryanodine receptors on the sarcoplasmic reticulum membranes and induces the release of intra-sarcoplasmic calcium. This mechanism is called calcium-induced calcium release and it increases the concentration of cytosolic free calcium (Figure 1.2). Free calcium binds to troponin and triggers a cascade of events that leads the sarcomeres to contract. Finally, calcium is sequestered back to sarcoplasmic reticulum and out of the cell, which leads to muscle relaxation.

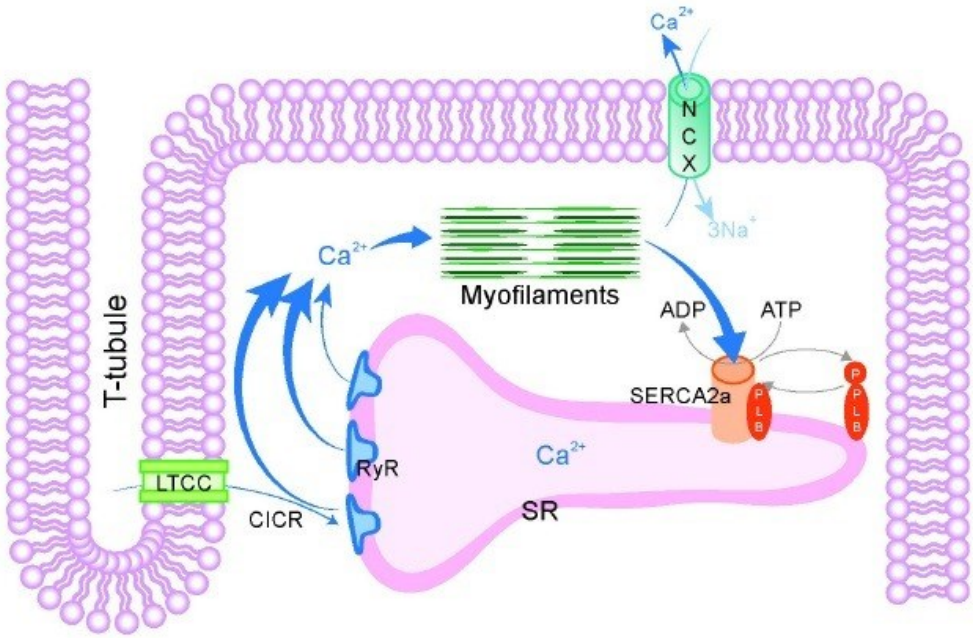

Figure 1.2: Calcium-induced calcium-release model (10).

1.2.2 Cardiomyocytes metabolism

The heart's need for energy is enormous; it beats more than 100,000 times per day and consumes more energy than any other organ (11). Almost 95% of ATPs synthesized in cardiomyocytes

come from mitochondrial oxidative phosphorylation (12), while the rest are obtained from glycolysis and GTP (via the Krebs cycle) (13, 14). Myocardial ATP pool turnover, under normal conditions, is about ten seconds, so the heart must continually produce enough ATP to maintain its contractile function (15). It is important for the heart muscle to adapt quickly to physiological changes and the availability of different substrates.

Cardiac metabolism is characterized by a large number of interdependent and highly regulated reactions that, together, orchestrate the energetic needs of the heart. Indeed, cardiomyocytes can use carbohydrates, lipids, ketone bodies, and amino acids as substrates for energy production (16). Glucose and fatty acids are the main energy substrates, while the contribution of others is minimal (Figure 1.3). Normally, in adult cardiomyocytes, fatty acids represent the main energy source, while glucose represents less than 10% of energy sources (17). However, these percentages are flexible and they depend on the physiological state of the heart (18). The alteration of substrate choice depends on substrate availability, oxygen supply and work load. For example, in a fasting state, fatty acids are the main energy source for the heart, while in a postprandial state, glucose becomes the main energy source (19). This equilibrium between glucose and fatty acids was first described by Randle et al. in 1963 (20). He demonstrates that an increase in cardiomyocytes' use of fatty acids is reciprocated by an inhibition of glucose utilization and vice versa. This is known as the Randle cycle and was later confirmed by McGarry et al. in 1977 (21).

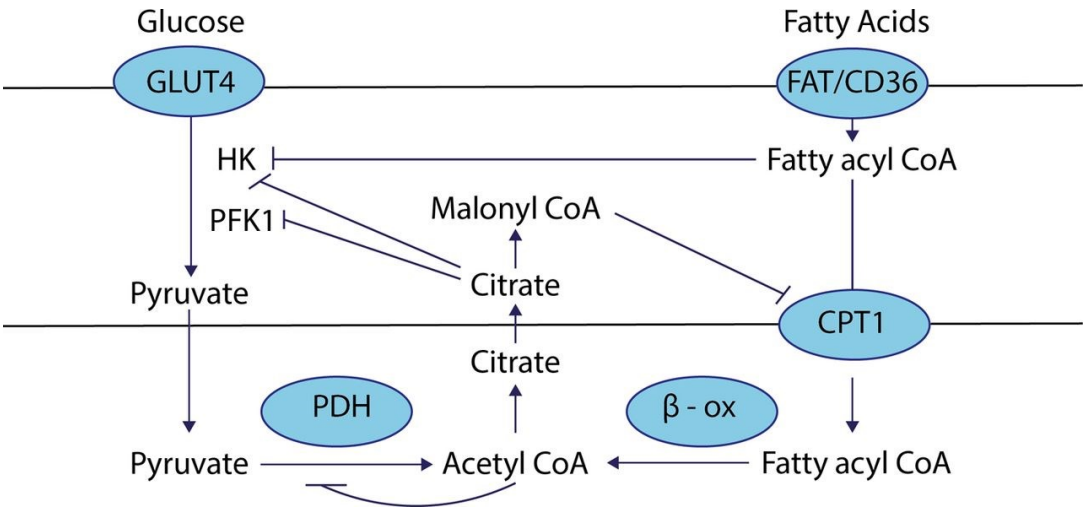

Figure 1.3: Fatty acids and glucose metabolism in cardiomyocytes (22).

Several mechanisms are involved in glucose/fatty acid counterbalance. First, fatty acids inhibit glucose oxidation through phosphofructokinase 1 (23) and pyruvate dehydrogenase (20). The mechanism involves citrate and acetyl-CoA respectively, which are both intermediates of fatty acid oxidation (FAO). Citrate inhibits phosphofructokinase 1 activity and thus inhibits glycolysis. Acetyl-CoA induces pyruvate dehydrogenase (PDH) kinase, which inhibits PDH activity and thus inhibits pyruvate oxidation.

Second, AMP-activated protein kinase (AMPK) is a metabolic sensor and plays a key role in energy homeostasis. AMPK can phosphorylate Acetyl-CoA carboxylase (ACC) and thus inhibits its activity (24) (25). ACC catalyzes malonyl-CoA synthesis, which is a potent inhibitor of Cpt1b. Since Cpt1b catalyzes the rate-limiting step of the FAO process, inducing AMPK activity leads to FAO upregulation.

Third, PPARs are master regulators of lipid metabolism and fatty acids are natural ligands of PPAR (26). When bound to fatty acids, PPARs translocate to the nucleus, where they heterodimerize with retinoid X receptors (RXRs) and bind PPAR response elements (PPREs). PPREs can be found in the promoters of many metabolic genes, including but not limited to: CD36 (27), Cpt1 (28), and PDK4 (29). CD36 translocates free fatty acids across the cell membrane and Cpt1 catalyzes the rate-limiting step for FAO. Therefore, PPARs are key factors of lipid metabolism in cardiomyocytes. Additionally, PDK4 phosphorylates and thus downregulates PDH activity, leading to inhibition of glucose oxidation.

Finally, insulin directly impacts FAO by modulating AMPK activity. Insulin inhibits AMPK activity, and thus inhibiting Cpt1b activity and FAO in cardiomyocytes (30). Insulin, at the same time, induces Glut-4 translocation and the transport of glucose into cytosol. In consequence, insulin augments glucose utilization and glucose oxidation (31).

1.2.3 Rat neonatal cardiomyocytes cultures

Cultures of rat neonatal cardiomyocytes formed the main model used in our studies. These cardiomyocytes were isolated directly from the ventricles of 1- to 3-day-old rats. Fibroblasts and myocytes are the main components of rat heart ventricles (5). For this reason, fibroblasts were separated from cardiomyocytes by incubating the cells' mixture for one hour. This was sufficient for fibroblasts to attach onto the plate but not enough for cardiomyocytes to do so. Cardiomyocytes were then transferred to another plate and incubated for seven days before performing experiments. We incubated for seven days, since the hearts of newborn mammals

adapt from the dramatic nutrient changes that occurs after birth, becoming less dependent on carbohydrates and more dependent on fatty acids (from colostrum and milk) as energy fuel (32). Simultaneously, capacity for FAO increases significantly during post-natal development (32, 33). Our cell cultures were almost 95% pure cardiomyocytes, which was confirmed through sarcomeric staining. This method provides a mean to study intracellular mechanisms in one specific cell type: cardiac myocytes. This model is known as the "redifferentiation model," where cardiac myocytes dedifferentiate when isolated from hearts before redifferentiating into spontaneously beating cells after incubating the cells in 2-10% fetal bovine serum for a few days (34).

We have used primary cardiomyocyte cultures, since they have many important advantages over cell line cultures. First, most cell lines come from tumor cells or transformed cells. These cells divide in an uncontrolled manner which is a behavior not found in primary cell culture. For example, H9C2 is a cell line derived from rat heart myoblasts. These cells can be passaged up to four months (35). However, primary rat cardiomyocytes do not divide at all. Second, physiological, morphological, and phenotypical traits found in primary cells are not always replicated in cell line cultures (36). This discrepancy is reflected in cellular responses and signaling pathways where primary cells isolated directly from tissue maintain most of the functions found *in vivo*, though this which is not the case for cell lines. A clear example is the H9C2 cell line, which is isolated from the ventricles of embryonic rats, though they exhibit many features of skeletal muscle (35).

It's important to mention that neonatal and adult cardiomyocytes are not identical. For example, some ion channels are not fully developed in neonatal cardiomyocytes (37). Additionally, some proteins (isoforms) implicated in contraction are not expressed in neonatal cardiomyocytes which is not the case for adult cardiomyocytes (37). Therefore, In vitro culture of Rat adult cardiomyocytes closely resemble the in-vivo myocardium of adult rats and hence it is a better model to study rat hearts. However, there are two main advantages for using neonatal over adult cardiomyocytes. First, culturing cardiomyocytes isolated from adult rats is laborious and cost intensive when compared to neonatal cardiomyocytes. Indeed, culturing neonatal cardiomyocytes yield more cells since they are less sensitive to the calcium-containing medium, a medium through which cells are reintroduced after dissociation (38). The disparity in calcium sensitivity is likely associated with Transverse tubule, since the Transverse tubule network is not fully developed until the adult period (39). Second, neonatal cardiomyocytes start beating spontaneously after they enter the dedifferentiation-redifferentiation cycle, unlike cardiomyocytes isolated from adult rats which require pacing to trigger contraction (40).

1.3 Fatty acids

1.3.1 Description and classification

Fatty acids are essential molecules in all organisms. They are carboxylic acids (COOH) attached to a hydrophobic aliphatic chain. They can be saturated or unsaturated depending on the absence or the presence of double bonds in their aliphatic chain. Unsaturated fatty acids are referred to as monounsaturated if they possess only one double bond in their aliphatic chain; they are

referred to as polyunsaturated if they possess two or more double bonds. Almost all double bonds found in native fatty acids have a cis configuration. A cis configuration bends the hydrocarbon chain, which affects the shape of the molecule and confers specific physical and chemical properties. A trans configuration is usually obtained after hydrogenation of the double bond. Additional classifications are used depending on the length of the carbon chain and the position of the double bonds. Concerning the number of carbons in the aliphatic chain, fatty acids are classified into four groups: 1) short chain fatty acids (less than five carbons), 2) medium chain fatty acids (6 to 12 carbons), 3) long-chain fatty acids (13 to 21 carbons), and 4) very long-chain fatty acids (22 carbons or more).

Since the physiological role of unsaturated fatty acids is more dependent on the relative position of the double bonds to the methyl group than saturated fatty acids, an alternative classification is used. The last methyl group from the carboxyl group is called omega (ω) and it is given the number 1. Omega-3 fatty acids are polyunsaturated fatty acids where three carbons from the methyl group form the first double bond, which means it is located between carbon 3 and carbon 4. Omega-6, omega-7, and omega-9 fatty acids also exist. There are three major omega-3 fatty acids: 1) alpha linolenic acid (ALA), 2) eicosapentaenoic acid (EPA) and 3) docosahexaenoic acids (DHA). The main source of ALA is fish oil and the protective role of omega-3s against cardiovascular disease is well established (41-43). Unlike omega-3s, omega-6s are proinflammatory, prothrombotic, and hypertensives (44).

The two most common fatty acids are palmitic and oleic acids (45). They are saturated and unsaturated fatty acids respectively. Palmitate has a carboxylic group and a 15-carbon aliphatic

chain for a total of 16 carbons. Oleate has a total of 18 carbons and one unsaturation between carbon 9 and carbon 10. A simplified nomenclature is used where the number of carbons followed by the number of unsaturated molecules are indicated. The positions of double bonds are specified by superscript numbers preceded with Δ. For example, palmitate is abbreviated 16:0 and oleate is abbreviated 18:1 (Δ^9). Additional well-known fatty acids in human diet include: arachidonic acid 20:4 ($\Delta^{5,8,11,14}$), stearic acid 18:0, and linoleic acid 18:2 ($\Delta^{9,12}$). It is important to note that saturated and monounsaturated fatty acids are metabolized differently in cardiomyocytes and significantly diverge in intracellular signaling (46, 47). For example, saturated fatty acids induce insulin resistance, while monounsaturated fatty acids induce insulin sensitivity in diabetic patients (48, 49). Additionally, palmitate causes mitochondrial dysfunction and increases mitochondrial reactive oxygen species, which is correlated with apoptosis in L6 skeletal muscle cells (50). Indeed, we showed that palmitate is a toxic fatty acid while oleate is neutral and even protective (51, 52).

Fatty acids are poorly soluble in water. Their hydrophobic characteristics are based on their molecular structure. Water solubility is positively correlated with the number of double bonds and negatively correlated with the length of the hydrocarbon chain. Therefore, fatty acids in organisms are rarely found free. In blood vessels, they are attached to a carrier protein: serum albumin (53). They are also conjugated to glycerol and converted to triacylglycerol then packed into lipoproteins and chylomicrons (54). Lipoproteins are spheres of decreasing diameter but with increasing density. Their center contains hydrophobic lipids such as triglycerides and cholesteryl ester and engulfed with apolipoproteins and amphiphilic lipids such as phosphatidylcholine. In the heart, lipoprotein lipase (LPL) acts on lipoproteins to release fatty

acids from triglycerides (55). The released fatty acids are transported into cardiomyocytes via passive diffusion (56) or via several membrane transporters, including CD36, FATP1/6, or FABPpm (57). Once inside the cells, Acyl-CoA-synthetase "activates" fatty acids by adding a CoA group. The CoA group confers hydrophilic properties onto fatty acids that permit them to further interact with hydrophilic cytosolic enzymes (58). For long-chain fatty acids, five isoforms of acyl-CoA synthetase exist in mammals (59-61). Each isoform differs in its subcellular location (62) and it has been shown that fatty acids' intracellular fate depends on the action of specific ACSLs (61). For example, heart-specific ACSL1 knockout in mice is associated with FAO impairment and cardiac hypertrophy (63).

In cardiomyocytes, lipid droplet synthesis and phospholipid synthesis are two other possible fates, beside oxidation, for fatty acids. Lipids are the main component of cell membranes (phospholipids) and they provide energy for various metabolic process. Fatty acids are stored as energy depots in triacylglycerol form. Using triacylglycerol instead of polysaccharides as energy depots has two main advantages. First, oxidation of fatty acids yields almost twice as much ATP compared to polysaccharides oxidation (64). Second, triacylglycerols are hydrophobic, unlike polysaccharides, which are hydrated when stored as glycogen or starch. Triacylglycerols are denser than polysaccharides, since cells can store more triacylglycerol (two grams of triacylglycerol per gram of polysaccharide) (3). Indeed, obese people can store enough fats to meet their energy needs for months, while the human body cannot store more than one day worth of energy in the form of glycogen (3).

Fatty acids also play an indispensable role as signaling molecules (for example eicosanoids) and as cofactors (65). Additionally, fatty acids consumed through dietary, participate in cell membrane synthesis. They reduce the melting point and augment membrane fluidity (66). Thus, they can modify some receptors' activity (67).

1.3.2 Fatty acid oxidation

1.3.2.1 β-oxidation

FAO occurs in peroxisomes and mitochondria. Even though a small fraction of fatty acids is oxidized in peroxisomes (32), mitochondria is considered the main site for β-oxidation. Small, medium, and long-chain fatty acids are oxidized in mitochondria, while the peroxisomal system is much more active on very long-chain fatty acids (68). Peroxisomal oxidation cannot degrade the fatty acid completely (69), but it shortens the fatty acid chain with a few cycles of β-oxidation. The shortened fatty acid translocates to mitochondria for further oxidation. Oxygen is the final electron acceptor in both mitochondria and peroxisome. However, one difference between the peroxisomal and mitochondrial oxidation pathways is that the former produces H_2O_2 while the latter produces H_2O (70, 71). An additional difference between the two is in ATP synthesis. ATP is generated in mitochondrial oxidation, which is not the case for peroxisomal oxidation (72).

Long-chain fatty acids cannot diffuse freely through mitochondrial membranes (73). Therefore, in cardiomyocytes, the CoA group of fatty acids is exchanged with the carnitine group through

CPT1B (Figure 1.4). CPT1B, which stands for carnitine-palmitoyl-transferase 1b, commits fatty acids to mitochondrial oxidation. The reaction catalyzed by CPT1B is the rate-limiting step for FAO, which regulates the number of fatty acids entering mitochondria (74, 75). The newly synthesized acyl-carnitine can diffuse freely through the outer mitochondrial membrane. Once it is in the mitochondrial intermembrane chamber, it is transported into a mitochondrial matrix with the help of carnitine/acylcarnitine translocase (CACT) (76). CACT exchanges carnitine with an acylcarnitine across the inner mitochondrial membrane. Subsequently, carnitine diffuses freely to the cytosol, where it is used by Cpt1b for the following reactions. Once inside the mitochondria, acyl-carnitine is converted back to acyl-CoA by Cpt2 (77).

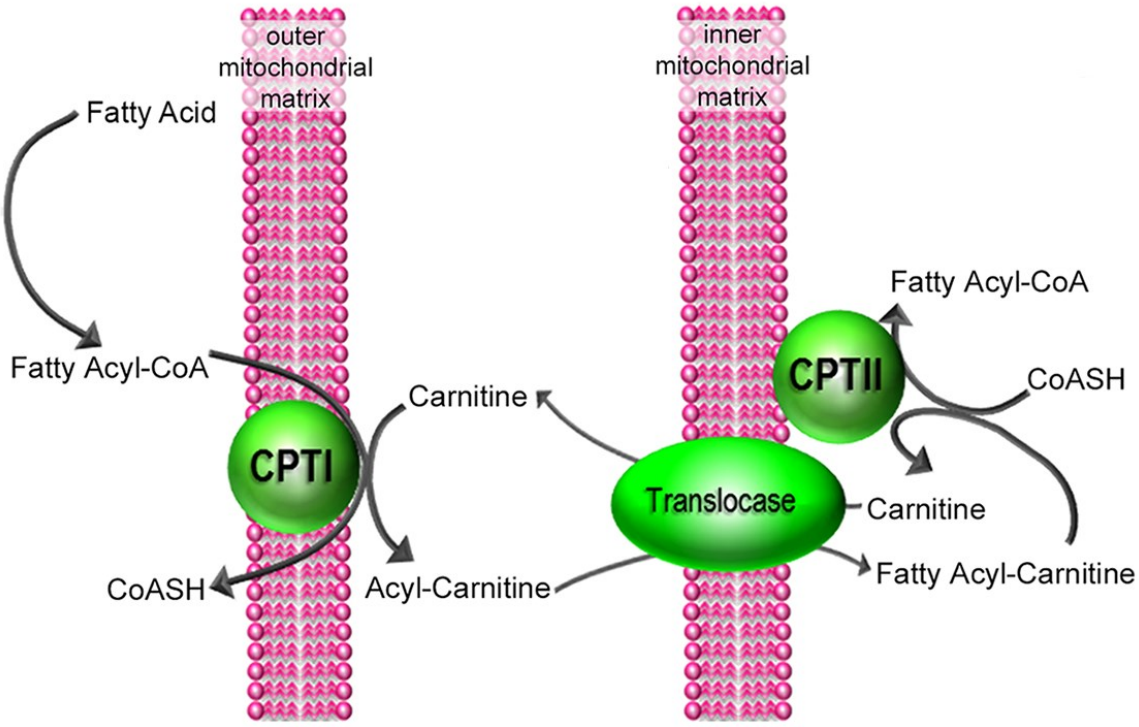

Figure 1.4: Initial steps of Fatty Acids Oxidation (adapted from (78)).

Mitochondrial acyl-CoA then enters the β-oxidation pathway. This pathway is a four-step reaction (Figure 1.5). The enzymes involved are in the following order (79): Acyl CoA dehydrogenase (ACAD), enoyl CoA hydratase, L-3-hydroxyacyl CoA dehydrogenase, and 3-ketoacyl CoA thiolase. ACAD catalyzes the first step of β-oxidation when it introduces a trans double bond between carbon number 2 and carbon number 3. This reaction is called dehydrogenation and it produces one molecule of $FADH_2$ as a by-product. Several members of the ACAD family have been found in the human genome and they are all involved in β-oxidation (80). Very long-chain acyl-CoA dehydrogenase, long-chain acyl-CoA dehydrogenase, medium chain acyl-CoA dehydrogenase, and short chain acyl-CoA dehydrogenase are responsible for the dehydrogenation of very long-chain acyl-CoA, long-chain acyl-CoA, medium chain acyl-CoA, and short chain acyl-CoA respectively. Enoyl CoA hydratase catalyzes the second step of β-oxidation. It adds one water molecule to the double bond between carbon 2 and carbon 3 and it converts 2-trans-enoyl-CoA to L-3-hydroxyacyl-CoA. L-3-hydroxyacyl CoA dehydrogenase follows enoyl CoA hydratase and it is responsible for the second dehydrogenation of the β-oxidation cycle. L-3-hydroxyacyl CoA dehydrogenase converts 3-hydroxyacyl-CoA to 3-oxoacyl-CoA. In this step, NAD+ is the electron acceptor. 3-ketoacyl CoA thiolase catalyzes the fourth and last step of the β-oxidation cycle when it reacts with one molecule of free CoA in order to split the original fatty acid into acetyl-CoA and acyl-CoA.

At the end of each β-oxidation cycle, an acetyl-CoA is produced and the fatty acid chain is shortened by two carbons. One additional molecule of the reducing equivalents nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) is produced (58). These reducing equivalents are transferred to the electron transport chain to undergo the oxidative

phosphorylation. The β-oxidation cycle continues until the fatty acid is entirely catabolized to acetyl-CoA (58).

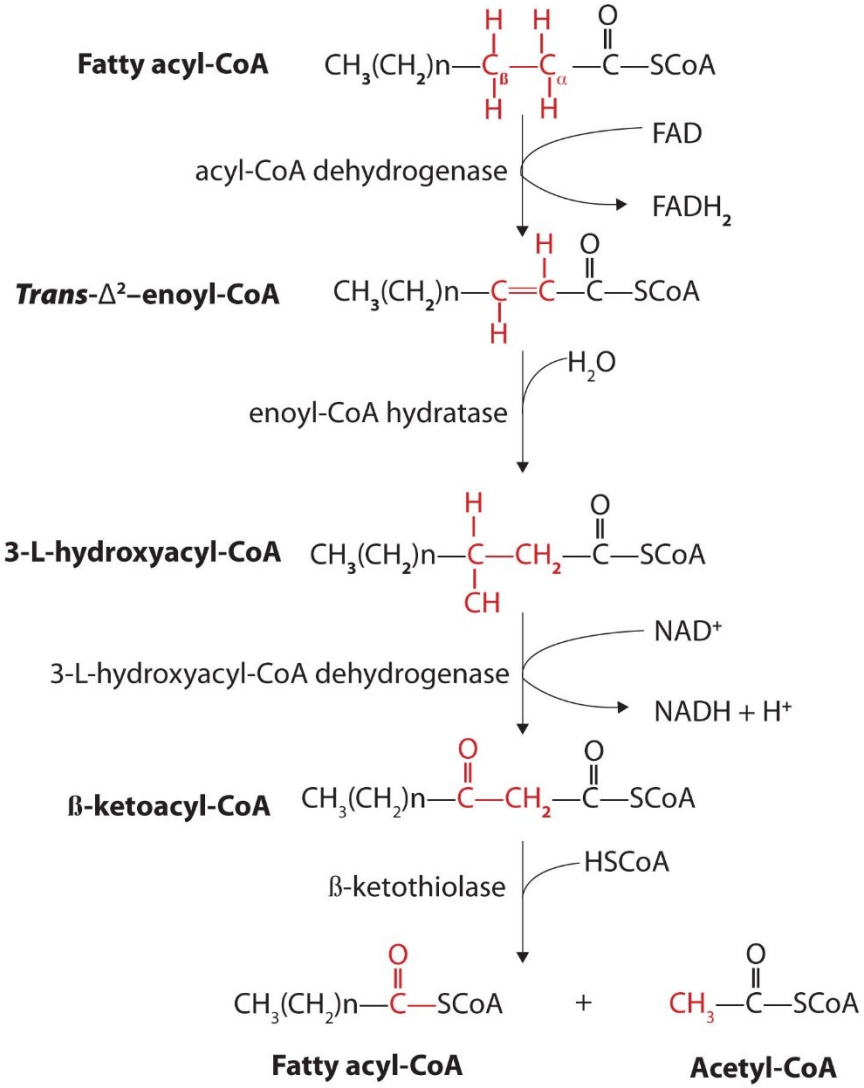

Figure 1.5: β-oxidation reaction of fatty acids (81).

If the fatty acid carbon chain is unsaturated, or if contains an odd number of carbons, then additional enzymes are required (58). The double bonds found in fatty acids usually have a cis

configuration which enoyl CoA hydratase cannot recognize. Therefore, in monounsaturated fatty acids, such as oleate, an additional enzyme is required such as enoyl CoA isomerase. Enoyl CoA isomerase converts the cis configuration in fatty acids to trans configuration. For polyunsaturated fatty acids, such as linoleic acid, two enzymes are employed: enoyl CoA isomerase and 2,4 dienoyl CoA reductase.

If the fatty acid chain contains an odd number of carbons, then the chain will be catabolized to several acetyl-CoA molecules and one propionyl-CoA molecule. Propionyl-CoA is metabolized to succinyl-CoA by a mechanism that involves three enzymes: 1) propionyl-CoA carboxylase, 2) methylmalonyl-CoA epimerase, and 3) methylmalonyl-CoA mutase. Succinyl-CoA is subsequently fed into the citric acid cycle (CAC) as an intermediate (82).

1.3.2.2 Citric acid cycle

Acetyl-CoA, the final product of β-oxidation, is further oxidized by the CAC, followed by oxidative phosphorylation. The citric acid cycle (CAC) and oxidative phosphorylation are mutual for both glycolysis and β-oxidation pathways. The CAC is also named Krebs cycle after the German researcher Hans Krebs who described the steps of the cycle (83). Like β-oxidation, the CAC takes place in the mitochondrial matrix. It is an eight-step reaction catalyzed by at least eight different enzymes: 1) citrate synthase, 2) aconitase, 3) isocitrate dehydrogenase, 4) α-ketoglutarate dehydrogenase, 5) succinyl-CoA synthetase, 6) succinate dehydrogenase, 7) fumarase, and 8) L-malate dehydrogenase (Figure 1.6) (58). First, a CoA group is cleaved from acetyl-CoA. Then citrate synthase combines oxaloacetate with acetate to form citrate. Next,

aconitase converts citrate to isocitrate. The latter is metabolized to α-ketoglutarate by isocitrate dehydrogenase, then to succinyl-CoA by α-ketoglutarate dehydrogenase. One molecule of CO_2 and two molecules of NADH are released during the last two steps. Succinyl-CoA synthetase converts succinyl-CoA to succinate and GTP. Next, succinate dehydrogenase metabolizes succinate to produced fumarate, which is then coupled with the synthesis of one $FADH_2$ molecule. Fumarase activity converts fumarate into to malate, which is further converted to oxaloacetate by L-malate dehydrogenase. A third molecule of NADH is formed (84). At the end of each cycle, acetyl-CoA yields two molecules of CO_2, three molecules of NADH, one molecule of $FADH_2$, and one molecule of GTP (85). CO_2 is cleared out of the cell as a waste, while NADH and $FADH_2$ enter the electron transport chain where they are used as precursors for oxidative phosphorylation.

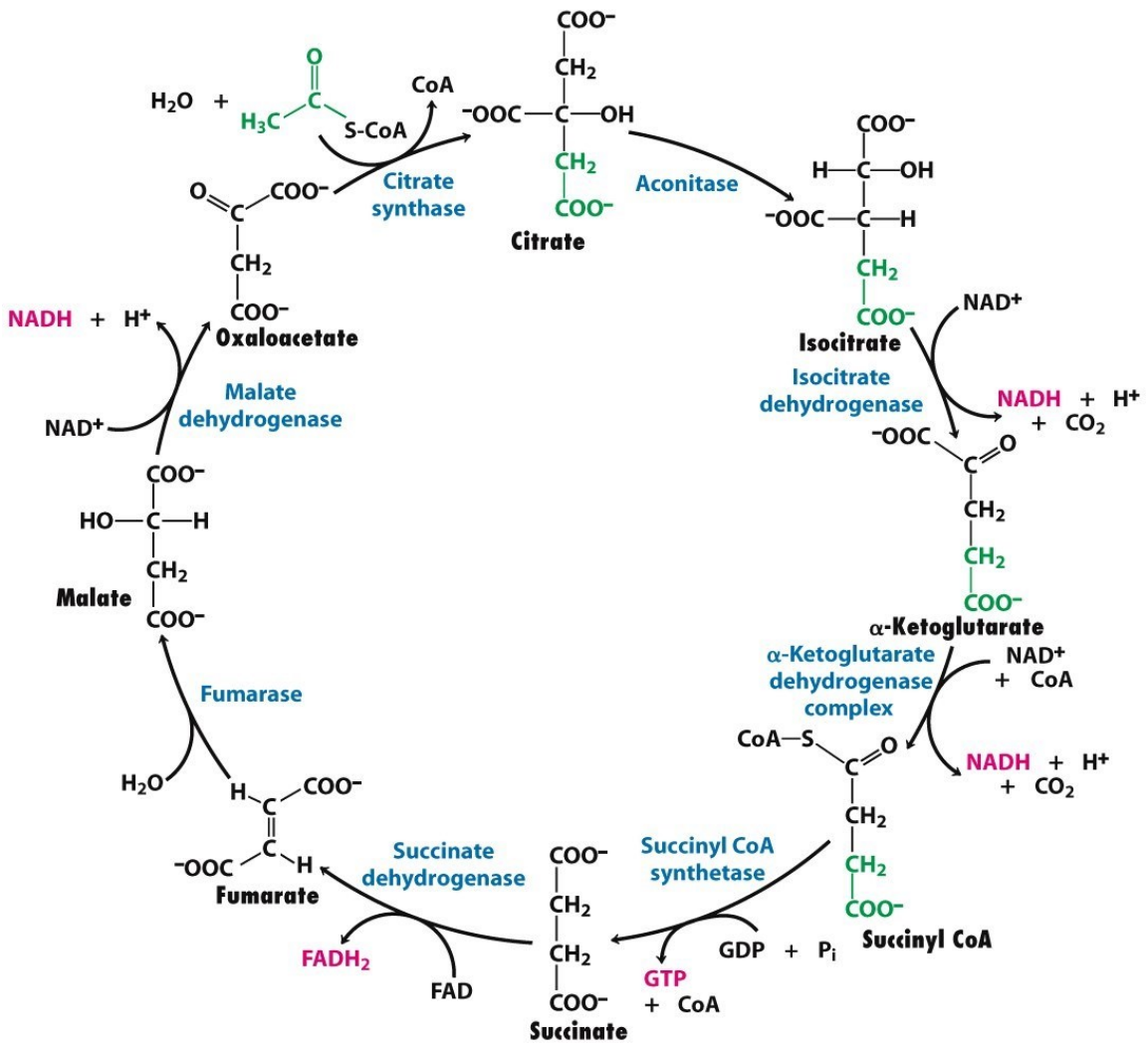

Figure 1.6: Overview of citric acid cycle (86).

In cardiomyocytes, the CAC is modulated by substrates availability and by the cell energy needs (87). For example, when the NADH/NAD^+ ratio is elevated, citrate synthase (88), isocitrate dehydrogenase (89), and α-ketoglutarate dehydrogenase (89) are downregulated. Additionally, CAC enzymes are regulated by the cycle intermediates such as oxaloacetate and acetyl-CoA.

CAC flux is regulated by anaplerotic and cataplerotic reactions (90). Anaplerosis is simply the regeneration of CAC intermediates while cataplerosis is the loss of CAC intermediates. Therefore, CAC intermediates can be used as precursors for cataplerotic reactions or they can be replenished by anaplerotic reactions. Some fatty acids such as palmitate do not contribute to anaplerosis (91), while glucose and pyruvate are important anaplerotic substrates (92, 93). Pyruvate carboxylation via pyruvate carboxylase or malic enzymes produces oxaloacetate and malate respectively (94). Pyruvate can be used as a substrate for α-ketoglutarate regeneration (93). Propionate and amino acids are also metabolites for anaplerotic reactions (93, 95). The extent of these reactions depends on various circumstances including substrate and oxygen supply. Indeed, the importance of anaplerosis was established when a perfused heart was supplied with ketone bodies as an energy substrate (96). A rapid deterioration in contractile function was observed and this decline was only reversed by supplementation of glucose and pyruvate (97).

1.3.2.3 Electron transport chain

NADH and $FADH_2$ generated in the CAC and in fatty acid beta oxidation are used to donate electrons for the mitochondrial respiratory chain. NADH and $FADH_2$ are oxidized to NAD+ and $FADH_2$+ respectively and the electrons released are captured by oxygen. Oxygen is transformed into water and this is coupled to the ATP synthesis. This process is called oxidative phosphorylation and it employs five complexes (NADH: ubiquinone oxidoreductase (or complex 1), succinate dehydrogenase (or complex 2), cytochrome bc1 complex (or complex 3), cytochrome c oxidase (or complex 4), and F0F1-type ATP synthase (or complex 5)) and two

electron carriers (ubiquinone and cytochrome c). The five protein complexes are bound to the mitochondrial inner membrane, unlike CAC enzymes, which are localized in the mitochondrial matrix (except for succinate dehydrogenase) (94-96). The first four complexes expulse H+ ions into the mitochondrial intermembrane chamber, creating a PH gradient. Therefore, the electron transport chain does not synthesize ATP directly. Instead, it generates energy by creating a proton gradient between mitochondrial inner and outer membranes. This proton gradient is capitalized by the last complex to generate ATP through chemiosmosis. The last complex, called F0F1 ATP synthase, returns most of the protons to the mitochondrial matrix, which enables it to synthesize ATP from ADP (98).

A small fraction of intermembrane protons "leaks" to the mitochondrial matrix through the uncoupling proteins. In cardiomyocytes, two main isoforms of uncoupling proteins exist: UCP-2 and UCP-3 (99). They transport protons back to the mitochondrial matrix without being coupled to ATP synthesis, hence their name. Instead of ATP, UCPs capitalize on the proton gradient by releasing energy in form of heat (100). UCP-1 is highly concentrated in brown adipose tissue and it plays a pivotal role in thermogenesis (101, 102).

Electrons carried by NADH are transferred to complex 1 in the electron transport chain, where flavoprotein is the electrons' acceptor. Electron flux is transferred from one complex to the other (complex 2, 3, and 4), inducing the reduction and the oxidation of several electron transporters: ubiquinone and cytochrome B, C1, C, A, and A3. Electron transfer is coupled to proton expulsion from the mitochondrial matrix to the intermembrane space. Oxygen, the final electron

acceptor, obtains its electrons from cytochrome A3, then combines with two molecules of H+ to generate H_2O.

Unlike NADH, $FADH_2$ transfers its electrons directly to complex 2, leading eventually to the synthesis of two molecules of ATP (instead of three molecules of ATP for NADH) (58).

ATP and ADP are transported through the mitochondrial membrane in opposite direction by antiport (translocase) (85). ATP exits the mitochondria to be used by the contractile units and ions pumps, while ADP enters the mitochondria to be used by oxidative phosphorylation. In healthy adult cardiomyocytes, the ATP pool is only sufficient for ten seconds (15). Therefore, the robust activity of mitochondrial oxidative phosphorylation is indispensable. For it to work flawlessly, mitochondrial oxidative phosphorylation needs a constant supply of oxygen (through the coronary circulation), as well as ADP, protons, and electrons. In prenatal conditions, rat hearts have low activity in CAC enzymes and the mitochondrial respiratory chain. However, a few days after birth, enzyme activity is upregulated severalfold (85, 103, 104).

At the end of the electron transport chain, the complete products of palmitate oxidation to carbon dioxide and water is:

Palmitoyl-CoA + 23 O_2 + 108 P_i + 108 ADP $\rightarrow$ CoA + 108 ATP + 16 CO_2 + 23 H_2O

1.3.3 TAG and lipid droplets synthesis

Triglycerides consists of two molecules attached together: glycerol and fatty acids. Glycerol is a three-carbon alcohol with a hydroxyl group for each carbon. Triglycerides can be found mainly in animal fats and vegetable oils. Most animal triglycerides are saturated triglycerides, which means they are composed of saturated fatty acids (58). These triglycerides are flexible and agglomerate together because they lack double bonds in their carbon chain. This is why lard and butter are solid at room temperature, while vegetable oils, in contrast, are liquid at room temperature since their triglyceride are unsaturated. Unsaturated triglycerides contain double bonds in their aliphatic chain. These double bonds usually have a cis configuration, causing the hydrocarbon chains to lose their flexibility and their ability to form agglomerates. In the food industry, unsaturated fatty acids found in vegetable oil are partially hydrogenated to improve their shelf life. A by-product of fatty acids hydrogenation is trans fats (105). These fats are unsaturated because their double bonds have a trans configuration. There is a clear association between trans-fat consumption and cardiovascular disease (106).

Upon entering the cell, fatty acids can be esterified and used for TAG synthesis. In cardiomyocytes, TAG is mainly synthesized from glycerol 3-phosphate. This pathway is a four-step reaction with each step catalyzed by a different enzyme. First, GPAT condenses acyl-CoA with glycerol 3-phosphate to form lysophosphatidic acid. This step is common for all glycerolipid synthesis and is considered the rate-limiting step in the TAG synthesis (107). Another acyl transferase, AGPAT, transfers a second acyl-CoA to lysophosphatidic acid. Phosphatase acts on phosphatidic acid (the product of the second step) and transforms it to

diacylglycerol (DAG) by removing a phosphate group. The final step produces TAG by condensing a third acyl-CoA with DAG. This step is catalyzed by DGAT and is the only committed step in TAG biosynthesis (108). Since DGAT is located on the ER membrane, TAG is considered a product of the ER (109). Another pathway for TAG synthesis involves the acylation of MAG. However, this pathway is not active in cardiomyocytes and is mainly found in hepatocytes and adipocytes.

TAG is a neutral lipid and its moderate presence in cardiac cytoplasm is harmless and might even be protective (110). Since DAG is associated with ER stress (111, 112), inflammation (113) and insulin resistance (114), the transformation of DAG to TAG by DAGT is considered a protective mechanism (115). Indeed, an increase in the DAG/TAG ratio is associated with increased cardiac lipotoxicity (116). Additionally, DGAT2 deficiency (DGAT2 $^{-/-}$) in newborn mice is a lethal condition associated with a severe decrease in TAG levels (117).

TAGs are stored in specific organelles called lipid droplets (LD). While the main site for the synthesis of LDs are adipocytes, LDs can also be found in almost all cells, including cardiomyocytes. LD is filled with TAG and cholesterol ester, which are engulfed with a monolayer of phospholipids (118). Additionally, a wide array of proteins is found on the outer layer of lipid droplets. Protein composition varies among cell types, which are categorized into nine groups (119): 1) PAT family, 2) lipid metabolism, 3) membrane traffic, 4) cytoskeleton, 5) chaperone, 6) ER, 7) mitochondria, 8) cell signal, and 9) miscellaneous. The PAT family, also known as perilipins, are the most abundant protein on LD membranes.

There are five isoforms of perilipin proteins numbered 1 to 5 (120). Perilipin 1 is dominant in white and brown adipocytes (121), while perilipin 2, 3, 4, and 5 can be found in cardiomyocytes (122). Perilipins are considered the gatekeepers of lipid droplets. They are phosphorylated by PKA and they play a major role in lipolysis by modulating the interaction between lipases (hormone sensitive lipase and adipose triglyceride lipase) and TAG in lipid droplets (123, 124). Initially, LDs were thought to play a passive role in energy and TAG. However, this role is far from the truth. LDs have been found to play a dynamic role in lipids' metabolism, lipotoxicity, cell membrane homeostasis, signalization pathways, and protein modification (125, 126). Additionally, in leucocytes, LDs are considered as inflammatory organelles (127). Since DGAT (the final enzyme in TAG synthesis) is bound on ER membranes, ERs are considered the site for LD biogenesis (109).

The exact mechanism for LD biogenesis is still unknown and several hypotheses have been proposed. The most accepted one suggests two steps for LD synthesis. First, TAG accumulates between the hydrophobic bilayers of ER membranes, and as TAG concentration increases, a len-shaped bulge starts to appear. Second, for reasons not well understood, TAG stops accumulating and LDs bud off through a mechanism similar to dewetting (128). Cytosolic LDs can also derive from existing LDs through fission. However, this process is not dominant (129). The stored triglycerides are ultimately hydrolyzed, then oxidized to meet the energy needs of cardiomyocytes. Interestingly, cardiomyocytes accumulate lipid droplets during fasting states; however, in a fed state, lipid droplets become scarce (130).

1.3.4 Phospholipids synthesis

The lipidic part of a cardiomyocyte membrane consists mainly of cholesterol and phospholipids. Phospholipids are a heterogeneous group of lipids, glycerophospholipids, and sphingomyelins that share a common role of maintaining membrane integrity. Cardiac sarcolemma is composed of lipid bilayers containing phospholipids which are asymmetrically distributed between outer and inner layers (or leaflets) (131). The mechanism causing this asymmetry is not well understood, but it likely involves two steps: translocase enzymes interact with the amino-groups of phospholipids and transfer them from outer to inner membranes (132), followed by an interaction between phospholipids with the intracellular cytoskeleton (133). Phospholipids' transversal diffusion from one leaflet to the other is slow; however, they can diffuse laterally very rapidly. Interestingly, negatively charged phospholipids are exclusively found on the inner membrane. This is important, since many membrane proteins have been shown to be modulated by their surrounding lipids (134, 135). Phospholipids are positioned in such a way that the hydrophilic groups are oriented towards the hydrophilic outside and the hydrophobic groups are oriented towards the membrane.

Glycerophospholipids contain glycerol as a backbone, where two fatty acids are attached in ester linkage to two carbons of glycerol. The third glycerol carbon is attached by phosphodiester linkage to a highly polar group. For example, the polar group in phosphatidylserine is serine and in phosphatidylethanolamine it is ethanolamine (58). Two major phospholipids are found on cardiac sarcolemma: phosphatidylcholine represents almost 45% of all membrane lipids, while phosphatidylethanolamine represents almost 35% (131). Occasionally, in some animal tissue,

the first glycerol carbon in glycerophospholipids is attached to fatty acids by ether linkage. This is the case for plasmalogen, a phospholipid that represents almost 30% of phosphatidylcholine in the human heart (136). Sphingomyelins, is another subtype of phospholipid. They consist of sphingosine as a backbone where one fatty acid and one polar group are attached to sphingosine by amide and phosphodiester linkage respectively. Phospholipids assume many roles besides maintaining membrane integrity. For example, sphingomyelin is highly concentrated in myelin, the protective layer that surrounds axons (58). Additionally, ceramides are considered the structural parents of all sphingolipids, where one fatty acid is attached to one sphingosine, and they are the main second messenger for sphingomyelin when it signals transduction. Ceramides are well known for their involvement in cardiac dysfunction (137). However, sphingosine-1-phosphate is one of ceramide's derivatives and has been shown to protect the heart against ischemia/reperfusion injury (138).

Additionally, phospholipids are hydrolyzed by phospholipase (A1, A2, C, and D) to generate lipid-signaling molecules known to modulate the function of cardiomyocytes. Inositol trisphosphate (IP3) and DAG are two molecules obtained by the hydrolysis of phosphatidyl inositol bisphosphate (PIP2) with phospholipase C (58). Inositol triphosphate (a phospholipid) triggers the release of Ca^{2+} from the endoplasmic reticulum and activates PKC. PKC regulates several cellular responses and plays a central role in signaling cascades (139). When their role is fulfilled, lipids on cellular membranes are recycled. Therefore, four types of phospholipase (A1, A2, C, and D) are used to hydrolyze the ester bonds and phosphodiester bonds in glycerophospholipids.

It is worth mentioning that cholesterols are also present on plasma membranes but their ratio to phospholipids is less than one (131). This ratio is even smaller in cardiomyocytes (131).

1.4 Diabetes and heart disease

1.4.1 Introduction

Diabetes is a heterogenous clinical syndrome, characterized by abnormal metabolism of glucose, lipids, and proteins. It is a chronic disease and clinically evident in cases of hyperglycemia (high blood sugar) which is caused by a lack of insulin secretion, high insulin resistance, or both. These complications deteriorate the quality of life and increase death rates. For example, when hyperglycemia is uncontrolled, diabetes leads to many complications, such as microvascular disease (retinopathy, nephropathy, and neuropathy) and macrovascular disease (cardiovascular disease, atherosclerosis, stroke, etc.). Unfortunately, the prevalence of diabetes is rising at alarming rate. In 1897, Sir William Osler considered diabetes a rare disease affecting 0.01% of the population in Europe and the United States (140). Today, about 420 million people have diabetes, according to the World Health Organization (WHO), and this number is expected to rise exponentially. Nationally, over 3 million Canadians are currently diagnosed with diabetes. WHO forecasts that this number will reach more than 4 million by 2030 (141).

In 2019, the expert committee of the American Diabetes Association established four diagnostic criteria to confirm diabetes (142). A patient is considered diabetic if one of these criteria is met:

1. Fasting plasma glucose is 7.0 mmol/L or higher.

2. Random plasma glucose is 11.0 mmol/L or higher + symptoms of diabetes.
3. Oral glucose tolerance test is 11.1 mmol/L or higher.
4. A1C test is 6.5% or higher.

For the fasting plasma glucose test, patients should withhold from food and drink for eight hours before testing their glycemia. If the result is 7.0 mmol/L or higher, the patient is considered diabetic. Alternatively, patients may ingest 75g of glucose in a sweetened drink and wait two hours before taking an oral glucose tolerance test (OGTT). If the test results are 11.1 mmol/L or higher, the patient is considered diabetic. Another way patients can be tested for diabetes is if they show symptoms of the disease (such as polyuria, polydipsia, etc.). In this case, a random test for glycemia is taken and a result of 11.0 mmol/L or greater concludes that the subject is diabetic. Finally, glycated hemoglobin (also referred to Hb1c, HbA1c, or A1C) is measured and if it represents more than 6.5% of total hemoglobin, then the subject is considered diabetic. A1C is a marker for chronic hyperglycemia that reflects the average level of systemic glycemia for the last two to three months. A1C is an important criterion for glycose homeostasis. According to the American Diabetes Association (ADA), its value is correlated with late complications of diabetes. It is enough to "fail" just one of these four tests to confirm the diagnosis. However, there is a grey area, called prediabetes, where the test results show high levels of glycemia but not high enough to fall above the specified limits. Prediabetes is a stage that occurs when healthy patients progress to diabetes. Not all prediabetic subjects will develop diabetes, but there is a high chance for it to happen. There are no clear symptoms of prediabetes; however, according to the ADA, having one of the following results indicates prediabetes: A1C levels of 5.7% to 6.4%, fasting plasma glucose levels of 6.1 mmol/L to 6.9 mmol/L, or OGTT levels of 7.8

mmol/L to 11 mmol/L. The last two tests are referred to "impaired fasting glucose" and "impaired glucose tolerance" respectively (Table 1).

	Fasting glucose (mmol/L)		Glucose tolerance test with 75g of glucose intake (mmol/L)
Impaired fasting glucose	6.1 to 6.9		N/O
Isolated impaired fasting glucose	6.1 to 6.9	and	<7.8
Isolated impaired glucose tolerance	<6.1	and	7.8 to 11.0
Impaired fasting glucose and impaired glucose tolerance	6.1 to 6.9	and	7.8 to 11.0
Diabetes	≥ 7.0	or	≥11.1

Table 1.1: Diabetes Canada diagnostic criteria for diabetes.

According to the American Diabetes Association, diabetes is classified into four categories:

1. Type 1 diabetes (T1DM) which is insulin dependent.
2. Type 2 diabetes (T2DM) which is insulin independent.
3. Gestational diabetes.
4. Other type of diabetes such as MODY (Maturity Onset Diabetes of the Young), NDM (Neonatal Diabetes Mellitus), etc

Almost 10% of diabetics have T1DM, while most of the rest have T2DM. These two types will be described in detail in the following sections.

Gestational diabetes, as well as other types of diabetes, can also be found; however, they are less widespread. Gestational diabetes develops during the second or the third trimester of pregnancy and affects around 10% of pregnant women. During pregnancy, physiological increase of insulin resistance is a characteristic of normal pregnancy. Consequently, to maintain euglycemia, pancreatic beta cells increase insulin secretion. Nonetheless, in 5% to 10% of pregnancies, insulin secretion remains constant and cannot compensate for the added insulin resistance. Therefore, this leads to gestational diabetes (143, 144). Increased risk of mortality and neonatal morbidity (hypocalcemia, hypoglycemia, and macrosomia) is associated with gestational diabetes (145, 146). Usually, after giving birth, glucose levels return to normal; however, these women are at high risk of developing T2DM.

The fourth type of diabetes includes many rare types, such as monogenic diabetes, or diabetes caused by the dysfunction of the exocrine pancreas, as well as insulin resistance caused by endocrinopathy and diabetes caused by chemical exposure. Monogenic diabetes is further divided into two categories, since this genetic disorder can cause insulin deficiency by affecting production or secretion or by causing the secretion of non-functional insulin (147). NDM and MODY are the two main forms of monogenic diabetes. Those diagnosed with MODY are usually under the age of 25, while NDM appears in newborns and infants six months old or younger. So far, eleven subtypes of MODY have been identified, all of them involving a single

mutation that may inhibit insulin synthesis or secretion (148). One of these identified genes codes for glucokinase. Glucokinase catalyzes glucose phosphorylation to form glucose 6-phosphate. Glucose 6-phosphate acts as a glucose sensor for beta cells and its level in the body influences insulin secretion. Individuals with MODY 2 have a mutation of this gene that causes in mild fasting hyperglycemia. Additional types of diabetes are those associated with mutations in insulin receptors. Symptoms include hyperinsulinemia and hyperglycemia. Carriers of these mutations can also be diagnosed with acanthosis nigricans, Donohue syndrome, and Rabson–Mendenhall syndrome.

Furthermore, any disorder that affects the exocrine secretion of the pancreas is capable of inducing diabetes: pancreatitis, cystic fibrosis, pancreatic carcinoma, etc. These pathologies reduce the total number of pancreatic beta cells and, as a result, reduce insulin secretion. Likewise, several endocrinopathies, such as acromegaly and Cushing syndrome, may lead to diabetes. These syndromes are characterized by increased levels of hormones (cortisol, glucagon, epinephrine) that antagonize insulin action.

Finally, certain drugs and chemicals (beta adrenergic agonist, diazoxide, cyclosporine, glucocorticoids, etc) induce diabetes as one of their many undesired side-effects.

Glucose is the main fuel for energy production in many organs and maintaining its concentration at a constant level is very important. Glucose levels are regulated by insulin and glucagon, which reduce or elevate glycemia respectively. The pancreatic islet's beta cells secrete insulin into the systemic circulation, while the pancreatic islet's alpha cells are responsible for glucagon

synthesis and secretion. Pancreatic islets, or islets of Langerhans, are clusters of approximately one thousand cells scattered all over the pancreas. Imbedded in the exocrine tissue, they represent approximately 1% of the total number of pancreatic cells. Among the cells found in these islets, we can count alpha cells (for glucagon secretion), beta cells (for insulin secretion), delta cells (for somatostatin secretion), and PP cells (for pancreatic polypeptide secretion). Beta cells represent 60% to 80% of Langerhans islet cells and are responsible for insulin production and secretion. Insulin is a hormone made of 51 amino acids that lowers glycemia by inducing glucose uptake and metabolism in the peripheral tissue. Alpha cells are the second most abundant cells in Langerhans islets and are responsible for glucagon secretion. Glucagon is a hormone made of 29 amino acids that raises blood glucose levels. Delta cells count for 2% to 8% of total Langerhans islet cells and are responsible for somatostatin secretion. Somatostatin (also known as the growth hormone-inhibiting hormone) is a peptide made of 14 amino acids that inhibits both insulin and glucagon secretion. It also regulates the relation between insulin and glucagon and therefore controls glycemia. Finally, PP cells are the least studied among Langerhans islet cells. In general, they negatively regulate the exocrine and endocrine functions of the pancreas.

1.4.2 Type 1 diabetes

T1DM, also called insulin-dependent diabetes, affects 5 to 10% of diabetics and is mainly caused by the destruction of pancreatic beta cells. Almost 75% of those affected with this type are 30 years old or younger. The clinical manifestation of T1DM is distinguishable by the sudden appearance of a wide array of symptoms: polydipsia, polyuria, and rapid loss of body

weight. These patients will also develop absolute insulinopenia due to the destruction of pancreatic beta cells. The etiology of T1DM is only partially identified; however, the causes of this disease are classified into two categories: immune-mediated causes and idiopathy causes. Many studies attribute multiple risk factors such as genetic predisposition, viral infection, and the geographic location of the patient (149-151). However, the most described cause includes the destruction of insulin-producing cells by the body's auto-immune system. This auto-immune process is characterized by mononuclear cells' filtration into the islets of Langerhans, which occurs many years before diagnosis. These cells are responsible for the autoimmune mechanism and prompt the appearance of several circulating autoantibodies directed against pancreatic beta cells: islet cell cytoplasmic antibodies, insulin auto-antibodies, glutamic acid decarboxylase auto-antibodies and tyrosine phosphatase-like protein IA2 auto-antibodies (152). The destruction of pancreatic beta cells precedes the development of hyperglycemia by many years and begins when one of these four auto-antibodies is detected in the patient's serum.

Unlike T2DM, patients with T1DM remain sensitive to insulin and can regain control of their blood glycemia by injecting insulin.

1.4.3 Type 2 diabetes

T2DM accounts for almost 90% of all diagnosed cases of diabetes and is one of the most widespread diseases in Canada. This pathology is triggered in adulthood, usually in individuals who are obese and older than 40 years of age. It affects 10% of Canadians, a percentage expected

to increase since the leading risk factors, obesity and a sedentary lifestyle, are more prevalent than ever.

The etiology of T2DM is complicated, since environmental and genetic factors are involved. The variance in the prevalence of T2DM among different countries can be explained by environmental factors. For example, age, sex, smoking, obesity, diet, and physiological activity are all etiological factors that contribute to the development of T2DM (153). Thus, lifestyle plays a major role in the etiology of T2DM. The majority of Type 2 diabetics are obese and their clinical profile includes abdominal obesity, hypertension, dyslipidemia, insulin resistance and dysglycemia (154). Individuals who suffer from these risk factors are five times more likely to develop T2DM. Fortunately, these risk factors can be avoided by exercising and losing weight, which reduces the incidence of T2DM by 40 to 70% (155-158).

T2DM is a polygenic disorder where heredity is a dominant etiological factor. Indeed, there is a 40% chance to develop T2DM in individuals who have one diabetic parent and a 70% chance if both parents are diabetic (159). Genes associated with T2DM are susceptibility genes. A mutation in the DNA sequence of these genes does not necessarily lead to T2DM, but it does increase the chances of developing diabetes. Dozens of single nucleotide polymorphisms (SNPs) have been identified by genome-wide association studies; however, these mutations explain less than 10% of disease heritability (160). As such, several variants confer T2DM risk, but only two genes encoding for calpain-10 (CAPN10) and transcription factor 7-like 2 (TCF7L2) are associated with this disease (161-163). These two putative genes, whose functions were unknown before, are linked to insulin expression and secretion in pancreatic beta cells (164,

165). We know now that CAPN10 is a calcium dependent protease and TCF7L2 is a transcription factor. The identification of such roles, which betters our understanding of the mechanism by which T2DM develops, demonstrates the importance of genome-wide association studies.

The exact mechanism that leads to T2DM is not fully elucidated, but insulin resistance always precedes the development of T2DM. Indeed, hyperglycemia seen in T2DM is mainly due to insulin resistance associated with impaired activity of insulin receptors Insulin becomes ineffective at stimulating glucose uptake by target cells (hepatocytes, myocytes, and adipocytes, etc.). These cells cannot consume or metabolize glucose properly and the resultant hyperglycemia causes a positive feedback for insulin production by beta cells, leading to systemic hyperinsulinemia (166). For this reason, insulin resistance at an early stage is characterized with compensatory hyperinsulinemia. Hence, T2DM is considered an insulin-independent disease. Hyperinsulinemia cannot persist forever and Beta cells cannot keep up with the high pace for a long time. As a result, pancreatic beta cells enter apoptosis and the pancreas start to fail. Consequently, insulin production decreases significantly, which causes T2DM to becomes more severe. Simultaneously, hyperglycemia induces glucotoxicity and a vicious cycle ensues that worsens T2DM (167).

Since hyperglycemia progresses slowly and remains asymptomatic, T2DM remains undetected and microvascular/macrovascular complications might be present during diagnosis (168, 169). The most common symptoms of T2DM include: polyuria, polydipsia, polyphagia, weight loss, vision blur, and fatigue (166).

1.4.3.1 Insulin resistance and glucotoxicity

Euglycemia is not only maintained by insulin secretion into the systemic circulation, but also by glucose uptake into peripheral tissues such as the liver, muscles, and adipose tissue, etc. A mismatch between glucose uptake and insulin secretion may indicate the presence of insulin resistance. Hyperglycemia seen in T2DM is mainly caused by inhibition of glucose transporter type 4 (GLUT-4) activity, which is a direct result of alterations in the function of insulin receptors (170). Cells' insulin sensitivity is directly correlated with the number of insulin receptors expressed on the cells' surface as well as the insulin's affinity to these receptors.

The accumulation of lipids plays a major role in the induction of insulin resistance (171). Insulin resistance is also induced by lipids' metabolites and inflammatory molecules that stimulate serine and threonine phosphorylation of insulin receptor beta units and insulin receptor substrates (IRS). Phosphorylation of serine and threonine by specific kinases seems to antagonize tyrosine phosphorylation. This mechanism is very likely responsible for insulin resistance (172). Additionally, non-esterified fatty acids (NEFAs), DAGs, acyl-CoAs, glucose, insulin, and cytokines (TNF-α, IL-1β) can also catalyze tyrosine dephosphorylation and therefore participate in insulin resistance. The main enzymes responsible for serine and threonine in IRS are protein kinase C (PKC), an inhibitor of NFKB kinase, MAPK, and c-Jun N-terminal kinase (JNK). JNK induces insulin resistance by phosphorylating serine 312 on IRSs and thus inhibits the interaction between insulin receptors and the phospho-tyrosine binding sites on IRSs. JNK can be activated by insulin through negative feedback as well as by fatty

acids and TNFα (171, 173-176). Therefore, chronic hyperinsulinemia leads to insulin resistance (176). This insulin resistance increases the level of circulating fatty acids, leading to DAG and acyl-CoA accumulation in liver and muscle cells. Consequently, these molecules activate PKC-θ and phosphorylate IRS1, exacerbating insulin resistance (177).

Other proteins, such as phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and Src homology 2 domain-containing inositol-5-phosphatase (SHIP) may also induce insulin resistance by inhibiting PI3K and its downstream signal.

Chronic hyperglycemia does not only induce insulin resistance but also inhibits insulin expression in several animal models (178-184). The mechanisms implicated include decreased binding of PDX-1 and MafA to their promoter's regions, as well as increased binding of C/EBPβ to its promoter (185, 186). Additionally, hyperglycemia promotes advanced glycation end-products (AGEs) formation. AGEs bind to their receptors' RAGEs (receptors for advanced glycation end-products) and lead to the synthesis of reactive oxygen species (ROS), which damages the function of pancreatic beta cells. In parallel, AGEs inhibit PDX-1 protein expression and insulin promoter activity (187, 188).

1.5 Diabetic Cardiomyopathy

1.5.1 Introduction

Cardiovascular disease is the main cause of mortality in diabetic patients. Diabetics represent 6 to 8% of the general population; however, they represent 12 to 30% of patients with heart failure (189). Additionally, several epidemiological studies have demonstrated that diabetes is an independent risk factor for heart failure (190-192). For example, the Framingham study has shown that heart failure is two to four times more likely to occur in diabetic men compared to non-diabetic men and 5 times more likely to occurs in diabetic women compared to non-diabetic women, even when controlled for age, obesity, dyslipidemia, hypertension, and coronary heart disease (192). Before 1972, cardiovascular mortality and morbidity found in diabetics was attributed to vascular disease. However, in 1972, Rubler had four diabetic patients who suffered from congestive heart failure without the presence of hypertension, congenital heart disease, coronary artery disease, atherosclerosis or alcoholism. Therefore, Rubler became the first to use the term diabetic cardiomyopathy (DCM) to diagnose his diabetes patients (193). In the following years, the notion of isolated DCM was gradually accepted by the cardiology community, and subsequent studies have confirmed its presence. Isolated DCM is defined by abnormal myocardial function and structure in diabetics without vascular disease, alcoholism, or other etiological factors. The existence of this pathological entity is supported by several epidemiological and preclinical studies that found early alterations in left ventricular structure and function during the onset of diabetes. These abnormalities are manifested with ventricular hypertrophies, as well as systolic and diastolic dysfunction. However, there is no specific treatment for DCM and the exact molecular mechanism that leads to DCM has not been fully

elucidated. DCM was the main subject of many preclinical studies in which researchers investigated its origin on diabetic models and underlined various pathophysiological mechanisms as potentially responsible (194). Since diabetes is associated with hyperlipidemia and cardiac steatosis, lipotoxicity is becoming the main culprit for DCM. In parallel, systemic hyperglycemia in diabetics contributes to the formation of AGEs, which alters myocardial protein structure and may be the cause of cardiac hypertrophy. It is also worth mentioning the presence of abnormal calcium homeostasis that may be the origin of abnormal excitation-contraction coupling. Finally, mitochondrial dysfunction, ER stress, increase ROS species, and inflammation have been identified as potential pathophysiological mechanisms for DCM. All these mechanisms lead to cardiomyocytes' apoptosis and necrosis and eventually heart failure, which is considered the final stage of DCM. Early diagnosis of diabetic patients with DCM allow the use of specific strategies to monitor and prevent the progression of DCM to heart failure. However, the clinical importance to identify DCM remains low, since there is no specific treatment. Therefore, research on mechanisms of DCM must be maintained in order to identify new therapeutic targets and develop new treatments.

1.5.2 Cardiac hypertrophy

Type 2 diabetics can have left ventricular hypertrophy without arterial hypertension (195). Indeed, cardiac hypertrophy is one of the early manifestations of DCM and is responsible for reduced systolic functions. Additionally, insulin resistance and T2DM are associated with concentric hypertrophy and diastolic dysfunction (196, 197). Hypertrophy is defined as an increase in volume of myocardial tissue, usually the left ventricle. This increase is caused by the

synthesis of new sarcomeres (contraction units) and, therefore, an increase in cardiomyocytes' volume. These new sarcomeres have two possible depositions. The first is parallel deposition, which makes the cells wider, the myocardium thicker, and the heart cavities smaller. This is known as concentric or pathologic hypertrophy and it is usually the outcome of chronic hypertension. The second is series deposition, which makes the cells longer, the myocardium larger, and the heart chambers bigger. This is known as eccentric or physiological hypertrophy and it is usually the outcome of regular exercising.

Hyperinsulinemia seen in T2DM may contribute to cardiac hypertrophy by upregulating the mitogenic response. Insulin mitogenic response occurs when the RAS-MEK-ERK1/2 pathway is activated, which leads to protein synthesis and cellular differentiation and proliferation. Activating this pathway is implicated in cardiac hypertrophy (198-200). Interestingly, even though adipocytes in diabetics are resistant to insulin's effect on glucose transport, it has been shown that insulin continues to activate and even upregulate the mitogenic pathway (201).

Furthermore, obesity contributes to cardiac hypertrophy independently of hypertension (202). For example, cytokines produced by adipocytes, such as leptins, are likely the trigger of this process. The mechanism is not fully characterized; however, it likely involves an endothelin-1-mediated ROS generation (203). Another adipokine, resistin, which is produced by macrophage, was shown to induce cardiac hypertrophy through IRS-1 and MAPK signaling pathways (204).

Another possible pathway for cardiac hypertrophy in diabetics is through hyperglycemia and angiotensin II (205-207). However, this pathway is unlikely to be the cause of cardiac hypertrophy seen in DCM, since it is usually preceded by hypertension.

1.5.3 Diastolic dysfunction

Left Ventricular Diastolic dysfunction is an independent risk factor for heart failure. Many investigators have demonstrated the presence of diastolic dysfunction in diabetic patients without congestive heart failure. For example, Schannwell et al. found a high prevalence of independent left ventricular diastolic dysfunction with preserved systolic function in Type 1 diabetic patients (208). Diastolic dysfunction is characterized by prolonged relaxation and an increase in ventricular pressure at the end of the diastole (209). Since ventricles cannot fill normally, it usually evolves into systolic dysfunction. Systolic dysfunction is defined as a decrease in the ejection fraction, or the incapability of the ventricles to pump enough blood to the peripheral organs. Hyperglycemia seen in diabetics contributes to diastolic dysfunction by upregulating PKC activity and increasing AGEs, polyol, and hexosamine production (210). These mediators modulate ryanodine receptors and SERCA (sarco/endoplasmic reticulum Ca2+-ATPase) and they impairs calcium accumulation in the endoplasmic reticulum during diastole (211-213). Furthermore, the β1 and β2 adrenergic receptors, which mediate positive inotropic effects, are downregulated in diabetics: hence, the sympathetic nervous system could also play a deleterious role in DCM. Simultaneously, the β3 adrenergic receptor, which possess a cardiodepressant effect on the ventricles, is upregulated in these similar

circumstances (214, 215). These changes may contribute to diastolic dysfunction by impairing ventricles relaxation.

1.5.4 Calcium dysregulation

Calcium is an essential element and a vital electrolyte necessary for maintaining a regular heartbeat rhythm. Intracellular and extracellular levels of calcium are tightly regulated, as are its storage and movement. It is mainly stored in the endoplasmic reticulum (ER). Calcium is also present in interstitial fluid, where it can enter the cells through ion channels. In human cardiomyocytes, there are mainly two types of calcium channels: L-type (long-lasting current) and T-type (tiny voltage threshold). In order to contract, calcium is released from its intracellular storage mainly through ryanodine receptor 2, found on the ER membrane and during relaxation, it is pumped into the ER by SERCA and out of the cells through the sodium-calcium exchanger (NCX) (Figure 1.7). SERCA is an ATP-dependent transporter found on the ER membrane and it has a molecular weight of 110 kDa. There are ten known SERCA isoforms obtained by alternative splicing of three SERCA genes: SERCA1, SERCA2, and SERCA3 (216). SERCA2a is predominantly expressed in cardiomyocytes and skeletal muscles (217). Controlled calcium current across the cell and ER membranes is crucial for cardiomyocytes' normal activity. Any alteration of this current causes dysregulation of calcium homeostasis, which perturbates the excitation-contraction coupling, and it may lead to DCM. Prolonged ventricular relaxation is one of DCM's characteristics. The main culprit is calcium that is not efficiently removed from cytosol. Indeed, several investigators have demonstrated that levels of intracellular diastolic calcium are elevated in cardiomyocytes isolated from diabetic rats in comparison to control rats

(218-220). Perturbation of calcium homeostasis seen in DCM is generally perpetrated through SERCA2 (221). Many research groups have demonstrated that SERCA2 expression or activity was reduced in diabetic models (219, 222-224). For example, according to Ligeti et al., the increase in concentration of intracellular calcium seen at the end of diastole in DCM was attributable to reduced SERCA activity, where the Vmax of this transporter was significantly reduced (225). Nevertheless, a negative contribution of NCX cannot be excluded, since it was shown that this transporter is also downregulated in diabetic hearts (218, 226). In the later stage of DCM, diastolic dysfunction progresses to systolic dysfunction where the frequency and amplitude of cytoplasmic calcium during contractions are weaker in diabetic hearts compared to non-diabetic hearts (227). This was explained by Teshima et al. who demonstrate that a downregulation of SERCA expression preceded downregulation of ryanodine receptor 2 (RyR2) (222).

The exact mechanism by which SERCA is downregulated in DCM is not fully elucidated but glucotoxicity is probably the main instigator. In fact, it was shown that SERCA activity was lower in cardiomyocytes incubated with a high level of glucose (25.5mM) compared to those incubated with a low concentration of glucose (5.5mM) (224). AGEs formation is an early manifestation of glucotoxicity, and Bidasee et al. underlined the presence of AGEs-bound SERCA2 after eight weeks of diabetes (228). Furthermore, when AGEs bind to their specific receptor RAGEs, the AGEs-RAGE system can modify myocardial calcium homeostasis (through SERCA, NCX, or other proteins) and alter ventricular relaxation, which progresses to DCM (229). SERCA2 can also be regulated by kinases such as Ca2+/calmodulin-dependent

protein kinase (CaMK). When SERCA2 is phosphorylated by CaKM, the Vmax of calcium uptake by ER is increased (230).

SERCA activity is also regulated by phospholamban (PLB). When bound to PLB, SERCA is inactive. However, PLB contains several phosphorylation sites, and phosphorylating PLB releases it from SERCA, which renders SERCA active. Studies have shown that PLB can be phosphorylated at Ser^{10} by PKC, at Ser^{16} by protein kinase A (PKA), and at Thr^{17} by CaMK. The concentration of PLB phosphorylated isoform is positively correlated with SERCA activity. Indeed, heart preparations isolated from diabetic rats are characterized with elevated levels of non-phosphorylated PLB (219, 228, 231). Therefore, SERCA activity can be indirectly regulated by kinases and phosphatases. Unsurprisingly, PKA- and CaMK-mediated phosphorylation of PLB was reduced in diabetic hearts (223, 231, 232).

Finally, oxidative stress is another factor believed to be implicated in calcium dysregulation. In fact, decreased expression of the SERCA2 protein in the cardiomyocytes of diabetic mice is completely prevented by overexpressing the antioxidant protein metallothionein (233).

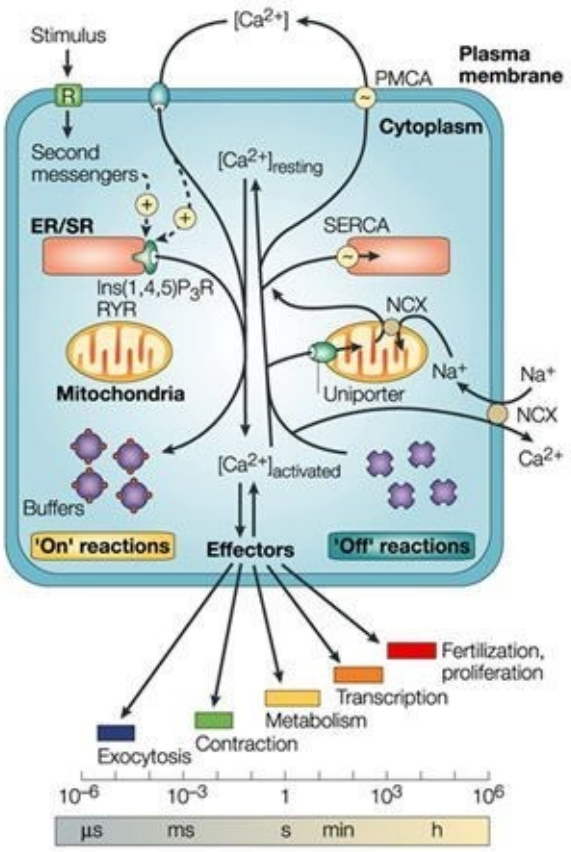

Figure 1.7: Calcium signaling and homeostasis (234).

1.5.5 Fibrosis

In 1972, Rubler et al. were the first to introduce the term diabetic cardiomyopathy. He used this term on four diabetic hearts where the autopsy and histology findings on these hearts revealed the presence of ventricular hypertrophy associated with myocardial fibrosis (193). Fibrosis is defined by the scarring and/or hardening of tissue caused by excessive deposition of collagen or other components of the extracellular matrix. Rubler et al.'s results were later confirmed by several studies that showed interstitial collagen and glycoproteins deposition in cardiac tissue

isolated from diabetic humans (235-237). Furthermore, an increase in interstitial fibrosis was demonstrated in the hearts of diabetic rats (238, 239). Indeed, the most important histopathological characteristic of diabetic hearts is perivascular and/or interstitial fibrosis. Additionally, myocardial fibrosis is frequently put forward to explain cardiac modifications seen in DCM.

In the heart, fibrosis can be reactive or reparative. For example, myocardial infarction is followed by the secretion of extracellular matrix components that protect against myocardial rupture and the loss of cardiac cells. This is called reparative fibrosis. In contrast, arterial hypertension induces reactive fibrosis characterized by excessive collagen secretion by fibroblasts which harden cardiac muscle and cause myocardial dysfunction (240). Fibrosis seen in DCM is mainly reactive fibrosis (241).

Exhibiting both hypertension and diabetes is considered a key factor that induces interstitial fibrosis. This concept was initially suggested in a study performed on rats and was later confirmed in humans. These authors demonstrated, through autopsy, that perivascular and interstitial fibrosis is more likely to be found in diabetics with hypertension compared to only diabetics or only hypertensive patients (237, 242).

The pathway leading to the development of fibrosis in DCM likely involves ROS synthesis and AGEs formation. Hyperglycemia and hyperlipidemia seen in diabetics induces ROS synthesis, which alters genes' expression and signal transduction, leads to oxidative stress, and eventually, activates necrosis and apoptosis. Protein alteration by AGEs can also be the origin of cardiac

stiffness known as fibrosis. AGEs facilitate cross-linking between collagen molecules, rendering these molecules more resistant against degradation. This favors the appearance of myocardial fibrosis (243). Hence, collagen accumulation is not only caused by an increase of its secretion but also by a decrease of its degradation. Furthermore, the renin angiotensin system is a key mediator for interstitial fibrosis seen in diabetics (244). Angiotensin 2, an inflammatory chemokine, is the main effector of the renin angiotensin system (245). Additionally, upregulation of the endothelin system in diabetic hearts can also play an important role in myocardial fibrosis. In conclusion, whatever the mechanism leading to the development of fibrosis in DCM, a decrease in myocardial compliance is the main functional consequence.

1.5.6 Steatosis

Cardiomyocytes of diabetic patients are characterized by altered lipid metabolism. Additionally, insulin resistance, a key characteristic of T2DM, is associated with increased need of cardiomyocytes for fatty acids as an energy source for its metabolic activities. Indeed, in normal hearts, around 75% of total metabolic energy comes from fatty acid oxidation (FAO). However, in T2DM, cardiomyocytes become even more dependent on fatty acids and FAO represents more than 90% of total energy production (246, 247). These circumstances are associated with cardiac steatosis, which is the accumulation of intramyocardial lipids. Indeed, intracellular triglyceride levels are increased in cardiomyocytes of diabetic patients, and cardiac steatosis was proposed as an important cause for DCM (248, 249). The accumulation of fatty acids in cardiomyocytes was demonstrated in diabetic rodents as well as in diabetic humans (249-252). The exact mechanism leading to cardiac steatosis in T2DM is still under investigation; however,

two major contributors are increased fatty acid uptake and hyperlipidemia, an increased concentration of serum lipids.

A key characteristic of hyperlipidemia in T2DM is high levels of plasma triglycerides (253). People with T2DM are at high risk to have hyperlipidemia, which is also one of the major risk factors for cardiovascular disease in T2DM. Insulin resistance and hyperlipidemia are highly correlated. Indeed, hyperlipidemia is usually found in prediabetics with insulin resistance and it has been found that treating Type 2 diabetics with thiazolidinediones (TZD) improves insulin activity and normalizes the concentration of plasma lipids (254, 255). In healthy subjects, insulin inhibits hormone sensitive lipase (HSL) in adipose tissue by decreasing its phosphorylation, which reduces the release of stored fatty acids from adipocytes into the systemic circulation (256). Therefore, the loss of insulin action on HSL in T2DM is associated with dyslipidemia and increased mobilization of lipids from adipocytes (257). Simultaneously, studies have demonstrated that apolipoprotein B (apoB) is overproduced by hepatocytes in T2DM (258-260). ApoB is an important protein found in both very low-density lipoproteins (VLDL) and low-density lipoprotein (LDL) that may explain the cause of hyperlipidemia seen in diabetics (261). Interestingly, events that promote hyperlipidemia in T2DM are positively correlated with increased fatty acid uptake by cardiomyocytes. In the heart, fatty acids can be transported into cardiomyocytes via several membrane transporters, including the cluster of differentiation 36 protein (CD36), the fatty acid transport protein 1/6 (FATP1/6), and the membrane-associated fatty acid binding protein (FABPpm) (57). These cells obtain their fatty acids mainly from lipoproteins where lipoprotein lipase (LPL) hydrolyzes triglycerides to free fatty acids. CD36 expression and activity as well as cardiac LPL expression are upregulated in diabetic settings

(262-265). Inducing cardiac steatosis by upregulating fatty acid uptake in transgenic mice was sufficient to instigate cardiomyopathy independently of hyperglycemia. In these transgenic mice, proteins involved in fatty acid uptake, such as long-chain acyl-CoA synthetase, FATP1, and glycosylphosphatidylinositol (GPI), a membrane-anchored form of lipoprotein lipase, were over-expressed leading to lipotoxic cardiomyopathy (266-268).

The incidence of cardiac steatosis is higher in the diabetic population compared to the general population. Cardiac steatosis precedes the onset of diastolic dysfunction and diabetic cardiomyopathy (248). The mechanism linking steatosis to DCM will be discussed in the following section on lipotoxicity.

1.5.7 Lipotoxicity

Free fatty acids and triglycerides are pathologically elevated in cardiac cells of diabetic patients. Lipotoxicity, which is defined by cellular dysfunction caused by overload of lipids (or its metabolites), is likely to play a central role in the development of DCM. Indeed, several research groups have demonstrated that lipotoxicity is a causal mechanism for DCM (194, 269, 270). Even though triglycerides are elevated in diabetic cardiomyocytes, their damaging effects are controversial. While some reports have indicated that cardiac triglyceride accumulation is associated with systolic and diastolic dysfunction, others have suggested that triglycerides are just markers for lipotoxic metabolites (248, 249, 271). TAG is likely to be a neutral lipid and lipotoxicity to be the result of buildup in other lipids such as ceramides, DAG, or oxidized phospholipids (194). In accordance with this, treating diabetic subjects with metformin or TZD

improved their cardiac function independently of changes in myocardial TAG content (272). Moreover, sequestering fatty acids (especially saturated fatty acids) into triglycerides, thus preventing the conversion of these fatty acids to toxic lipids, protected cardiomyocytes against ER stress and cell death (273, 274).

Saturated fatty acids, such as palmitic acid and stearic acid, are largely involved in DCM development. They induce ER stress and apoptosis in many cell types, including cardiomyocytes (274-277). Borradaile et al. found that palmitate incorporates between the phospholipid layers of the ER membrane, dilating the membrane and leading to the disruption of its structure and loss of its integrity (278). Saturated fatty acids promote inflammation in a wide array of cell types by increasing pro-inflammatory cytokine secretion (IL-6 and TNF-α) (279, 280). They lower cardiolipin (anionic phospholipid) concentration on the mitochondrial membrane, leading to apoptosis in rat neonatal cardiomyocytes (281). Additionally, saturated fatty acids increase ROS production which activates PKC signaling pathways (282). PKC regulates several proteins' activity through phosphorylation. Some of these target proteins are: JNK, mitogen-activated protein kinase (MAPK), and nuclear factor kappa B (NF-κB). Recent studies have demonstrated that fatty acid metabolites can activate PKC-θ, which consequently phosphorylates and activates IκB kinase (283). IκB phosphorylates the serine residue of IRS-1 and inhibits its capacity to bind to phosphoinositide 3-kinase (PI3K). The insulin-signaling cascade is therefore impaired and the translocation of glucose transporter GLUT4 to the membrane is inhibited. Glut4 is the primary transporter of glucose in cardiomyocytes (284).

PI3K is an important component of the insulin signaling pathway. Insulin-mediated inhibition of AMPK activity is lost in lipotoxic conditions in cardiomyocytes (285). The mechanism likely involves PI3K, which is downstream of AMPK (286). Since AMPK regulates fatty acid oxidation, lipotoxicity is also associated with impaired FAO (52, 287). Impairing FAO exacerbates DCM, since it promotes cardiac steatosis and lipotoxicity (288-291). A decrease in FAO is associated with increased accumulation of ceramides (291, 292).

Ceramides are well known inducers of lipotoxicity and cellular dysfunction in many cell types, including cardiomyocytes (293, 294). They activate inflammatory signaling pathways and induce production of reactive nitrogen species (RNS) (269). They also impair insulin signaling by impeding GLUT4 translocation (295). The ceramides level is elevated in skeletal muscles of obese subjects (296). Lowering ceramide concentration improves insulin resistance and lipotoxicity (297). For example, lipotoxicity in transgenic mice overexpressing glycosylphosphatidylinositol (GPI), a membrane-anchored form of lipoprotein lipase, was prevented by inhibiting ceramide biosynthesis (298). Additionally, overexpressing ceramidase, an enzyme that cleaves ceramides, prevents insulin resistance mediated by saturated fatty acids (299). However, Listenberger et al. found that inhibiting ceramide biosynthesis was not enough to completely prevent palmitate-induced cell death in hamster ovary cells (300). The authors pointed to ROS as a mechanism for palmitate lipotoxicity.

DAG and ROS signaling pathways share the same downstream kinase: PKC. DAG accumulation activates PKC, and both molecules induce insulin resistance in obese patients (301). Feeding mice a high fat diet for ten weeks impaired insulin-induced glucose oxidation,

which was positively associated with DAG accumulation (302). DAG accumulation was secondary to increased GPAT activity and decreased DGAT activity in the working hearts of these mice. Indeed, cardiac overexpression of DGAT, which transforms DAG to TAG, decreased DAG levels and protected mice hearts against lipotoxicity (303). GPAT, on the other hand, is one of several enzymes that catalyze DAG synthesis. Toxicity associated with an increased expression of GPAT is not only related to DAG accumulation, but also to elevated levels of lysophosphatidic acid (304).

Interestingly, there are three isoforms of DAG and only one of them, sn-1,2 DAG, has the ability to activate PKCs (305-307). The other two isoforms, sn-2,3 DAG and sn-1,3 DAG, cannot bind to PKC. One difference between oleate and palmitate is the ratio of sn-1,2 DAG to sn-1,3 DAG found in cardiomyocytes treated with these fatty acids. The ratio was higher in palmitate compared to oleate (287). This may explain the difference between saturated fatty acids and unsaturated fatty acids regarding their toxicity. Indeed, palmitate activates the DAG/PKC pathway, causing a pleiotropic effect on cellular dysfunction (308, 309). This was not the case in oleate-treated cells. Oleate, an unsaturated fatty acid, is not only non-toxic in cardiomyocytes isolated from neonatal rats, but also protected these cells from palmitate-mediated lipotoxicity (52). Additionally, oleate improved the lipid profile and prevented mitochondrial dysfunction, insulin resistance, and inflammation in myocytes (310, 311). Diets rich in oleic acid are also associated with reduced cardiovascular events (312).

1.5.8 Hyperglycemia

Chronic hyperglycemia seen in patients with undiagnosed prediabetes or uncontrolled diabetes has multiple consequences. In fact, chronic hyperglycemia is likely the main cause of some diabetic complications such as nephropathy, neuropathy and retinopathy (313). Furthermore, epidemiological studies have demonstrated a clear association between hyperglycemia and cardiovascular disease. Interestingly, this association can be seen even when glucose levels are lower than those found in diabetic patients (314). Cellular glucotoxicity is described as the deleterious effects of hyperglycemia on cells' structure and function in different body tissue (315). Glucotoxicity, the consequence of chronic hyperglycemia, appears in a slow and progressive manner (186). The concept of glucotoxicity is usually used to describe the effect of T2DM on pancreatic beta cells (316). However, here we will describe the mechanism of glucotoxicity on the function of cardiac cells instead. So far, hyperglycemia stimulates three important mechanisms in cardiomyocytes that contribute to glucotoxicity: ROS formation, the hexosamine biosynthesis pathway, and AGEs formation. These mechanisms have a common ending: insulin resistance and cell death (317-319).

Hyperglycemia induces ROS synthesis. An increased level of ROS mediates insulin resistance by inhibiting GLUT4 translocation to the membrane. Several serine/threonine kinases are stimulated by ROS which alters IRS-1 activity and initiates the pro-inflammatory signaling pathway by phosphorylating l'IKKβ and NF-κB (320, 321). Treatment with antioxidants, such as lipoic acid prevents deleterious effects of ROS on glucose transport *in vivo* and *in vitro* (322, 323).

An increased flux of glucose in the hexosamine biosynthesis pathway is another mechanism where hyperglycemia may contribute to insulin resistance (324). The rate-limiting enzyme in this pathway is glutamine:fructose-6-phosphate amidotransferase (GFAT) and the final product is UDP-N-acetylglucosamine (UDP-GlcNAc). O-GlcNAc transferase, an enzyme responsible for proteins' post-translational modification, uses UDP-GlcNAc as its substrate. Hexosamine biosynthesis pathways induce insulin resistance and vascular complication by causing modifications on O-GlcNAc. Indeed, overexpressing GFAT in mice causes insulin resistance (325). Thus, an increase in O-GlcNacylation can alters insulin signaling and inhibits GLUT4 translocation to the membrane (324, 326).

AGEs formation is stimulated by hyperglycemia and oxidative stress (321). AGEs are proteins or lipids that undergo glycation after contact with sugar (327). AGEs inhibits insulin mediated tyrosine phosphorylation of IRS-1 and IRS-2, altering PI3K and Akt activity (328). Simultaneously, AGEs binds to their specific receptor RAGE which activates the transcription factor NF-κB and stimulates ROS production (329, 330).

1.6 Mechanism implicated in diabetic cardiomyopathy

1.6.1 ER stress

1.6.1.1 ER stress and the unfolded protein response

The endoplasmic reticulum (ER) can be described as a network of membranes in the form of tubules and sacks. It represents almost 50% of all membranes in eukaryotic cells. ER is a highly dynamic organelle that regulates many vital functions to maintain cellular homeostasis. Calcium concentration inside the ER is around three times higher compared to its intracellular concentration, making ER an important storage site for calcium. Nevertheless, the main function of the ER is the synthesis of lipids and proteins. Inside the ER, lipids and proteins are synthesized separately in two distinct zones: the smooth ER and the rough ER. Rough ER is rich with ribosomes, which give it a rough appearance when seen on an electronic microscope, hence the name. The smooth ER, which lacks ribosomes, is the site of lipid metabolism and synthesis, while the rough ER is the site of protein synthesis and maturation (331). Protein maturation is a critical process in insuring a proper functioning of these proteins. An important maturation step for newly synthesized proteins is "protein folding." When it folds, a protein acquires its nascent three-dimensional structure. Before being exported to the Golgi apparatus, the nascent proteins undergo the maturation step while interacting with different classes of chaperone proteins. Even though these chaperone molecules are found in several organelles, some of them are exclusive for the ER such as glucose-related peptide 78 (GRP78). Chaperone molecules interreact with

the hydrophobic sequence of nascent proteins, which prevent their aggregation and facilitate their proper folding (332).

Perturbation in ER homeostasis decreases its capacity to support proteins' maturation. If protein synthesis exceeds protein maturation, an aggregate of unfolded proteins will start to accumulate, causing ER stress. The presence of ER stress triggers a physiological response called the unfolded protein response (UPR) (333). The UPR aims to: (1) reduce protein synthesis to prevent the accumulation of unfolded proteins, (2) increase expression of ER chaperons to stimulate protein folding, (3) promote the export of irreversibly misfolded proteins and, subsequently, their degradation in the proteasome, (4) set off apoptosis if the previous steps fail to solve ER stress (334). Indeed, unresolved ER stress is involved in the development of many pathologies, such as T2DM and diabetic cardiomyopathy (335, 336). The involvement of ER stress in diabetic cardiomyopathy will be discussed in detail in the following section.

The unfolded protein response is a signaling pathway divided into three distinct branches (Figure 1.8). These branches are regulated by three transmembrane proteins: protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK), activating transcription factor 6 (ATF6), and inositol requiring enzyme 1 (IRE1). Under a normal physiological state, these three proteins are bound to GRP78 on the ER membrane, which keeps them inactive. The accumulation of unfolded proteins will recruit GRP78 and causes the dissociation of GRP78 with UPR effectors. This dissociation activates the UPR (337).

The IRE1 pathway can be described as follows. IRE1 is a transmembrane protein that has an endoribonuclease (RNase) activity on its C-terminal cytosolic domain and a serine/threonine kinase activity (338). The first branch discovered in UPR was the IRE1 branch and the IRE1 remains the most studied protein in ER stress. Two isoforms of IRE1 are found in mammalian cells: IRE1α and IRE1β. IRE1β is expressed only in intestinal epithelial cells, while IRE1α is ubiquitously expressed in all cells, including cardiomyocytes (339, 340). Once it is released from GRP78, IRE1 homodimerizes and auto-phosphorylates, thanks to its kinase activity (341). Activated IRE1 initiates mRNA splicing of the XBP-1 transcription factor (XBP-1 stands for X-box binding protein 1) and removes 26 nucleotides from its mRNA. Interestingly, the translation of spliced XBP-1 generates a longer protein compared to non-spliced mRNA (342). The splicing causes a frame shift in its codon sequence that moves the stop codon downstream of its original position. Spliced XBP-1 translocates to the nucleus and activates several genes involved in protein maturation and folding (343, 344). It equally induces the expression of many genes involved in protein degradation and apoptosis (343).

The PERK pathway can be described as follows. PERK is a serine/threonine kinase that phosphorylates eukaryotic initiation factor 2 alpha (elF2-α). Once released from GRP78, PERK is dimerized and activated by autophosphorylation (342). Consequently, PERK phosphorylates and thus inhibits elF2-α. Inhibition of elF2-α causes a global decrease protein translation. Interestingly, some protein translations, such as activating transcription factor 4 (ATF4), are upregulated. ATF4 has an open reading frame (ORF) in its 5' untranslated region (5' UTR); therefore, it is translated in a 5' cap independent manner, allowing it to be translated more efficiently (345). In the early stage of ER stress, ATF4 expression promotes cellular survival by

regulating genes involved in oxidative stress resistance and amino acid import. However, prolonged ER stress induces apoptosis through the ATF4/CHOP pro-apoptotic signaling pathway (338). Indeed, one of ATF4's target genes is the CCAAT/enhancer binding protein (C/EBPs), a homologous protein (CHOP) that is also a transcription factor that upregulates genes involved in apoptosis (346).

The ATF6 pathway can be described as follows. There are two isoforms of ATF6: ATF6α and ATF6β (347). Both are ubiquitously expressed; however, since most researchers have focused on ATF6α, the names ATF6 and ATF6α were used interchangeably. When bound to GRP78, ATF6 is inactive (343). Once released from GRP78, ATF6 is transported to the Golgi apparatus, where it is cleaved by two proteases: site 1 protease (S1P) and site 2 protease (S2P) (343). Following the cleavage, the N-terminal fragment is released, then translocated to the nucleus to act as a transcription factor. Interestingly, ATF6 belongs to the ATF/CREB family, since its structure and activation mechanism are similar to other proteins in the CREB family (CREB stands for cAMP response element binding protein) (348). Hence, the ATF6 structure contains a leucine zipper domain that mediates the binding of ATF6 to cAMP response element (CRE) and activation of target genes (349, 350). It also induces the expression of genes where the promoter contains UPRE (unfolded protein response element) and ERSE (ER stress response element) (351). For example, the expression of chaperons' molecules, such as GRP-78, are upregulated in order to increase protein folding and decrease protein aggregation in the ER. CHOP, XBP-1, and proteins implicated in endoplasmic-reticulum-associated protein degradation (ERAD) are also induced by ATF6 (342, 343, 352).

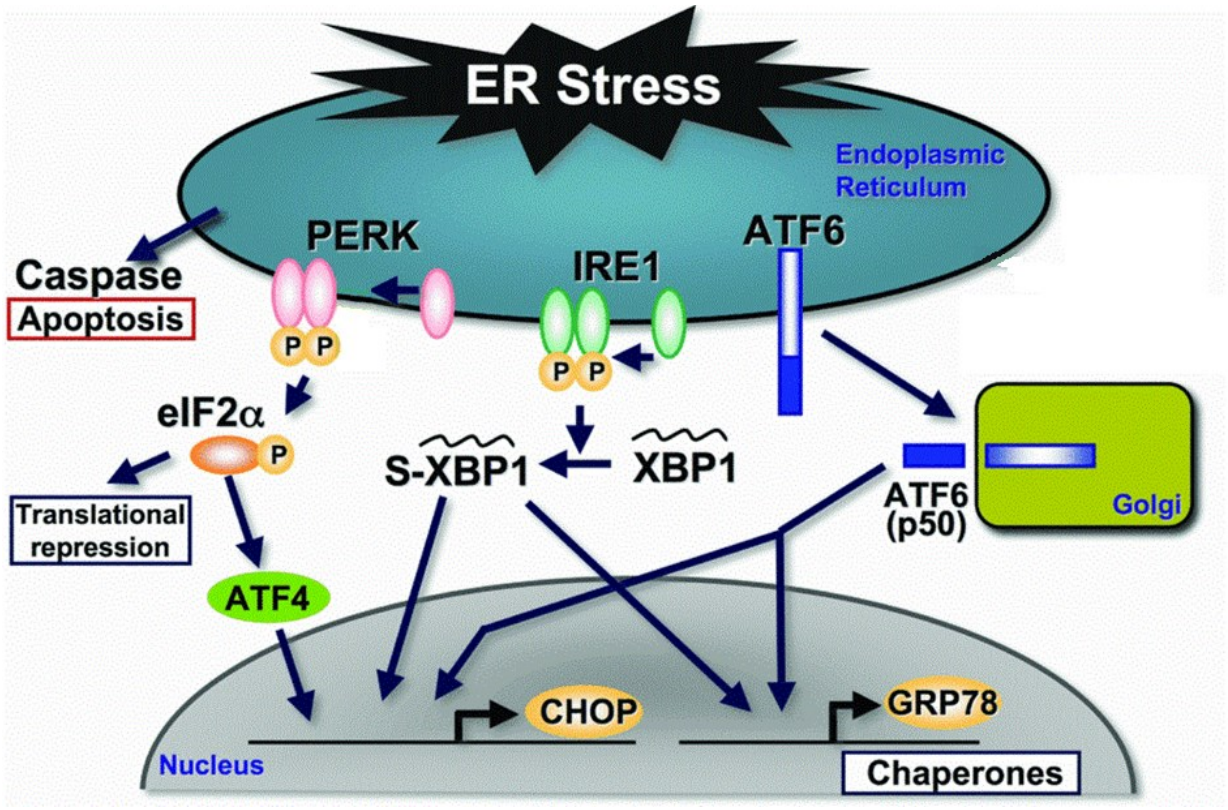

Figure 1.8: The three arms of ER stress [adapted from (353)].

1.6.1.2 ER stress in diabetic cardiomyopathy

ER stress is central to the pathogenesis of several degenerative diseases including Parkinson's and Alzheimer's (354, 355), several cancers (356), metabolic disorders such as obesity and insulin resistance (357, 358), and cardiac diseases such as ischemia, atherosclerosis, hypertrophy, and heart failure (359).

In cardiomyocytes, the ER has the additional role of storing calcium and regulating calcium homeostasis; hence, it is called the sarcoplasmic reticulum (SR). Since the primary function of cardiomyocytes is to contract, the SR is relatively-well developed, making the cells highly

vulnerable to ER stress. As an example, thapsigargin, a non-selective inhibitor of SERCA channels, is a well-known powerful inducer of ER stress in cardiomyocytes—in other words, the mechanism for thapsigargin-mediated dysfunction and the death of cardiac cells (360). Tunicamycin, an inducer of ER stress because it inhibits protein glycation, causes cell death in cultured cardiomyocytes (361). Indeed, ER stress is an important risk factor for cardiovascular disease and some researchers have focused on attenuating ER stress as a therapeutic target (359).

The relationship between ER stress and cardiovascular disease is based on many studies done *in vivo* and *in vitro*. Exacerbating ER stress through hypercholesterolemia or hypertriglyceridemia renders the heart more vulnerable to ischemia (362). ER stress is associated with heart dilatation and hypertrophy, since depleting the CHOP gene attenuates hypertrophy and cardiac dysfunction. The proapoptotic pathway involving CHOP, JNK, and caspase-12 is induced by ER stress and activated in failing hearts (363). Hypoxia induces CHOP expression, and ER stress is upregulated within the myocardial infarction area (364, 365).

In diabetics, ER stress is a major contributor to cardiac dysfunction, since it can be triggered by characteristics usually seen in diabetic hearts, such as free fatty acids, hyperglycemia, and inflammation (366). Indeed, Miki et al. found markers of ER stress in Otsuka Long-Evans Tokushima Fatty (OLETF) rats were upregulated in a genetic model of T2DM (367). Similarly, unfolded protein response is triggered in the myocardium of streptozotocin-induced Type 1 diabetic mice (368).

Free fatty acids are likely to be the main instigator of ER stress in diabetic patients. ER is an important regulator of lipid metabolism and triglyceride synthesis (369). Accumulation of newly synthesized TAG in the ER membrane bilayer is an obligatory step in lipid droplet synthesis. A perturbation in this process caused by an increase in intracellular fatty acids may trigger ER stress. For example, accumulation of saturated fatty acids perturbates ER membrane fluidity and inhibits SERCA activity, leading to ER stress (370). Palmitate, a saturated fatty acid, is a powerful inducer of ER stress in cultured human and rodent cardiomyocytes (274, 371). Palmitate also induces apoptosis, most likely through ER stress-mediated cell death since CHOP and JNK were upregulated in a mouse model of lipotoxic cardiomyopathy (372).

Culturing cardiomyocytes in a high-glucose medium (28mM) activates ER stress and cell death (373). *In vivo*, hyperglycemia alters calcium homeostasis and causes ER stress that contributes to diabetic cardiomyopathy (374). The authors used transgenic, non-obese Type 2 diabetic rats, where only the PERK and the ATF6 branches of ER stress were activated (374). These findings are in agreement with other studies that found improving glucose intolerance and hyperglycemia alleviates ER stress (375).

Inflammation is another possible instigator of ER stress in diabetic cardiomyopathy. However, it is still not clear whether inflammation induces ER stress or vice versa. Chronic infection and cytokine secretion is associated with ER stress and cell apoptosis (376). While some researchers demonstrated that certain cytokines induce different branches of the UPR (such as IL-1β, which induces PERK phosphorylation and XBP1 splicing), others have established that ER stress induces sterile inflammation (377, 378).

1.6.1.3 ER stress-induced apoptosis

The main purpose of UPR and ER stress is to maintain ER homeostasis. Unfortunately, if ER persists and remains unresolved, it activates pro-apoptotic pathways. Inducing apoptosis via ER stress occurs through the activation of CHOP by PERK/elF2α, activation of the Ask1/JNK pathway by IRE1, and the release of calcium from ER via Bax/Bcl2 (379).

The PERK/eIF2α branch of the UPR is the main pathway for CHOP activation. CHOP is a transcription factor that targets several genes, such as GADD34, TRB3, and Bcl-2 (380, 381). GADD34 exacerbates ER stress by interacting with protein phosphatase 1 (PP1) and upregulating eIF-2α. This is associated with apoptosis and cell death (381). Bcl-2 regulates apoptosis by inducing the pro-apoptotic pathway or inhibiting the anti-apoptotic pathway. Interestingly, Chop can activate apoptosis by eliminating the anti-apoptotic effect of Bcl-2 (380). Unlike GADD34 and Bcl-2, TRB3 can regulate Chop activity. When ER stress is temporary and brief, TRB3 downregulates Chop activity. However, when ER stress persists for a longer period, TRB3 mediates apoptosis (382).

IRE-1 can also activate apoptosis through ASk1-mediated upregulation of JNK and p38 mitogen-activated protein kinases (p38 MAPK) (383). This signaling mechanism is highly reliant on the Bcl-2 protein family. Bcl-2 proteins are divided into two groups: 1) the pro-apoptotic sub family consisting of proteins such as BAX, BAK, BAD, BIM, BID, and HRK, as well as 2) the anti-apoptotic sub family consisting of proteins such as Bcl-2 and Bcl-XL (384).

The pro-apoptotic protein must dimerize before being activated, while the anti-apoptotic proteins bind to the monomers of pro-apoptotic proteins and thus inhibit them. The pro-apoptotic proteins are activated once dissociated from the anti-apoptotic proteins. Therefore, to induce cell death, BID and BIM translocate to the mitochondrial membrane and bind to BAX and BAK. These four proteins are hence activated, altering the permeability of the mitochondrial membrane and releasing cytochrome c (385). Cytochrome c initiates a cascade of events that activate caspase-9 and, subsequently, the pro-apoptotic caspase pathway.

The anti-apoptotic Bcl-2 proteins are regulated by IRE1 via an Ask1/JNK signaling cascade. Following this, JNK phosphorylates and thus dissociates the anti-apoptotic Bcl-2 from the monomers of pro-apoptotic Bcl-2. These latter proteins are hence activated (386).

As a side note, the same protein family, Bcl-2, is involved in calcium homeostasis. The BAX inhibitor-1 (BI-1) proteins are found on the ER membrane and function as a calcium leak channel. Just like its name suggest, BI-1 is an inhibitor of BAX and, therefore, an inhibitor of BAX-mediated apoptosis (387, 388).

1.6.2 Inflammation

Inflammation is a defense mechanism that organisms have developed to protect themselves against pathogens, micro-organisms and their products, and certain internal elements such as abnormal cells. Inflammation is regulated by the immune system, which is subdivided into innate immunity (non-specific) or acquired immunity (specific). Innate immunity is gained at

birth and consists of barriers such as skin as well non-specific physiological processes involving leucocytes and their products. As for acquired immunity, it must evolve as the system encounters different kinds of invaders, setting up defense procedures such as the secretion of antibodies (by lymphocytes). Even though inflammation is a defense mechanism, it may cause cellular damage at an intensity dependent on the affected organ or tissue. Organs disposing internal redundancy (kidneys, for example, which have several independent renal glomeruli) or having a high capacity for cell regeneration are much more protected against inflammation compared to the heart, which lacks these characteristics. When the heart's exposure to inflammation persists, an onset of cardiomyopathy may occur (389).

Diabetes is associated with a low-grade inflammatory state and an increase of pro-inflammatory circulating cytokines (390). The first evidence suggesting the presence of inflammation in diabetic patients is traced back to more than a hundred years ago, when glycemia and glycosuria were reduced by administering sodium salicylate (391). Currently, diabetes is characterized by a low concentration of circulating inflammatory mediators such as acute phase proteins (fibrinogen, haptoglobin, and C-reactive protein), as well as cytokines and chemokines[(TNF-α, IL-6, IL-1β, and monocyte chemoattractant protein 1 (MCP1)] (392, 393). Furthermore, it is now established that TNF-α is a key component in the insulin resistance seen in diabetics (394, 395).

The low-grade inflammatory state found in diabetic patients is due, at least, to macrophage infiltration in adipose tissue and islets of Langerhans (396, 397). Unfortunately, macrophage infiltration of cardiac tissue is much less studied. However, one research group found that

cardiomyopathy succeeds macrophage infiltration of the heart (398). Macrophage secretion of pro-inflammatory cytokines induces insulin resistance via the activation of JNK and NF-κB inflammatory pathways (399).

Nevertheless, increased evidence now links low-grade inflammation in the heart to metabolic dysregulation (400). More precisely, saturated fatty acids and other lipids induce *in situ* inflammation, independently of circulating macrophage and cytokines (51, 279). This is in accordance with atherosclerosis, an inflammatory-based disease of which lipids are the main instigators (401). Activation of pro-inflammatory pathways by free fatty acids can be demonstrated by three events. First, cytosolic accumulation of fatty acids is associated with increased accumulation of intramyocellular DAGs and the activation of several isoforms of PKC (402). PKC is well known for its ability to activate the IκB kinase (IKK), a kinase upstream of NF-κB, in many cell types including cardiomyocytes (403, 404). Therefore, DAG-mediated activation of PKC may be an upstream event for NF-κB upregulation. On the other hand, PKC seems to upregulate TNF-α activity (405). Consequently, TNFα creates a feedback amplification loop by promoting lipid accumulation and steatosis via the suppression of AMPK (406). Second, toll-like receptor 4 (TLR-4) is essential for the production of inflammatory cytokines. It has been proposed that fatty acids are endogenous ligands for TLR4; hence, the TLR-4 inflammatory pathway can be triggered by fatty acid accumulation, linking lipotoxicity to IKK and NF-κB (407, 408). Third, ER stress induced by long-chain fatty acids may lead to IKK and JNK upregulation and, therefore, the generation of an inflammatory response (357).

Schilling et al. proposed that lipid-induced inflammation is the main culprit for cardiac dysfunction in diabetes and obesity (398). Additionally, in T2DM, obesity-related inflammation and lipid abnormalities are usually present before the onset of hyperglycemia (409). Indeed, dysregulation in lipid metabolism remains the most important factor of inflammation in T2DM and diabetic cardiomyopathy (400). Since cardiac lipid metabolism is mainly regulated by peroxisome proliferator-activated receptors (PPARs), a type of transcription factor, the next section will examine the relationship between PPARs and inflammation in diabetic cardiomyopathy.

1.6.2.1 PPARs

The metabolic flexibility of the heart is essential for maintaining its contractile function. Long-term alteration of the heart's metabolic profile involves the transcriptional regulation of genes implicated in cardiac metabolism. PPARs and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) were among the first transcription factors identified that cardiomyocytes use to regulate the heart's metabolism (410). The three different PPAR members (PPARα, PPARβ/δ, and PPARγ) are part of the nuclear receptors superfamily. Once a ligand binds and activates them, they form a heterodimer with RXR. RXR is a nuclear receptor activated by retinoic acid. The heterodimer PPAR-RXR induces the expression of its target genes by binding to a specific promoter region: PPRE. The PPAR transcription factors are known for their role in fatty acid metabolism and inflammation (411-413). TZD, a specific activator of PPARγ, is beneficial against Type 2 diabetes and insulin resistance (414).

Long-chain fatty acids are among the main ligands for PPARs. Once activated, PPARα induces the expression of proteins implicated in fatty acid uptake (CD36 and FATP1), fatty acids' esterification and translocation (FABP and ACSL), and fatty acid mitochondrial oxidation (Cpt1 and ACADL). The uncoupling proteins found on the mitochondrial membrane (UCP1 and UCP2), as well as PDK4, an enzyme involved in glucose oxidation, are also regulated by PPARα (400).

The anti-inflammatory action of PPARs has been discovered thanks to a drug known as fibrate during the 1990s. Fibrates are PPARα ligands that lower hyperlipidemia and fight inflammation by lowering IL-6 secretion (415, 416). Interestingly, co-administering PPARα and PPARδ agonists to cultured cardiomyocytes attenuated palmitate-mediated lipotoxicity by downregulating IL-6 expression (51). Additionally, some non-steroidal drugs are simultaneously PPAR agonists and anti-inflammatory (417). This is in line with other findings that state PPARγ performs direct anti-inflammatory actions by reducing TNF-α and the secretion of other interleukins (418, 419).

1.6.3 Oxidative stress and ROS

Under normal aerobic metabolism, oxygen undertakes a series of oxidation-reduction reactions leading to the production of water (420). During this process, intermediates called reactive oxygen species (ROSs) are formed. Their synthesis involves a fundamental mechanism of removal (oxidation) or the addition of an electron (reduction) by one of the many pro-oxidant enzymes. Under normal physiological conditions, ROS concentration is low since ROS

synthesis is highly controlled by enzymatic and non-enzymatic antioxidant systems (421). An imbalance in favor of ROS synthesis (or that of other oxidants) versus anti-oxidant molecules causes oxidative stress. The term ROS refers to several groups of oxygen-reactive metabolites such as free radicals or other non-free radicals (Table 2). Free radicals such as superoxide anion ($O_2^{\cdot -}$), a hydroxyl radical (.OH), are highly reactive molecules since they contain one or more non-paired electrons (422).

Type	Free radicals	Non- radicals
ROS	Superoxide, $O_2^{\cdot}$ Hydroxyl, OH^- Peroxyl, $RO_2^{\cdot}$ Alkoxyl, RO^-	Hydrogen peroxide, H_2O_2 Ozone, O_3
RNS	Nitrogen monoxide, NO Nitrogen dioxide, NO_2^-	Nitrous acid, HNO_2 Peroxynitrite, $ONOO^-$

Table 1.2: Selected examples of free reactive species (adapted from (423)).

ROSs are mainly formed during the electron transport chain and as a by-product of lipid oxidation in mitochondria or peroxisome. In cardiomyocytes, saturated fatty acids significantly increase ROS generation (287, 424). ROSs are cytotoxic since they can damage the cell membrane and alter enzyme activity, thereby altering signaling cascades and promoting damaging processes such as inflammation and apoptosis. These species oxidize proteins, nucleic

acids, and lipids, and they react with nitric oxide to produce peroxynitrite. The latter reacts with tyrosine residues in proteins to produce nitrotyrosine. Nitrotyrosine is a marker of oxidative stress in tissue and is associated with apoptosis (425).

Inflammation and ROS are highly associated. ROS can instigate an inflammatory response by activating stress-sensitive kinases (PKC, JNK, MAPK, and IKK), consequently activating nuclear transcription factors such as activator protein 1 (AP-1) and NF-κB. These transcription factors induce the expression of genes associated with inflammatory markers, including cytokines (TNF-α and IL-6) (426). A positive feedback loop is thus generated since these inflammatory markers can promote oxidative stress and ROS production (427).

The concentration of anti-oxidants, such as glutathione, is low in obese patients with Type 2 diabetes when compared to healthy subjects (428). Furthermore, glutathione activity is negatively associated with cardiovascular events (429). Oxidative stress starts early and its severity increases progressively in the hearts of Streptozotocin-induced (STZ) diabetic rats (430). It is also suggested that ROSs contribute to the progression of DCM. In the heart, ROSs are involved in the development of cardiac fibrosis and hypertrophy, two characteristics of DCM (431, 432). Finally, ROSs are associated with contractile dysfunction and heart failure (433).

1.6.4 Mitochondrial dysfunction

1.6.4.1 Fatty acids oxidation

In diabetic models, cardiac LPL and CD36 activity are upregulated, which is associated with increased intramyocardial fatty acid accumulation. CD36 plays a major role in FA uptake, since more than 50% of fatty acids are transported into cardiomyocytes through a CD36-dependent mechanism. Indeed, T2DM and DCM are associated with hyperlipidemia and cardiac steatosis. Lipid accumulation in diabetic hearts is mainly due to the mismatch between fatty acid uptake and mitochondrial capacity to oxidize these fatty acids (Figure 1.9). The inability of mitochondria to oxidize the increased supply of fatty acids is at least partially due to mitochondrial dysfunction. In accordance with this, evidence suggests that mitochondrial dysfunction is an early event that occurs during diabetes and DCM (434). Although cardiomyocytes appear to respond to this mitochondrial dysfunction by activating the mitochondrial biogenesis program via mechanisms that are both dependent and independent of the PGC-1α transcription factor, cardiac steatosis remain a key characteristic of T2DM and DCM (248, 435).

Whether FAO oxidation is upregulated or downregulated in diabetic patients remains a controversial subject, since inconsistent results have been reported in different models of diabetes. While some studies show that FAO is upregulated in diabetic models, others have demonstrated a complete or partial impairment of FAO.

Arguments for increased FAO in DCM comes from several research groups where FAO has been shown to be upregulated in db/db and ob/ob mice, preceding the development of insulin resistance and cardiac dysfunction (370). Additionally, cardiac-specific overexpression of PPARα were associated with cardiac dysfunction similar to what is observed in human DCM (436). Furthermore, with the help of positron emission tomography, it was shown that patients with Type 2 diabetes have increased fatty acids uptake and β-oxidation (437). Finally, ATGL overexpression protects against STZ-induced cardiomyopathy, which was associated with reduction of β-oxidation in aerobic working heart perfusions (372).

This suggests that inhibiting FAO is protective against DCM, which might be true in certain conditions. For example, Type 2 diabetic patients display insulin resistance and impaired glucose oxidation. Therefore, inhibiting FAO might sensibilize cardiomyocytes to glucose oxidation, since glucose oxidation and FAO are inversely regulated by a metabolic process referred to as the Randle cycle. Additionally, glucose oxidation is more advantageous compared to palmitate oxidation during hypoxia or ischemia, when oxygen levels are low (438, 439). This can be explained when we take into consideration that each substrate of fatty acids or glucose has a different ratio of energy produced to oxygen consumed. This ratio, expressed as ATP/O, is 3.17 for glucose and 2.83 for palmitate (440). It means that for each oxygen molecule consumed, glucose generates 3.17 ATP and palmitate generates 2.83 ATP. Since diabetes is a significant predictor for ischemic stroke, and since ischemic heart failures are increased in Type 2 diabetic patients, inhibiting FAO may be beneficial (441, 442).

Despite the positive prospect of inhibiting FAO in DCM, this option is not viable as a long-term treatment for diabetic hearts. Long-term reduction of FAO leads to the accumulation of fatty acids in cytosol and, hence, lipotoxicity. This notion is supported by many studies showing the detrimental effects of inhibiting FAO in diabetic settings. For example, inhibiting PPARδ expression to downregulate FAO was associated with cardiac steatosis, cardiomyopathy, and ventricular dysfunction (443). Similarly, mice with long-chain acyl-CoA dehydrogenase (LCAD) deficiency have impaired FAO and develop cardiomyopathy, which has been associated with cytosolic lipids accumulation (444). This notion is further supported by two studies that investigated the outcome of the short-term and long-term effects of CPT1B deficiency in mice. CPT1B deficiency protected mice from insulin resistance in the short-term; however, the long-term effect causes CPT1B deficient mice to develop severe insulin resistance (445, 446).

The fact that cardiac steatosis is associated with T2DM and DCM suggests that FAO is impaired in these cases and that stimulating FAO may protect against cardiac dysfunction by preventing lipid accumulation (447). Arguments confirming this hypothesis come from several studies where PPAR receptors are genetically modulated. Knocking out PPARα in mice overexpressing PPARγ induced FAO and enhanced cardiac function when compared to mice only overexpressing PPARγ (448). Similarly, knocking out CD36 in PPARα transgenic mice protected these mice from cardiac dysfunction (449). In support of this, samples taken from the atria of Type 2 diabetic patients display reduced capacity to oxidize fatty acids. Supplementing ZDF rats with resveratrol was associated with improved oxidation of palmitoyl-CoA and reduced oxidative stress (450, 451). Finally, we have demonstrated in cultured cardiomyocytes

that inhibiting FAO is associated with cell death, while upregulating FAO protected these cardiomyocytes from palmitate-mediated lipotoxicity (52).

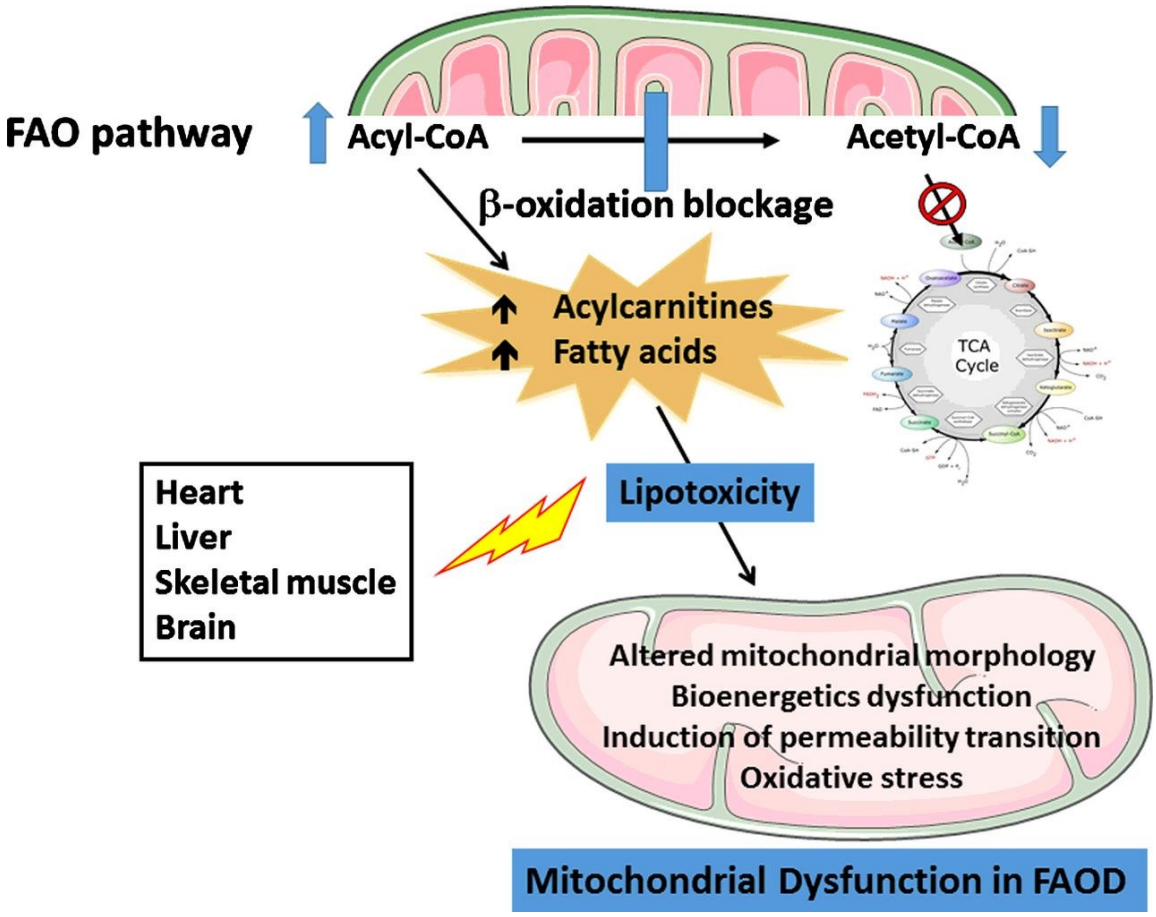

Figure 1.9: Inhibiting FAO is associated with lipotoxicity and mitochondrial dysfunction (FAOD stands for fatty acids oxidation disorders) (452).

1.7 Apoptosis

Programmed cell death, or apoptosis, is an essential component of normal cell proliferation and development and also a response to certain abnormal states. Apoptosis occurs when cells trigger self-destruction in response to a signal. It is a physiological cell death, genetically programmed, necessary for the survival of multicellular organisms. Hence, it is in constant balance with cell proliferation. Apoptosis was identified in diabetic hearts (244). Cardiac apoptosis occurs when moderate stress is repetitive or chronic such as in DCM (453). Indeed, we have discussed in previous sections several mechanisms where diabetes may lead to heart failure and cell death independently of vascular disease or other etiological factors.

Apoptosis takes several hours to complete, but it can be prevented by inhibitors of cytokine synthesis such as pentoxifylline), by antidepressants such as sertraline, or by inhibitors of cyclo-oxygenase-2 (454, 455). There are two possible pathways for apoptosis that both lead to the caspase pathway: the intrinsic pathway and the extrinsic pathway. These two pathways are distinguished by the initiating proteins that trigger them and by adapter proteins involved in each of them (Figure 1.10).

1.7.1 Intrinsic pathway

Several factors can activate the intrinsic pathway, namely as the accumulation of unfolded proteins in ER, DNA damage, and hypoxia. The intrinsic pathway is also called the mitochondrial intrinsic pathway since it involves the release of cytochrome c from

mitochondria. Once released, cytochrome c interacts with procaspase-9 and APAF-1 to form the apoptosome. Apoptosome recruits the effector caspases: caspase-3, 6, and 7 (456). Caspase-3 is the more potent compared to the other caspases (457). However, Morishima et al. have demonstrated a caspase signaling pathway that is specific to ER stress and independent of cytochrome c (458). It starts when caspase-12 activates caspase-9 which in turns activates caspase-3. Additionally, caspase-12 mediates an ER-specific apoptosis pathway (459).

As a side note, some caspases are implicated in inflammation. For example, caspase-1, -4, -5 and -12 are associated with inflammation and usually activate the assembly of inflammasomes (460). Inflammasomes facilitate the secretion of pro-inflammatory proteins (IL-1β, IL-18, and IRFs) that mediate the antiviral response.

1.7.2 Extrinsic pathway

The extrinsic pathway is activated when specific ligands bind to cell death receptors. These ligands are TNF, CD-95 (cluster of differentiation 95), TRAIL (TNF-related apoptosis-inducing ligand), and TL1A (TNF-like ligand 1A). The receptors belong to the TNF family, which includes: TNFR1 (TNF-receptor 1), Fas/APO-1, DR-3 (death receptor 3), DR-4, and DR-5.

These receptors are coupled to one of these two adapter proteins: FADD (Fas-associated death domain) or TRADD (TNFR-associated death domain). When ligands bind to their receptors, monomers of initiator caspases are recruited, causing their dimerization with the adapter proteins. These dimers are the biologically active form of initiator caspases. Once activated,

initiator caspases cleave and hence activate the effector caspases, which exert their proteolytic functions (456). Additionally, the initiating caspase-8 activates the effector caspases and cleaves BID which in turn activates the intrinsic pathway (456).

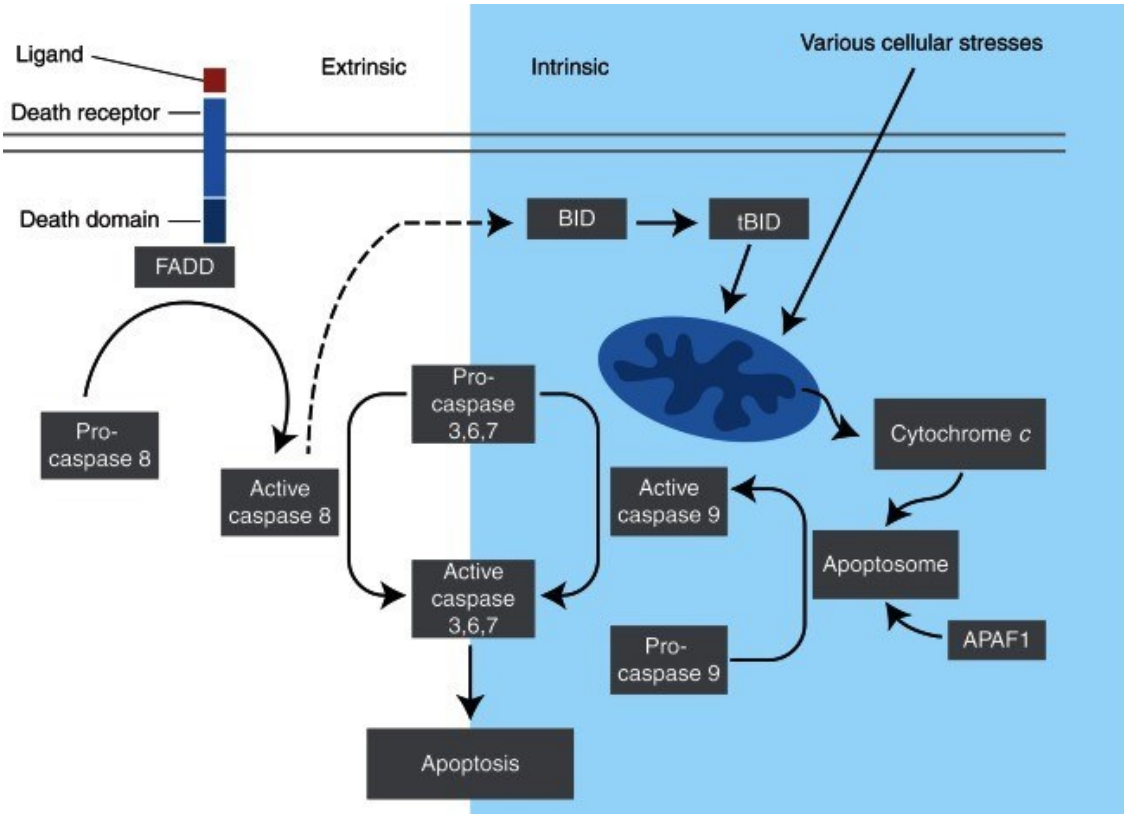

Figure 1.10: Extrinsic and intrinsic pathways of apoptosis (456).

1.8 Rationale, hypothesis and objectives

Cardiovascular disease is the number-one killer of diabetic patients (461). Additionally, diabetes is an independent risk factor for heart failure. Indeed, diabetic patients are two to five times more likely to die from heart failure compared to the general population (462). Several studies

have shown cardiac dysfunction in these diabetic patients without the presence of hypertension, coronary artery diseases, or other etiological factors. This is known as diabetic cardiomyopathy (DCM). The specific cause for DCM is multifactorial however, recent studies tend to point at lipotoxicity as a possible causal mechanism (463). First, diabetic patients often exhibit hyperlipidemia and cardiac steatosis (248). Second, intramyocardial lipid accumulation causes lipotoxicity and leads to cell death with ensuing cardiac dysfunction (464).

The two main fats in the human diet are oleate, a monounsaturated fatty acid, and palmitate, a saturated fatty acid (465). Importantly, lipotoxicity is mainly caused by saturated fatty acids, while unsaturated fatty acids have been described as neutral and even protective (274). The exact mechanism of action of saturated fatty acids is yet to be fully elucidated, but there is evidence that supports the role of calcium dysregulation, alterations in membrane phospholipids, loss of nuclear integrity, loss of mitochondrial membrane potential, impaired cardiolipin synthesis, oxidative stress, and impaired fatty acid oxidation (281, 466-473).

However, in diabetic settings, the exact molecular mechanism where lipotoxicity induces cardiomyopathy is still under investigation. The first part of this thesis aims to characterize and assess the qualitative aspects of lipid accumulation in cultured rat neonatal cardiomyocytes. More precisely, we assessed the effect of palmitate and oleate on ER stress, cell viability, inflammation, and FAO. The results of the first part of our research led us to hypothesize that FAO is attenuated in diabetic mice and that enhancing FAO is a protective pathway in diabetic cardiomyopathy that attenuates cardiac steatosis and, hence, lipotoxicity. As such, we hypothesized that FAO is decreased in palmitate-treated cardiomyocytes and enhancing FAO

should attenuate palmitate-mediated cell death. Our hypothesis was also tested in STZ diabetic mice.

The specific objectives are twofold: (1) to elucidate the mechanism of action and the effect of palmitate and oleate on ER stress, apoptosis, inflammation, oxidative stress and fatty acid oxidation. (2) to investigate the relationship between diabetic cardiomyopathy and FAO.

We have proceeded with a methodical approach using cultured cardiomyocytes and progressed to a diabetic mice model. We combined *in vitro* techniques in cardiomyocytes with *in vivo* studies in STZ mice in order to investigate FAO and the role of this metabolic pathway in diabetic cardiomyopathy.

Chapter 2: Saturated fatty acids induce endoplasmic reticulum stress in primary cardiomyocytes

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2.1 Authors' contributions

Taha Haffar performed most of the experiments, helped analyze the data, and assisted with writing the manuscript. Félix-Antoine Bérubé-Simard performed some experiments. Jean-Claude Tardif contributed to the design of the study. Nicolas Bousette conceived and designed the study, performed some experiments, supervised the work, and wrote the manuscript.

2.2 Context

Lipotoxicity play a key role in the pathogenesis of diabetic cardiomyopathy. The toxicity of different types of fatty acids is not equivalent. Oleate and palmitate are the two most abundant fatty acids in the human diet. Additionally, palmitate is found toxic in many cell types, while oleate is not. Preliminary findings in our lab reveal a clear difference in the quality and quantity of oleate and palmitate intramyocellular accumulation. Since fatty acids are mainly stored in lipid droplets and since lipid droplets are initially formed between the leaflets of the endoplasmic reticulum, we have investigated the relationship between lipotoxicity and ER stress. We found that palmitate, a toxic fatty acid, induces ER stress and ER-stress mediated cell death in primary neonatal rat cardiomyocytes. We also found that oleate, a nontoxic fatty acid, does not induce ER stress.

2.3 Abstract

Introduction: Diabetes is a major contributor to cardiovascular disease. There is a growing body of evidence pointing towards intra-myocellular lipid accumulation as an integral etiological factor. Here we aimed to determine the effect of two common fatty acids on lipid accumulation and cellular stress in primary cardiomyocytes.

Methods: We evaluated lipid accumulation biochemically (by triacylglyceride assay and radiolabeled fatty acid uptake assay) as well as histologically (by BODIPY 493/503 staining) in mouse and rat neonatal cardiomyocytes treated with saturated (palmitate) or mono-unsaturated (oleate) fatty acids. Endoplasmic reticulum (ER) stress was evaluated by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting. Cell viability was assessed by propidium iodide staining.

Results: We found that both oleate and palmitate led to significant increases in intracellular lipid in cardiomyocytes; however there were distinct differences in the qualitative nature of BODIPY staining between oleate and palmitate treated cardiomyocytes. We also show that palmitate caused significant apoptotic cell death and this was associated with ER stress. Interestingly, co-administration of oleate with palmitate abolished cell death, and ER stress. Finally, palmitate treatment caused a significant increase in ubiquitination of Grp78, a key compensatory ER chaperone.

Conclusion: Palmitate causes ER stress and apoptotic cell death in primary cardiomyocytes and this is associated with apparent differences in BODIPY staining compared to oleate treated

cardiomyocytes. Importantly, the lipotoxic effects of palmitate are abolished with the co-administration of oleate.

2.4 introduction

Diabetes is a major contributor to cardiovascular disease. In fact, diabetes is an independent risk factor for coronary artery disease and hypertensive heart disease. However, diabetics can also develop isolated diabetic cardiomyopathy, characterized by ventricular dysfunction in diabetics without obvious macro-vascular disease or other etiological factors including alcoholism. Diabetic cardiomyopathy, with or without superimposed vascular disease, predisposes diabetic patients to heart failure. Indeed, the incidence of heart failure is four times higher in diabetic patients than the general population [1]. Furthermore, diabetes is an independent risk factor for death in patients with heart failure [2].

The specific cause of diabetic cardiomyopathy is likely multifactorial, however amounting evidence now suggests that intra-myocellular lipid accumulation is an important contributory factor. Several studies have demonstrated excess myocardial lipids or, cardiac steatosis, in diabetic humans and mice [3-7]. Indeed the cardiac lipotoxicity resulting from elevated intra-cellular lipids is believed to lead to cell death with ensuing cardiac dysfunction. The two main fats in the human diet are oleate, a monounsaturated fatty acid, and palmitate, a saturated fatty acid. Importantly, lipotoxicity is predominantly due to saturated fatty acids like palmitate,

whereas unsaturated fatty acids have been described as “lipo-protective”. A multitude of studies have demonstrated the toxic effects of palmitate in primary cardiomyocytes and cardiomyocyte cell lines. While the exact mechanism of palmitate has not yet been completely elucidated there is evidence to support the role of impaired β-oxidation [8-10], calcium dysregulation [11], loss of mitochondrial membrane potential [12], oxidative stress [13], alterations in membrane phospholipids [14], impaired cardiolipin synthesis [15], and finally loss of nuclear integrity [16].

Our aim here was to assess the qualitative nature of lipid accumulation in oleate and palmitate treated primary neonatal cardiomyocytes (NCMs) and their effects on endoplasmic reticulum (ER) stress and cell viability. ER stress results in the activation of several stress response genes including inositol requiring enzyme-1 (Ire1), and activating transcription factor-6 (Atf6), which are normally retained in the inactive state by glucose regulated protein 78 (Grp78/Bip), an ER chaperone. Ire1 functions as an endoribonuclease which catalyzes the splicing of X-box binding protein-1 (Xbp1) mRNA. Spliced Xbp1 mRNA is translated into a nuclear transcription factor essential to the ER stress response. In addition, Atf6 release from Grp78 results in migration of this protein to the Golgi apparatus where proteases act to release the amino terminus. This amino-terminal portion of Atf6 is also a nuclear transcription factor.

Here we found distinct differences between oleate and palmitate induced lipid staining and ER stress activation in both mouse and rat NCMs thus providing mechanistic insight into the role of lipotoxicity in diabetic cardiomyopathy.

2.5 Methods

Preparation of fatty acid-albumin complex solutions: For proper fatty acid transport into cells, we used bovine serum albumin (BSA) dissolved in 150mM NaCl as vehicle. Sodium Oleate (dissolved in methanol) or palmitate (dissolved in 150mM NaCl solution heated to 70°C) were mixed with 0.17mM BSA/150mM NaCl solutions (6:1molar ratio). Conjugation of oleate, or palmitate, to BSA was performed by gentle agitation at 37 °C for 1 hour and conserved at -80°C. In all experiments, NCMs were treated with either oleate or palmitate for 24 hours unless otherwise indicated.

Isolation and culture of primary NCMs: Hearts were aseptically harvested from new born (1-2 day old) CD-1 mouse or Sprague-Dawley rats pups and washed with Hanks buffer solution (137mM NaCl, 5.36 mM KCl, 0.81mM MgSO4, 5.55mM dextrose, 0.44mM KH2PO4, 0.34mM Na2HPO4, 20mM HEPES, and 50 μg/ml gentamicin). Next, ventricular tissue was isolated, cut in small pieces and washed 3 times in Hanks buffer solution. Ventricular tissue was digested by repeated incubations (for 10-20 mins at 37 °C) in Hanks buffer solution containing 50U ml collagenase-2 and 0.36μM CaCl2 until fully digested. Following each digestion round, cells were added to ice-cold fetal bovine serum (FBS). Post-digestion FBS suspensions were centrifuged at 800g for 5 min (4°C). The cell pellets were re-suspended in culture media (DMEM/ F12, 10% FBS) and plated on a 10 cm cell culture dish for 1 hour at 37 °C to let cardiac fibroblasts adhere to the plate. Non-adhering cardiomyocytes were then re-plated in appropriate cell culture plates. Cells were incubated for a minimum of 120 hours with daily replacement of

culture media (DMEM/F12, 2% FBS, 100μM bromodeoxyuridine). Bromodeoxyuridine was added to inhibit growth of cardiac fibroblasts. All animal experimentations were performed according to the guidelines of the Canadian Council on Animal Care and they were specifically approved by the institutional animal care committee at the Montreal Heart Institute. All experiments were carried out in rat NCMs unless otherwise indicated.

Histology: NCMs were seeded onto 18mm round coverslips coated with 2% gelatin. NCMs were treated as described and then fixed in 3% paraformaldehyde for 30 min. at room temperature. Cells were then stained with 1μM BODIPY 493/503 (Invitrogen), 1μM Rhodamine Phalloidin (invitrogen), and 1μM DAPI (Invitrogen).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments: Total RNA was isolated from NCMs. RNA extraction, cDNA synthesis and qPCR were performed using respective commercial kits (Qiagen) and Eco Ilumina real-time qPCR system. Data was analysed using the 2-ΔΔCt method [17]. PCR results are presented as the expression of the indicated gene relative to an endogenous control gene (Rpl34) and normalized to the control group. Data are presented as mean ± standard error (SE). Samples were analyzed in triplicate in three separate experiments. Primers were designed to span exons using the NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in supplemental table S2.1. Xbp1 primers do not amplify the non-spliced isoform, they only amplify the spliced Xbp1 isoform.

Western blot analysis: Total cellular protein was isolated from rat (or mouse where indicated) NCMs with isolation buffer (250mM Sucrose, 50mM Tris, 1μM PMSF (protease inhibitor), 1μM DTT, and Proteinase inhibitor cocktail (ROCHE)). Protein concentrations were measured by Bradford assay. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed with the following antibodies: anti-Grp78 (SC-13968, Santa Cruz biotechnologies), anti-ATF6 (sc-22799, Santa Cruz biotechnologies), anti-Xbp1 (SC-7160, Santa Cruz Biotechnologies), anti-Chop (SC-7351, Santa Cruz biotechnologies), anti-Caspase3 (ab2302 (abcam)). Equal protein loading conditions were utilized for all blots, which was verified by Ponceau staining of the membrane.

Co-immunoprecipitation: Immunoprecipitations were carried out using protein A/G-agarose beads (Thermo Scientific). Briefly, freshly treated NCM lysate homogenates were incubated for 4 hours under continuous rotation at 4˚C with anti-Grp78 antibody (SC-13968, Santa Cruz biotechnologies) in binding buffer (140 mM NaCl, 14 mM KCl, 0.1% Triton X-100 with 0.01% BSA). Simultaneously, protein A/G-agarose beads were blocked in 0.1% BSA in binding buffer for 2 h. Following this the antibody-protein complex formation was added to the agarose beads, and rotated overnight at 4°C. Samples were washed three times and eluted in 0.1 M glycine (pH 2.4). Immunoprecipitates were probed with anti-ubiquitin antibody (1/400, Santa-Cruz Biotechnology) and anti-Grp78 antibody (SC-13968, Santa Cruz biotechnologies).

Fatty acid uptake assay: NCMs were treated with either BSA (51μM), C14- radiolabelled oleate (100μM) or C14-radiolabelled palmitate (100μM) for 2 hours. Following this cells were washed twice with phosphate buffered saline (PBS), harvested by trypsinization and homogenized. Cell

lysates were added to scintillation fluid and samples were counted with the Scintillation counter (Beckman LS6500). Data are presented as μmol/min based on the counts per minute (CPMs) from isolated cells relative to CPMs from total amount of radiolabeled fatty acid that was added to the cells (divided by 120 since the cells were exposed for 2 hours).

Triacylglyceride (TAG) assay: Mouse NCMs were treated with BSA (n=3), 300uM (n=3) oleate or 300uM palmitate (n=3) for 24 hours and then assayed for TAG levels using the adipogenesis assay kit from Biovision (Catalog #K610-100) as per the manufacturer's instructions. Values are presented as nmol glycerol (determined via a standard curve derived from increasing concentrations of pure glycerol) and normalized to protein concentration (determined using the bicinchoninic acid (BCA) assay) for each sample. Assays were done in duplicate.

Cell viability assay: Propidium iodide (PI) exclusion assays were carried out to evaluate cell viability. Briefly, NCMs were treated with palmitate (and oleate where indicated) for the indicated time points (n=4 per timepoint). After a 30 min incubation with PI at 37 °C, we measured fluorescence (535nm excitation / 617nm emission) with the Synergy2 fluorescence plate reader (Bio-Tek). Assays were done in triplicate.

Statistical analysis: Numerical data were presented as the mean ± SE. Student's T-test was used for two group experiments, while we performed one-way ANOVA with the Tukey post-hoc test for multiple group comparisons. P-values of <0.05 were considered statistically significant.

2.6 Results

2.6.1 Palmitate induces cell death of primary NCMs, which is prevented by the addition of Oleate

Treatment of NCMs with 300μM palmitate caused a time dependent cell death as evidenced by increased PI staining, a marker of late cell death. The palmitate induced cell death was significantly increased at 18 hours and progressed thereafter until at least the 24-hour time point (Figure 2.1A). Importantly, 8 hours of palmitate did not cause cell death while there was only a trend for increased cell death following 16 hours exposure. Because oleate has been shown to be protective against palmitate [18], we tested the effect of co-administration of various concentrations of oleate on the cytotoxic effect of palmitate. Interestingly, we found that concentrations as low as 50μM oleate were sufficient to completely abolish the cytotoxic effect of palmitate (Figure 2.1B).

2.6.2 Distinct lipid staining in palmitate treated NCMs

The marked difference in cell death between oleate and palmitate prompted us to evaluate if there is a difference in lipid accumulation in NCMs caused by these two fatty acids. Excess non-metabolized fatty acids can be converted to TAG and stored in lipid droplets in mammalian

cells.

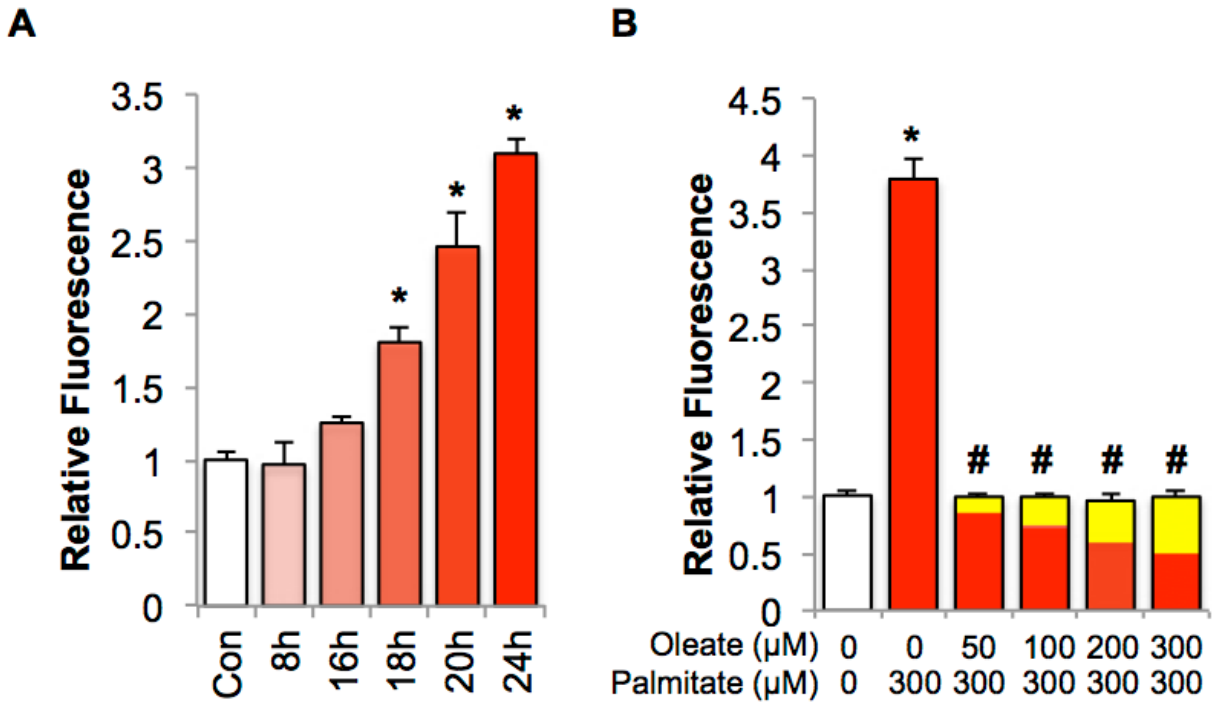

Figure 2.1: Palmitate causes time dependent cell death in NCMs, which is prevented by the co-administration of oleate. (A) Graph demonstrating the relative fluorescence of cells stained with PI after treatment with 300μM palmitate for the indicated time points. Control cells were treated with 51μM BSA in serum free media. (B) Graph demonstrating the relative fluorescence of cells stained with PI after treatment with either palmitate (represented by the color red) or palmitate + oleate (represented by red and yellow) at the indicated concentrations. *Indicates $p<0.05$ vs. control NCMs. # Indicates $p<0.05$ vs. NCMs treated with palmitate alone.

To characterize the degree of oleate and palmitate induced lipid droplets in NCMs, we treated cells with either 400μM oleate or 300μM palmitate for 24 hours and then stained the cells with BODIPY 493/503, a stain for neutral lipids, as well as Rhodamine Phalloidin, an actin stain (Figure 2.2). Importantly we show that our NCM cultures are relatively pure as the majority of cells exhibited the striated sarcomeric staining pattern characteristic of cardiomyocytes. In agreement with the high degree of cell death following 24 hours of palmitate, we observed a

loss of sarcomeric integrity in NCMs treated with palmitate (Figure 2.2G). We found that both oleate and palmitate led to increased BODIPY493/503 staining in NCMs. However there were some key differences in the staining pattern induced by the two different fatty acids. Firstly, oleate treated cells exhibited abundant BODIPY staining of spherical objects that were consistent with the morphology of typical lipid droplets (Figure 2.2D-F). On the other hand, BODIPY staining of palmitate treated NCMs was much fainter and more diffuse in appearance. (Figure 2.2G-I). Indeed the depicted micrographs of palmitate treated NCMs required brightness and contrast enhancement to visualize the BODIPY staining, despite being subjected to the identical staining protocol (similar results from >3 separate experiments). Secondly, while spherical stained objects could also be visualized in palmitate treated NCMs, suggesting the presence of lipid droplets, there were also many stained objects that appeared nonspherical or irregular in shape, which is inconsistent with the morphology of lipid droplets and hence may represent non lipid droplet staining. Thus the difference in BODIPY staining between the two fatty acids suggests a difference in the chemical or physical nature of the resulting lipid accumulation.

In contrast to treatment with palmitate alone, the co-treatment of 50μM oleate and 300μM palmitate to NCMs for 24 hours resulted in a marked difference in cellular histology (Figure 2.2J-L). Firstly, phalloidin staining showed high integrity of sarcomeres similar to control and oleate treated NCMs. In addition, we found that oleate +palmitate co-treatment altered the BODIPY staining with an apparent decrease in the faintly diffuse staining pattern characteristic of palmitate treated NCMs and an increase in small brightly staining spherical objects characteristic of oleate treated NCMs. Because there is a substantial degree of cell death in

NCMs treated with palmitate for 24 hours, we aimed to determine if the apparent difference in BODIPY staining was due to cell death. Therefore we evaluated histology of NCMs treated with 300μM palmitate for 8 hours (Figure 2.3), a time point that is not associated with cell death. Indeed, at this time-point the palmitate treated NCMs exhibited sarcomeric integrity similar to control or oleate treated cells (Figure 2.3A,D,G). However, NCMs treated with palmitate for 8 hours exhibited a similar staining pattern as observed for NCMs treated with palmitate for 24 hours (Figure 2.3H), suggesting that the altered BODIPY staining in palmitate treated NCMs was a characteristic of palmitate treatment and not an indirect result of cell death.

To verify that the altered BODIPY staining in palmitate treated NCMs was not due to alterations in lipid uptake, we measured fatty acid uptake and total cellular triglyceride levels. We found that there was no difference in uptake of radio-labeled oleate vs. palmitate (Figure 2.4A) and that both oleate and palmitate led to a ~3 fold increase in TAG levels (Figure 2.4B) confirming that both oleate and palmitate were being incorporated into the cells to similar degrees.

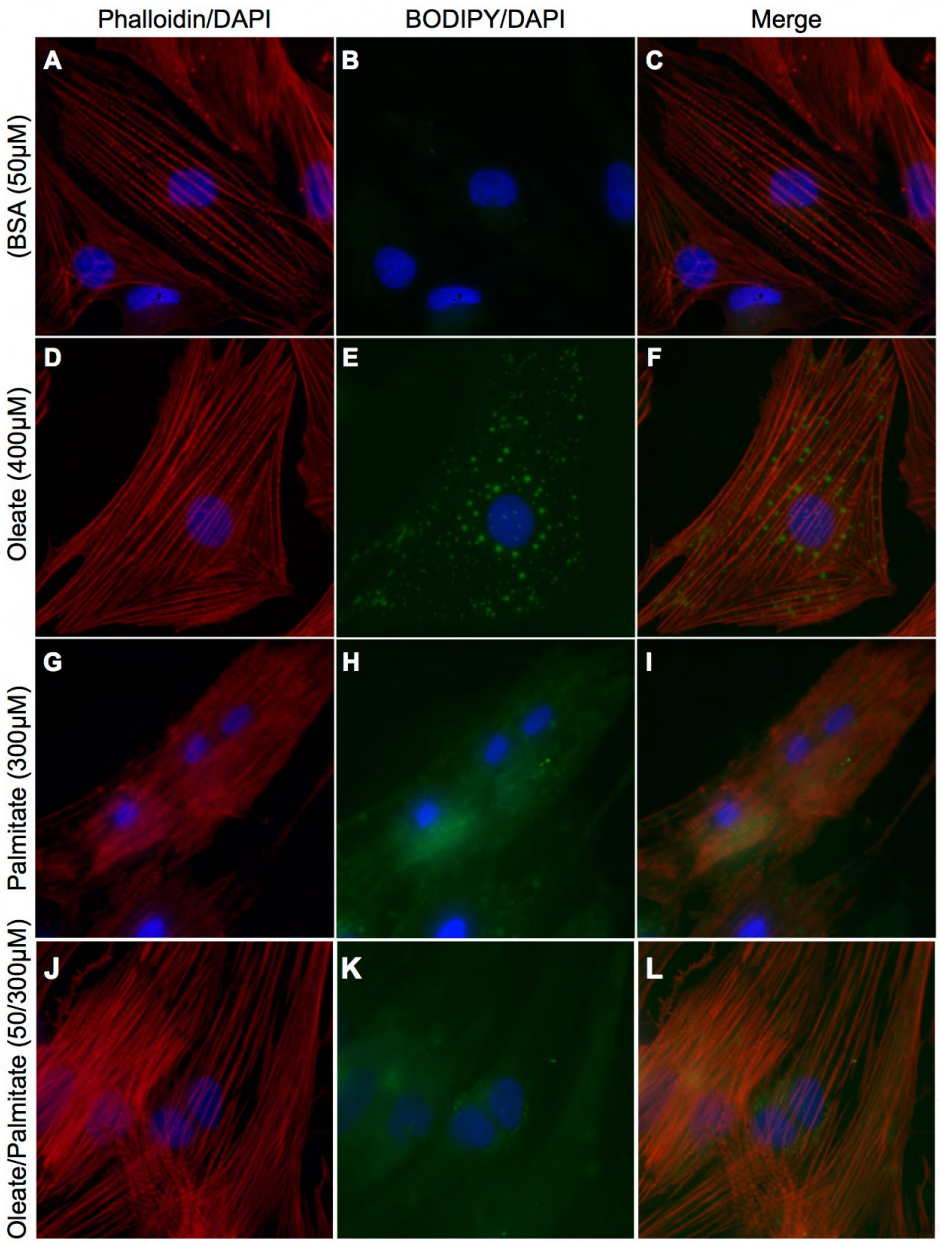

Figure 2.2: Marked differences in BODIPY staining in NCMs treated with oleate, palmitate, or oleate + palmitate for 24 hours. Images of NCMs treated with either BSA (control, **A-C**), 400μM oleate (**D-F**), 300μM palmitate (**G-I**), or 50μM oleate + 300μM palmitate (**J-L**) for 24 hours. All cells were stained with Rhodamine Phalloidin (actin stain, left column) and BODIPY 493/503 (neutral lipid stain, middle column) and DAPI (nuclear stain). Right column represents merged image of left and middle columns. All images exhibit cells at 1000x magnification.

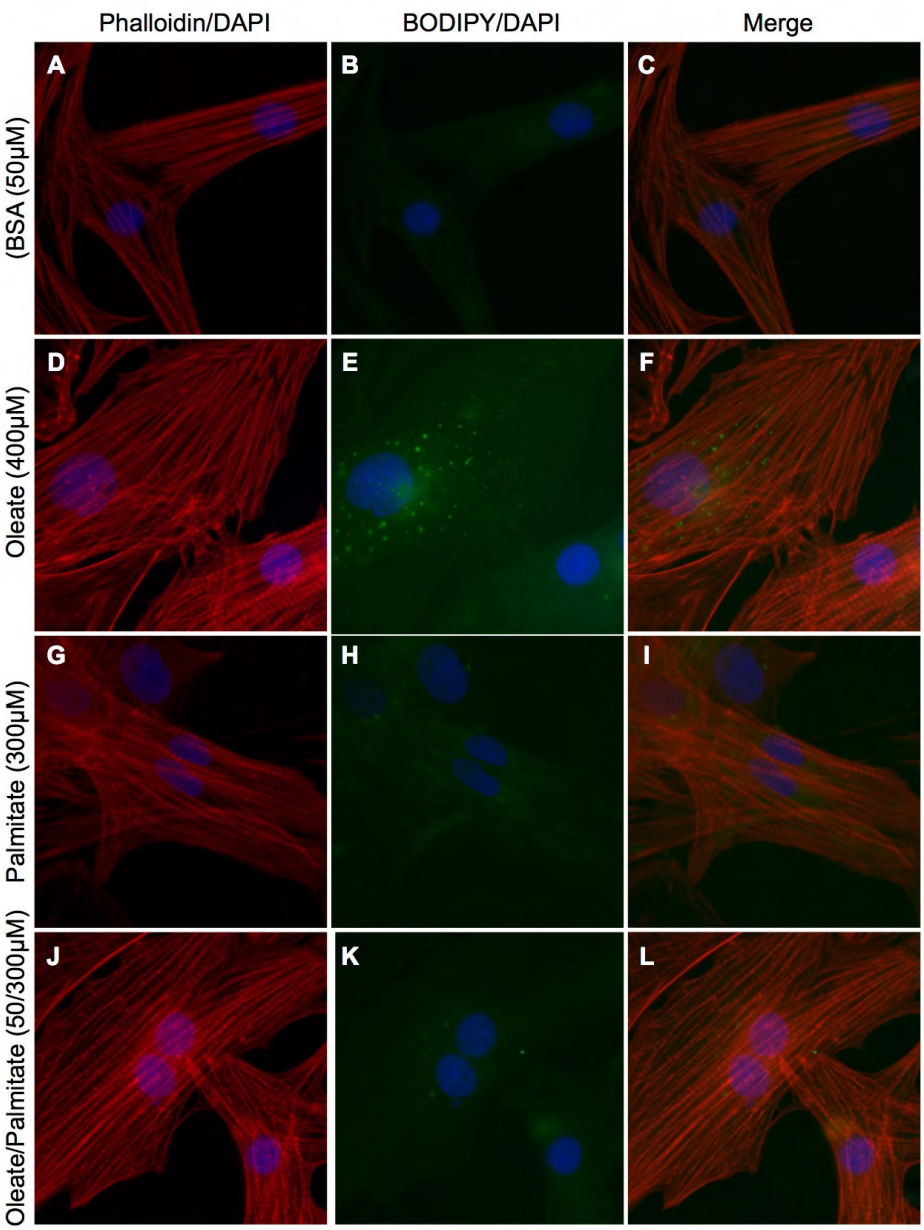

Figure 2.3: Marked differences in BODIPY staining in NCMs treated with oleate, palmitate, or oleate + palmitate for 8 hours. Images of NCMs treated with either BSA (control, **A-C**), 400µM oleate (**D-F**), 300µM palmitate (**G-I**), or 50µM oleate + 300µM palmitate (**J-L**) for 8 hours. All cells were stained with Rhodamine Phalloidin (actin stain, left column) and BODIPY 493/503 (neutral lipid stain, middle column) and DAPI (nuclear stain). Right column represents merged image of left and middle columns. All images exhibit cells at 1000x magnification.

2.6.3 Palmitate induces ER stress

The altered BODIPY staining in palmitate treated cells suggested that perhaps the intracellular lipid may also be accumulating in the ER in addition to lipid droplets. Indeed, palmitate has been previously shown to cause pathological changes to the ER membrane by increasing the degree of saturation of ER phospholipids and consequent ER stress in a cardiomyoblast cell line [19]. In accordance with this, numerous studies have shown that palmitate induces ER stress, but this has not been shown in either mouse or rat primary NCMs. Therefore we aimed to characterize ER stress in NCMs. We found that palmitate caused a significant increase in mRNA levels of key ER stress mediators including Grp78, spliced Xbp1, DnaJ (Hsp40) homolog, subfamily B, member 9 (Dnajb9, a marker of Xbp1 activity), Atf4, and Atf6 in both rat and mouse NCMs (Figure 2.5A & Supplemental Figure S2.1A). Importantly, the induction of all of these ER stress markers exhibited both dose and time dependence in palmitate treated NCMs (Supplemental figure S2.2). In addition, we also found that palmitate induced time dependent activation of cleaved and full length Atf6 (Figure 2.5B). Interestingly, Xbp1 protein (~30kDa) derived from the un-spliced Xbp1 mRNA decreased over time, indicating increased activity of Ire1. On the other hand, Xbp1 protein (~50kDa) derived from the spliced mRNA, increased over time indicating an increase in the active form of Xbp1 in palmitate treated NCMs (Figure 2.5C). It is important to mention that the protein derived from spliced Xbp1 mRNA is actually larger than the protein derived from the un-spliced mRNA because the splicing introduces a frame-shift which displaces the stop codon.

2.6.4 Palmitate induces apoptotic cell death

Severe or prolonged ER stress is believed to lead to activation of apoptotic pathways. CCAAT/enhancer binding protein (c/ebp) homologous protein (Chop) is a transcription factor implicated in ER stress induced apoptosis. Therefore we evaluated Chop expression and activation in palmitate treated NCMs. We found significantly increased mRNA levels of Chop in NCMs (Figure 2.5A). Furthermore, the palmitate mediated induction of Chop mRNA exhibited both time and dose dependency (Supplemental figure S2.2). Because Chop is active in the nucleus we evaluated nuclear levels of Chop protein. Interestingly, we show a dose dependent increase of Chop protein levels in the nuclear containing subcellular fraction of palmitate treated NCMs (Figure 2.6A). In addition we also demonstrate a time dependent increase of total Chop protein in palmitate treated NCMs (Figure 2.6B). Chop activation should lead to induction of proapoptotic pathways. Therefore we assessed the degree of caspase-3 activation in palmitate treated NCMs. Indeed, we found that caspase-3 cleavage was significantly increased after 16 and 18 hours of palmitate exposure in NCMs compared to both oleate treated and control NCMs (Figure 2.6C). Importantly, the time point of Caspase-3 cleavage coincides with the commencement of cell death in these cells.

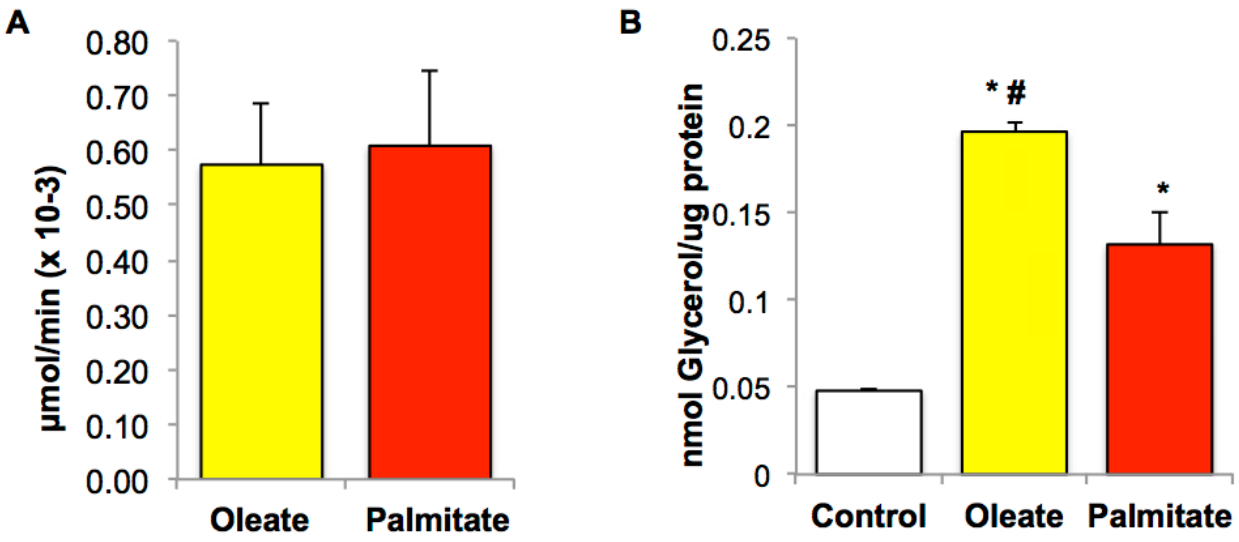

Figure 2.4: Oleate and palmitate exhibit similar uptake rates and intracellular lipid accumulation in NCMs. (**A**) Graph demonstrating the µmol fatty acid uptake/minute from cells treated with C14- radiolabelled oleate (100µM) or C14- radiolabelled palmitate (100µM) for 2 hours. (**B**) Graph demonstrating significant increase in intracellular triglyceride levels in mouse NCMs treated with 300µM oleate (n=3) or 300µM palmitate (n=3) compared to cells treated with BSA (control, n=3). * Indicates $p<0.05$ vs. control NCMs. # Indicates $p<0.05$ vs. palmitate treated NCMs.

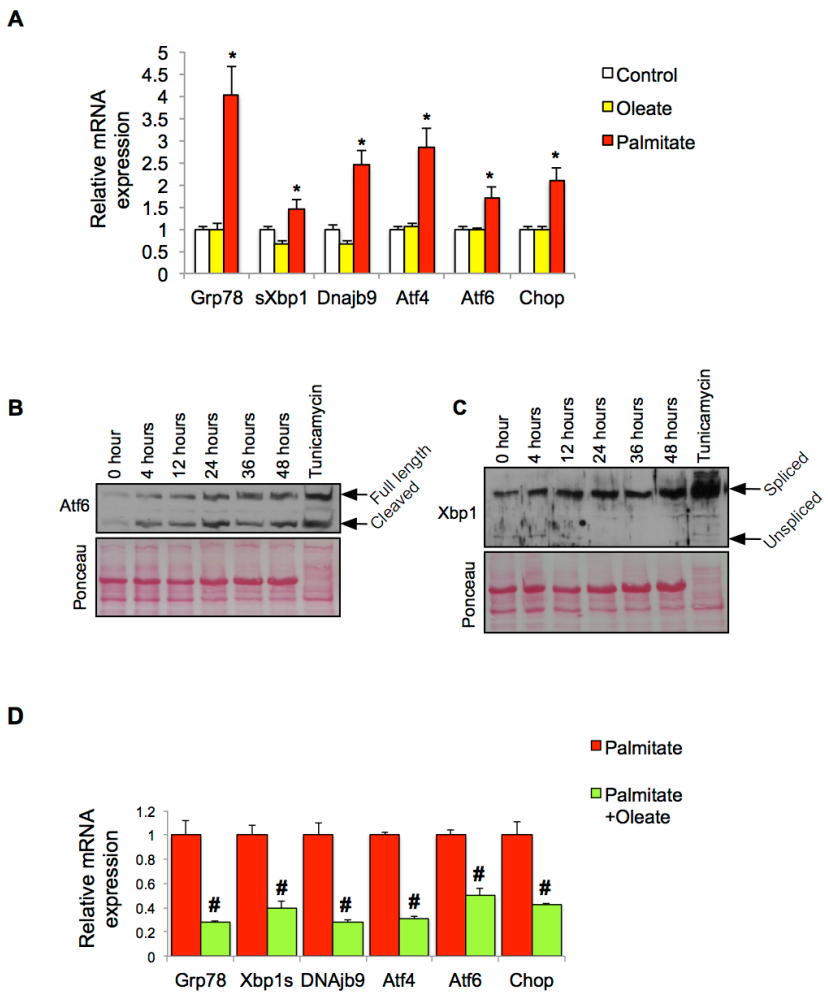

Figure 2.5: Palmitate induces ER stress in NCMs. (A) Graph demonstrating the mRNA levels of 6 key markers and mediators of ER stress in NCMs treated with either BSA (control), 400µM oleate or 300µM palmitate for 24 hours (n= 9/group). (**B**) Western blot demonstrating the time dependent increase in full length and cleaved Atf6 protein following administration of 300µM palmitate for the indicated time points. NCMs were also treated with tunicamycin (0.1µg/ml) for 24 hours as a positive control. (**C**) Western blot demonstrating the time dependent increase in Xbp1 protein derived from the spliced mRNA (~54kDa) and the time dependent decrease in Xbp1 protein derived from the unspliced mRNA (~30kDa) in NCMs treated with 300µM palmitate for the indicated times. NCMs were also treated with tunicamycin (0.1µg/ml) for 24 hours as a positive control. (**D**) Graph demonstrating the mRNA levels of 6 key markers and mediators of ER stress in NCMs treated with 300µM palmitate, or 50µM oleate + 300µM palmitate for 24 hours (n= 3/group). * Indicates $p<0.05$ vs. control NCMs. # Indicates $p<0.05$ vs. palmitate treated NCMs.

2.6.5 The protective effect of oleate is associated with a decrease in ER stress

Previous studies have demonstrated that unsaturated fatty acids, like oleate, prevent the toxicity associated with saturated fatty acids. In agreement with this, we showed that as little as 50μM oleate can prevent palmitate induced cell death. Expectedly, oleate alone, did not induce the mRNA levels of any of the ER stress markers in rat or mouse NCMs (Figure 2.5A and Supplemental Figure S2.1B). Furthermore, co-treatment of NCMs with 50μM oleate +300μM palmitate was sufficient to significantly attenuate ER stress compared to NCMs treated with palmitate alone (Figure 2.5D). Altogether these data demonstrate that palmitate but not oleate, induces ER stress and apoptosis in primary NCMs.

2.6.6 Palmitate causes increased ubiquitination of Grp78

Grp78 is an important ER chaperone and a key marker of ER stress. In accordance with this we show that palmitate significantly induces Grp78 mRNA levels (Figure 2.7A), and this effect is both dose and time dependent (Supplemental Figure S2.2A-B). However, surprisingly Grp78 protein levels were only marginally elevated in palmitate treated rat NCMs (Figure 2.7B). As a positive control we evaluated the effect of tunicamycin, a known ER stressor, on Grp78 mRNA and protein expression. We found that tunicamycin (0.1μg/ml) substantially increased Grp78 mRNA (> 10-fold, Figure 2.7A) and protein levels (Figure 2.7B). The fact that palmitate

increased Grp78 mRNA but not protein suggested that perhaps the protein was being degraded. To test if this was via the ubiquitin-proteasome pathway we evaluated the degree of Grp78 ubiquitination in palmitate treated NCMs vs. untreated control and vs. tunicamycin treated cells. We immunoprecipitated Grp78 and then blotted for both Grp78 (to demonstrate equivalent loading of Grp78 immuno-precipitates) and for ubiquitin (Figure 2.7C). Semiquantitative analysis of the immunoblots demonstrated that the Grp78 immuno-precipitate isolated from NCMs treated with 200μm palmitate exhibited a significantly increased ubiquitin signal compared to either control, tunicamycin, or low dose (50μM) palmitate treated NCMs (Figure 2.7C-D).

2.7 Discussion

Hearts of patients with diabetic cardiomyopathy have increased lipid levels compared to healthy non-diabetic patients [4,5,7,20,21]. The resulting lipotoxicity is likely to contribute to the increased cardiac events, heart failure and death in this patient population. Palmitate, a saturated fatty acid, is one of the most abundant lipids in human diets, and has been shown to cause lipotoxicity in a variety of cell types. In contrast, oleate, another major lipid in human diets, is non-toxic and has even been shown to reverse toxicity induced by palmitate.

While there is a large body of evidence demonstrating the toxic effects of palmitate *in vitro*, no studies have investigated the effect of oleate and palmitate specifically on lipid accumulation in

NCMs. Here we show that oleate and palmitate led to distinctive BODIPY staining in spontaneously contracting mammalian cardiomyocytes. Specifically, BODIPY staining of oleate treated NCMs resulted in the appearance of distinct spherical objects, which is consistent with the presence of lipid droplets.

On the other hand, BODIPY staining in palmitate treated NCMs was much fainter and less distinct. Although, spherical objects could be discerned in palmitate treated NCMs suggesting the presence of lipid droplets, they were much fainter than in oleate treated NCMs. In addition, there was also a large degree of staining of non-spherical or irregularly shaped objects, which is inconsistent with the morphology of lipid droplets. It is unlikely that this faint diffuse staining is background staining since it was not observed in control NCMs treated with BSA.

Interestingly we also show that 50μM oleate completely abolished palmitate induced cell death and ER stress; and this was associated with a change in lipid staining. Indeed co-administration of oleate and palmitate to NCMs attenuated the appearance of the faint diffuse staining pattern characteristic of palmitate only treated NCMs. Instead we observed the staining of small spherical objects similar to cells treated with oleate alone. The cause for this alteration in lipid staining is currently unknown but may be related to the increased induction of TAG formation by oleate compared to palmitate. Indeed, it has been previously suggested that oleate attenuates palmitate-induced lipotoxicity by inducing the formation of TAG, thus sequestering saturated fatty acids into inert moieties [40]. Specifically, the incorporation of saturated fatty acids into TAG, and subsequent storage of this TAG in lipid droplets, may reduce their bioavailability.

This attenuated bioavailability is thus expected to decrease their subsequent metabolism into toxic metabolites and/or their incorporation into phospholipids and hence attenuate ER stress. In support of this, oleate has been shown to be a better substrate for TAG synthesis than palmitate in skeletal muscle cells [41]. Here we show that oleate produces brightly staining objects consistent with the morphology of lipid droplets, while palmitate produces a staining pattern that is fainter and more diffuse with the presence of irregularly shaped objects. This suggests that perhaps BODIPY has higher affinity for oleate derived TAG compared to DAG produced by palmitate. The nature of the irregularly shaped stained objects and the cause for the faint/diffuse staining pattern will be the focus of future studies. However, a potential caveat of these findings is that perhaps oleate and palmitate are differentially stained by BODIPY and hence the difference in staining pattern is due to differences in affinity of the dye for the respective fatty acids rather than differences in actual lipid accumulation.

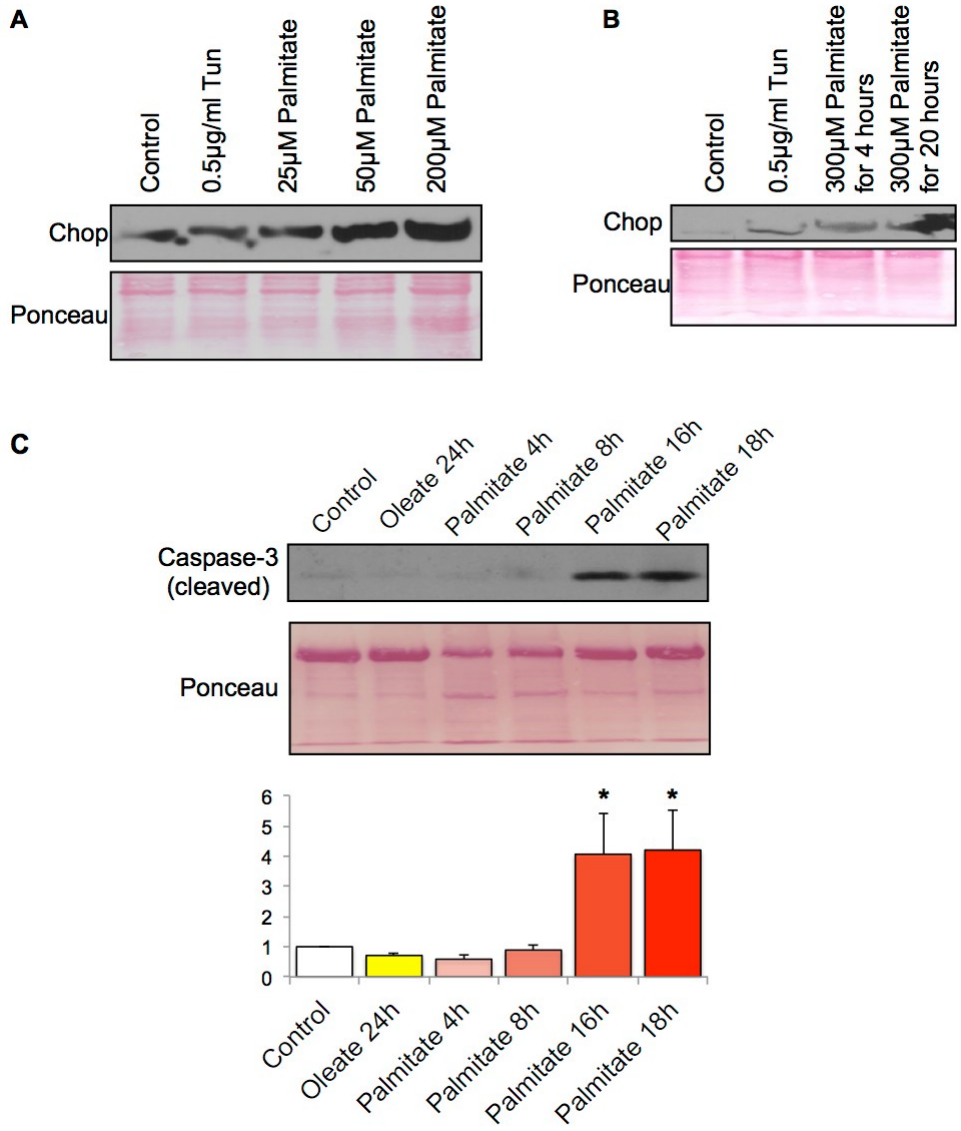

Figure 2.6: Palmitate induces apoptotic cell death in NCMs. (A) Western blot demonstrating the increase in Chop protein in the nuclear subcellular fraction of NCMs treated with palmitate for 24 hours at the indicated concentrations. Tunicamycin (0.5µg/ml for 24 hours) treated NCMs were used as positive control. **(B)** Western blot demonstrating time dependent increase in Chop protein in whole cell lysates of NCMs treated with 300µM palmitate for the indicated times. Tunicamycin (0.5µg/ml for 24 hours) treated NCMs were used as positive control. (**C**) Western blot and graph demonstrating increased levels of cleaved caspase-3 in NCMs treated with 300µM palmitate for the indicated time points compared to either BSA (Control), or 400µM oleate. * indicates $p<0.05$ vs. control and oleate treated NCMs.

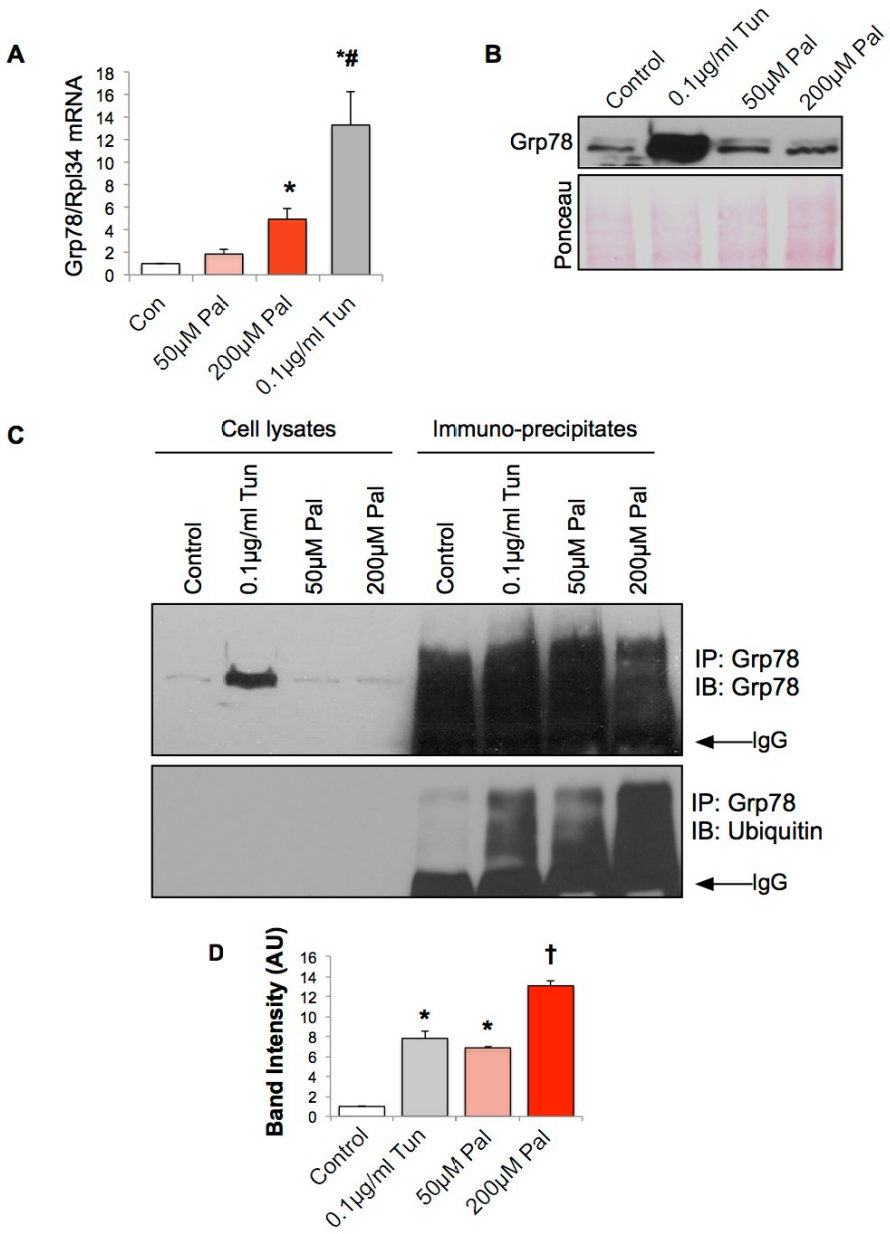

Figure 2.7: Palmitate causes the ubiquitination of Grp78 protein in NCMs. (**A**) Both palmitate and tunicamycin significantly induce Grp78 mRNA levels. (**B**) Western blot demonstrating that tunicamycin strongly up-regulates Grp78 protein levels but palmitate does not. (**C**) Western blots demonstrating that Grp78 immuno-precipitated from NCMs treated with 200μM palmitate exhibit significantly increased ubiquitination. The IgG bands in (C) represents the antibody that is used to immunoprecipitate Grp78. (D) Graph demonstrating results from densitometry analysis of ubiquitin immuno-blot in (C). * indicates $p<0.05$ vs. control. # indicates $p<0.05$ vs. palmitate. † indicates $p<0.05$ vs. all other conditions.

The altered lipid staining in palmitate treated NCMs suggested to us that perhaps lipid may not be completely stored in lipid droplets resulting in potential accumulation of lipid in the ER. The accumulation of lipid in the ER has major implications for lipotoxic mechanisms. Indeed, lipid accumulation in the ER can perturb ER membrane physiology thereby leading to ER stress [19, 22]. ER stress has been shown to be induced by palmitate in different cell types [13, 19, 23-29], but this has not been previously demonstrated in primary NCMs. Altogether, we show that palmitate causes a robust induction of markers of ER stress with ensuing apoptosis in NCMs and this is associated with altered lipid staining patterns.

ER stress is an important pathological process in a variety of cardiovascular disorders including diabetic cardiomyopathy [30-32]. ER stress results in activation of a compensatory mechanism referred to as the unfolded protein response (UPR). As the name suggests, it can occur as a result of a build-up of unfolded proteins, but also results from other stress stimuli including calcium dysregulation [33], and membrane perturbations [19, 22]. Here we demonstrated activation of the UPR in palmitate treated mouse and rat NCMs. Indeed, we show significant increases in spliced Xbp1 mRNA (a marker of Ire1 activity) as well as full length and cleaved Atf6 protein levels in NCMs treated with 300μM palmitate. Of note, palmitate did not change the ratio of cleaved to un-cleaved Atf6, suggesting that palmitate induced ER stress did not cause an increase in the activity of the S1 and S2 proteases of the Golgi apparatus responsible for cleavage of Atf6. In addition, we showed that palmitate significantly induced active Xbp1 protein in NCMs. Finally we also demonstrated that palmitate induced significant increases in the expression of Dnajb9 mRNA (a marker of Xbp1 activity) as well as Grp78, Atf4 and Atf6

mRNA levels. Importantly, palmitate induction of all of the latter factors exhibited dose and time dependency.

The ER stress-dependent apoptotic pathway is believed to occur through the activation of Chop [34]. Atf6, a mediator of ER stress, has been shown to induce Chop expression [35]. This is in accordance with our data demonstrating increased expression and activation of both Atf6 and Chop in palmitate treated NCMs. While Chop up-regulation is consistently associated with apoptosis [36, 37], the underlying mechanism has not been fully elucidated. There is evidence to suggest that Chop downregulates bcl-2 [38], and/or up-regulates death receptors [39]. We show here that palmitate leads to significant cell death after 18 hours exposure which is preceded by cleavage of caspase-3 at 16 hours suggesting that the mechanism of cell death is at least partially apoptotic.

It is important to bear in mind that the UPR is actually a compensatory response aimed at restoring ER function in part by up-regulation of ER chaperones. Indeed loss of key UPR chaperones results in cell death [42, 43], whereas their overexpression is protective [44, 45]. Interestingly, we found that palmitate treated NCMs exhibited only marginally elevated Grp78 protein levels despite a prominent induction of Grp78 mRNA levels. This suggests that there is an important post-transcriptional regulation of Grp78 in palmitate treated NCMs. In fact, we show significantly increased ubiquitination of Grp78 following high dose palmitate, suggesting this protein is actively degraded by the ubiquitin-proteasome pathway in palmitate treated NCMs. Loss of this important chaperone likely contributes to palmitate induced cell death. Palmitate has been shown to induce the ubiquitinproteasome pathway via PKC activation in

hepatocytes leading to degradation of anti-apoptotic proteins and subsequent lipoapoptosis [46]. Future studies are required to determine whether this is the mechanism for ubiquitination of Grp78 in NCMs.

Here we aimed to characterize the effect of palmitate in NCMs. We found that palmitate led to significant increases in intracellular lipid accumulation, ER stress, and cell death. However the qualitative nature of the BODIPY staining in palmitate treated NCMs differed markedly from that of the oleate treated NCMs which may be due to differences in their inherent capacity to form DAG vs. TAG. Further studies are required to fully characterize the nature of palmitate induced lipid accumulation in cardiomyocytes and how this translates into ER stress.

2.8 Acknowledgements

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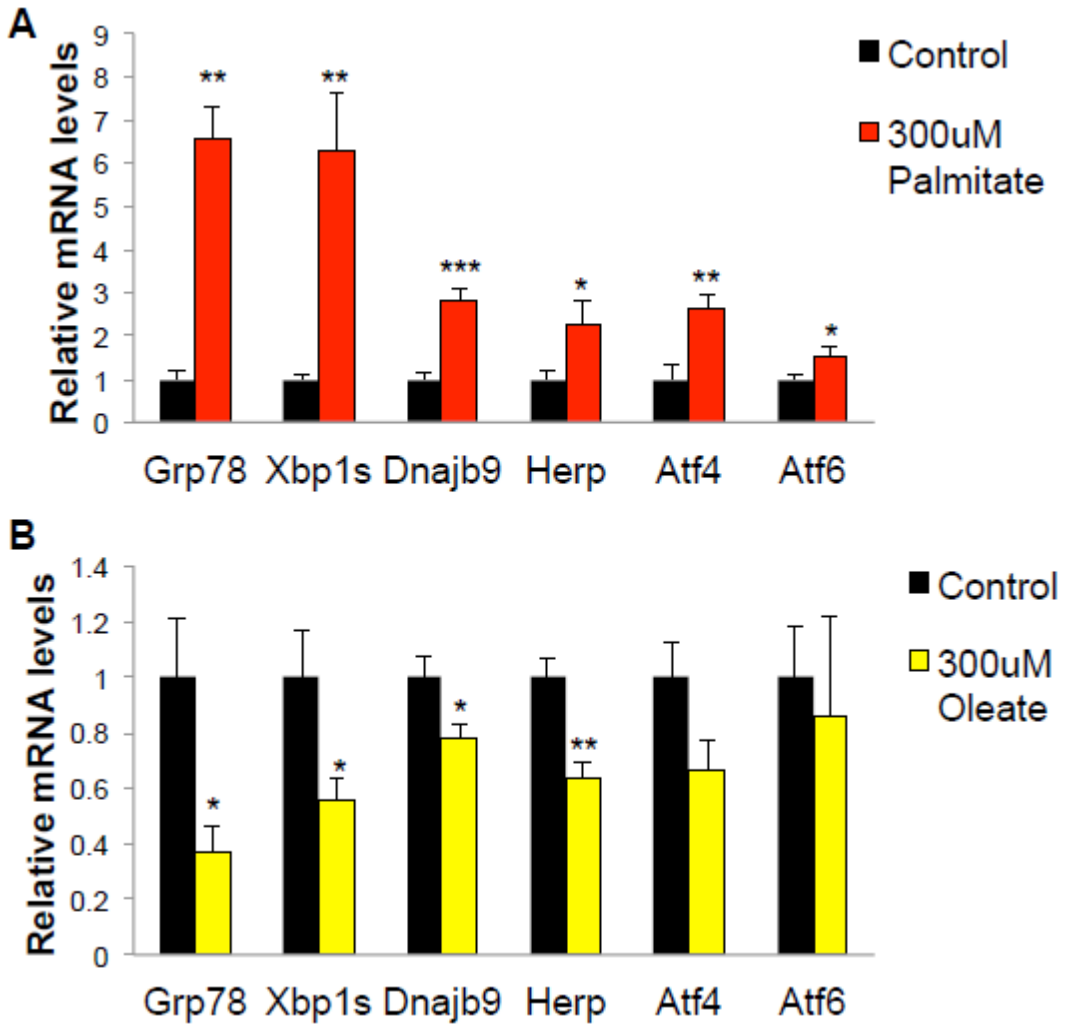
Conflict of interest statement: Authors state no conflict of interest.

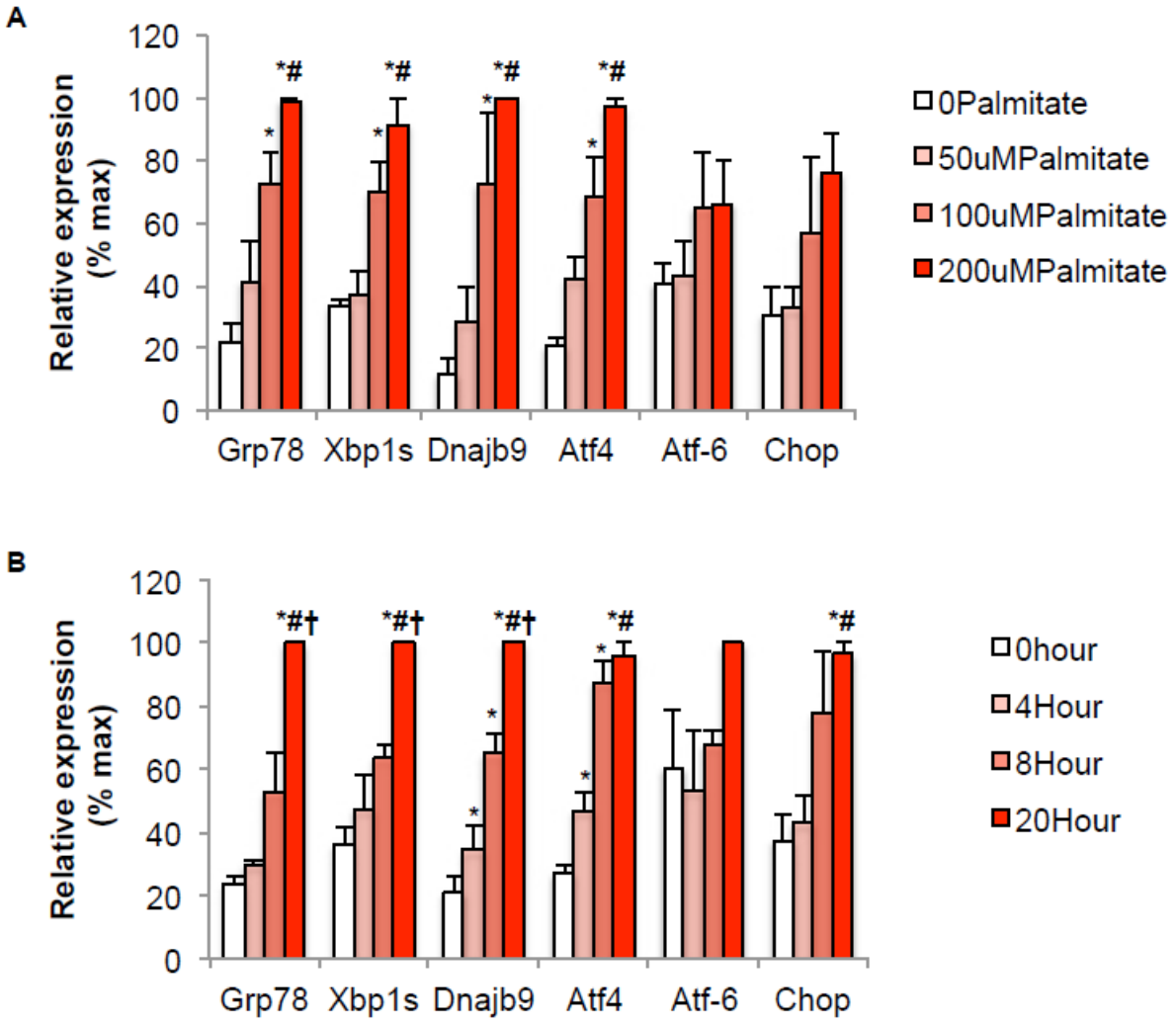
2.9 Supplementary data

Gene	Primer sequence
mAtf4-F	ATG GCC GGC TAT GGA TGA TG
mAtf4-R	TCT GGC ATG GTT TCC AGG TC
mAtf6a-F	ACC ATA GCA AGC AGC CAC A
mAtf6a-R	CTG GAA TGG GAC CAC CTG AC
mChop F	GAG GAG GAA GAG CAA GGA AGA AC
mChop R	TTC TCC TTC ATG CGT TGC TTC C
mDnajb9 F	CGC CCT GTG GCC CTG ACT TG
mDnajb9 R	AGC TTT CAG GGG CAA ACA GCC A
mGrp78-F	CGA TAC TGG CCG AGA CAA CA
mGrp78-R	GAC GAC GGT TCT GGT CTC AC
mHerp F	GGC ATC CCT GAG CGC AGT CG
mHerp R	TCA GGT GGG CCT TGA GGC GA
mRpl34 F	AGC ACC TAA ATC TGC ATG TGG CG
mRpl34 R	TAA GGA AAG CCC GCT TGA TCC TG
mXbp1 F(primer spans the splice site and therefore only amplifies spliced Xbp1)	TGA GTC CGC AGC AGG TGC A
mXbp1 R	AGG CAA CAG TGT CAG AGT CC
rAtf4 F	TTC CGG GAC AGA TTG GAT GTT GGA
rAtf4 R	CAT GTG TCA TCC AAC GTG GCC AAA
rAtf6-F	GTA CTG AGG AGA CAG CAG CG
rAtf6-R	GCC TCT GGT TCT CTG ACA CC
rChop F	AGT CTC TGC CTT TCG CCT TTG AG
rChop R	TGC AGG GTC AAG AGT AGT GAA GG
rGrp78-F	TTC CGA GGA ACA CTG TGG TG
rGrp78-R	GTC AGG GGT CGT TCA CCT TC
rDnajb9 F	TTG GTC AGA ACC AGA ACA CTC GG
rDnajb9 R	GTA CTG TGC GTC GAT TGG TGC TA
rRpl34-F	TGC TGT GAG ACC CAA AGT CCT CA
rRpl34-R	TAA GGA AAG CCC GCT TGA TCC TG
rXbp1-F (primer spans the splice site and therefore only amplifies spliced Xbp1)	TGAGTCCGCAGCAGGTGCA
rXbp1 R	GCATTCTGGACAAGTTGGACCC

Supplemental Table S2.1: List of primers



Supplemental figure S2.1: Palmitate induces ER stress in mouse cardiomyocytes (mNCMs). (A) Graph demonstrating the mRNA levels of 6 key markers and mediators of ER stress in mNCMs treated with 300μM palmitate for 24 hours vs. control mNCMs treated with BSA.* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. (B) Graph demonstrating the mRNA levels of 6 key markers and mediators of ER stress in mNCMs treated with 300μM oleate for 24 hours vs. control mNCMs. Rpl34 was used as housekeeping control gene.



Supplemental figure S2.2: Palmitate Palmitate induces markers of ER stress in a dose and time dependent manner in rat neonatal cardiomyocytes (rNCMs). (A) Graph representing the degree of mRNA expression of Grp78, spliced Xbp1, Dnajb9, Atf-4, Atf-6, and Chop relative to the housekeeping gene Rpl34. Expressed as percent of maximum values in rNCMs treated with either 0, 50, 100, or 200μM Palmitate for 25 hours. (B) Graph representing the degree of mRNA expression of Grp78, spliced Xbp1, Dnajb9, Atf-4, Atf-6, and Chop relative to the housekeeping gene Rpl34. Expressed as percent of maximum values in rNCMs treated with 300μM Palmitate for either 0, 4, 8, or for 20 hours. * indicates $p<0.05$ vs. control (0 hour or 0 μM palmitate). # indicates $p<0.05$ vs. 50μM palmitate or 4 hours palmitate. † indicates $p<0.05$ vs. 8 hours palmitate.

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Chapter 3: Cardiomyocyte lipotoxicity is mediated by Il-6 and causes downregulation of PPARs

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3.1 Authors contribution

Taha Haffar performed all the experiments, helped to analyze the data, and assisted with writing the manuscript. Félix-Antoine Bérubé-Simard helped with some experiments. Nicolas Bousette designed the study, supervised the work, and wrote the manuscript.

3.2 Context

We have previously found that palmitate induces ER stress, while oleate does not. Since PPARs play a key role in lipid metabolism, we wanted to investigate the effect of oleate and palmitate on the expression and activity of PPARs. PPAR activity was determined by measuring the expression of PPAR target genes (CPT1B, ACADL, and ACSL). Our results show that twenty-four-hour treatment of palmitate inhibits PPAR expression and activity in primary rat neonatal cardiomyocytes. Since PPARs are implicated as regulators of inflammatory response, we have examined the expression of two major cytokines: IL-6 and TNFα. We found that palmitate lipotoxicity is associated with an increased expression of both IL-6 and TNFα mRNA. Interestingly, the protective effect of oleate against palmitate-mediated lipotoxicity is associated with the attenuation of IL-6 but not TNFα, suggesting Il-6's more important role in propagating palmitate-mediated inflammatory response.

3.3 Abstract

Here we sought to evaluate the effect of palmitate on cytokine and PPAR activity/expression. We investigated the effect of BSA conjugated palmitate and oleate on PPAR activity, PPAR-a and d expression, as well as the expression of cytokines and key factors responsible for b-oxidation by qRT-PCR and western blotting in primary rat neonatal cardiomyocytes (NCMs). Furthermore we evaluated the effect of anti-inflammatory actions of AICAR and PPAR agonists on cytokine expression and cell death in palmitate treated NCMs. We found that palmitate caused down regulation of PPARs and increased cytokine expression and cell death, all of which was significantly attenuated by the co-administration of either AICAR or PPAR agonists. This work supports the pro-inflammatory actions of intracellular lipid and provides further insight into the pathological mechanism of cardiac lipotoxicity as occurs in diabetic hearts.

3.4 Introduction

Diabetic cardiomyopathy is associated with cardiac lipid accumulation. Indeed, studies have shown a build-up of intramyocellular lipids or, cardiac steatosis, in diabetic hearts and this is believed to lead to cardiac lipotoxicity [1-5]. A key to understanding lipotoxicity is a delineation of how lipid metabolic pathways are perturbed in cardiac disease.

PPARs regulate the expression of several metabolic genes and are therefore key determinants of lipid metabolism. PPAR-d deletion in the heart impairs b-oxidation and causes cardiomyopathy suggesting critical importance of this protein [6]. Similarly, PPAR-a null mice also exhibit cardiac functional defects [7,8]. In contrast PPAR activation can attenuate cardiac pathologies due to a variety of stress stimuli [9,10]. Interestingly, diabetic rats have been shown to have attenuated PPAR activity in the heart [11] as well as decreased PPAR-a and d protein levels [12,13].

One of the key PPAR target genes is Carnitine palmitoyl transferase-1b (Cpt1b, muscle isoform) since it catalyzes the rate limiting step in b-oxidation, namely the transport of fatty acids across the outer mitochondrial membrane. Cpt1b is transcriptionally regulated by PPAR-a and d. On the other hand, activity of Cpt1b is regulated by the metabolic intermediate malonyl-CoA. Malonyl- CoA is synthesized by Acetyl-CoA carboxylase (Acc2 is the predominant isoform in the heart) while it is degraded by Malonyl-CoA decarboxylase (Mcd). Acc2 on the other hand is regulated by the master metabolic regulator AMP-activated protein kinase (AmpK). The importance of Cpt1b in normal cardiac physiology is underscored by a recent study showing that Cpt1b deletion leads to adult mortality associated with severe hypertrophy [14]. Furthermore overexpression of Cpt1b in skeletal muscle has been shown to enhance b-oxidation and attenuate high fat diet induced insulin resistance [15].

Inflammation is an important pathological component of diabetes. Several studies have demonstrated the association between inflammation and diabetic cardiomyopathy [16-22]. Diabetics frequently exhibit systemic inflammation as evidenced by increased circulating levels

of TNF-α, and Il-6 [23-25]. In addition, there is evidence to suggest that intracellular lipids can induce cardiac inflammation *in situ* independently of circulating cytokines. Indeed, palmitate, a saturated fatty acid, induces increased expression of TNF-a and Il-6 levels in skeletal muscle cells [26,27], adipocytes [28], endothelial cells [29], keratinocytes [30], and hepatocytes [31].

Here we sought to further our understanding of cardiac lipotoxicity by evaluating the effects of palmitate, a toxic saturated fatty acid, on PPAR activity and expression as well as cytokine expression in primary cardiomyocytes. We show that palmitate causes down-regulation of PPARs which in turn impairs the expression of key proteins involved in b-oxidation. In addition, we show that the impaired PPAR levels may be due to induction of Il-6, and that anti-inflammatory treatments which attenuate cytotoxicity are associated with attenuated Il-6 expression. This report describes the effects of palmitate on PPAR and cytokine expression in primary rat neonatal cardiomyocytes (NCMs) thus providing new information on the mechanism of cardiac lipotoxicity.

3.5 Methods

Preparation of fatty acids: Bovine serum albumin (BSA) in 150 mM NaCl was used as fatty acid vehicle. Sodium oleate was dissolved in methanol and then added to 0.17 mM BSA (6:1 M ratio). Oleate was conjugated to BSA by gentle agitation at 37 °C for 1 h and then stored at -80 °C until used. Palmitate was dissolved in 150 mM NaCl at 70 °C. The latter solution was then

slowly added to 0.17 mM BSA at 37 °C (6:1 M ratio). The palmitate-BSA conjugate was agitated for 1 h at 37 °C and then stored at -80 °C until used.

Primary cardiomyocyte harvest and cell culturing: All animal care protocols were approved by and conformed with the guidelines of the institutional animal care and use committees at the Montreal Heart Institute. Hearts from 1 to 2 day old neonatal Spraguee Dawley rats were removed aseptically and washed with Hanks buffer solution containing 137 mM NaCl, 5.36 mM KCl, 0.81 mM MgSO4, 5.55 mM dextrose, 0.44 mM KH2PO4, 0.34 mM Na2HPO4, 20 mM HEPES, and 50 mg/ml gentamicin (Gibco). Ventricular tissue digestion was carried out through repeated incubations of the tissue with Hanks buffer solution containing 50 U/ml collagenase-2 (Worthington, NJ) and 0.36uM CaCl2 for 5e10 min or until solution became noticeably turbid. Once turbid, the digestion solution was added to ice-cold fetal bovine serum (FBS), taking care to leave the non-digested ventricular tissue in the initial tube. This was repeated until ventricular tissue was completely digested. The resultant cell suspension was plated on a Falcon Primaria cell culture dish (Becton Dickenson) for 1 h at 37 °C to let cardiac fibroblasts adhere to the plate. After 1 h non-adherent cardiomyocytes were re-plated on the Primaria plate for 120 h. Cells were subject to daily media replacement with DMEM/F12 (50:50; Gibco, ON) with 2% FBS and 100 mM bromodeoxyuridine to inhibit growth of any contaminating cardiac fibroblasts.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR): Total cellular RNA was isolated from mouse or rat neonatal cardiomyocytes using RNEasy (Qiagen). cDNA was synthesized using a commercially available kit (Qiagen). qPCR was performed using SYBR

Green Master mix from Qiagen and the Eco Ilumina real-time qPCR system. Primer sequences are listed in supplemental table S1. Data was analyzed using the 2-DDCt method [32]. All PCR results represent the expression of the gene of interest relative to endogenous control (Rpl34) normalized to the control group, and are presented as mean ± standard error (SE). Primers were designed to span exoneexon regions to avoid amplification of contaminating DNA and primer specificity was verified by blasting all sequences using the NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Western blot analysis: Total cellular protein was harvested from rat neonatal cardiomyocytes using protein isolation buffer (250 mM Sucrose, 50 mM Tris, 1 mM PMSF (protease inhibitor), 1 mM DTT, and Proteinase inhibitor cocktail (ROCHE)). Protein concentrations were determined using Bradford assay and equal protein loading conditions were used and verified by Ponceau staining of the membrane. Proteins were transferred to PVDF membranes and probed with the following antibodies: anti-Acadl (Sc-82466, Santa Cruz biotechnologies), anti-Cpt1b (GWB-MQ462C, GenWay Biotech), anti-PPAR-a (SC-9000, Santa Cruz Biotechnologies), anti-PPARd
(PA1-823A, Pierce antibodies).

Viability assays: We evaluated cell viability by propidium iodide (PI) exclusion assays. Briefly, rat NCMs were treated with palmitate for indicated time points (n ¼ 4 per timepoint). The treated cells were then incubated with PI for 30 min at 37 °C. Fluorescence was measured (535 nm excitation/617 nm emission) using the Synergy2 fluorescence plate reader from Bio-Tek. Assays were done in duplicate.

Statistics: All data are presented as mean ± standard error. Student's T-test was used for comparison of two groups, while one-way ANOVA with the Tukey post-hoc test was used for multiple group comparisons. P-values of <0.05 were considered statistically significant.

3.6 Results

3.6.1 Time dependent effects of palmitate on PPARs

Because of the importance of PPARs in lipid metabolism and hence cardiac lipotoxicity, we sought to evaluate the effect of palmitate on PPAR activity in primary rat neonatal cardiomyocytes (NCMs). PPAR activity was measured indirectly by assessing the mRNA expression of PPAR target genes including Acyl-CoA dehydrogenase, long chain (Acadl), Acyl-CoA synthetase (Acsl1), and carnitine palmitoyltransferase (Cpt1b) [33]. To validate these as legitimate PPAR target genes in NCMs we administered PPAR-a and d agonists (Wy-14643 and GW501516, respectively) to NCMs for 24 h. We found that both PPAR agonists significantly induced the expression of all 3 genes (Supplemental Fig. S3.1). Having validated these as legitimate PPAR targets in NCMs, we assessed the time dependent expression of these genes in palmitate treated NCMs. Interestingly, we found that 300 mM palmitate initially increased PPAR activity as evidenced by significantly increased expression of the 3 genes after 4 and/or

8 h exposure (Fig. 3.1A). However, after 24 h of palmitate exposure, we found that mRNA expression of PPAR target genes had significantly decreased from peak values down to levels below baseline. On the other hand, oleate, a non toxic unsaturated fatty acid, significantly induced PPAR activity for more than 24 h (Fig. 3.1B). To assess if the attenuated mRNA expression translated into decreased protein levels we assessed protein levels for Acadl and Cpt1b. Interestingly, Cpt1b (Fig. 3.2A), but not Acadl (Supplemental Fig. S3.2A), was significantly decreased after 24 h palmitate exposure. To determine if the decrease in PPAR activity was due to decreased PPAR abundance we performed Western blots for PPAR-a and PPAR-d. Interestingly, NCMs treated with 300 mM palmitate for 24 h showed significant reductions in both PPAR-a (Supplemental Fig. S3.2B) and -d (Fig. 3.2B) isoforms.

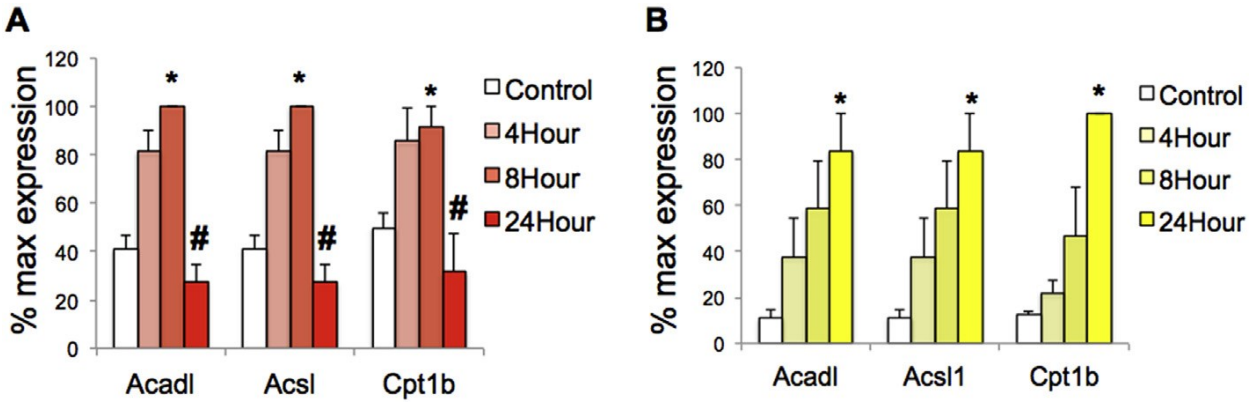

Fig. 3.1 PPAR target gene expression is induced early on, and then repressed later, in palmitate treated neonatal cardiomyocytes (NCMs). Graphs showing the % maximum expression of Acyl-CoA synthetase (Acsl), Acyl-CoA dehydrogenase (Acad), and Carnitine palmitoyl transferase (Cpt1b) in NCMs treated with (A) 300 mM palmitate or (B) 400 mM oleate for the indicated times. Control cells were treated with BSA (fatty acid vehicle) for 24 h. Values represent expression at indicated time relative to maximum expression of all time points (normalized to housekeeping gene Rpl34). * indicates $p < 0.05$ vs. control. # indicates $p < 0.05$ vs. 8 h time-point.

3.6.2 Lipotoxicity induces marked cytokine expression in primary cardiomyocytes

Because of the well established link between PPARs and inflammatory cytokines we sought to determine if palmitate induced the expression of cytokines in primary cardiomyocytes. Palmitate has been shown to induce inflammatory markers in a variety of cell types but this has not been previously shown in NCMs. Therefore, we evaluated TNF-α and Il-6 mRNA expression in NCMs treated with 300 mM palmitate for 24 h. We found that palmitate led to a robust induction of both TNF-α (>20 fold) and Il-6 mRNA (>200 fold) levels (Fig. 3.3A-B) and this was dose dependent (Fig. 3.3C).

Because both AICAR (an AmpK activator) and PPAR agonists have previously been shown to attenuate cytokine expression in other cell types; we were interested if these treatments could attenuate palmitate induced cytokine expression in primary cardiomyocytes. In addition, we also tested the effect of oleate on palmitate induced cytokine expression because of the well established capacity for oleate to protect against palmitate induced lipotoxicity. Therefore, we tested the effect of oleate (50 mM), AICAR (2 mM), and PPAR agonists [Wy-14643 (240 mM) or GW501516 (10 mM)] on expression of TNF-α and Il-6 in palmitate treated NCMs. Interestingly, we found that all four treatments had different effects on TNF-a mRNA expression. Specifically, oleate had no effect, AICAR caused a significant decrease, while PPAR agonists caused nonsignificant increases in TNF-α mRNA levels (Fig. 3.3D). In contrast, all four treatments concordantly led to significant decreases in Il-6 levels (Fig. 3.3E).

Oleate has been previously shown to attenuate palmitate mediated cell death but the effect of AICAR and PPAR agonists on palmitate induced cell death has not been previously described in primary cardiomyocytes. Therefore, we evaluated viability in cells treated with palmitate ± AICAR, Wy-14643, or GW501516. We found that all treatments significantly attenuated palmitate induced cell death (Fig. 3.4A and B).

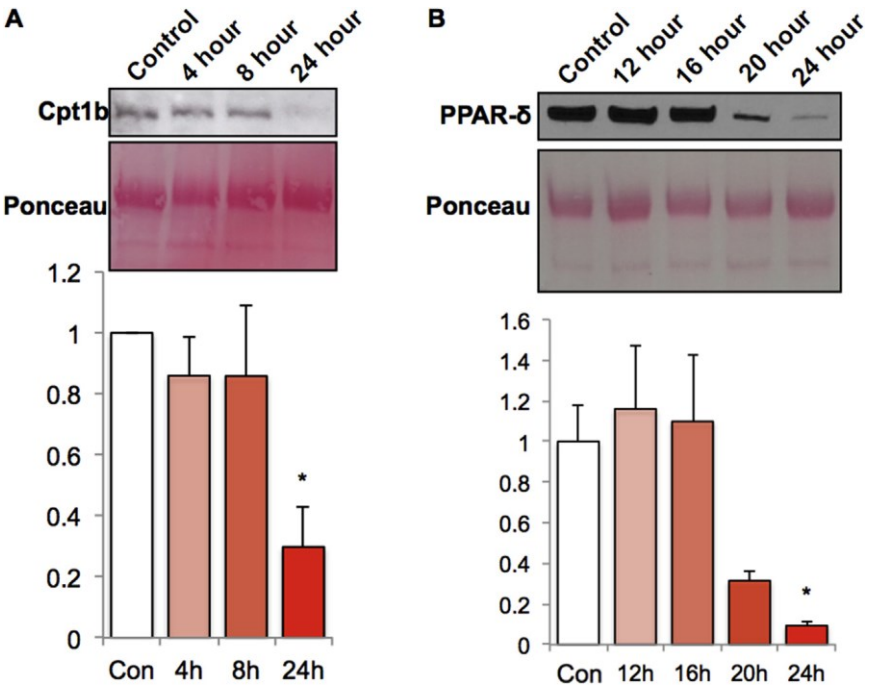

Fig. 3.2 Palmitate induces the down regulation of Cpt1b and PPAR-d protein levels. (A) Western blot and associated ponceau staining of membrane demonstrate that Cpt1b protein significantly decreases after 24 h of palmitate exposure in NCMs. Graph at bottom represents quantification of Cpt1b protein abundance relative to total protein as determined by ponceau stain from 3 separate experiments (B) Western blot and associated ponceau staining of membrane demonstrate that PPAR-d protein significantly decreases after 24 h of palmitate exposure in NCMs. Graph at bottom represents quantification of PPAR-d protein abundance relative to total protein as determined by ponceau stain from 3 separate experiments. In all cases Control cells were treated with BSA (the fatty acid vehicle) in serum free media for 24 h * indicates $p < 0.05$ vs. control.

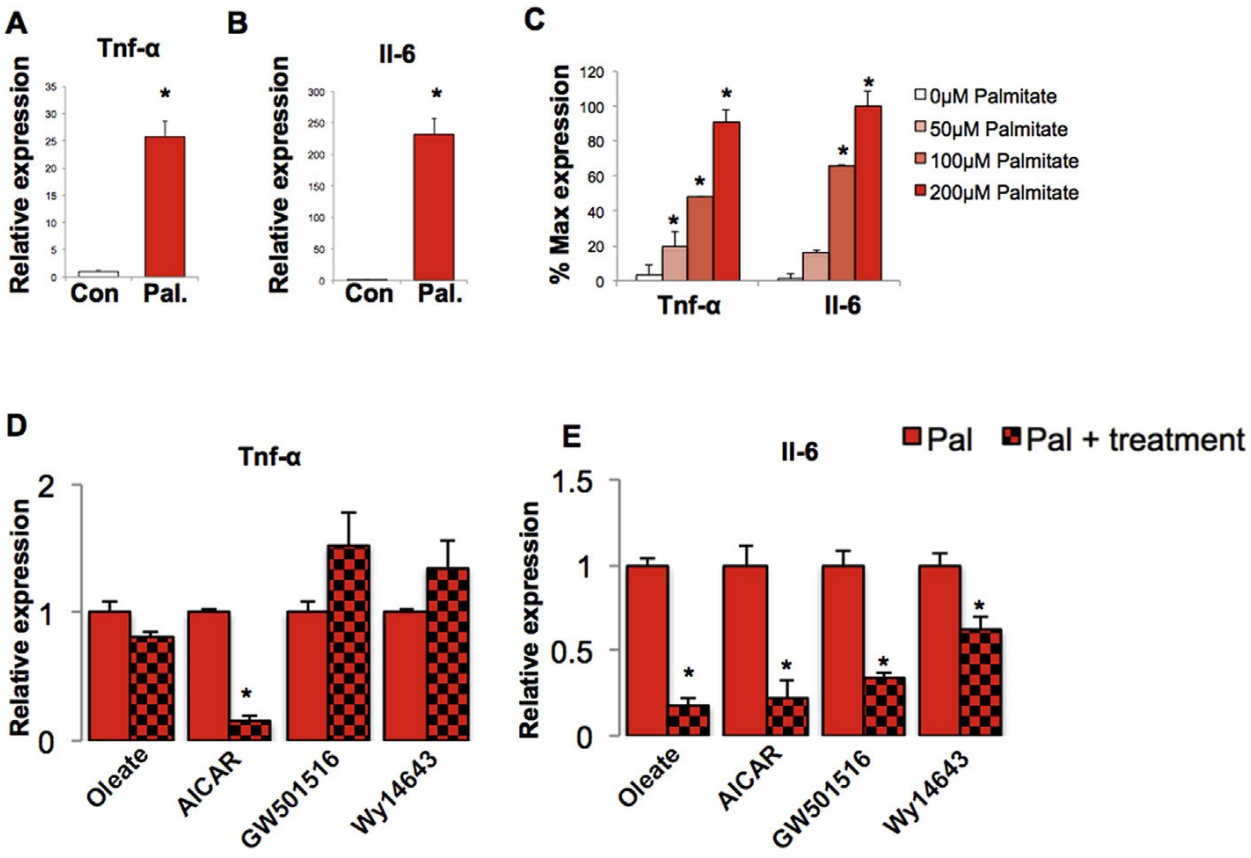

Fig. 3.3 Palmitate induces the expression of inflammatory mediators Tnf-a and Il-6 in neonatal cardiomyocytes (NCMs). (A-B) Graphs showing the expression of Tumor necrosis factor-alpha (Tnf-a) and Interleukin-6 (Il-6) relative to housekeeping gene Rpl34 in NCMs treated with 300 mM palmitate for 24 h (compared to BSA treated control NCMs). (C) Graph showing the % maximum expression of Tnf-a and Il-6 (relative to housekeeping gene Rpl34) in NCMs treated with palmitate at the indicated concentration. (D) Graphs showing the expression of Tnf-a (relative to housekeeping gene Rpl34) in NCMs treated with palmitate ± the indicated treatment for 24 h (E) Graphs showing the expression of Il-6 (relative to housekeeping gene Rpl34) in NCMs treated with palmitate ± the indicated treatment for 24 h * indicates $p < 0.05$ vs. control.

3.7 Discussion

Diabetes is a major contributor to cardiovascular disease. Indeed, the dyslipidemia in diabetics significantly increases risk for atherosclerosis. However, the accumulation of lipid within cardiomyocytes themselves, as opposed to in the vessels that supply them, may be equally pathological. Furthermore, the two pathological conditions can occur simultaneously, hence the substantial increase in heart failure and death by cardiovascular disease in diabetics.

Here we were interested in delineating the pathological mechanisms of lipotoxicity in an *in vitro* model of cardiac lipotoxicity. Indeed, we utilized primary rat neonatal cardiomyocytes which have the advantage of continuous spontaneous contraction which more closely mimics the high energy demand, and hence the energy metabolism of the beating heart, than would cardiomyocyte cell lines like H9c2 or AC16 cells. Although HL-1 cells can be differentiated to spontaneously contract, in our hands we found that this was highly variable.

Using primary cardiomyocytes we found several important aspects regarding mechanisms of cardiac lipotoxicity. Specifically, we show that (1) lipotoxicity causes impairment of PPAR activity due to loss of PPAR protein abundance and this results in Cpt1b downregulation; (2) cardiomyocyte lipotoxicity is associated with induction of cytokines, (3) oleate, AICAR and PPAR agonists significantly decrease Il-6 but not TNF-α levels in palmitate treated cardiomyocytes and this is associated with attenuated cell death. Together this data points to a key role for Il-6 in the pathological mechanism of cardiac lipotoxicity.

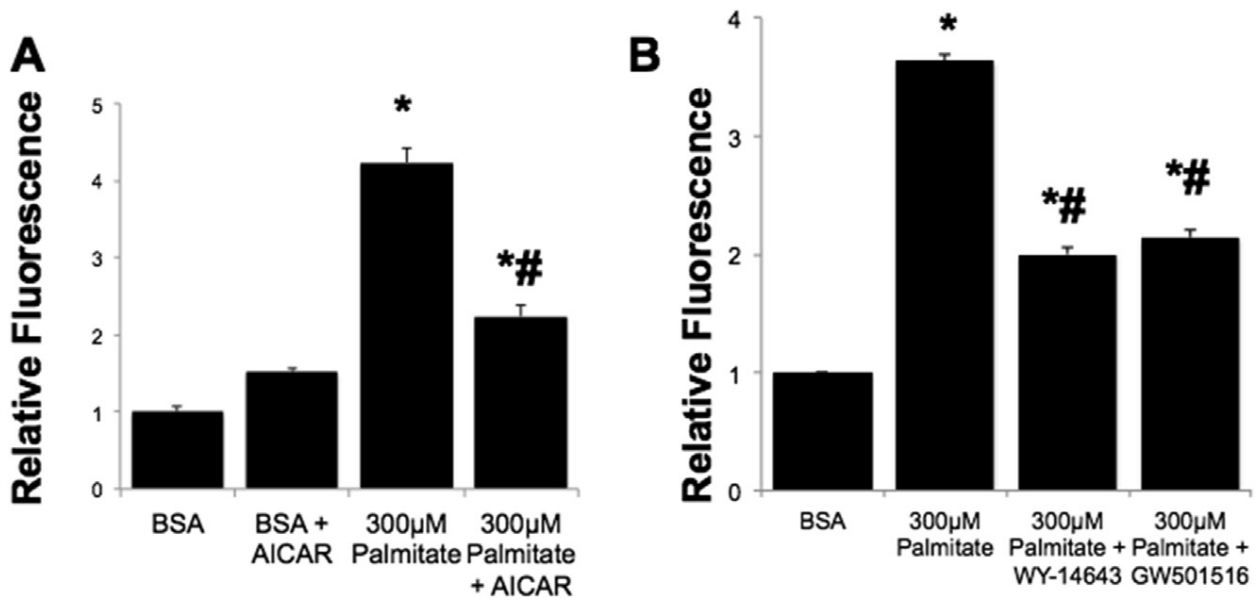

Fig. 3.4 Palmitate induced cell death in neonatal cardiomyocytes (NCMs) is attenuated with treatments that reduce Il-6 expression. Graphs (AeB) demonstrate relative fluorescence (y-axes) from NCMs treated as indicated in x-axes, followed by staining with propidium iodide, which is excluded from live cells but taken up by dead/dying cells. * indicates $p < 0.05$ vs. BSA treated NCMs (control). # indicates $p < 0.05$ vs. palmitate treatment alone.

Palmitate causes PPAR down-regulation A key finding of this current study was that palmitate induced PPAR activity at early time points but this activation was lost after 24 h. In contrast oleate, the non-toxic fatty acid resulted in persistent PPAR activation. Indeed it is conceivable that persistent PPAR activity is necessary for the cell to manage the high lipid load and that loss of this metabolic capacity results in accumulation of toxic metabolites and cell death. The observed early PPAR activation by palmitate suggests that palmitate can act as a PPAR ligand, but that it simultaneously activates other pathways that ultimately lead to PPAR degradation. Indeed we show that both PPAR-a and d are decreased in NCMs treated with palmitate for 24 h. The PPAR degradation results in decreased expression of genes responsible for b-oxidation of lipids thus allowing for the build-up of lipotoxic intermediates which

ultimately result in cell death. Several lines of evidence suggest that the increased cytokine expression could be responsible for the PPAR degradation. For instance, inflammation was suggested to be the cause of attenuated PPAR activity in hearts of diabetic mice [12]. More specifically, Il-6 has been shown to decrease PPAR-a and/or g activity and abundance in adipocytes and hepatocytes [34-38], but this has not been previously shown in cardiomyocytes. In addition, Tnf-a transgenic mice were shown to have decreased expression of PPAR-a and b-oxidation genes in the heart, resulting in decreased fatty acid oxidation [39]. Interestingly, they found that Tnf-a didn't have any direct effects on oxidation in cultured cardiomyocytes, but instead they attributed the impairment in oxidation in the Tnf-a transgenic hearts to the activation of the Tgf-b-Smad3 pathway. Unfortunately they did not evaluate Il-6 levels in that study, but since both Tgf-b [40,41] and Tnf-a [42] have been shown to induce Il-6, this supports the hypothesis that Il-6 is the key inducer of PPAR degradation. It is also important to note that the Il-6 expression precedes PPAR degradation, further supporting it as a cause rather than a consequence of metabolic dysfunction. Future studies will be focused on the mechanism of PPAR degradation in lipotoxic environments.

We also demonstrate that the loss of PPARs results in decreased Cpt1b protein levels, suggesting that PPAR down-regulation impairs oxidation through down-regulation of Cpt1b. The loss of Cpt1b protein after 24 h of palmitate exposure suggests that either Cpt1b protein half-life is very short and that protein levels mirror mRNA levels or that palmitate leads to the active degradation of Cpt1b protein. Importantly, although Acadl mRNA levels were down after 24 h; protein levels were unchanged, suggesting that this protein either has a longer half life than Cpt1b or that it is not specifically targeted for degradation.

Considering that our hypothesis revolves around the key beneficial role PPARs play in mitigating lipotoxicity, it is important to note the paradoxical finding that activation of PPAR-a by transgenic overexpression causes diabetic cardiomyopathy due to lipotoxicity. However it must be kept in mind that in these latter mice PPAR-a is expressed at supra-physiological levels (~50e100 fold) potentially leading to more lipid uptake than can be handled by the oxidative or storage capacities of hearts in these mice. Interestingly, PPAR-d overexpressing mice did not exhibit lipid accumulation or develop cardiomyopathy. Both PPAR-a and PPAR-d induce β-oxidation, however the key difference is the fact that PPAR-a induces lipid uptake to a much greater degree than PPAR-d [43]. Thus perhaps the ratio of PPAR-a vs. d activity can also have an impact on lipid accumulation and hence lipotoxicity in the heart.

Lipotoxicity induced cytokine expression, which was attenuated by oleate, AICAR and PPAR agonists Palmitate has been shown to induce the expression of cytokines in a variety of cell types, but this has not been previously shown in NCMs. Here we showed that palmitate robustly induced the expression of both TNF-a and Il-6. This is highly relevant considering the important role inflammation has in the pathology of diabetes. Interestingly, oleate has been shown to attenuate palmitate induced Il-6 release from skeletal muscle cells [44]. Indeed, we showed that the protection from cell death afforded by oleate, AICAR, and PPAR agonists were consistently associated with a reduction in Il-6, but not Tnf-a. This suggests that the primary pathological culprit involves Il-6 signaling. Further studies will be required to demonstrate the mechanism of Il-6 induction in lipotoxic conditions.

3.8 Acknowledgments

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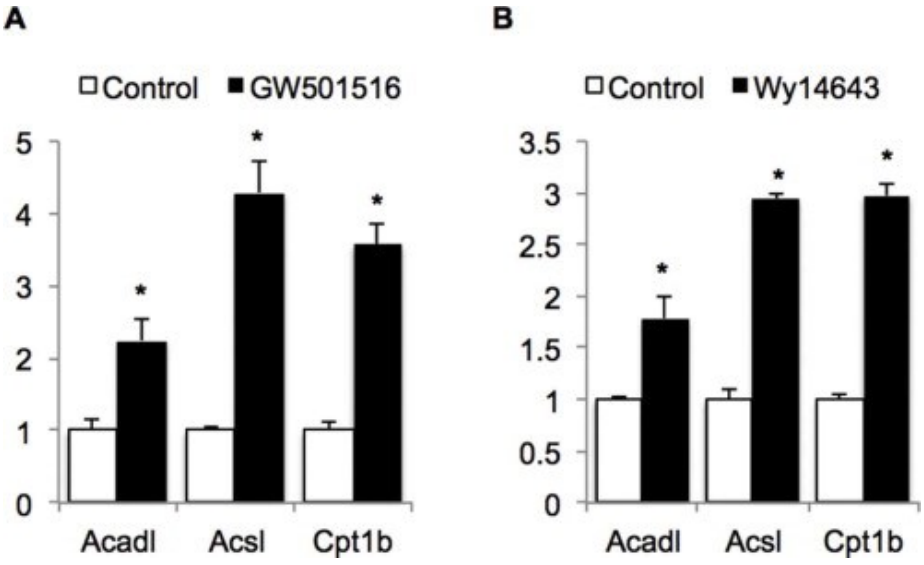
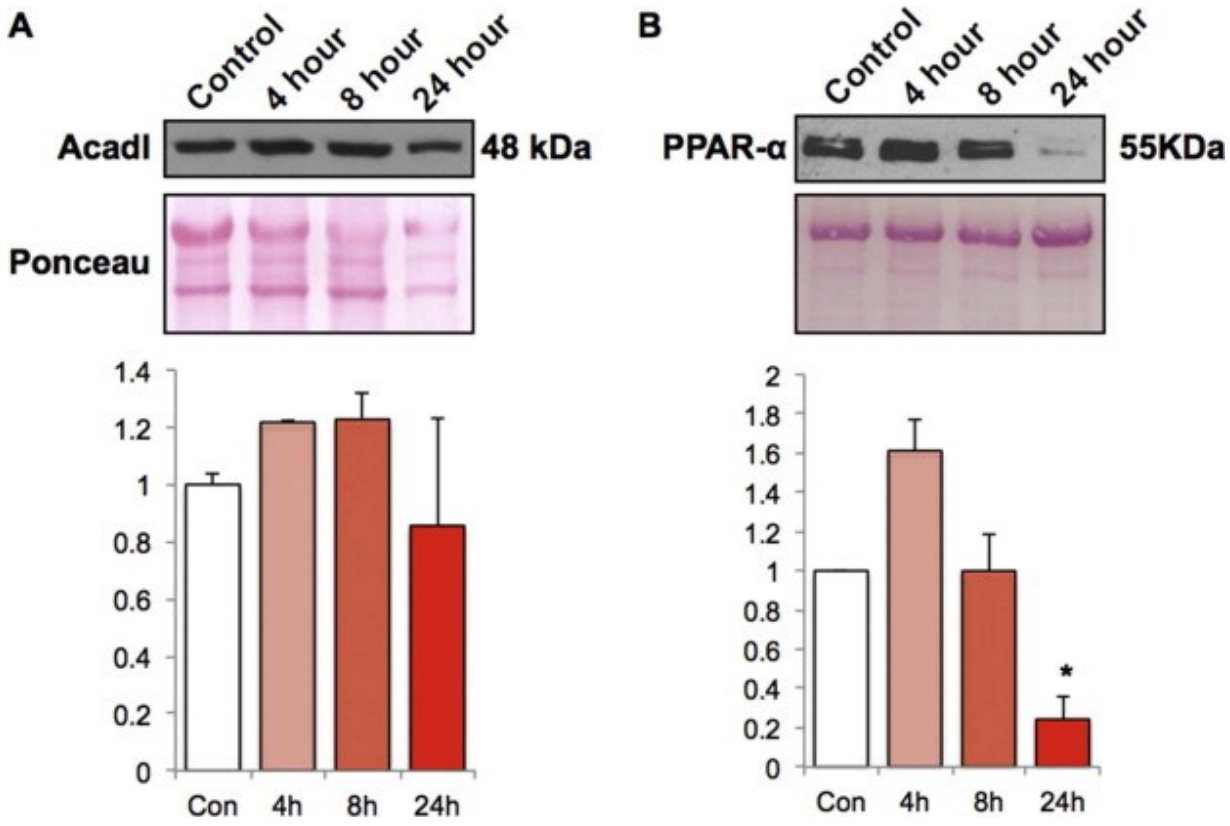
Conflict of interest: None.

3.9 Supplementary data



Supplemental Fig. S3.1: Acadl, Acsl, and Cpt1b are legitimate PPAR target genes in rat primary cardiomyocytes (NCMs). NCMs were treated with either 1 μM GW501516 or 60 μM Wy14643 in serum free media for 24 h. Control cells were only treated with serum free media. * indicates $p < 0.05$ vs. control.



Supplemental Fig. S3.2: Protein levels of Acadl and PPAR-α in NCMs treated with palmitate. (A) Western blot and associated ponceau staining of membrane demonstrate that Acadl protein remains relatively stable over 24 h of palmitate exposure in NCMs. Graph at bottom represents quantification of data from 2 seperate experiments (B) Western blot and associated ponceau staining of membrane demonstrate that PPAR-α protein significantly decreases after 24 h of palmitate exposure in NCMs. Graph at bottom represents quantification of data from 3 seperate experiments. In all cases Control cells were treated with BSA (the fatty acid vehicle) in serum free media for 24 h * indicates $p < 0.05$ vs. control.

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Chapter 4: Impaired fatty acid oxidation as a cause for lipotoxicity in Cardiomyocytes

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Keywords: Diabetic cardiomyopathy, Lipotoxicity, Fatty acid oxidation

4.1 Authors contribution

Taha Haffar performed most of the experiments, helped analyze the data, and assisted with writing the manuscript. Félix-Antoine Bérubé-Simard performed the knockdown studies. Nicolas Bousette conceived and designed the project, supervised the work, and wrote the manuscript.

4.2 Context

PPARs are master regulators of fatty acid metabolism. Our previous study demonstrated that palmitate lipotoxicity inhibits PPAR expression and activity. CPT1B, an enzyme that catalyzes the rate-limiting step of fatty acid oxidation, is a PPAR target gene. Hence, we hypothesized that palmitate lipotoxicity is associated with inhibition of fatty acid oxidation. Indeed, our results show a clear inhibition of fatty acid oxidation in primary rat neonatal cardiomyocytes treated with 300uM palmitate. Oleate, a non-toxic fatty acid, does not inhibit fatty acid oxidation. Interestingly, inducing fatty acid oxidation by carnitine reduces palmitate lipotoxicity. On the other hand, inhibiting fatty acid oxidation by etomoxir causes oleate to become toxic.

4.3 Abstract

A major cause for diabetic cardiomyopathy is excess lipid accumulation. To elucidate mechanisms of lipotoxicity mediated diabetic heart disease we need to further our understanding of how lipid metabolism is altered in the diabetic heart. Here we investigated the role of lipid clearance by oxidation as a regulator of lipid-mediated toxicity (lipotoxicity).

We evaluated the effect of pre-treating rat neonatal cardiomyocytes (NCMs) with either oleate (monounsaturated fatty acid) or palmitate (saturated fatty acid) on fatty acid oxidation (FAO) by measuring 14C eCO_2 production. We evaluated carnitine palmitoyltransferase (Cpt1b) expression by western blotting and mitochondrial membrane potential by quantitative and qualitative fluorescence analyses using the JC-1 dye. We inhibited the Cpt1b pharmacologically using etomoxir and genetically by knocking down its expression using LentiVector mediated transduction of siRNAs targeting the Cpt1b gene.

We found that palmitate had a slower clearance rate from NCMs than oleate, and this was associated with a significant decrease in FAO. This impairment in FAO was not the result of either loss of Cpt1b protein or mitochondrial integrity. Enhancing FAO with either oleate or carnitine was associated with a significant attenuation of palmitate mediated lipotoxicity. In contrast impairing FAO in oleate treated NCMs caused lipotoxicity.

Here we demonstrate that a major difference between non-toxic unsaturated fatty acids and toxic saturated fatty acids is there ability to stimulate or inhibit fatty acid oxidation, respectively. This has important implications for diabetic cardiomyopathy since diabetic hearts consistently exhibit elevated lipid accumulation.

4.4 Introduction

Cardiac disease is the primary cause for morbidity and mortality in the diabetic population [1]. Diabetes is an independent risk factor for atherosclerosis and hypertension. However, Diabetes also causes cardiomyopathy independently of the latter vascular pathologies. This diabetic cardiomyopathy is due to excess lipid accumulation and the ensuing lipotoxicity [2-7]. To understand mechanisms of lipotoxicity mediated diabetic heart disease we need to further our understanding of how lipid metabolism is altered in the diabetic heart.

Long chain fatty acids are made up of three main categories including saturated fatty acids (e.g. palmitate), mono-unsaturated fatty acids (e.g. oleate), and poly-unsaturated fatty acids (e.g. linoleate). Oleate and palmitate are the two most common fatty acids in the human diet. Importantly, in vitro studies have shown that lipotoxicity is due mainly to saturated fatty acids, whereas unsaturated fatty acids like oleate are non-toxic and can even be protective.

The importance of lipid metabolism in the heart is underscored by the fact that the heart derives ~75% of its energy from fatty acids. However, this number increases to >90% in the diabetic setting. The diabetic heart is characterized as having an excess of intracellular lipid (also known as cardiac steatosis) [8-12]. Lipids accumulate in the heart because uptake exceeds lipid clearance. The two main pathways for lipid clearance are lipid export and fatty acid catabolism through oxidation. While lipid export does occur from cardiomyocytes [13], it is likely a small fraction of lipid clearance compared to oxidation. Interestingly, studies have shown that β-oxidation is increased in models of diabetic cardiomyopathy despite the accumulation of lipid. The fact that diabetic hearts consistently exhibit elevated lipid accumulation suggests that either the increased b-oxidation is insufficient to handle the elevated uptake; or that the oxidation is incomplete. Indeed, β-oxidation represents only the first half of fatty acid oxidation (FAO). The second half being the oxidation of acetyl-CoA to CO_2 in the citric acid cycle. Impaired citric acid cycle activity could lead to a build up of acetyl-CoA, which is converted to malonyl-CoA, a key substrate in fatty acid biosynthesis.

Here we investigated the effect of palmitate, a saturated fatty acid, on FAO in primary cardiomyocytes. We found that palmitate impaired FAO, and that enhancing FAO attenuated palmitate mediated cell death. Conversely, impairing FAO induced cell death in primary cardiomyocytes treated with the non-toxic fatty acid oleate.

4.5 Methods

Rat neonatal cardiomyocyte (NCM) harvest and culturing: NCMs were harvested from 1-day-old Sprague-Dawley rat pups as previously described [15]. NCMs were fed daily with DMEM/F12 þ 2% iFBS þ 100 mM bromodeoxyuridine (Sigma). Cells were cultured for a minimum of six days before treated to promote differentiation.

Fatty acid clearance assay: We evaluated the rate of fatty acid clearance by treating NCMs with 300 mM oleate or palmitate (275 mM cold fatty acid þ 25 mM hot 14C fatty acid). After 24 h we measured CPMs using a Scintillation counter (Beckman LS6500). CPMs were normalized to total CPMs produced by 25 mM radiolabeled fatty acid not exposed to cells.

Viability assays: We determined cell viability by the propidium iodide (PI) exclusion assay. Briefly, following treatment, NCMs were incubated with PI for 30 min at 37 °C. Fluorescence was measured (535 nm excitation/617 nm emission) using the Synergy2 fluorescence plate reader from Bio-Tek. Assays were done in triplicate.

Fatty acid oxidation assays: NCMs were treated with non-radiolabelled BSA, oleate or palmitate for 8 h then with 25 mM radio-labelled 14C-oleate for 2 h. Media from treated NCMs were transferred to glass tubes containing center wells and stopper tops. NaOH soaked Whatman filter paper was placed in the center well after addition of the cell media. Concentrated HCL was added to the media to release the CO_2 which was captured by the basic filter. Filter papers

were transferred to scintillation vials containing Aquasol II (Perkinelmer, NE9529). Radiolabelled CO_2 was counted by the Scintillation counter (Beckman LS6500).

Mitochondrial membrane potential assay: NCMs were incubated with warm Krebs-Ringer with HEPES buffer (KHR) containing 7.6 mM JC-1 (Sigma, cat# 1130-5) for 10 min. After incubation, cells were washed twice with warm KHR before reading the fluorescence (530 nm excitation/590 nm emission for red dye and 485 nm excitation/528 nm emission for green dye) using the Synergy2 fluorescence plate reader from Bio-Tek and a fluorescence microscope (Olympus IX83).

Western blotting: Total cellular protein was harvested from NCMs using RIPA protein isolation buffer containing Proteinase inhibitor cocktail (ROCHE). Protein concentrationwas determined using the Bradford assay. Proteins were transferred to activated PVDF membranes and probed with Cpt1b antibody (Geneway, cat# GWB-MQ462C). Equal protein loading conditions were utilized and verified by Ponceau staining of the membrane.

qRT-PCR: Total cellular RNA was isolated using RNEasy isolation kit (Qiagen) and cDNA synthesis was carried out using the Quantitect reverse transcription kit (Qiagen). Real-time RT-PCR was carried out using the Eco illumina PCR cycler. Primers were designed using the NCBI primer BLAST tool. Exon/exon junctions were selected to prevent amplification of genomic DNA. PCR amplifications were done using the Bryt green master mix (Promega). Cpt1b was normalized to the housekeeping gene Rpl34 and quantification was carried out using the DDCt method. Primer sequences: rCpt1b-F, TCGAGTTCAGAAACGAACGC, rCpt1b-R,

GTGTGTCTCCTGGTCTCAGC, rRpl34-F, TGCTGTGAGACCCAAAGTCCTCA, rRpl34-R, TAAGGAAAGCCCGCTTGATCCTG.

Knockdown studies: LentiVector mediated transduction of NCMs was carried out as previously described [14]. Briefly, HEK-T293 cells were transfected with plasmids expressing packaging proteins (psPAX2), envelope proteins (pMD2.G) and siRNA expressing plasmids (pLenti-siRNA, cat# i057517, ABMGood). 48 h after transfection cellular media containing virus were harvested and centrifuged to remove cells. NCMs were treated with 10 mg/ml polybrene for 90 min and then exposed to virus containing media for 21 h 6 days after transduction cells were treated as described below.

Statistical analysis: Data are presented as the mean ± standard error. Student's Ttest was used for comparison of experiments with two groups. For three or more group comparisons we performed one-way ANOVA with the Tukey post-hoc test. P-values of <0.05 were considered statistically significant.

4.6 Results

4.6.1 Palmitate impairs fatty acid oxidation in primary cardiomyocytes

We previously demonstrated that palmitate led to a marked difference in the qualitative nature of lipid staining compared to oleate treated NCMs [15]. Indeed, palmitate caused a diffuse lipid staining pattern, while oleate produced canonical lipid droplet staining. To assess if this qualitative difference in lipid accumulation also exhibited quantitative differences we measured the amount of oleate or palmitate remaining after a 24-h period. Interestingly, there was a significant increase in palmitate remaining after 24 h compared to oleate (Fig. 4.1A) suggesting that palmitate was not oxidized with the same efficiency. In support of this, a previous report demonstrated that palmitate decreased fatty acid oxidation (FAO) following 20 h exposure [16]. However, from our own experience we knew that cell death was already rampant in NCMs treated with palmitate for 20 h [15]. Therefore, we aimed to evaluate if FAO was altered before the onset of cell death (i.e. after 8 h exposure of palmitate). Importantly, for all FAO experiments, cells were pre-treated with either palmitate or oleate and then the degree of CO_2 production was measured in both groups using only radiolabelled [14C]-oleate. Therefore differences in CO_2 production were the result of pre-treatments and not an inherent difference in the cells ability to oxidize different fatty acids. Interestingly, we found that pre-treatment of NCMs with 300 mM palmitate for 8 h caused a marked decrease in FAO compared to oleate

(300 mM) pre-treated NCMs (Fig. 4.1B). Therefore, palmitate impairs FAO in cardiomyocytes which may contribute to lipid accumulation and hence lipotoxicity.

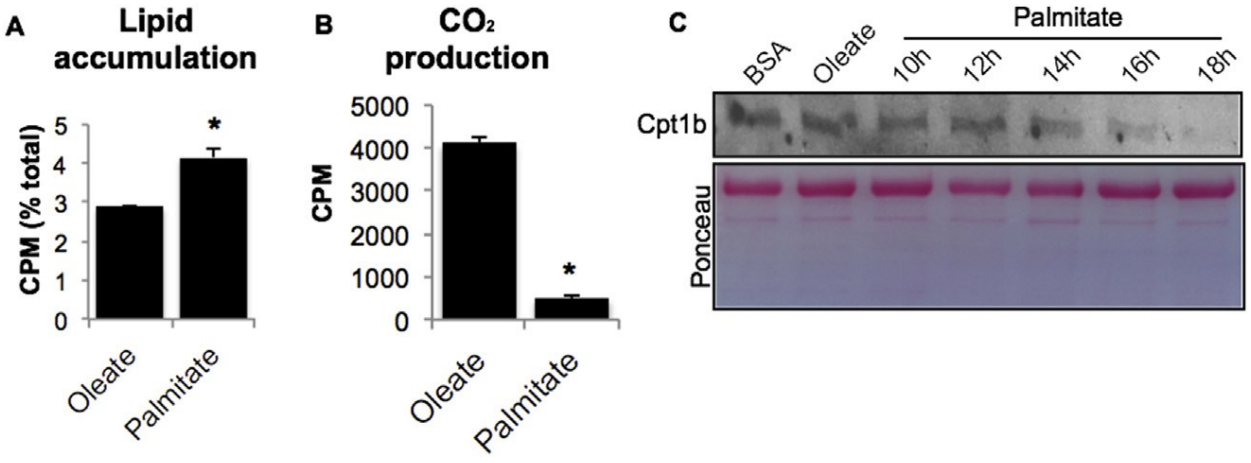

Fig. 4.1 Palmitate impairs fatty acid oxidation in primary rat neonatal cardiomyocytes (NCMs). (A) Graph demonstrating significantly increased palmitate remaining in NCMs 24 h after administration of either 300 mM oleate or 300 mM palmitate suggesting impaired clearance of palmitate compared to oleate. (B) Graph demonstrating significant decrease in the production of CO_2 in palmitate compared to oleate pre-treated NCMs (300 mM each, 8-h treatment). (C) Western blot demonstrating that palmitate induces the time dependent decrease in Cpt1b protein levels but this occurs after the impairment of fatty acid oxidation. Ponceau staining of membrane demonstrates equal protein loading conditions. *indicates $p < 0.05$ vs. oleate treated NCMs.

4.6.2 Impaired FAO is not due to loss of carnitine palmitoyltransferase (Cpt1b) or loss of mitochondrial integrity

Cpt1b is the rate-limiting enzyme in FAO. We previously demonstrated that palmitate caused a marked loss of Cpt1b protein levels in NCMs after 24 h [17]. To determine if this was the cause

of the decreased FAO we performed a time-course analysis for Cpt1b protein levels in palmitate treated NCMs. Interestingly, we found that palmitate caused a time dependent decrease in Cpt1b protein levels but this began only after 14e16 h (Fig. 4.1C). Therefore the decrease in FAO was not the result of decreased Cpt1b levels.

Next we considered that perhaps palmitate is resulting in loss of mitochondrial integrity. To test this we assessed mitochondrial membrane potential (MMP) in palmitate treated NCMs. Indeed, palmitate caused a loss of MMP after 16-h exposure to palmitate (Fig. 4.2A and B). However the MMP was unaffected by 8 h exposure to palmitate, suggesting that this was not the cause of impaired fatty acid oxidation.

4.6.3 Enhancing fatty acid oxidation attenuates palmitate-mediated lipotoxicity

If palmitate mediated impairment of FAO is contributory to the observed lipotoxicity then enhancing oxidation should attenuate palmitate induced cell death. We previously demonstrated that as little as 50 mM oleate can abolish lipotoxicity induced by 300 mM palmitate [15]. Therefore we tested the effect of 50 mM oleate on FAO in palmitate treated NCMs. Indeed, we found that pre-treating NCMs with 50 mM oleate + 300 mM palmitate significantly enhanced FAO compared to NCMs pre-treated with 300 mM palmitate alone (Fig. 4.3A).

Next we evaluated if addition of carnitine, a known inducer of Cpt1b could enhance FAO in palmitate treated NCMs. Indeed, NCMs pre-treated with 300 mM palmitate + 250 mM carnitine for 8 h exhibited significantly increased FAO compared to NCMs pretreated with palmitate alone (Fig. 4.3B). In support of the protective role FAO has against lipotoxicity, co-administration of carnitine significantly attenuated palmitate mediated cell death in NCMs (Fig. 4.3C).

4.6.4 Impairing fatty acid oxidation causes lipotoxicity

To verify that it is truly the impairment of FAO that is contributing to lipotoxicity we tested the effect of impairing FAO in cells treated with oleate, a non-toxic mono-unsaturated fatty acid. To impair FAO we treated NCMs with etomoxir, an irreversible inhibitor of Cpt1b, the rate limiting step in FAO. Specifically, NCMs were treated with either 300 mM oleate or 300 mM oleate + 300 mM etomoxir. We found that etomoxir significantly impaired FAO in oleate treated NCMs (Fig. 4.4A). Next we tested the effect of etomoxir on cell viability. Interestingly, neither oleate nor etomoxir caused cell death on their own but when combined they led to a significant degree of cell death (Fig. 4.4B).

To complement this assay and to rule out the possibility of offtarget effects of etomoxir we assessed the effect of silencing Cpt1b expression on viability of oleate treated NCMs.We confirmed Cpt1b knockdown at both the mRNA and protein level (Fig. 4.4C and D). Similarly

to the etomoxir data, we found that Cpt1b KD only led to cell death in cells treated with oleate but not in BSA (vehicle control) treated NCMs (Fig. 4.4E).

4.7 Discussion

Diabetic hearts exhibit pronounced lipid accumulation. As such, a major contributor to the pathology of diabetic cardiomyopathy is lipotoxicity. Here we sought to further our understanding of the mechanisms of lipotoxicity by investigating a potential cause for lipid accumulation. We found that palmitate impairs fatty acid oxidation in NCMs. Importantly, we utilized NCMs, which spontaneously contract in culture and thus more closely mimic the high metabolic demands of the beating heart than do cell lines. We found that the palmitate-induced impairment of FAO was not due to loss of Cpt1b or mitochondrial integrity. We also demonstrated that enhancing FAO attenuated palmitate mediated lipotoxicity while decreasing FAO caused oleate, which is normally non-toxic, to induce cell death of NCMs. Therefore we provide strong evidence that lipotoxicity is due in part to impaired FAO.

Here we found that significantly more palmitate remained in NCMs after 24 h compared to cells exposed to the molar equivalent of oleate. This suggests that palmitate clearance is slower than oleate. To test if this was due to impaired FAO we evaluated the degree of CO_2 production in NCMs pre-treated with either oleate or palmitate for 8 h. This time-point is not associated with cell death from either fatty acid. We found that palmitate pre-treatment severely inhibited CO_2

production from fatty acid oxidation. Importantly, we quantified CO_2 production, which is a measure of complete fatty acid oxidation and not just b-oxidation.

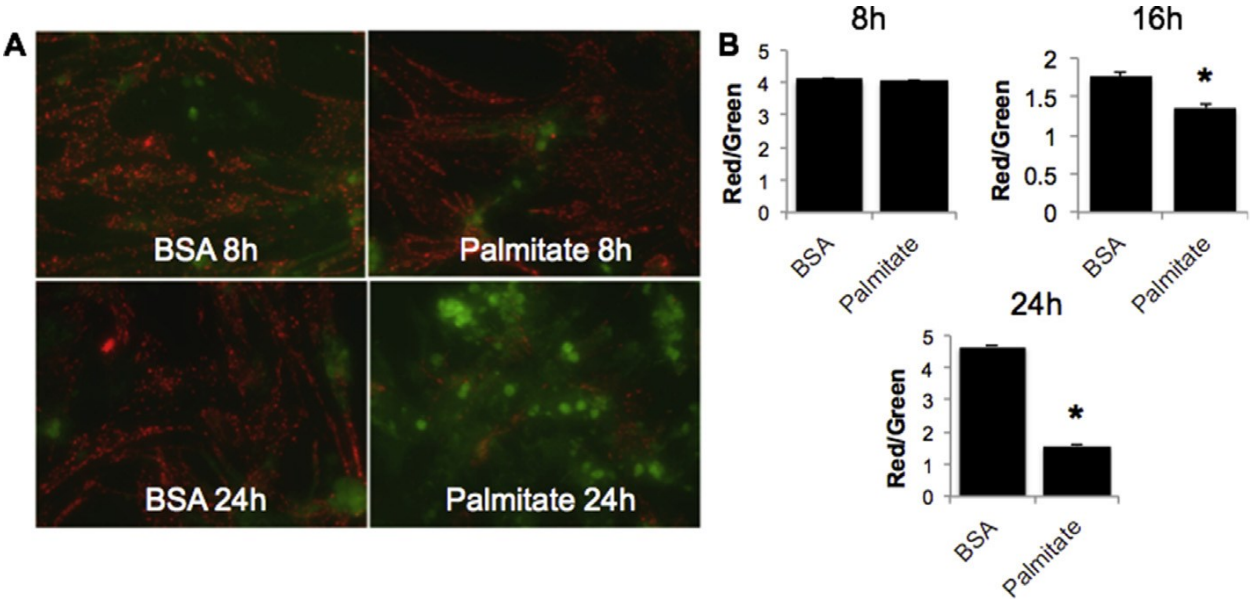

Fig. 4.2 Palmitate causes a time dependent loss of mitochondrial membrane potential in NCMs. (A) Fluorescent images of NCMs with either bovine serum albumin (BSA, 51 mM, vehicle control) or palmitate (300 mM) for the indicated time points and then stained with JC-1 dye. Red staining indicates mitochondria with polarized membranes whereas green staining indicates loss of mitochondrial membrane potential. (B) Graphs demonstrating the quantification of the red and green fluorescence in JC-1 dye stained NCMs treated with either BSA (51 mM, vehicle control) or palmitate (300 mM) for the indicated time points. A decrease in the red/green fluorescence ratio is an indicator of loss of mitochondrial membrane potential. *indicates $p < 0.05$ vs. BSA treated NCMs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Because we previously found that Cpt1b protein was decreased following 24 h exposure to palmitate [17], we evaluated Cpt1b protein levels and found that they only began to decrease after ~14-16 h palmitate, which is long after the time at which we saw severely impaired FAO

(i.e. 8 h). We also tested for mitochondrial integrity by measuring the MMP using the JC-1 dye. We found that palmitate caused a loss of MMP, however the loss of MMP only began after the observed decrease in FAO. The loss of FAO capacity results in decreased formation of citric acid cycle derived reducing equivalents, which in turn can lead to loss of the proton gradient across the inner mitochondrial membrane. Therefore this suggests that loss of FAO may actually be causing the loss of MMP. The loss of MMP will translate to loss of mitochondrial integrity, which may in turn explain the loss of Cpt1b protein levels. Indeed we see a close time correlation between the loss of MMP and decreases in Cpt1b levels. The cause of palmitate-induced impairment of FAO is currently unclear but could include inhibition of Cpt1b activity or perhaps impairments in the expression or activities of b-oxidation and/or citric acid cycle enzymes (discussed below).

Next we tested the possibility that this impaired FAO might be causing toxicity. Indeed we found that increased FAO induced by either oleate or carnitine were both associated with attenuated palmitate mediated cell death. Interestingly, oleate abolished palmitate mediated lipotoxicity while carnitine only attenuated it. This is in line with the fact that oleate has multiple effects including increased fatty acid oxidation, anti-ER stress [15], and anti-inflammatory effects [17], while carnitine is only an inducer of increased FAO through activation of Cpt1b.

We further supported the notion that lipotoxicity is due at least in part to impaired FAO by showing that oleate which is not only non-toxic, but actually protective against palmitate, can actually cause lipotoxic cell death if FAO is inhibited. We showed this with both a pharmacological and genetic approach, thus further validating our hypothesis. The exact

mechanism of cell death in oleate treated NCMs that have impaired FAO is still unclear, but likely involves an accumulation of lipotoxic intermediates such as diacylglyceride.

The potential role of impaired FAO as a contributor to lipotoxicity in diabetic cardiomyopathy may have been overlooked thus far because many studies have shown that b-oxidation is increased in mouse models of diabetic cardiomyopathy. In fact, it has been suggested that the high b-oxidation leads to cardiac dysfunction through an elevated degree of oxidative stress. Indeed, PPAR-a transgenic mice, which overexpress PPAR-a specifically in the heart, exhibit increased b-oxidation, lipid accumulation, oxidative stress and cardiomyopathy [3], which resembles the pathology of the diabetic heart. Furthermore, some in vivo studies have shown benefit in cardiac function by inhibiting b-oxidation [18,19], likely through enhanced glucose oxidation and the consequent improvement in cardiac efficiency. Importantly, in vivo studies using inhibitors of fatty acid oxidation have been only short term, thereby ignoring the harmful effects of long-term inhibition of fatty acid oxidation (i.e. enhanced lipid accumulation). Moreover, the mere association of increased b-oxidation with cardiac dysfunction in PPAR-a transgenic mice does not necessarily indicate causality, especially in light of the overt lipid accumulation.

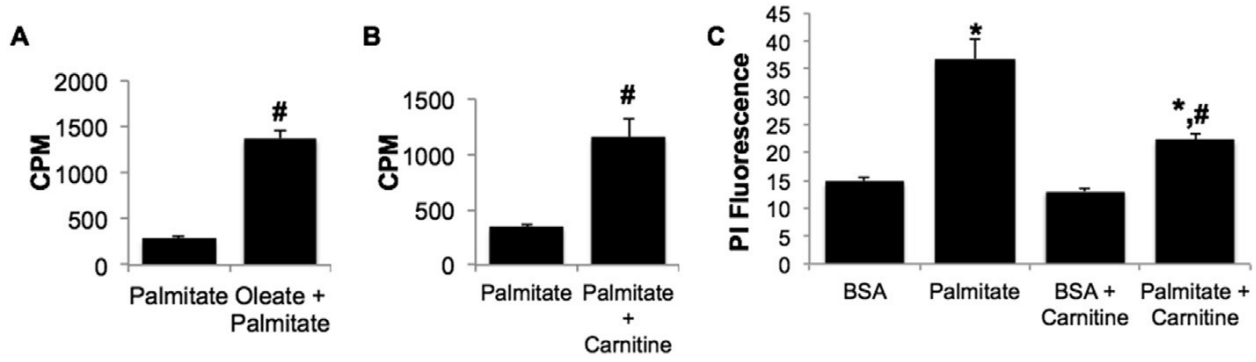

Fig. 4.3 Enhancing fatty acid oxidation attenuates palmitate mediated lipotoxicity in primary NCMs. (A) Graph demonstrating significantly increased [14C]eCO_2 production and hence fatty acid oxidation in NCMs pre-treated with 300 mM palmitate þ 50 mM oleate for 8 h compared to NCMs pre-treated with palmitate alone. (B) Graph demonstrating significantly increased [14C]eCO_2 production and hence fatty acid oxidation in NCMs pre-treated with 300 mM palmitate þ 250 mM carnitine for 8 h compared to NCMs pre-treated with palmitate alone. (C) Graph demonstrating significantly increased propidium iodide (PI) fluorescence (an indicator of cell death) in palmitate (300 mM for 24 h) treated NCMs that is significantly reduced by the co-administration of 250 mM carnitine. *indicates $p < 0.05$ vs. BSA control, #indicates $p < 0.05$ vs. palmitate alone.

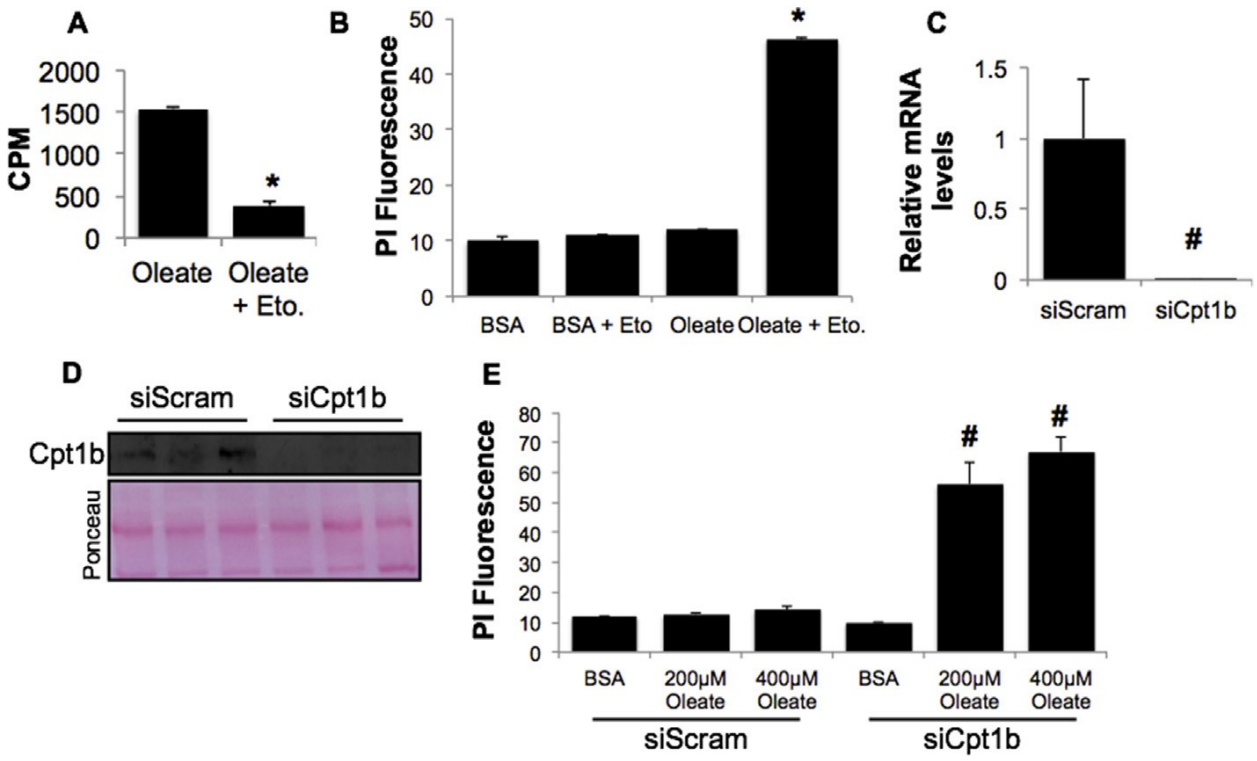

Fig. 4.4 Inhibiting fatty acid oxidation causes cell death in NCMs. (A) Graph showing significantly reduced $14CeCO_2$ production and hence fatty acid oxidation in NCMs pre-treated with 300 mM oleate þ 300 mM etomoxir (Eto) compared to NCMs pre-treated with oleate alone. (B) Graph demonstrating propidium Iodide (PI) fluorescence (y-axis) in NCMs treated as indicated in x-axis (BSA ¼ 51 mM; oleate ¼ 300 mM; Eto. ¼ 300 mM). Increased fluorescence indicates increased cell death. (C) Graph demonstrating the mRNA expression of Cpt1b relative to housekeeping gene Rpl34 as determined by qRT-PCR in NCMs transduced with LentiVectors expressing either scrambled siRNAs (neg. control) or siRNAs targeting Cpt1b. (D)Western blot demonstrating the protein expression of Cpt1b in NCMs transduced with LentiVectors expressing either scrambled siRNAs (neg. control) or siRNAs targeting Cpt1b. Ponceau shows equal loading. (E) Graph demonstrating PI fluorescence (y-axis) in NCMs treated as indicated in x-axis. Increased fluorescence indicates increased cell death.*indicates $p < 0.05$ vs. oleate alone. #indicates $p < 0.05$ vs. corresponding siScram control.

On the other hand, a strong argument against the potential toxicity of enhanced fatty acid oxidation comes from 3 important studies involving genetic modulation of the PPAR receptors.

Indeed these studies have shown that enhanced fatty acid oxidation was actually associated with enhanced cardiac function and survival, while impaired fatty acid oxidation was associated with lipid accumulation and cardiomyopathy. Firstly, knockout of CD36/FAT (fatty acid translocase), the key fatty acid uptake protein in cardiomyocytes, significantly attenuates cardiac dysfunction and mortality in PPAR-a transgenic mice despite persistent elevated boxidation [20]. Secondly, PPAR-a knockout in mice with cardiac overexpression of PPAR-g caused elevated b-oxidation but enhanced cardiac function and survival, compared to mice with PPAR-g overexpression alone [21]. Finally, PPAR-d knockout mice exhibit impaired fatty acid oxidation, lipid accumulation, and cardiomyopathy [22]. As such, it is possible that although diabetic cardiomyopathy is associated with elevated b-oxidation, it may not be elevated to its full potential. Indeed, there may be some factors that are driving increased FAO, while others are simultaneously inhibiting it. Therefore further elevation of FAO may theoretically decrease lipid accumulation and hence lipotoxicity.

There also exists the possibility that the observed increase in β-oxidation in diabetic cardiomyopathy does not equate to an increased degree of complete oxidation, due to impairments in the citric acid cycle (CAC). The notion of an impaired CAC is supported by a study that demonstrated that while citrate levels were normal, isocitrate and a-ketoglutarate levels were decreased in the diabetic heart [23]. They demonstrated that the decrease in intermediates was due to an impaired aconitase activity, the enzyme responsible for the conversion of citrate to isocitrate. Another study has shown decreased levels of CAC enzymes in diabetic mouse hearts [24]. Finally, perhaps even more important than the decrease in lipid clearance resulting from an impaired CAC would be the loss of the proton gradient and ATP

synthesis. This would in turn cause loss of mitochondrial membrane potential, release of cytochrome C, and ultimately activation of the intrinsic apoptotic pathway.

In conclusion, here we show that lipotoxicity is due at least in part to impaired FAO. Indeed palmitate a toxic saturated fatty acid impairs FAO and causes cell death. Enhancing FAO in palmitate treated cardiomyocytes attenuates cell death. Conversely, inhibition of FAO causes oleate to become a toxic fatty acid. This work thereby provides a stimulus for further investigation into the possibility of enhancing fatty acid oxidation in diabetic patients as a therapeutic modality.

4.8 Acknowledgements

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Chapter 5: Lipotoxic Palmitate Impairs the Rate of β-Oxidation and Citric Acid Cycle Flux in Rat Neonatal Cardiomyocytes

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Key Words: Metabolism, Lipotoxicity, Fatty acids, Cpt1b, β-oxidation, Citric acid cycle

5.1 Authors contribution

Taha Haffar performed most of the experiments, helped analyze the data, and assisted with writing the manuscript. Ali Akoumi performed some experiments. Nicolas Bousette conceived and designed the study, supervised the work, and wrote the manuscript.

5.2 Context

Fatty acid oxidation is a three-step process. First, fatty acids are transported into mitochondria to be oxidized. Second, fatty acids enter β-oxidation and generate acetyl-CoA. Third, acetyl-CoA enters the citric acid cycle to be further catabolized. CO_2 is generated during the citric acid cycle. Our previous results show a decrease in CO_2 production in palmitate-treated cardiomyocytes compared to oleate-treated cardiomyocytes. Hence, we examined each step in order to elucidate the mechanism of palmitate-mediated inhibition of fatty acid oxidation. Treating cardiomyocytes with palmitate for eight hours was enough to significantly attenuate fatty acid oxidation but not long enough to inhibit CPT1B activity. Our results suggest that palmitate-mediated inhibition of fatty acid oxidation is downstream of CPT1B. Interestingly, we found that acetyl-CoA levels, the final product of β-oxidation, are decreased in palmitate-treated cells. Simultaneously, isocitrate dehydrogenase activity and net aconitase activity (two enzymes that catalyze two steps of CAC) were impaired in palmitate-treated cells.

5.3 Abstract

Background/Aims: Diabetic hearts exhibit intracellular lipid accumulation. This suggests that the degree of fatty acid oxidation (FAO) in these hearts is insufficient to handle the elevated lipid uptake. We previously showed that palmitate impaired the rate of FAO in primary rat neonatal cardiomyocytes. Here we were interested in characterizing the site of FAO impairment induced by palmitate since it may shed light on the metabolic dysfunction that leads to lipid accumulation in diabetic hearts. ***Methods:*** We measured fatty acid oxidation, acetyl-CoA oxidation, and carnitine palmitoyl transferase (Cpt1b) activity. We measured both forward and reverse aconitase activity, as well as NAD+ dependent isocitrate dehydrogenase activity. We also measured reactive oxygen species using the 2', 7'-Dichlorofluorescin Diacetate (DCFDA) assay. Finally we used thin layer chromatography to assess diacylglycerol (DAG) levels. ***Results:*** We found that palmitate significantly impaired mitochondrial β-oxidation as well as citric acid cycle flux, but not Cpt1b activity. Palmitate negatively affected net aconitase activity and isocitrate dehydrogenase activity. The impaired enzyme activities were not due to oxidative stress but may be due to DAG mediated PKC activation. ***Conclusion:*** This work demonstrates that palmitate, a highly abundant fatty acid in human diets, causes impaired β-oxidation and citric acid cycle flux in primary neonatal cardiomyocytes. This metabolic defect occurs prior to cell death suggesting that it is a cause, rather than a consequence of palmitate mediated lipotoxicity. This impaired mitochondrial metabolism can have important implications for metabolic diseases such as diabetes and obesity.

5.4 Introduction

Cardiac disease is the primary cause for morbidity and mortality in the diabetic population [1]. Since diabetes impairs glucose uptake and oxidation there is a compensatory increase in fatty acid uptake. This in turn leads to cardiac lipid accumulation and ensuing lipotoxicity. Several studies have pointed to the role of lipotoxicity in diabetic cardiomyopathy [2-7]. Furthermore, several studies have shown a correlation between the degree of lipid accumulation and cardiac dysfunction in human diabetic hearts [8-11].

Lipids accumulate in the diabetic heart because uptake exceeds lipid clearance. Lipid clearance in the heart is primarily dependent on fatty acid oxidation (FAO). FAO begins by carnitine palmitoyl transferase (Cpt1b) mediated FA uptake into mitochondria. Once inside the mitochondrion fatty acids are oxidized by the progressive removal of 2 carbons with each cycle of β-oxidation. The resultant 2-carbon acetyl-CoA is fed into the citric acid cycle (CAC) where it is oxidized to CO_2 with the consequent production of reducing equivalents, including NADH and FADH2. The reducing equivalents are then fed into the electron transport chain (ETC), which drives oxidative phosphorylation.

Some models of diabetic cardiomyopathy including leptin or leptin receptor deficient mice have shown that β-oxidation is actually increased in the diabetic heart [12, 13]. However studies in other models show either no change [14, 15] or decreased oxidation [16, 17]. These discrepancies might be explained by differences in species, concentration of fatty acid used to

measure oxidation, and/or animal age. For instance, two studies evaluating diabetic humans as well as a study of a porcine model of diabetes all showed decreased FAO with diabetes [18-20], possibly indicating a difference between rodents and higher order mammalians. Another study showed that diabetic rat hearts exhibited significantly enhanced FAO at low concentrations of FA, but FAO was significantly depressed at high concentrations [21]. This is important because serum FA levels are generally higher in diabetics. Finally, age and associated pathological sequelae may be an important determinant of FAO in diabetics as older diabetic rats exhibited significant reductions in FAO compared to both age matched controls and younger diabetic rats [22]. Another potentially limiting factor is that many of these studies only measured β-oxidation, which is merely the first half of FAO and does not take into consideration downstream steps including acetyl-CoA oxidation in the CAC nor the components of the electron transport chain (ETC). Thus it is interesting that several studies show impaired mitochondrial function in the heart of diabetic humans and animals as evidenced by decreased fatty acid driven respiration [20, 23-27]. These latter studies implicate potential deficiencies in either events upstream of β-oxidation, such as mitochondrial uptake (i.e. Cpt1b activity) or downstream events including citric acid cycle flux or the electron transport chain. In support of the notion that diabetes causes metabolic derangements downstream of β-oxidation, Lin et al. found an impairment in net aconitase enzyme activity in the diabetic rat heart [28]. Aconitase is a key CAC enzyme responsible for the conversion of citrate to isocitrate.

It should be noted that an increase in β-oxidation without a concomitant increase in citric acid cycle flux would nonetheless compromise metabolism of the heart by impairing energy production and/or by providing a surplus of substrates for re-synthesis of fatty acids. Regardless

of whether or not fatty acid oxidation is increased in diabetic hearts, the fact remains that diabetic hearts accumulate lipid suggesting that the degree of FAO, whatever it may be, is not sufficient in the face of elevated FA uptake. This leads to the question as to whether increasing FAO would attenuate lipotoxicity in diabetic cardiomyopathy.

Interestingly, we have shown that increasing fatty acid oxidation is associated with attenuated lipotoxicity in primary cardiomyocytes [29]. Specifically we previously showed that palmitate, a lipotoxic FA, impaired fatty acid oxidation in primary rat neonatal cardiomyocytes (NCMs) and that enhancing fatty acid oxidation attenuated palmitate-mediated lipotoxicity. In contrast, we also demonstrated that impairing fatty acid oxidation induced lipotoxicity. Here we aimed to determine how palmitate impairs FAO in primary cardiomyocytes. We found that palmitate impairs both β-oxidation and citric acid cycle flux, but not the uptake of FA into mitochondria.

5.5 Materials and Methods

Rat neonatal cardiomyocytes (NCM) culture: NCMs were isolated from hearts of 1-day-old rats. Hearts were isolated and tissue was washed several times in digestion buffer containing 137 mM NaCl, 5.36 mM KCl, 0.81mM MgSO4, 5.55 mM dextrose, 0.44 mM KH2PO4, 0.34 mM Na2HPO4, 20 mM HEPES, and 50 μg/ml gentamicin. Tissue was then incubated in digestion buffer with collagenase (50units/ml). Cells in suspension resulting from the tissue digestion were transferred to ice cold FBS every 10-20 min. This step was repeated until all

tissue was digested. Cells in FBS were pelleted and then re-suspended in DMEM containing 10% FBS. Cells were plated in petri dish for 1h at 37 ˚C to allow fibroblasts to adhere. The non-adherent cardiomyocytes were then plated at a density of ~40,000 cells/cm2. NCMs were cultured in DMEM/F12 + 2% FBS + 100 μM bromodeoxyuridine for a minimum of 7 days prior to treatments to allow for maturation and the switch from primarily glucose oxidation to primarily fatty acid oxidation.

Fatty acid preparation: Oleate (Sigma, O7501) was solubilized in anhydrous methanol while palmitate (Sigma, P9767) was solubilized in 150 mM NaCl by heating to 70˚C. Both fatty acids were then complexed to bovine serum albumin (BSA) in a 6:1 ratio as previously described [29].

Fatty acid oxidation (FAO) assay: Cells were pretreated with 300 μM oleate or 300 μM palmitate for 8 h. Media was removed and cells were washed 2 times with warm PBS. Radiolabelled oleate (American Radio-chemicals, ARC 0297) or palmitate (American Radio-chemicals, ARC 0172A) were solubilized in non-bicarbonate assay buffer (114 mM of NaCl, 4.7 mM of KCl, 1.2 mM of KH2PO4, 1.2 mM of MgSO4, 0.5% fatty acid free BSA) and cells were incubated with 0.4μCi of 14C- oleic acid or 14C- palmitic acid for 2 hours. To determine CO_2 production 6N HCl was added to cell media and CO_2 was captured by a filter paper soaked in 2M NaOH. The filter paper was then added to a scintillation vial with scintillation fluid and radioactivity was read with the Beckman LS6500 scintillation counter.

To obtain acid soluble metabolites (ASM) cardiomyocytes were collected by trypsinization and re-suspended in 1M perchloric acid and then homogenized using 25G gauge needle. Samples

were centrifuged at 17,000g and the supernatant was added to scintillation fluid and counted using Beckman LS6500 scintillation counter.

To obtain water soluble metabolites (WSM), cardiomyocytes were re-suspended in 250M sucrose/ 50M tris buffer and homogenized with 25G gauge needle. Water saturated butanol was added to each sample and butanol/water phases were separated by centrifugation at 1000g for 5 min. The water phase was added to scintillation fluid and counted using Beckman LS6500 scintillation counter. The radioactivity from samples was normalized to radioactivity of loading control for each radiolabelled fatty acid (i.e. 14C-oleate and 14C- palmitate) and to protein concentration. Data are expressed as picomols/μg protein/min.

Acetyl-CoA oxidation assay: This assay was done identically to the fatty acid oxidation assay described above except cells were exposed to 14C-Acetate for 1 hour (American Radio-chemicals, ARC0101) instead of radiolabelled fatty acids. Radioactivity from samples was normalized to radioactivity of loading control and to protein concentration. Data are expressed as picomols/μg protein/min.

Aconitase activity assay: Aconitase reverse activity was measured by quantifying the synthesis rate of cis-aconitate (Abcam, ab109712). Briefly, crude mitochondria were isolated from cardiomyocytes using buffer containing 10 μM tris-Mops, 1 μM EGTA/tris and 200 μM sucrose. 50 μg of crude mitochondria were re-suspended in aconitase preservation buffer with isocitrate. Cis-aconitate level was measured by reading the absorbance at 240 nm for 30 min at 37 ˚C. Aconitase forward activity was carried out identically however citrate was used as substrate in

place of isocitrate. Data are presented as change (Δ) in absorbance from baseline to the end of the experiment.

Isocitrate dehydrogenase (IDH) activity assay: IDH activity was measured (Abcam, ab102528). Briefly, cardiomyocytes were treated with oleate or palmitate for 8 h then isolated by trypsinization. Cells were re-suspended in supplied buffer containing NAD+ and NADH formation was determined by measuring the absorbance at 450 nm for 30 minutes at 37 ˚C. Data are presented as change (Δ) in absorbance from baseline to the end of the experiment.

Carnitine palmitoyl transferase (Cpt1b) activity assay: Cpt1b activity assay was carried out as previously described [30]. Briefly, cardiomyocytes were treated with 300 uM palmitate or 300 uM oleate for 8 h followed by [1-C14] Carnitine for 30min. Radiolabeled acyl-carnitine was separated from radiolabeled carnitine using butanol-water phase separation. Acyl-carnitine levels, indicating cpt1b activity, were measured by a scintillation counter (Beckman LS6500). Specifically, organic phase radioactivity was normalized to loading control radioactivity and protein concentration and expressed as picomol/μg protein /min.

2', 7'-Dichlorofluorescin Diacetate (DCFDA) assay: The DCFDA assay was carried out as previously described with slight modifications [31]. Briefly, cardiomyocytes were treated with oleate or palmitate for 8 h and then cells were washed twice with Krebs- Ringer HEPES buffer then incubated with 10 μM DCFH-DA. Fluorescence was read (485 nm excitation and 530 nm emission) for 30 min at 37 ˚C using the Synergy2 microplate reader. Data are presented as change (Δ) in fluorescence from baseline to the end of the experiment.

Thin Layer Chromatography (TLC): Lipid samples were isolated by the Bligh and Dyer method then spotted on a silica gel plate. The silica plate was then placed in a TLC tank allowing the lipids to migrate with the mobile phase by capillary action. The TLC tank was equilibrated with 150ml of 75:75:1.5 Chloroform: DiethylEther: Acetic acid. Lipid spots were then visualized on silica plate by rhodamine labeling. Mono-, Di-, & Triglyceride Mix (Sigma, 1787-1AMP) was used as standard.

Western blotting: Western blotting was carried out as previously described, [29, 32] with the anti-IDH3a antibody (1/200 dil. Santa Cruz biotechnology, SC-514358), 4-HNE antibody (1/1000, Abcam, ab46545) Histone H3 antibody (Santa Cruz biotechnology, SC-10809), and the α-tubulin (Santa Cruz biotechnology, SC-23948).

Fatty acid uptake assay: Cardiomyocytes were pre-treated with either 300 μM oleate or palmitate for 8 hours. Following this cardiomyocytes were exposed to 0.5UCi/ml of radiolabeled oleate or palmitate (in a buffer containing 114 mM of NaCl, 4.7 mM of KCl, 1.2 mM of KH2PO4, 1.2 mM of MgSO4) for 15 min. Cells were then washed twice in ice-cold PBS and then isolated by trypsinization. Uptake of radiolabelled fatty acids was measured by scintillation counter (Beckman LS6500) and normalized to protein abundance.

Statistical analysis: All data are presented as mean ± standard error. Statistics were carried out with StatGrapher software. Students T-test was used for two-group comparisons. Multiple groups were compared using one-way ANOVA with Tukey post-hoc test. Data involving

multiple groups and multiple conditions (e.g. TLC analysis) were analysed by two-way ANOVA with Tukey post-hoc test.

5.6 Results

5.6.1 Palmitate impairs complete FAO but not Cpt1b activity

Here we demonstrated that an 8-hour treatment of 300 μM palmitate impaired complete FAO in primary neonatal cardiomyocytes (NCMs) compared to cells treated with an equivalent time and dose of oleate (Fig. 5.1A). Specifically, NCMs treated with palmitate had a marked decrease in CO_2 production compared to oleate treated cells. A similar degree of impairment was observed in cells treated with palmitate + carnitine compared to cells treated with oleate + carnitine (Fig. 5.1B). To verify that the difference was not due to altered uptake rates between cells pre-treated with oleate compared to those pre-treated with palmitate, we evaluated uptake rates of radiolabelled oleate or palmitate following 8 hour pre-treatment of either 300 μM oleate or 300 μM palmitate, respectively. We found no difference in uptake rates between the two different pre-treatments (Fig. 5.1D).

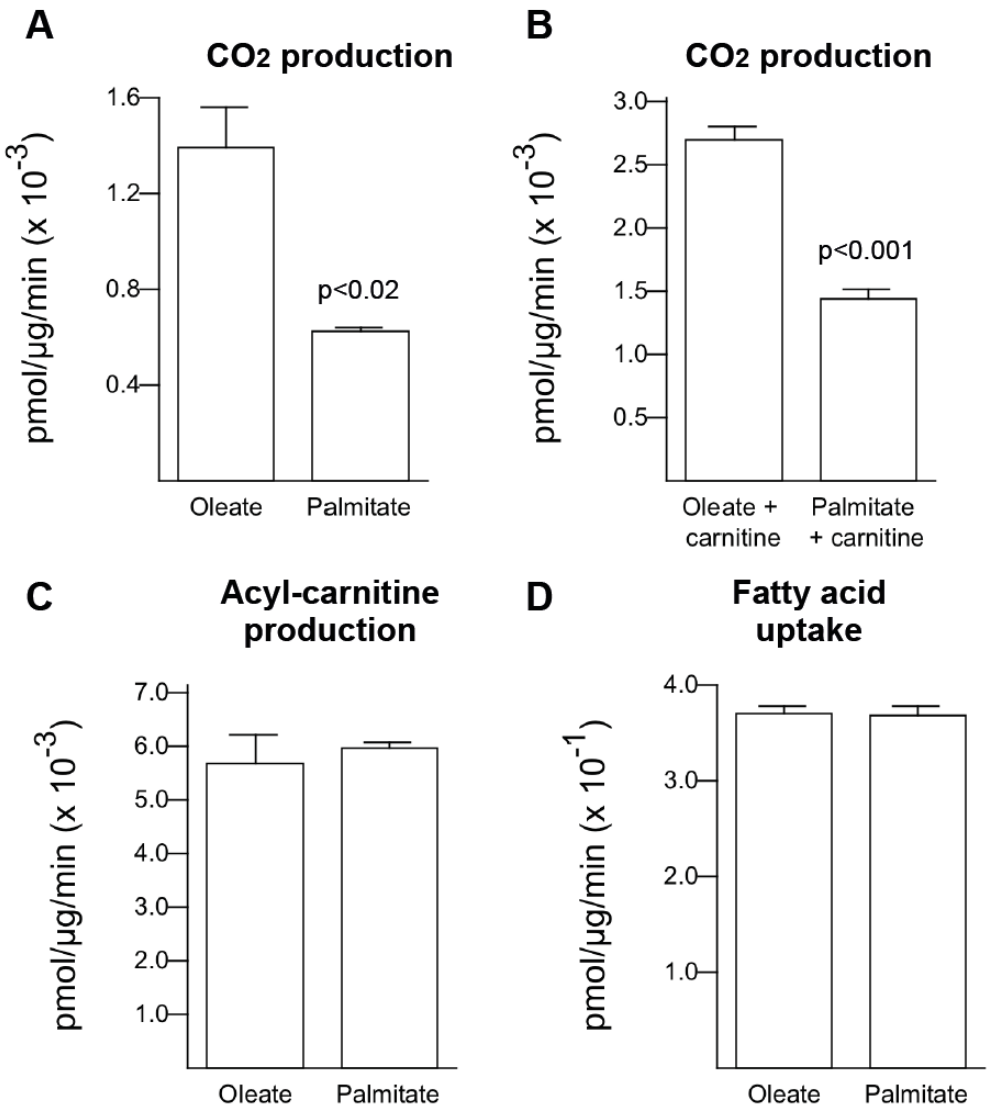

Fig. 5.1 Palmitate impairs complete fatty acid oxidation in primary neonatal cardiomyocytes (NCMs). (A) Graph showing significantly decreased CO_2 production in NCMs treated with 300 µM palmitate for 8 hours compared to those treated with 300 µM oleate. (B) Graph showing significantly decreased CO_2 production in NCMs treated with 300 µM palmitate + 250 µM carnitine for 8 hours compared to those treated with 300 µM oleate + 250 µM carnitine. (C) Graph showing no difference in Cpt1b activity (measured as acyl-carnitine production) between NCMs treated with 300 µM palmitate compared to NCMs treated with 300 µM oleate for 8 hours. (D) Graph showing no difference in uptake of radiolabelled oleate or palmitate in NCMs pre-treated with either 300 µM oleate or 300 µM palmitate for 8 hours, respectively.

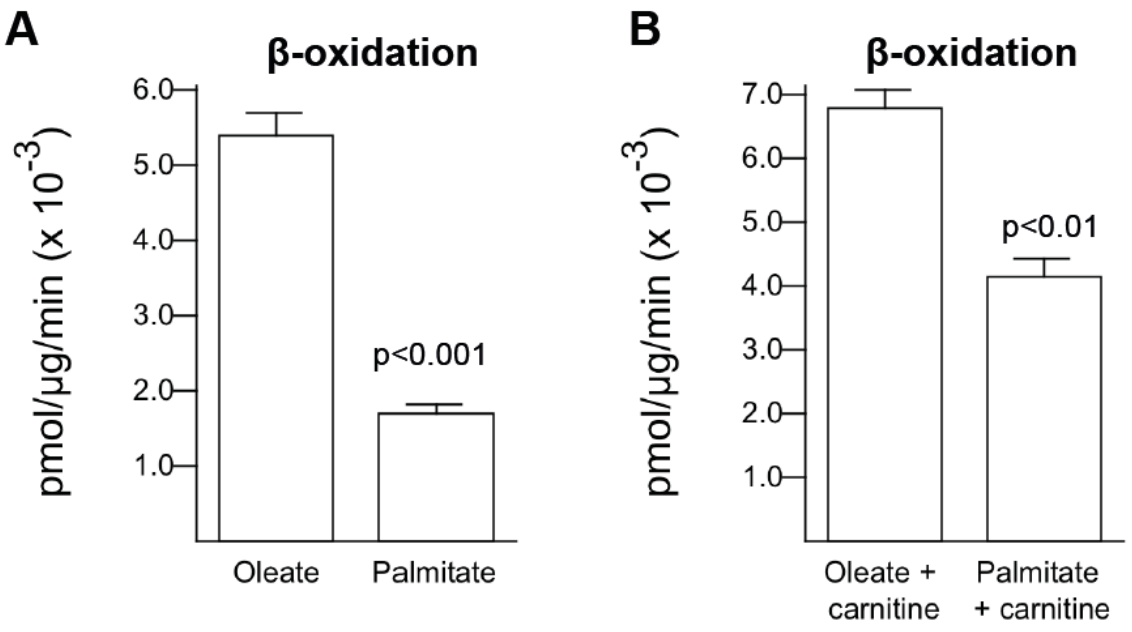

Fig. 5.2 Palmitate impairs ß-oxidation in primary neonatal cardiomyocytes (NCMs). (A) Graph showing significantly decreased water soluble metabolites (WSMs) from NCMs treated with 300 µM palmitate for 8 hours compared to those treated with 300 µM oleate. (B) Graph showing significantly decreased WSMs from NCMs treated with 300 µM palmitate + 250 µM carnitine for 8 hours compared to those treated with 300 µM oleate+ 250 µM carnitine.

We previously showed that Cpt1b protein content was not altered under these conditions [33]. Therefore, here we assessed if palmitate decreased Cpt1b activity rather than expression. We evaluated Cpt1b activity by measuring the production of acyl-carnitines in NCMs pre-treated with either oleate or palmitate. We found that Cpt1b activity was not altered by palmitate (Fig. 5.1C). This is supported by the observation that co-administration of carnitine increases FAO to the same degree in either oleate or palmitate treated cells (i.e. both ~2 fold over basal levels of each respective fatty acid without carnitine, data not shown). This suggests that palmitate mediated impairment of FAO occurs downstream of Cpt1b.

Similar results were found in H9C2 cardiomyoblasts. Indeed, palmitate significantly reduced CO_2 production but did not have any effect on Cpt1b activity (data not shown).

5.6.2 Palmitate impairs β-oxidation

To test the effect of palmitate on β-oxidation we quantified acid soluble metabolites (ASM), an established technique for assessing the degree of β-oxidation by quantifying the production of fatty acid metabolites with 6 or less carbons. Because the radiolabelled fatty acid contains the label on the first carbon, measured ASM metabolites should represent acetyl-CoA produced by β-oxidation of fatty acids and/or intermediates of the citric acid cycle derived from said acetyl-CoA. We found that pre-treatment with palmitate led to a small non-significant decrease in ASM production compared to cells pre-treated with oleate (data not shown) suggesting that β-oxidation may be impaired by palmitate. However, we found that etomoxir only partially prevented radioactivity in ASM samples. Indeed, ASM samples from cells treated with etomoxir only had a ~2 fold reduction in radioactivity (data not shown). In contrast, etomoxir nearly abolished the production of radioactive CO_2 (~10 fold decrease, data not shown). Because etomoxir is an inhibitor of Cpt1b, it prevents fatty acids from entering mitochondria and thus prevents mitochondrial β-oxidation. Therefore, the presence of high radioactivity in the ASM samples from cells treated with etomoxir suggests that oleate and palmitate are being oxidized to a significant degree in peroxisomes or alternatively, that full–length fatty acids are partially

soluble in 1 M perchloric acid. This data implies that in our hands, ASM analysis is not entirely specific for mitochondrial β-oxidation.

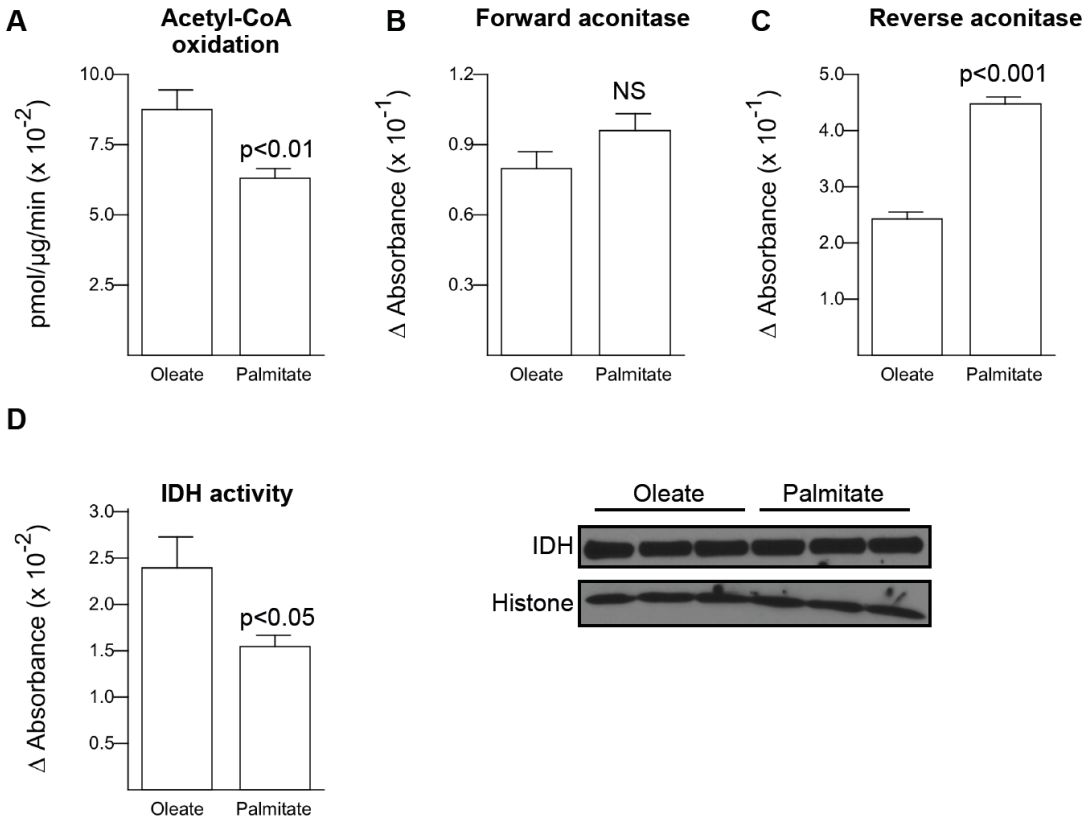

Fig. 5.3 Palmitate impairs citric acid cycle (CAC) flux in primary neonatal cardiomyocytes (NCMs). (A) Graph showing significantly decreased CO_2 production from radiolabelled acetyl-CoA in NCMs treated with 300 µM palmitate for 8 hours compared to those treated with 300 µM oleate. (B) Graph showing no significant difference in forward aconitase activity in NCMs treated with 300 µM palmitate for 8 hours compared to those treated with 300 µM oleate. (C) Graph showing significantly increased reverse aconitase activity in NCMs treated with 300 µM palmitate for 8 hours compared to those treated with 300 µM oleate. (D) Graph showing significantly decreased isocitrate dehydrogenase (IDH) activity in NCMs treated with 300 µM palmitate for 8 hours compared to those treated with 300 µM oleate. (E) Western blot showing no difference in IDH3a protein levels in NCMs treated with 300 µM palmitate for 8 hours compared to those treated with 300 µM oleate. Histone was used as loading control.

Therefore we established a novel technique in which we measured water-soluble metabolites by separating full-length fatty acids in an organic phase while measuring water-soluble metabolites (WSMs) in the aqueous phase. WSMs include acetyl-CoA produced by β-oxidation of fatty acids as well as any of the downstream citric acid cycle intermediates derived from the latter acetyl-CoA. We were able to nearly abolish radioactivity in WSM samples from cells treated with etomoxir suggesting that this technique is much more specific for measuring mitochondrial β-oxidation (data not shown). Interestingly, we found that treating NCMs with 300 μM palmitate for 8 hours led to a significant decrease in WSMs compared to NCMs treated with 300 μM oleate for the same time (Fig. 5.2A), suggesting that palmitate impairs β-oxidation in NCMs. Co-administration of carnitine did not rescue the palmitate mediated decrease in WSM production (Fig. 5.2B). Similar results were found in H9C2 cardiomyoblasts. Indeed, palmitate significantly reduced WSM production in H9C2s (data not shown).

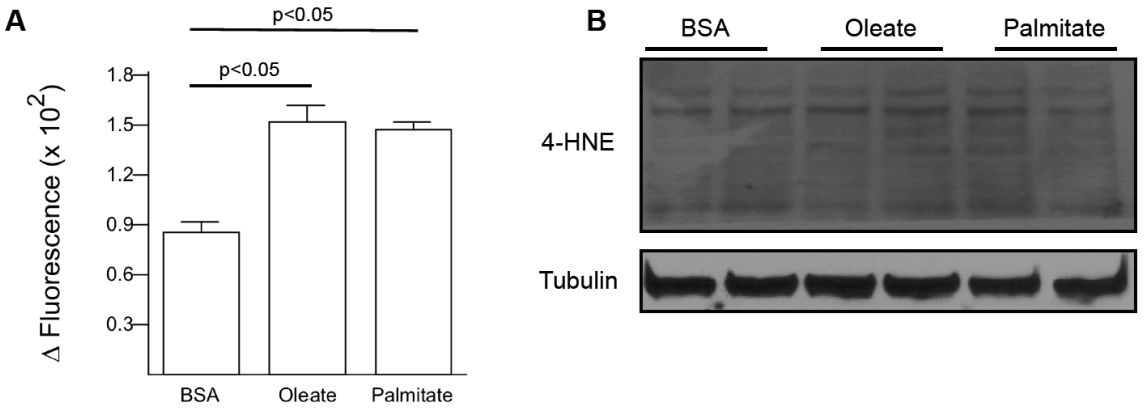

Fig. 5.4 300 μM oleate or palmitate for 8 hours induces increased ROS generation but not overt oxidative stress in primary neonatal cardiomyocytes (NCMs). (A) Graph showing significantly increased DCFDA fluorescence in NCMs treated with 300 μM oleate or 300 μM palmitate for 8 hours compared to those treated with

BSA. (B) Western blot showing no difference in 4-HNE immunoreactivity in NCMs treated with BSA, 300 μM oleate, or 300 μM palmitate for 8 hours. Tubulin was used as loading control.

5.6.3 Palmitate impairs citric acid cycle flux

To assess if palmitate had any effect on the citric acid cycle (CAC) we measured the rate of acetyl-CoA oxidation. Acetyl-CoA is oxidized by the citric acid cycle which is downstream of, and thus independent of β-oxidation. Therefore acetyl-CoA oxidation is a measure of citric acid cycle flux. Interestingly, we found a significant decrease in acetyl-CoA oxidation in 300μM palmitate treated NCMs compared to NCMs treated with 300 μM oleate (Fig. 5.3A) suggesting that palmitate also impairs citric acid cycle flux.

Aconitase is a key proximal CAC enzyme that has been previously shown to be differentially regulated in diabetic hearts [28], therefore we were interested in evaluating if palmitate had any effect on this enzyme. Aconitase catalyzes the conversion of citrate to isocitrate through the intermediate formation of aconitate. We measured both forward activity (citrate to aconitate) and reverse activity (isocitrate to aconitate) in NCMs treated with either oleate or palmitate for 8 hours. We found that there was no significant difference in forward activity between oleate and palmitate treated NCMs (Fig. 5.3B). In contrast, palmitate led to a significantly enhanced reverse activity (Fig. 5.3C). This increased reverse activity consequently causes a net decrease in isocitrate levels thus contributing to decreased CAC flux.

Next we evaluated IDH activity because it is widely considered the rate-limiting step of the CAC [34]. We found that there was a significant decrease in IDH activity in palmitate treated NCMs compared to oleate treated NCMs (Fig. 5.3D). To determine if the attenuated IDH activity was due to decreased protein levels, we performed a western blot to assess levels of IDH3, the mitochondrial isoform. Not surprisingly considering the short exposure time of palmitate (i.e. 8 hours), there was no decrease in IDH protein levels in palmitate treated NCMs compared to oleate treated NCMs (Fig. 5.3E). Therefore this indicates that palmitate negatively regulates IDH activity but not expression.

5.6.4 Palmitate does not impair FAO through oxidative stress

Several reports indicate that oxidative stress has deleterious effects on mitochondrial enzymes [35-38], therefore we assessed if this might be the causal mechanism for palmitate induced FAO impairment. To test this we measured ROS generation using the redox sensitive dye DCFDA [31]. Interestingly we found that pre-treatment with either oleate or palmitate caused significant increases in ROS generation compared to control cells treated with BSA alone (Fig. 5.4A). To evaluate if this increased degree of ROS generation translated into oxidative stress we evaluated the degree of 4-Hydroxynonenal (4-HNE) protein adduct formation in cells treated with either BSA, oleate or palmitate. Interestingly there was no increase in 4-HNE protein adducts in either oleate or palmitate treated cells (Fig. 5.4B), indicating that the observed increase in ROS

generation was not sufficient to overcome the cells' endogenous antioxidant system and suggests that this increase in ROS is not the cause of palmitate mediated FAO impairment.

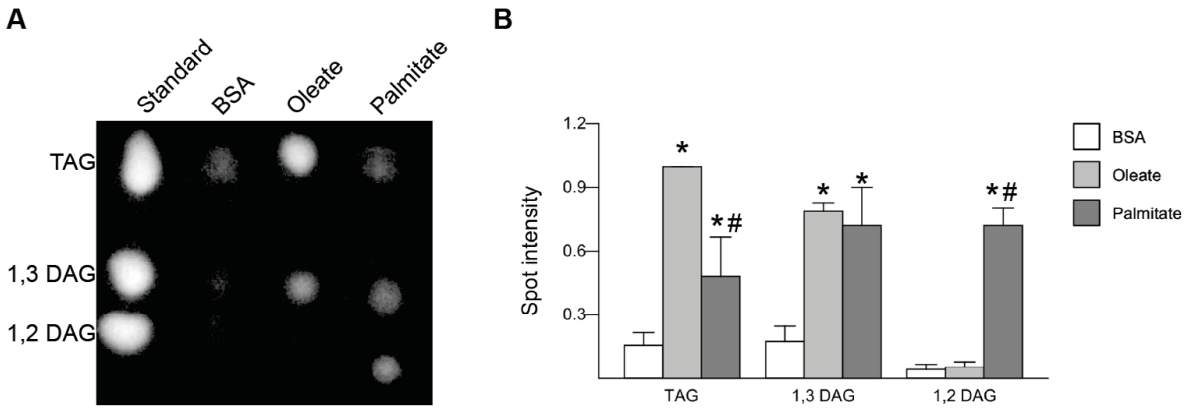

Fig. 5.5 Palmitate induces increased 1,2 diacylglycerol (DAG) in primary neonatal cardiomyocytes (NCMs). (A) Image of representative silica plate spotted with lipids isolated from cells treated with either BSA, 300 μM oleate or 300 μM palmitate for 24 hours. (B) Graph showing significantly increased 1,2 DAG in NCMs treated with 300 μM palmitate compared to those treated with either BSA or 300 μM oleate. Graph also shows significantly increased TAG in NCMs treated with 300 μM oleate compared to NCMs treated with either BSA or 300 μM palmitate. * indicates $p < 0.05$ vs. BSA treated NCMs. # indicates $p < 0.05$ vs. NCMs treated with 300 μM oleate.

5.6.5 Palmitate causes the accumulation of sn1,2 Diacylglycerol (1,2 DAG)

Here we showed that aconitase is differentially regulated in the same manner as observed in diabetic hearts by Lin et al. [28]. That is, they found that aconitase exhibited significantly enhanced reverse activity without a difference in forward activity just as we have reported here for palmitate treated NCMs. In that study they attributed this differential regulation to PKC-β

mediated phosphorylation of aconitase. Because PKC is a conventional isoform, which is activated by DAG, we were interested in assessing if palmitate induced an increase in intracellular DAG compared to BSA (control) or oleate treated NCMs. Interestingly, we found that palmitate exhibited significantly more 1,2 DAG than BSA and oleate treated NCMs (Fig. 5.5A-B). On the other hand both oleate and palmitate induced an increase in 1, 3 DAG compared to BSA control cells.

Together this data shows that palmitate significantly impairs both β-oxidation and the citric acid cycle, but does not affect Cpt1b activity. This has important implications for diabetic cardiomyopathy because impairment in FAO can lead to lipid accumulation and lipotoxicity.

5.7 Discussion

We previously demonstrated that palmitate lipotoxicity in cardiomyocytes was associated with impaired FAO and that enhancing FAO decreased lipotoxicity [33]. In addition, we also previously showed that inhibiting FAO by blocking Cpt1b either pharmacologically or genetically caused oleate, which is normally non-toxic, to induce cell death. This latter work suggests that impaired FAO can contribute to lipotoxicity likely by promoting the accumulation of toxic lipid intermediates. This led us to investigate which steps of FAO palmitate impairs. Here we showed that palmitate significantly impaired β-oxidation and citric acid cycle flux, with no effects on Cpt1 activity. Cpt1b catalyzes the rate-limiting step in FAO. As such it is a key

site for regulation of FAO. However, we found that Cpt1b activity was not different between oleate and palmitate treated NCMs suggesting that this is not the site of palmitate induced impairment. In support of this, we found that addition of carnitine increased oleate and palmitate oxidation to equivalent degrees (i.e. both were induced ~2 fold compared to basal levels of each respective fatty acid without carnitine).

Since Cpt1b activity was not affected by palmitate, we next evaluated the degree of β-oxidation in NCMs treated with either oleate or palmitate. To this end we measured the abundance of acid soluble metabolites in cells treated with oleate and palmitate (as described in methods). We found a small reduction in ASM levels from NCMs treated with palmitate compared to those treated with oleate. However, as described in the results above, we found that ASM analysis may not be entirely specific for mitochondrial β-oxidation. To overcome this limitation we adapted the protocol used for Cpt1b activity assay in which free carnitine is separated from acylated carnitine through the use of organic/aqueous phase separation. By separating cell components into an aqueous phase and an organic phase we can separate full length fatty acids in the organic phase from acetyl-CoA and other water soluble metabolites (such as intermediates of the citric acid cycle) in the aqueous phase. Indeed this assay appears to be more specific for mitochondrial β-oxidation since we nearly abolish the production of WSMs in NCMs treated with oleate + etomoxir. Through the WSM analysis we were able to confirm a significant decrease in β-oxidation in NCMs treated with palmitate compared to those treated with oleate. This has important implications for lipotoxicity of the heart. Indeed we have previously demonstrated that impairing FAO causes toxicity in NCMs treated with non-toxic oleate.

Therefore inhibition of β-oxidation may be an important cause for palmitate-mediated lipotoxicity.

The mechanism of palmitate-mediated inhibition of β-oxidation is currently unknown but may be related to palmitate mediated post-translational modifications (PTMs). Indeed fatty acids have been shown to induce PTMs in mitochondrial proteins [39]. These PTMs include phosphorylations, acetylations and palmitoylations [28, 40, 41]. Currently there is a strong debate as to whether post-translational acetylation increases or decreases β-oxidation enzyme activity. Indeed studies have shown that acetylation decreased LCAD activity and that Sirt3, a deacetylase could restore function [42, 43]. On the other hand β-hydroxyacyl CoA dehydrogenase (HADH) has been shown to be activated with acetylation [44]. Which enzymes are post-translationally modified and what types of PTMs are regulating these enzymes will be the focus of future studies.

Next we assessed citric acid cycle flux in NCMs by measuring oxidation of acetyl- CoA. Interestingly, we found that palmitate significantly impaired oxidation of acetyl- CoA compared to oleate. Because acetyl-CoA is the product of β-oxidation, its oxidation is dependent only on citric acid cycle activity and not mitochondrial β-oxidation activity. Therefore this indicates that palmitate also inhibits the citric acid cycle. The citric acid cycle is a central hub for cellular metabolism. Indeed it is responsible for oxidation of acetyl- CoA not only from FAs but also from glucose derived pyruvate. The reducing equivalents produced through the latter oxidation of acetyl-CoA are necessary to maintain mitochondrial membrane potential and therefore drive oxidative phosphorylation. Also it is an important source of substrates for a variety of

biosynthetic pathways. Therefore any impairment in this cycle can have profound effects on cellular metabolism. Interestingly, we found that reverse aconitase activity was significantly enhanced while forward activity was unchanged in NCMs treated with palmitate compared to those treated with oleate. The increase in reverse activity (i.e. production of aconitate from isocitrate) reduces the isocitrate levels necessary for the downstream isocitrate dehydrogenase (IDH). This reduction in substrate for IDH thus can reduce CAC flux leading to decreased oxidation of acetyl-CoA and decreased reducing equivalents. This not only impairs energy production by decreasing NADH levels necessary for ETC activity and oxidative phosphorylation but also potentially results in increased levels of substrates for de novo fatty acid synthesis.

The finding of palmitate induced increase in reverse aconitase activity with unchanged forward activity is actually very relevant to in vivo diabetic cardiomyopathy. Indeed Lin et al. [28] actually found the same phenomenon in hearts of diabetic rats. Indeed they found that diabetic rats exhibited significantly increased reverse aconitase activity with normal forward activity and that this was due to PKC-β mediated phosphorylation of aconitase. PKC-β is a conventional PKC isoform which is activated by diacylglycerol (DAG). Therefore we evaluated if palmitate induced an increase in DAG compared to oleate. Interestingly, we found that palmitate resulted in significantly less TAG but significantly more DAG than oleate in NCMs. Moreover we found that the real difference was in the levels of the sn1,2 DAG isomer and not the sn1,3 DAG isomer. This is of utmost importance because previous studies have shown that it is the 1,2 and not the 1,3 DAG isomer that is responsible for PKC activation [45]. Together this data shows that CAC

flux is impaired by palmitate at least in part by a net decrease in aconitase mediated isocitrate production and that this may be occurring through PKC mediated phosphorylation of aconitase.

Next we assessed if isocitrate dehydrogenase activity was affected by palmitate. Interestingly we found that palmitate significantly attenuated IDH activity as well. Importantly, the decrease in IDH activity in palmitate treated cells was independent of the altered aconitase activity because the IDH activity assay measured the formation of α-ketoglutarate from supplied, not endogenous, isocitrate. To verify that this was not due to targeted protein degradation, we assessed IDH protein levels. Not surprisingly, considering the short 8-hour incubation time, we found no difference in protein levels. Reduced IDH activity has a major impact on CAC flux as it catalyzes an irreversible reaction producing both CO_2 and NADH. The mechanism of IDH inhibition by palmitate is currently unknown.

Oxidative stress is a major contributing factor to diabetic cardiomyopathy and several studies have shown that oxidative stress is deleterious to mitochondrial enzyme function [35-38]. Therefore we assessed ROS generation using the DCFDA assay and oxidative stress by determination of 4-HNE immunoreactivity. 4-HNE immunoreactivity is a marker for oxidative stress because lipid peroxidation by ROS leads to increased levels of 4-HNE adducts on cellular proteins [46]. Interestingly we found that palmitate significantly increased ROS generation. However, this is unlikely the cause of IDH inhibition for 2 reasons. Firstly, the degree of ROS generation was equivalent to oleate induced ROS generation. Since IDH activity was decreased in palmitate compared to oleate treated NCMs yet both oleate and palmitate induced ROS generation to similar degrees, it is unlikely that this as a cause for inhibition. Secondly we found

no evidence of oxidative stress in oleate or palmitate treated cells (as determined by 4-HNE immunoreactivity) compared to control (BSA treated) cells indicating that the elevated ROS observed did not overwhelm the cells endogenous anti-oxidant potential. This is supported by a previous study which showed that palmitate mediated cell death was not due to ROS [47]. Together this data demonstrates that palmitate attenuates both β-oxidation and citric acid cycle flux likely in part through DAG mediated PKC activation. The fact that palmitate is one of the most abundant fatty acids in human diets suggests that this may be a contributing factor to the lipotoxicity observed in diabetic cardiomyopathy.

5.8 Acknowledgements

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Disclosure Statement: No conflict of interest.

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Chapter 6: Overexpression of Cpt1b reduces both ER stress and oxidative stress in STZ/HFD mice and in palmitate treated T293

6.1 Context

Our previous studies accentuate the detrimental effect inhibiting fatty acid oxidation has on cardiac cell function. Since diabetic cardiomyopathy is associated with lipotoxicity and cardiac steatosis, we wanted to investigate the relationship between FAO and diabetic cardiomyopathy using a diabetic mice model. These mice were injected with streptozotocin to induce diabetes and fed a high fat diet. Subsequently, mitochondria were isolated from the heart tissue and the rate of fatty acid oxidation was measured. Our results show that the chronic feeding of a high fat diet impaired the fatty acid oxidation of diabetic mice, leading to cardiac dysfunction.

6.2 Introduction

Diabetes is a major risk factor for multiple disorders including microvascular disease (neuropathy, nephropathy and retinopathy) and macrovascular disease (coronary artery disease, stroke). Additionally, diabetic patients with cardiovascular disease have poorer prognosis

compared to non-diabetic patients with cardiovascular disease (1). Diabetic patients are at high risk to develop diabetic cardiomyopathy (2, 3). Diabetic cardiomyopathy is defined by cardiac dysfunction without coronary artery disease, hypertension. Several metabolic pathways and mechanisms have been hypothesized as the cause for DCM however an increasingly popular theory is pointing toward cardiac lipid accumulation and lipotoxicity (4, 5). Diabetic patients exhibit hyperlipidemia (6) which predispose to increase lipid uptake and cardiac lipid overload known as cardiac steatosis (7). Unresolved cardiac steatosis eventually leads to lipotoxicity (8). lipotoxicity is defined by cellular dysfunction caused by lipid overload. Therefore, reducing fatty acid accumulation in the heart can be a therapeutic target.

We have previously shown that attenuating fatty acid accumulation by inducing Fatty Acid Oxidation (FAO) in rat neonatal cardiomyocytes is protective against palmitate mediated lipotoxicity (12). FAO occurs in peroxisomes and mitochondria. Small, medium and long chain fatty acids are oxidized in mitochondria, while the peroxisomal system is much more active on very long chain fatty acids. Oxygen is the final electron acceptor in both mitochondria and peroxisome however one difference between the peroxisomal and mitochondrial oxidation pathways is that the former produces H2O2 while the latter produces H2O. H2O2 is a strong oxidant therefore the ratio of mitochondrial to peroxisomal FAO can have an important impact on the degree of oxidative stress in the cell.

To be oxidized in mitochondria, fatty acids need to be attached to carnitine by Carnitine palmitoyl transferase 1 (Cpt1). Three isoforms of Cpt1 are currently known: cpt1a, cpt1b and cpt1c. Cpt1b is mainly found in muscle and in the heart. Cpt1b catalyzes the rate limiting step

for FAO. We have previously shown that inducing Cpt1b activity with carnitine (a cpt1b co-factor) elevates FAO and protects rat neonatal cardiomyocytes from palmitate lipotoxicity (12). Additionally, we previously found that inhibiting Cpt1b activity with etomoxir inhibits FAO and induces steatosis and cell death (12). The protective role of FAO was further validated in diabetic rat models (13, 14). Palmitate complete oxidation in muscle tissue was reduced by 70% in streptozotocin (STZ) injected rats compared to control (13). Interestingly, AMP-activated protein kinase (AMPK) induced muscle FAO in STZ rats (13) and prevented Zucker Diabetic Fatty (ZDF) diabetic rats from developing diabetes (14).

Here we aim to investigate the role of cardiac FAO in the setting of diabetic cardiomyopathy using an in vivo model of DCM (STZ/HFD mice). Since Cpt1b catalyzes the rate limiting step for FAO in cardiomyocytes, we overexpressed it in C57BL/6 mice using AAV9. We found that overexpressing Cpt1b in STZ/HFD mice is associated with attenuation of ER stress and oxidative stress. We also observed similar results in T293 cell line where inducing FAO by overexpressing Cpt1b protects T293 from palmitate lipotoxicity.

6.3 Methods

Diabetes was induced in C57BL/6j Mice by injecting streptozotocin (S0130, sigma) (intraperitoneal injections) for 5 consecutive days (40g/kg). on the 6th day, diabetes was confirmed by measuring blood glucose after 4h of fasting. Mice were considered diabetic if

fasting glucose was higher than 13mM. diabetic mice were then fed a regular chow diet (control mice) or fed with high fat / high carbohydrate diet (D12079B, research diets) for 4 weeks (acute mice) or 16 weeks (chronic mice). 4 weeks before sacrifice, mice were injected with either AAV9 GFP or AAV9 Cpt1b.

AAV9 GFP and AAV9 Cpt1b were prepared using pds GFP (company) and AAV Cpt1b vectors (AAV0007511, abm) respectively. Briefly, T293 cell line (derived from the Human Embryonic Kidney 293 cell line) was transfected with GFP or AAV cpt1b plasmid for 48h and virus were isolated from the cells by freezing and thawing (3 cycles). AAV9 virus released from cells were overlaid on a gradient of iodixanol (25%, 40% and 60%) then separated from contaminant by ultracentrifugation at 103000g for 3h at 4 °C. AAV9 virus collected from the 40% iodixanol was further concentrated using concentrator tubes (UFC905024, Millipore). AAV9 concentration was determined by coomassie staining and by PCR and each mouse was injected 50billion virus particle through intravenous tail injection.

Serum glucose levels: isolated blood was left on the counter without shaking for 10 minutes before centrifuging for 10 minutes at 200g at 4 °C. Serum which is the upper serum was extracted then glucose levels were measured using OneTouch Ultra 2 glucose measuring system.

Serum TAG levels: serum was isolated from mice as described previously then TAG levels were measured using an adipogenesis kit (K610-100, biovision). Briefly, TAG are hydrolyzed to glycerol were glycerol is detected by fluorescence (excitation 535nm / emission 587nm).

FAO: hearts were isolated from diabetic and control mice then homogenized in STE buffer (0.25M sucrose, 10mM Tris-HCl and 1mM EDTA) on ice using a douncer. Homogenates were transferred to 15ml tubes containing tissue oxidation buffer (100mM sucrose, 10mM Tris-HCl, 5mM KH2PO4, 0.2mM EDTA, 80mM KCl, 1mM MgCl2, 0.1mM Malate, 0.05mM CoA, 1mM DTT, 2mM Carnitine, 2mM ATP, 297uM oleate and 3uM radiolabeled oleate C14). FAO was set off by transferring the reaction mixture into 37 °C water bath. After 2h, FAO was terminated by transferring the tubes to ice. A whatman #1 filter paper soaked with NaOH 2N was suspend in the tube using center wells and stopper top. 1.5ml of 6N HCl was injected into the reaction and CO_2 released was trapped by the filter papers. Filters were transferred to 20ml vials then dissolved in Aquasol and CPM was counted using Beckman LS6500 scintillation counter.

DCFDA: cells were transfected using PEI with pscram or AAV cpt1b for 24 hours. Following transfection, cells were treated with BSA or 300uM palmitate for 1h or 16h. cells were washed with Krebs- Ringer HEPES (KHR) buffer then incubated with 5uM of DCFHDA. Fluorescence was read for 30min at 485nm excitation and 530nm emission using synergy plate reader.

JC1: cells transfected with pscram or AAV cpt1b and 24h post transfection cells were treated with BSA or 300uM palmitate. Cells were washed with KHR buffer then incubated with 7.5uM JC-1 (sigma, cat# 1130-5) for 10min. After incubation, cells were washed twice with warm KHR before reading the fluorescence (530 nm excitation /590 nm emission for red dye and 485 nm excitation /528 nm emission for green dye) using the Synergy2 fluorescence plate reader from Bio-Tek.

Western blot: proteins were harvested from T293 cells using RIPA buffer (50mM tris-HCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 150mM NaCl, 2mM EDTA and proteinase inhibitor cocktail (Roche)) or from heart homogenates using lysis buffer (250mM Sucrose, 50mM Tris, 1μM PMSF (protease inhibitor), 1μM DTT, and Proteinase inhibitor cocktail). Protein concentrations were determined using Bradford assay and they were probed with the following antibodies: ATF6 (SC-22799, Santa Cruz, 1/400), Chop (SC-7351, Santa Cruz 1/400), Tubulin (SC-23948, Santa Cruz, 1/400) Nitrotyrosine (SC-32757, Santa Cruz, 1/400), GAPDH (SC-30317, Santa Cruz, 1/400) and Cpt1b (GWB-MQ462C, Geneway, 1/2000).

Viability assays: Cells were treated with oleate or palmitate then incubated with 2.5uM propidium iodide for 30 minutes. Fluorescence was measured (535 nm excitation / 617 nm emission) using the Synergy2 fluorescence plate reader from Bio-Tek.

PCR: RNA was isolated from heart homogenates using triazol. cDNA was synthesized using 5X All-In-One RT MasterMix (G390, abcam). qPCR was performed using SYBR Green Master mix from Qiagen and the Eco Ilumina real-time qPCR system. Data was analyzed using the 2-ΔΔCt method. Results represent the expression of the gene of interest relative to endogenous control (Rpl34) normalized to the control group, and are presented as mean ± standard error (SE). Primers were designed to span exon-exon regions to avoid amplification of contaminating DNA and primer specificity was verified by blasting all sequences using the NCBI Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Statistical analysis: All data are presented as mean ± standard error. Statistics were carried out with StatGrapher software. Students T-test was used for two-group comparisons. Multiple groups were compared using one-way ANOVA with Tukey post-hoc test. Data involving multiple groups and multiple conditions were analysed by two-way ANOVA with Tukey post-hoc test. P-values of <0.05 were considered statistically significant.

6.4 Results

6.4.1 STZ mice exhibit hyperglycemia, hyperlipidemia, ER stress and oxidative stress.

The pathogenesis of diabetic cardiomyopathy is complex and multifactorial therefore each mouse model used to study DCM have its advantages and disadvantages. Here we wanted to confirm the efficacy of STZ injection followed with HFD feeding to induce DCM in mice. Since diabetic cardiomyopathy is associated with hyperglycemia, hyperlipidemia, ER stress and oxidative stress we evaluated these traits in our STZ/HFD model. here We demonstrate elevated levels of serum glucose in STZ/HFD mice compared to control mice fed with normal chow diet (fig 6.1A). Additionally, TAG serum concentration was also elevated in STZ/HFD mice compared to control mice (fig 6.1B). To assess if this increase in serum lipid levels is associated with intramyocellular lipid accumulation, we evaluated lipid droplet abundance and found elevated levels in cardiomyocytes of STZ/HFD mice compared to control mice (fig 6.1C & fig

6.1D). Since steatosis is associated with ER stress we measured 2 key cardiac ER stress mediators, ATF6 and Chop, by western blot (fig 6.1E). Atf6 cleavage (an indicator of Atf6 activity) and Chop protein expression were induced in cardiomyocytes of STZ/HFD mice. Cleaved Atf6 is significantly elevated in STZ/HFD mice compared to control (fig 6.1 F). Additionally Chop levels are increased significantly in STZ/HFD compared to control mice (fig 6.1G). Oxidative stress is a common pathological marker in diabetic cardiomyopathy, therefore we measured nitrotyrosine protein expression in STZ/HFD and control mice by western blot (fig 6.1H). We found higher nitrotyrosine protein expression in STZ/HFD model compared to control model (fig 6.1I).

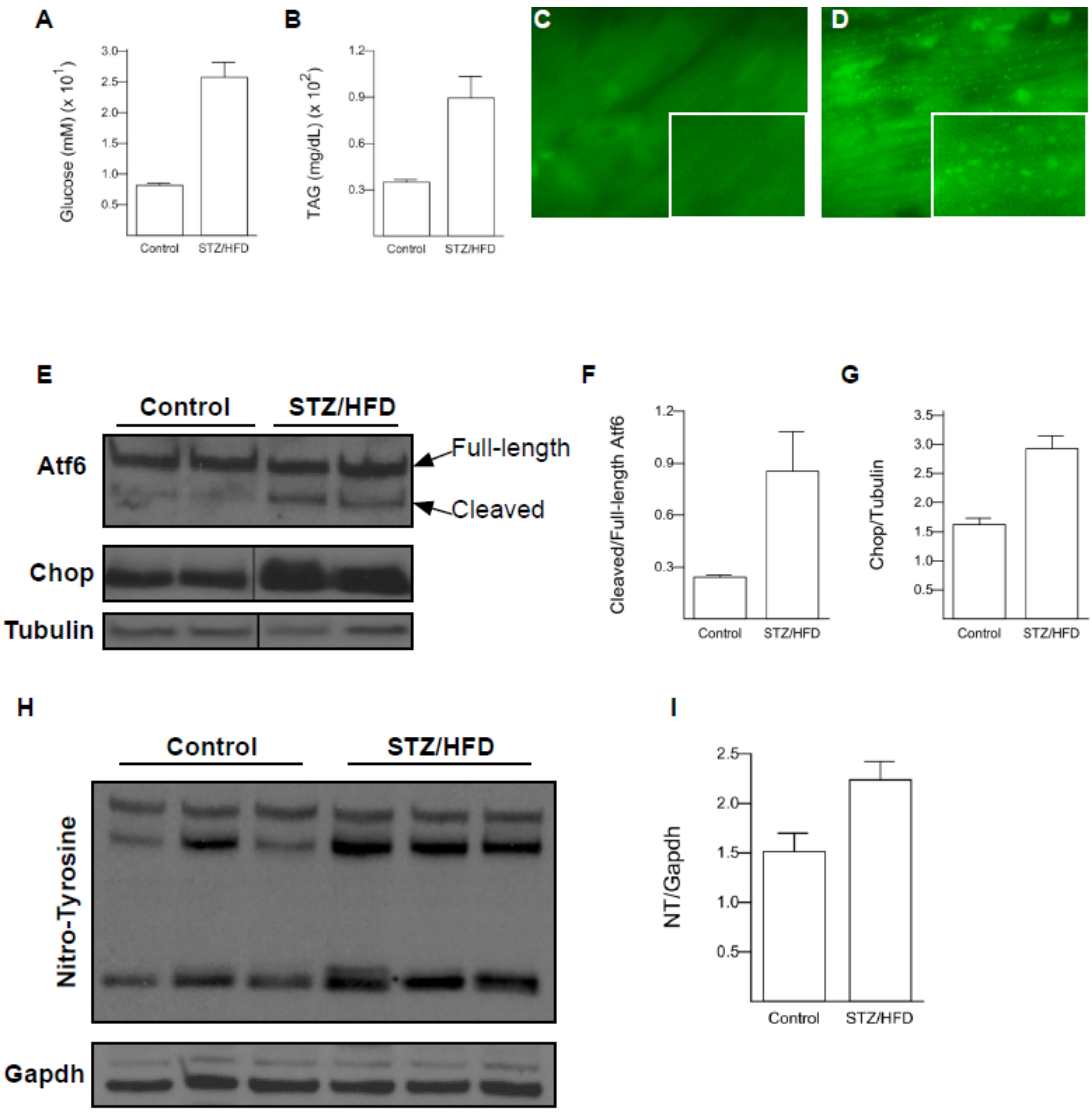


Figure 6.1: High-fat diet feeding (HFD) leads to hyperglycemia, hyperlipidemia, ER stress and oxidative stress in STZ mice. (A) Graph demonstrating significantly elevated fasting serum glucose in STZ/HFD mice compared to saline injected controls (B) Graph demonstrating significantly elevated serum TAG levels in STZ/HFD mice compared to saline injected controls. (C-D) Images of BODIPY493/503 stained cardiac sections from control and STZ/HFD mice showing increased lipid deposition (bright green spots) in STZ/HFD mice. (E) Western blots demonstrating expression of proteins involved in ER stress (Atf6 and Chop). (F-G) Graphs demonstrating qRT-PCR results for mRNA expression levels of genes involved in ER stress (Atf6 and Chop). (H) western blot depicting protein levels of Nitro-Tyrosine (a marker of oxidative stress) in control and STZ/HFD mice. (I) Graph demonstrating quantification of Nitro-Tyrosine protein levels in control and STZ/HFD mice.

6.4.2 Fatty acid oxidation is impaired in chronic STZ/HFD mice models.

Mitochondrial fatty acids oxidation is altered in many cardiac pathologies including diabetic cardiomyopathy. Therefore we aimed to assess FAO in our STZ/HFD model. Feeding STZ mice with high fat diet for 4 weeks (acute mice) was not enough to see significant reduction of cardiac FAO (fig 6.2b). Interestingly, chronic feeding of High fat diet for 18 weeks (chronic) caused a significant decrease in FAO compared to control mice (fig 6.2a).

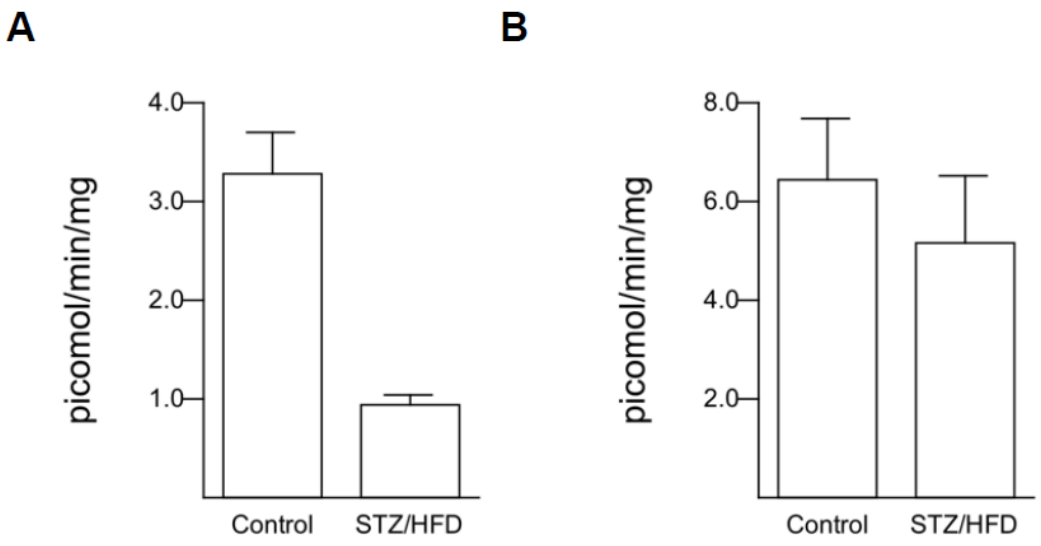


Figure 6.2: Chronic feeding of high-fat diet causes a significant decrease of Fatty acids oxidation in STZ mice. (A) Graph showing the level of complete FAO (CO_2 production) in mitochondria isolated from control and STZ/HFD mice. (B) Graph showing equal production of CO_2 in mitochondria isolated from control and STZ/HFD mice.

6.4.3 Cpt1b overexpression is protective in STZ/HFD mice.

Cpt1b is an enzyme responsible for the rate limiting step of FAO therefore we aimed to regulate Cpt1b protein expression using AAV9 vectors. we selectively overexpressed Cpt1b in mice hearts by injecting them, 4 weeks before sacrifice, with AAV9 GFP (control) or AAV9 Cpt1b. Successful AAV9 transfections were confirmed by measuring Cpt1b protein expression in cardiac tissue lysates by western blot (fig 6.3a). We showed increased cardiac Cpt1b protein expression in STZ-Cpt1b mice compared to STZ-GFP mice (fig 6.3a). Interestingly, AAV9 Cpt1b rescued mice from STZ/HFD induced FAO inhibition (fig 6.3b) and restored FAO to levels equal to control. Rescuing FAO by AAV9 Cpt1b was associated with downregulation of nitrotyrosine post-translational modification of proteins (fig 6.3c) suggesting a protective effect against oxidative stress.

6.4.4 Palmitate induces ER stress and oxidative stress in T293 cells.

Here we aim to support our in vivo data in T293 cells treated with palmitate. Consistent with our previous data, we found that palmitate induces cells death (fig 6.4a) which was associated with oxidative stress (fig 6.4b) and ER stress (Figure 6.4C, 6.4D, 6.4E, 6.4F). Additionally, palmitate inhibits FAO in T293 cells compared to cells treated with oleate (fig 6.4g).

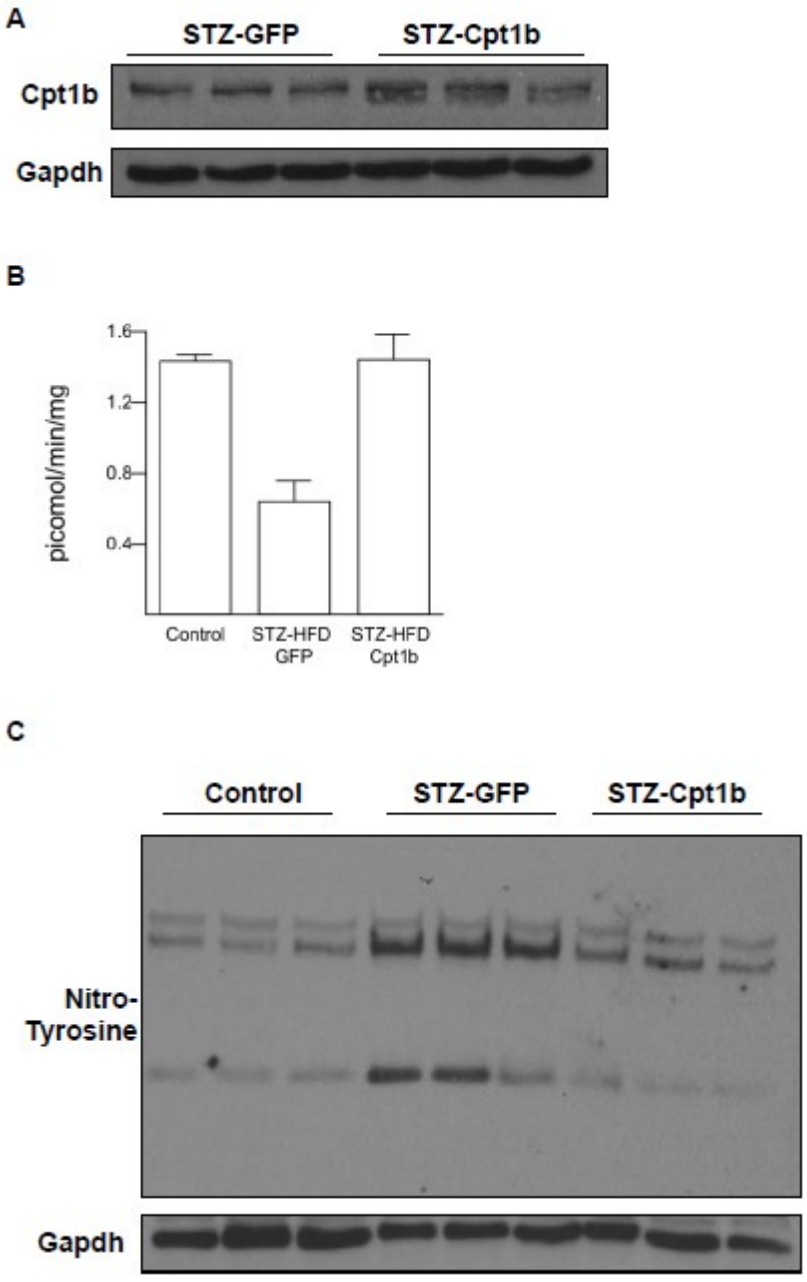


Figure 6.3: Overexpressing Cpt1b using AAV9 Cpt1b rescued mice from STZ/HFD induced FAO inhibition. (A) Western blot demonstrating increased expression of Cpt1b in cardiac tissue from mice injected with AAV9-Cpt1b compared to mice injected with AAV9-GFP. (B) Graph demonstrating decreased FAO (CO_2 production) in mitochondria isolated from STZ/HFD mice compared to control. FAO was restored by increasing Cpt1b expression. (C) western blot showing increased Nitro-Tyrosine expression in STZ-GFP mice compared to control. Nitro-Tyrosine protein expression was reduced in following tail-vein injection of AAV9-Cpt1b in STZ mice.

6.4.5 Overexpressing Cpt1b protects against palmitate induced lipotoxicity.

Since we found that Cpt1b was protective in STZ/HFD mouse model, we wanted to investigate the mechanism of Cpt1b mediated protection in T293 cells. We overexpressed Cpt1b in HEK-293T cells by plasmid transfection (Figure 6.5A-B). Interestingly, Cpt1b overexpression protected against palmitate mediated cell death (Figure 6.5C). additionally, Cpt1b OE significantly reduced palmitate mediated ROS generation (Figure 6.5D) and this was associated with a trend for decreased ER stress gene expression (Atf6, Chop, sXbp1) (supplementary Figure 2). Finally, Cpt1b overexpression significantly enhanced FAO in palmitate treated cells (Figure 6.5E).

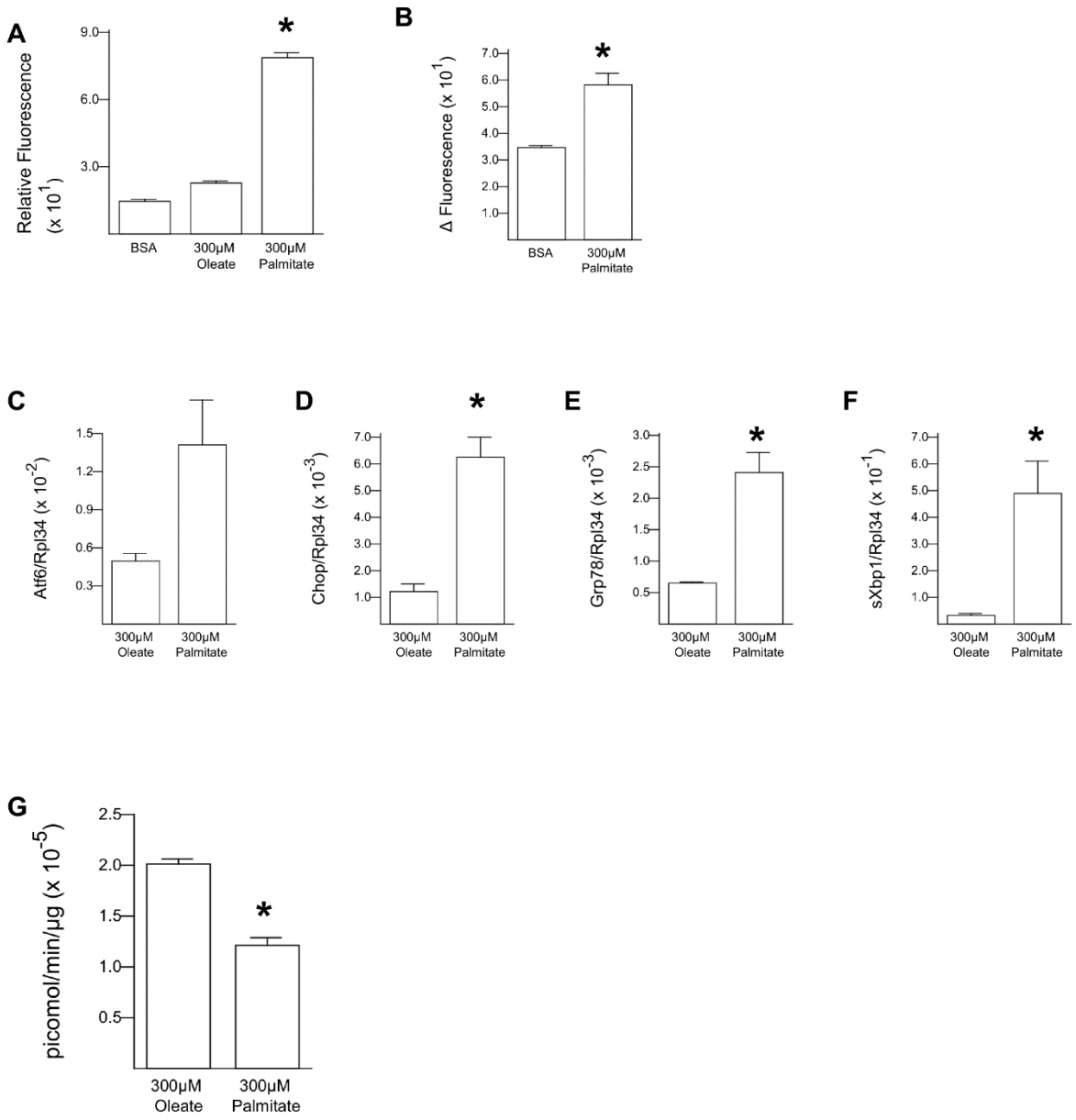


Figure 6.4: Palmitate induces ER stress and oxidative stress in T293 cells. (A) significantly increased PI fluorescence (a marker of cell death) in palmitate treated T293 cells compared to cells treated with 300uM oleate of BSA. (B) graph showing an increased oxidative stress in palmitate treated cells compared to control. (C-F) Graphs demonstrating qRT-PCR results for mRNA expression levels of genes involved in ER stress (Atf6, Chop, GRP78 and sXbp1). (G) Palmitate impairs complete FAO (CO_2 production) in T293 cells compared to oleate treated cells.

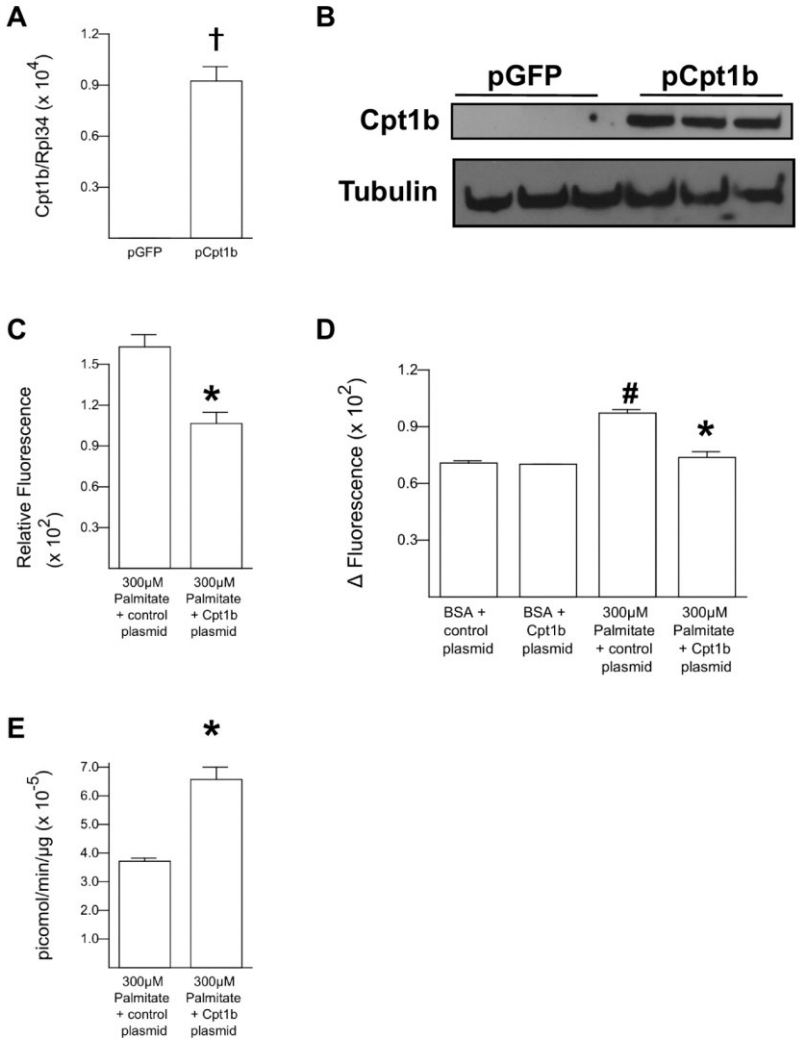
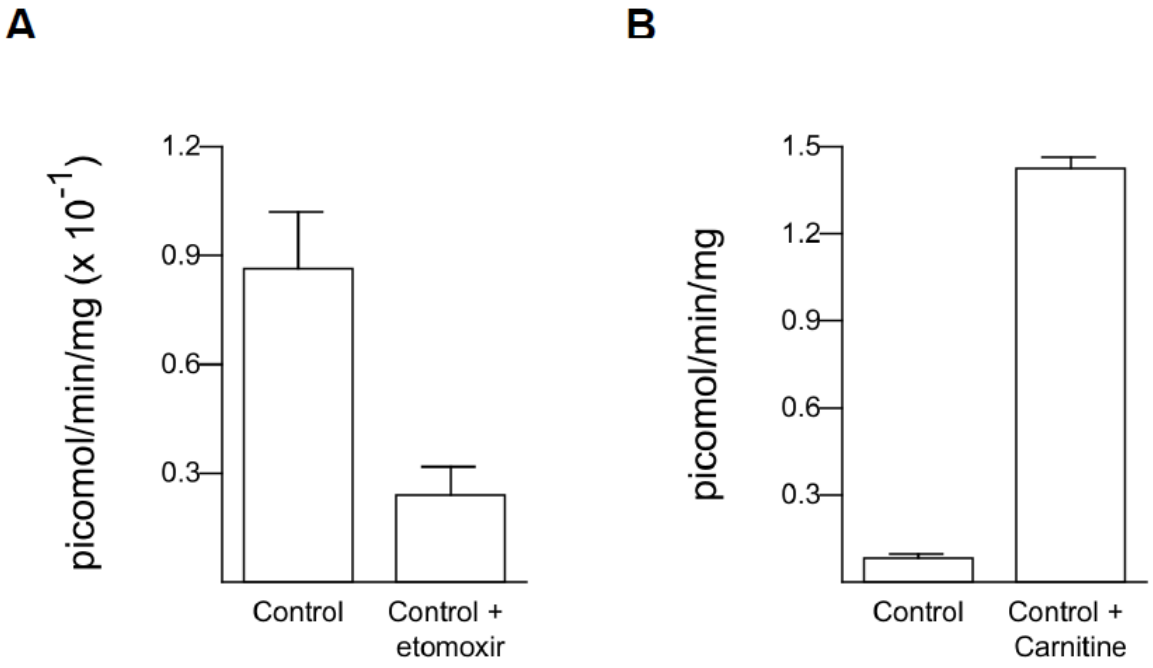
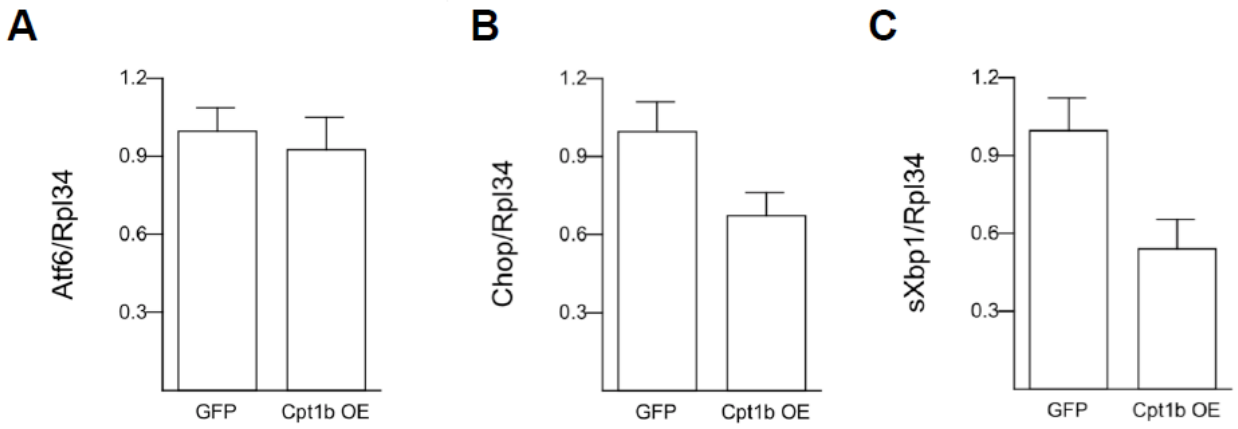


Figure 6.5: Enhancing fatty acid oxidation attenuates palmitate mediated lipotoxicity in T293 cells. (A) Graphs demonstrating significantly elevated Cpt1b mRNA (relative to the housekeeping gene Rpl34) in Cpt1b OE T293 cells. (B) Western blot demonstrating elevated Cpt1b protein levels in T293 cells transfected with Cpt1b plasmid. (C) Graph demonstrating that Cpt1b OE significantly attenuates cell death induced by 300μM palmitate. Cell death was measured by propidium iodide fluorescence and normalized to control cells. (D) Graph showing the level of oxidative stress in T293 cells treated with BSA or 300uM palmitate and transfected with control plasmid or Cpt1b plasmid. (E) Graphs demonstrating significant increase in the production of CO_2 in T293 transfected with Cpt1b plasmid. † indicates $p<0.05$ vs. T293 cells transfected with pGFP. * indicates $p<0.05$ vs. T293 cells treated with 300uM palmitate and transfected with control plasmid. # indicates $p<0.05$ vs. T293 cells treated with BSA and transfected with control plasmid.

6.5 Supplementary data



Supplemental figure S6.1: Cardiac mitochondria isolated from STZ/HFD mice are sensitive to etomoxir and carnitine. (A) Graph demonstrating significantly decreased [14C]-CO_2 production and hence fatty acid oxidation in mitochondria isolated from cardiac tissue and treated with etomoxir (B) Graph demonstrating significantly increased [14C]-CO_2 production and hence fatty acid oxidation in mitochondria isolated from cardiac tissue and treated with carnitine.



Supplemental figure S6.2: Overexpressing Cpt1b attenuates Atf6, Chop and sXbp1 genes expression. Graphs demonstrating the mRNA levels of 3 key markers and mediators of ER stress in T293 cells transduced with AAV GFP plasmid or AAV Cpt1b plasmid.

Chapter 7: Discussion and conclusion

7.1 Discussion

Lipotoxicity in cardiomyocytes isolated from rats and diabetic mice is the general theme presented in this thesis. The five studies used in this thesis aim to characterize the sequential molecular events in which lipotoxicity leads to diabetic cardiomyopathy. In the first four studies, we have used an in-vitro model, where primary cardiomyocytes were isolated from neonatal rats and mice. These cells mimic the behavior of their in-vivo counterparts, since they contract spontaneously and are hence characterized by the same high-energy demand seen in beating hearts. Therefore, these cells are more advantageous to cell line cultures such as H9C2 and AC16, which rarely contract under our conditions. In the last study, an in-vivo model is employed that consists of streptozotocin-induced diabetic mice fed a high fat diet for four weeks (acute) or 18 weeks (chronic).

We decided to investigate lipotoxicity as a potential pathogenic mechanism for diabetic cardiomyopathy, since the hearts of diabetic patients are characterized by steatosis (249, 251). Additionally, hyperlipidemia seen in the blood vessels supplying the heart likely contributes to atherosclerosis and other cardiovascular events. Therefore, lipotoxicity and the accumulation of fatty acids in diabetic hearts is likely the main culprit for cardiac diseases seen in the diabetic population, such as cardiomyopathy and heart failure. To investigate lipotoxicity in diabetic hearts we used palmitate, a well-known toxic fatty acid. Palmitate and oleate are the two most

abundant fatty acids in the human diet. Contrary to palmitate, oleate was shown to be neutral in many cell types.

One clear difference between rat neonatal primary cardiomyocytes treated with oleate or palmitate is the shape of lipid droplets seen after staining these cells with BODIPY. Oleate leads to clear and distinct lipid droplets while palmitate leads to faint and diffuse lipid droplets. Palmitate-treated cells also display abnormal and irregular-shaped staining that is far removed from the shape of lipid droplets. Interestingly, adding oleate to cardiomyocytes treated with palmitate protected these cells from palmitate lipotoxicity. This was associated with changes of BODIPY staining where lipid droplets became more clear and distinct. The main constituent of lipid droplets is TAG. TAG is considered a neutral lipid, while DAG is well known for its toxicity in many cell types. BODIPY binds to TAG. The qualitative and quantitative increase of BODIPY staining in cardiomyocytes co-treated with oleate and palmitate compared to cardiomyocytes treated with palmitate alone indicates an increase of TAG levels, which might explain the protective mechanism of oleate on palmitate-treated cells. Indeed, other studies have suggested that oleate protects against palmitate lipotoxicity by inducing TAG synthesis and thus sequestering palmitate into a neutral lipid (110). This reduces the bioavailability of palmitate to be incorporated into toxic lipids such as DAG and ceramides.

Since the first steps of lipid droplet synthesis occur in the ER, and since our previous results indicate the lack of clear staining of lipid droplets in palmitate-treated cells, we decided to investigate the possibility of lipid accumulation in the ER. Accumulation of lipids in the ER disrupts ER function and thus triggers ER stress. Previous studies done by other researchers

show that ER stress is induced in palmitate-treated cells (275, 278, 474-476). Consistently, we show that palmitate leads to robust ER stress in primary rat neonatal cardiomyocytes. This ER stress was associated with cell death and altered lipid staining.

ER stress is a common pathological process found in diabetic cardiomyopathy (368, 477). ER stress leads to the activation of the unfolded protein response (UPR). UPR aims to restore ER homeostasis by ensuring proper protein folding. Therefore, ER stress is activated when unfolded proteins accumulate in the ER. ER stress can also be stimulated through other pathways, such as perturbation of calcium homeostasis and disruption of ER membrane structure (278, 478, 479). In our first study, we demonstrated clear activation of ER stress in cardiomyocytes isolated from mice and rats and treated with palmitate. More precisely, we demonstrated significant increase of cleaved Atf6 and spliced Xbp1. In addition, we showed a significant increase of GRP78, Atf4, and Atf6 mRNA levels. This increase caused by palmitate incubation was time- and dose-dependent.

Unresolved ER stress promotes cell death. The mechanism likely involves Chop expression and activation (480). Our results support this, since we found significant increase of Atf6 and Chop expression and activation in cardiomyocytes treated with palmitate. Chop induces cell death by upregulating the expression of pro-apoptotic proteins and by downregulating the anti-apoptotic proteins (481, 482). This is in accordance with our results, where we showed increased protein expression of caspase-3 at sixteen hours, which preceded cell death. These results indicate that palmitate-mediated cell death is at least partially due to apoptosis.

It important to note that UPR is a compensatory mechanism that aims to restore ER homeostasis. Hence, upregulation of chaperon molecules such as GRP78 is considered a protective mechanism during ER stress (483, 484). Interestingly, we found that palmitate significantly increases the expression of GRP78 at the mRNA level but not the protein level. This indicates the existence of post-transcriptional or post-translational mechanism impeding the expression of GRP78 proteins in palmitate-treated cardiomyocytes. Indeed, we identified the ubiquitination of GRP78 in cardiomyocytes incubated with palmitate suggesting that GRP78 is specifically degraded by the ubiquitin proteasome pathway. We found the loss of this chaperone contributes to palmitate-mediated cell death. The activation of the ubiquitin proteasome pathway by palmitate is probably done in a PKC-dependent manner, since other studies have shown this in hepatocytes and we have demonstrated in subsequent studies that cardiomyocytes treated with palmitate are characterized by increased levels of DAG, a known inducer of PKC (485).

PPARs are key metabolic regulators of fatty acid metabolism. Since fatty acids are natural ligands of PPARs, we set to examine the effect of palmitate and oleate on PPAR expression and activity. Additionally, we have investigated the effect of these two fatty acids on cytokine levels, considering that PPARs modulate the inflammatory response.

One of the key findings of this thesis is that palmitate induces the activity of PPAR receptors in cardiomyocytes for a relatively short duration. This activation does not persist for longer times and is lost after the twenty-four-hour time point. In contrast, oleate induction of PPAR activity persisted beyond the twenty-four hours. This accentuates the difference between palmitate lipotoxicity and oleate's neutral, and even protective, effect. Indeed, some of PPAR's target

genes are implicated in fatty acid catabolism, and inducing PPAR activity by oleate improves the cell's ability to clear intracellular fatty acids and hence lipid accumulation. However, treating cardiomyocytes with palmitate inhibits PPAR activity at the twenty-four-hour time point and contributes to intramyocellular lipid accumulation, resulting in the accumulation of toxic fatty acids and lipotoxicity.

The fact that palmitate increases PPAR activity at early time points suggests that palmitate is a ligand for PPAR. It appears also that other mechanisms occur simultaneously that hinder palmitate-mediated activation of PPAR at later time points. Indeed, we found that the expression of PPARα and PPARδ proteins are downregulated after prolonged incubation of cardiomyocytes with palmitate.

CPT1B is a key enzyme in fatty acid oxidation that catalyzes the rate-limiting step of β-oxidation. CPT1B is a PPAR target gene in cardiomyocytes. We found that the degradation of PPAR proteins in cells treated with palmitate is associated with decreased levels of CPT1B mRNA and decreased expression of CPT1B proteins. Contrary to CPT1B , ACADL retains elevated protein levels despite the inhibition of ACADL mRNA expression. This suggests that PPARs-mediated inhibition of fatty acid oxidation occurs through downregulation of CPT1B.

Our previous findings accentuate the protective role of PPARs in mitigating myocardial lipid accumulation and lipotoxicity by upregulating the oxidation of fatty acids. It is important to note that our findings might seem contradictory to the results of other research groups. For instance, Finck et al. found that the hearts of PPARα transgenic mice exhibit cardiac steatosis and

cardiomyopathy. However, the level of PPARα in these transgenic mice is 50 to 100 times higher compared to normal physiological levels. These abnormal levels may be the cause of cardiac dysfunction and steatosis seen in these transgenic hearts. Indeed, PPARα is known to induce both fatty acid uptake and fatty acid oxidation (436). The supra-physiological level of PPARα in these transgenic mice is likely causing the uptake of fatty acids to a higher degree than fatty acid oxidation. PPARδ, in contrast, induces fatty acids oxidation to a higher degree than fatty acid uptake, and over-expressing PPARδ in mice hearts did not predispose them to heart failure.

It has been shown that palmitate induces the expression of cytokine in several cell types including cardiomyocytes. Additionally, several reports have indicated that cytokines lead to PPARs protein degradation. First, inflammation causes PPARs downregulation in diabetic hearts (486). Second, IL6 was the main culprit of PPARα and PPARγ inhibition in adipocytes and hepatocytes; however, this was not confirmed in cardiomyocytes (487-490). Interestingly, TNFα transgenic mice exhibit reduced activity of PPARα receptors and reduced ability to oxidize fatty acids (491). The authors did not confer this effect to TNFα, but rather attributed the impairment of fatty acid oxidation in the heart of these transgenic mice to the Tgfβ-Smad3 pathway. The authors did not examine the level of IL6 in these transgenic hearts, but other studies have shown that TNFα and TGFβ induces IL6 expression (492, 493). Therefore, we sought to investigate the effect of palmitate on IL6 and TNFα levels. We found a robust increase of both IL6 and TNFα levels in palmitate-treated cells. Additionally, this increase was dose-dependent. Another key finding of this thesis is that oleate and other anti-inflammatory molecules reduced palmitate-mediated cell death by only reducing IL6 and not TNFα. Indeed,

oleate, AICAR, WY-14643, and GW-501516 all reduced cell death, while the level of IL6 had a varying effect on TNFα. Therefore, our results indicate that IL6, rather than TNFα, is the main culprit for palmitate-mediated inhibition of PPAR expression and cell death, a finding that is in accordance with the studies mentioned before. Furthermore, the increase of IL6 levels occurs before the degradation of PPAR proteins, further supporting our hypothesis that IL6s play a key role in antagonizing palmitate-mediated lipotoxicity.

The prominent decrease of PPARs activity and Cpt1b expression led us to investigate the effect of palmitate on fatty acid oxidation in rat neonatal cardiomyocytes. The inability of fatty acids to be catabolized through oxidation likely prompt fatty acids to accumulate in cytosol, causing steatosis. Therefore, we measured intracellular fatty acids in cardiomyocytes treated with oleate or palmitate, finding that palmitate leads to a significant increase in intracellular fatty acids compared to oleate. This suggests that palmitate clearance was diminished, in contrast to oleate.

Next, we measured the rate of complete fatty acid oxidation in cardiomyocytes incubated with oleate and palmitate for eight hours. We chose this time point since it is not associated with cell death. We found that palmitate, in contrast to oleate-treated cells, significantly impaired fatty acid oxidation.

Since we have previously shown that CPT1B protein expression is downregulated after twenty-four-hour exposure to palmitate, we wanted to see if this impairment of FAO at an eight-hour time point is also associated with impaired CPT1B expression. We found that CPT1B protein expression begins to decrease after fourteen to sixteen hours of palmitate exposure. We also

tested the integrity of the mitochondrial membrane using the JC-1 dye and found that the loss of the mitochondrial membrane's potential occurs simultaneously with the loss of CPT1B expression after the impairment of FAO. This indicates that the decrease of CPT1B proteins is likely a secondary effect of mitochondrial membrane damage. Moreover, the impairment of FAO and the depletion of the citric acid cycle-derived reducing equivalents is probably the cause of mitochondrial membrane damage, since it precedes it.

The fact that FAO impairment occurs before cell death and mitochondrial damage in palmitate-treated cells lead us to hypothesize that upregulating FAO protects cardiomyocytes from palmitate lipotoxicity. Indeed, co-treating cardiomyocytes with palmitate and oleate or palmitate and carnitine increased the rate of FAO and protected these cells from palmitate-mediated cell death. Interestingly, oleate completely abolished palmitate-mediated cell death, while carnitine significantly attenuated it. This is in line with the fact that carnitine only induces FAO, while oleate has pleotropic effects, such as induction of FAO, attenuation of ER stress, and attenuation of inflammation (51, 274).

Another piece of evidence pointing towards the protective effect of oleate against palmitate lipotoxicity through increasing FAO comes from the following experiments. We inhibited FAO pharmacologically and genetically in cardiomyocytes treated with oleate. As expected, oleate, which is non-toxic, became toxic under these circumstances. This further confirms our hypothesis that palmitate lipotoxicity is mainly due to the inhibition of FAO. The exact events causing oleate to become toxic following FAO inhibition are still not clear, but they likely involve the accumulation of toxic intermediates such as DAG.

Our results indicating that increasing FAO protects against lipotoxicity might seem inconsistent with other studies where the heart of diabetic mouse models exhibit increased β-oxidation, which is associated with oxidative stress, cardiac dysfunction, and cardiomyopathy. Indeed, the hearts of PPARα transgenic mice are characterized by increased β-oxidation, cardiac steatosis, and cardiomyopathy, which resembles the pathology of diabetic hearts (494). Furthermore, other in-vivo studies demonstrated that inhibiting β-oxidation improved glucose oxidation which is beneficial in some diabetic mouse models (495, 496). However, these studies were only short-term and the authors did not investigate the long-term effects of inhibiting cardiac FAO on heart function. Additionally, the association between increased β-oxidation and cardiomyopathy in PPARα transgenic mice does not necessarily mean causality, especially since these mice exhibit cardiac steatosis.

On the other hand, results confirming the beneficial effects of increasing FAO in diabetic hearts come from three studies that modulated PPARs in mice. Indeed, these studies demonstrated that improving FAO was inversely associated with diabetic cardiomyopathy, while inhibiting FAO was associated with intramyocardial lipid accumulation and cell death. First, a knockout of CD36 in PPARα transgenic mice was associated with improved cardiac function, despite elevated β-oxidation (449). Second, overexpressing PPARγ in mice hearts while simultaneously knocking out PPARα caused increased β-oxidation and reduced lipid accumulation, which was associated with improved cardiac function (448). Third, PPARδ-deficient mice exhibit cardiac steatosis, cardiomyopathy, and an increased rate of β-oxidation (448). Therefore, it seems that the increase of β-oxidation is not the cause but rather a consequence of intramyocardial lipid

accumulation in diabetic mouse models. Additionally, these results suggest that the increase of β-oxidation is not reaching its full potential in preventing cardiac steatosis.

Another notion, that β-oxidation does not equate to complete fatty acid oxidation, is often ignored. Indeed, the rate of FAO depends on fatty acid uptake into mitochondria, the rate of β-oxidation, and the rate of CAC. Inhibition of FAO is set to happen if one of these steps is downregulated. Therefore, if the CAC is downregulated, an increase of β-oxidation does not necessarily mean an increase of FAO. In accordance with this, Baseler et al. found that CAC enzymes are downregulated in diabetic mice (497). Additionally, Lin et al. found that isocitrate and α-ketoglutarate levels were decreased in diabetic hearts, despite the normal level of citrate indicating an impairment of energy metabolism and the CAC in these hearts (498).

After demonstrating that palmitate-mediated lipotoxicity at least partially occurs by inhibiting FAO, our next aim was to determine which step of FAO is impaired by palmitate. We showed that palmitate had no effect on CPT1B activity; however, it impaired β-oxidation and the CAC in primary rat neonatal cardiomyocytes. As a matter of fact, we measured CPT1B activity in cardiomyocytes treated with oleate and palmitate for eight hours and found no significant difference between these two conditions. We chose an eight-hour time point since we found previously that it was long enough for palmitate to inhibit complete fatty acid oxidation.

Following the uptake of fatty acids into the mitochondria, fatty acids enter β-oxidation to yield acetyl-CoA. Since we wanted to measure the rate of β-oxidation in palmitate- and oleate-treated cells, we measured "acid soluble metabolites." Hypothetically, acetyl-CoA are soluble in acid,

while long-chain fatty acids such as oleate and palmitate are not supposed to be soluble in acid. However, this was not the case in our experimental conditions, where the acids' soluble metabolites were contaminated with oleate and palmitate. Fortunately, acetyl-CoA and fatty acids can be separated using an aqueous/organic separation method. Acetyl-CoA is water soluble, while fatty acids are soluble in the organic phase. In our hands, fatty acid contamination of the water phase was insignificant. Therefore, we used the water/organic separation method to measure the level of acetyl-CoA, which was correlated with the rate of β-oxidation. Interestingly, we found that β-oxidation was inhibited in palmitate-treated cells, in contrast to oleate-treated cells. This is important since it demonstrates that palmitate lipotoxicity is mediated by the inhibition of β-oxidation. As a side note, inhibiting β-oxidation with etomoxir nearly abolished the detection of acetyl-CoA in the aqueous phase, confirming the specificity of the aqueous/organic separation method.

The molecular mechanism of β-oxidation inhibition by palmitate is still unknown, but there is a strong line of evidence pointing towards post-translational modification (PTM) (499). Indeed, palmitate induces the acetylation, phosphorylation, and palmitoylation of mitochondrial proteins (498, 500, 501). However, whether post-translational acetylation of enzymes implicated in β-oxidation increases or decreases their activity is still under debate. For example, acetylation of long-chain acyl-CoA dehydrogenase (LCAD) inhibits its activity, while Sirt3, a deacetylase, restores its function (502, 503). On the other hand, acetylation of β-hydroxyacyl-CoA dehydrogenase (HADH) increases its activity (504).

Acetyl-CoA produced from β-oxidation enters the CAC to be further oxidized. Reducing equivalents such as NADH and FADH2 are some by-products created during the CAC. These reducing equivalents are critical for oxidative phosphorylation, and, hence, they maintain mitochondrial membrane potential. An inhibition of the CAC can lead to disastrous consequences on oxidative phosphorylation, mitochondrial membrane integrity, and mitochondrial function. Additionally, acetyl-CoA produced from glucose oxidation enters the CAC to be further oxidized. Hence, an inhibition of the CAC impedes energy metabolism and promotes cardiomyocytes' dysfunction, since the energy demand of these cells is high. We found that cardiomyocytes incubated with palmitate have reduced acetyl-CoA oxidation compared to cells incubated with oleate, indicating that palmitate inhibits the CAC. Indeed, we found that palmitate-treated cells have increased aconitase reverse activity while the aconitase forward activity is not altered. Therefore, since the level of isocitrate, the product of aconitase activity (both forward and reverse), is reduced in these cells, a reduced level of substrates for isocitrate dehydrogenase is indicated.

Our results are in line with other studies done on diabetic cardiomyopathy. Line et al. found that aconitase forward activity was unchanged in diabetic mouse hearts, while aconitase reverse activity was increased (498). They attributed this increase to PKCβ-mediated phosphorylation of the aconitase enzyme. PKCβ is one of the conventional isoforms of PKC and is activated by DAG. We measured the ratio of DAG/TAG in palmitate-treated cardiomyocytes and found it higher compared to oleate-treated cardiomyocytes. Additionally, PKCβ is activated by one isoform of DAG (1,2 DAG), which interestingly was the isoform elevated in our cardiomyocytes

incubated with palmitate (505). Taken together, our data shows that the CAC is inhibited by palmitate, an event at least partially caused by PKC-mediated inhibition of aconitase activity.

Next, we measured isocitrate dehydrogenase (IDH) activity in palmitate-treated cardiomyocytes. We found its activity is inhibited in these cells when contrasted to cardiomyocytes treated with oleate. It is important to note that our method of measuring IDH activity is independent of aconitase activity and the level of endogenous isocitrate, since we supplement these cells with exogenous isocitrate. This further confirms findings that palmitate inhibits the CAC, since the step catalyzed by IDH is irreversible. To verify if IDH activity is reduced because of IDH protein degradation, we measured IDH protein expression in cardiomyocytes incubated with palmitate for eight hours. We found that eight hours of palmitate incubation was not enough to achieve results, indicating the existence of another mechanism to explain IDH reduced activity.

IDH activity might be downregulated by oxidative stress. Several studies have demonstrated that mitochondrial proteins can be damaged through oxidative stress (506-508). Additionally, oxidative stress has been suggested to play a role in pathophysiology of diabetic cardiomyopathy. Therefore, we measured ROS using the DCFDA assay and measured oxidative stress by determining the immunoreactivity of 4-HNE. 4-HNE is a marker of oxidative stress because lipid peroxidation by reactive oxygen species leads to elevated 4-HNE adducts on proteins (509). Interestingly, we found that ROS levels are elevated in palmitate-treated cells when contrasted to the control. However, this is unlikely to be the mechanism for IDH reduced activity, since oleate and palmitate elevated ROS to similar levels. Additionally, there was no

evidence for oxidative stress in oleate- or palmitate-treated cardiomyocytes, indicating that increased levels of ROS were not enough to overtake the endogenous anti-oxidative system. This is in line with other studies where palmitate lipotoxicity was shown to be independent of ROS (510). Altogether, we demonstrated that palmitate inhibits the CAC, which is at least partially due to DAG-mediated activation of PKC. This is important, since palmitate is a major constituent of fatty acids in the human diet and thus a likely cause of lipotoxicity seen in diabetic cardiomyopathy.

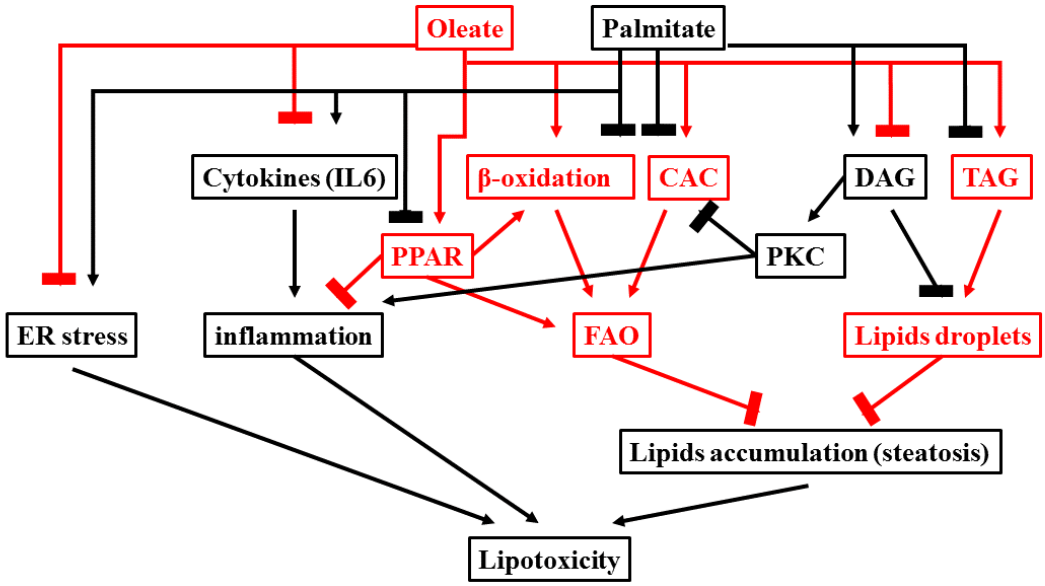


Figure 7.1: Diagram summarizing the results of this thesis. The black arrows represent pathways associated with lipotoxicity. The red arrows represent pathways associated with attenuation of lipotoxicity

Finally, our in-vitro results led us to investigate the alteration of mitochondrial fatty acid oxidation in a diabetic mouse model. We have used an STZ/HFD model since it contains many

advantages for studying DCM in Type 2 diabetes. STZ causes the destruction of pancreatic β-cells, which leads to hypoinsulinemia. This causes the heart to be less dependent on glucose and more dependent on fatty acids as an energy source. Additionally, hypoinsulinemia causes the release of fatty acids from adipose tissue, since insulin is an inhibitor of adipose triglyceride lipase (511). This predisposes the heart to hyperlipidemia and further advances cardiac steatosis. Cardiac steatosis is an independent risk factor for DCM. It is important to note that we use multiple low-dose STZ injections, which have low levels of toxicity on the liver and kidney and therefore no secondary effect on the heart. An additional advantage of our model is that we feed our STZ mice with a high-fat diet to ensure higher dependency on fatty acids and to induce steatosis.

We confirmed that our diabetic mouse model exhibits hyperglycemia, a key characteristic of T2DM, and hyperlipidemia, which is highly prevalent in T2DM. ER stress and oxidative stress are implicated in the pathophysiology of several cardiovascular diseases, including DCM. Indeed, oxidative stress is a major risk factor for DCM, and several studies have showed a clear association between oxidative stress and heart failure (512, 513). We measured ROS levels using DCFDA fluorescent staining. We also measured oxidative stress by semi-quantifying nitrotyrosine's post-translational modification of proteins. We found a significant increase of ER stress and oxidative stress in our STZ/HFD mice compared to control mice. Since our in-vitro results show a clear association between lipotoxicity and the inhibition of FAO, we measured the rate of FAO in mitochondria isolated from the heart of STZ/HFD mice. Interestingly, we found that mice who have been diabetic for 18 weeks and chronically fed an HFD during that time exhibit a significant reduction of complete FAO in cardiac cells. It has

been suggested that induction of FAO is protective in diabetic settings (514, 515). Since CPT1B catalyzes the rate-limiting step of β-oxidation, overexpressing CPT1B should theoretically increase FAO. We found that overexpressing CPT1B is protective and was associated with attenuation of ER stress and oxidative stress in our diabetic mouse model. To verify if the protective effect of CPT1B is through increasing FAO, we overexpressed CPT1B in a T293 cell line. We chose this cell line since it is easy to use to modulate CPT1B expression. Interestingly, overexpressing CPT1B in T293 cells induced FAO and protected these cells from palmitate lipotoxicity. Therefore, our results suggest that overexpressing CPT1B and enhancing FAO is a potential remedy for DCM.

The exact molecular mechanism elucidating the protective effect of enhancing FAO is not fully elucidated. However, it has been suggested that inducing FAO promotes lipid clearance, causing lipids to be channeled away from other metabolic pathways that increase toxic metabolites, such as DAG and ceramides. Indeed, inhibiting FAO with etomoxir resulted in the accumulation of muscle DAG in C57bl6 mice (516). This is supported by another study that found increasing the export of lipids from the heart by overexpressing apolipoprotein B was protective in diabetic settings (517).

Other research groups found that STZ mice exhibit higher rates of β-oxidation, which might seem to contradict our results. However, two things need to be taken into consideration. First, an increase in β-oxidation is not equal to an increase in total FAO. Our method quantifies the rate of FAO by measuring the production of CO_2 from the citric acid cycle. Therefore, even if the rate of β-oxidation is increased, complete FAO will be inhibited if the citric acid cycle is impaired. Indeed, we have previously shown that palmitate inhibits FAO at least partially due

to its inhibition of the CAC (287). This is supported by others who have found that the CAC is impaired in several settings of lipotoxicity and diabetes (518-520). Second, an increase in β-oxidation may be due to an increase of the availability of substrates (lipids) and not due to an intrinsic increase in mitochondrial metabolism. Indeed, this was demonstrated in db/db mice (521). This might explain the difference between the results found in our preparations, which involve using isolated mitochondria, and the preparations of others, which involve perfusing working hearts.

An important feature of our in-vivo study is that we divided our STZ/HFD mice into two different groups: acute and chronic. Acute mice were fed an HFD for four weeks, while chronic mice were fed an HFD for 18 weeks. In this way, we took into consideration two distinct stages of DCM progression. We found a clear difference between acute and chronic mice, where four weeks of high-fat feeding was not enough to induce ER stress, oxidative stress, or to inhibit FAO. In our settings, therefore, the time needed to induce highly prevalent characteristics of DCM was higher than four weeks. This is important because our long-term experiment, which lasted 18 weeks and showed that STZ/HFD mice exhibit decreased FAO, is critically different from the short-term experiments done by other research groups, which found that STZ mice exhibit increased rates of β-oxidation. Indeed, the increase of β-oxidation at early time points of diabetes is likely a physiological response, while the decrease of fatty acid oxidation is a pathological effect of prolonged diabetes.

7.2 Conclusion

In conclusion, the results presented in this thesis identify new molecular mechanisms of lipotoxicity in primary neonatal rat cardiomyocytes, in primary neonatal mouse cardiomyocytes, and in cardiac tissue of diabetic mice. These results help explicate the mechanism of lipotoxicity in diabetic hearts and shed new light on saturated fatty acids as major a contributor in the pathophysiology of diabetic cardiomyopathy.

We found that palmitate induces intracellular lipid accumulation, ER stress, and cell death. Additionally, palmitate inhibits PPAR activity, which was associated with IL6 and TNFα induction and the inhibition of fatty acid oxidation. A key finding in this thesis is that palmitate toxicity is attenuated by inducing fatty acid oxidation, while oleate becomes toxic by inhibiting fatty acid oxidation. Furthermore, we demonstrated that palmitate likely impairs β-oxidation and the citric acid cycle through DAG-mediated PKC activation. This is important, since it might explain how the western diet, which is high in saturated fats, can contribute to the lipotoxicity observed in diabetic cardiomyopathy. In accordance with this, we found that the hearts of diabetic mice that were chronically fed a high-fat diet exhibit oxidative stress and reduced capacity to oxidize fatty acids.

Unfortunately, this project is far from being able to elucidate the exact mechanism of lipotoxicity in diabetic cardiomyopathy. However, I hope it will be a stepping stone for future studies that focus on impaired fatty acid oxidation as a cause for lipotoxicity in diabetic cardiomyopathy.

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