Comparative Analysis of Trans-Fats and Alpha-Linolenic Acid Administration on Cardiomyocyte Viability during Ischemia/Reperfusion Injury

By

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Abstract

Ischemic heart disease is the largest cause of death due to cardiovascular origins. A better understanding of the mechanisms responsible for ischemic heart disease increases the potential for therapies. This will lead to decreased mortalities in Canada and around the world. Nutritional interventions have gained increasing attention as causes or treatments for cardiovascular disease. For example, trans fats (TFAs) have both beneficial and deleterious effects on cardiovascular disease [1]. In this study, we would like to examine this phenomenon. We contrast the effects of two different TFAs on cardiomyocyte viability. We compare the industrially produced trans-fat elaidic acid (EA) and the ruminant trans-fat vaccenic acid (VA) on apoptotic and autophagic markers during non-ischemic (control), ischemic (ISCH) and ischemia/reperfusion (IR) conditions. Rat cardiomyocytes are exposed to medium containing fatty acids conjugated with bovine serum albumin for 24 hours. VA and EA have no significant effect on biomarkers of apoptosis or cell death. Interestingly, a similar effect is observed with autophagic and apoptotic markers of LDLr-/- mice whose diets were supplemented with VA or EA. Cells pre-treated with EA prior to 60 minutes of simulated ISCH and 120 minutes of IR increased cell death compared to control through augmented apoptosis. VA decreases the number of dead cells during ISCH and IR. However, the apoptotic parameters remain unchanged. We also observe that VA decreases oxidized phospholipid content in non-ischemic conditions. We conclude that not all TFAs are deleterious to the heart. EA is toxic to cardiomyocytes with or without ISCH or IR whereas VA is cardioprotective during IR and ISCH conditions. We believe VA decreases oxidized phospholipid content to produce this cardioprotective effect.

For the purposes of comparison, we examined the effects of α-linolenic acid (ALA), an essential polyunsaturated fatty acid found in foods like flaxseed. Omega-3 fatty acids have been

associated with improved cardiovascular outcomes [2]. Here, isolated adult rat cardiomyocytes from male Sprague Dawley rats were exposed to medium containing ALA for 24 hours and then exposed control, ISCH or IR conditions. Cell death increases during ISCH and IR. An increase in DNA fragmentation and caspase-3 activity was observed in both the ISCH and IR conditions. Pre-treatment of the cells with ALA subsequently inhibits cell death during ISCH and IR challenge and significantly reduced both DNA fragmentation and caspase-3 cleavage during ISCH and IR. Cardiomyocyte resting Ca^{2+} increased and Ca^{2+} transients decreased during ISCH or I/R but ALA pre-treatment did not improve either parameter significantly. We hypothesize that apoptosis is initiated through phosphatidylcholine oxidation within the cardiomyocytes. Pre-treatment of cells with ALA resulted in a significant incorporation of ALA within cardiomyocyte phosphatidylcholine. Two pro-apoptotic oxidized phosphatidylcholine (OxPC) species, 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) were significantly increased during both ISCH and IR. ALA pre-treatment significantly decreased the production of POVPC and PGPC during ISCH and I/R. It is concluded that ALA protects the cardiomyocyte from apoptotic cell death during simulated ISCH and IR by inhibiting the production of specific pro-apoptotic OxPC species. In summary, we observe a differential effect of ALA, VA and EA on parameters of cardiomyocyte viability during ISCH or IR.

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Purity, patience, and perseverance are the three essentials to success and, above all, love.

-Swami Vivekananda

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Abbreviations

AA: Arachadonic Acid

ALA: Alpha (α)-Linolenic Acid

ATGL: Adipose Tissue Triacylglycerol Lipases

ATP: Adenosine Triphosphate

Bax: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma-2

cLA: Conjugated Linoleic Acid

CPT: Carnitine palmitoyltransferase

CVD: Cardiovascular Disease

DHA: Docosahexanoeic Acid

EA: Elaidic Acid

EPA: Eicosapentaenoic Acid

FABPpm: Fatty Acid Binding Protein (Plasma Membrane)

FAT/CD36: Fatty Acid Translocation/Cluster of Differentiation 36

FATP: Fatty Acid Transport Protein

FFA: Free Fatty Acid

GLUT: Glucose Trasporter

HSL: Hormone Sensitive Lipase

IHD: Ischemic Heart Disease

IR: Ischemia/Reperfusion

ISCH: Ischemia

iTFAs: Industrial Trans Fats

KDDiA-PPC: 1-palmitoyl-2-(9'-keto-10'-dodecene-dioyl)-sn-glycero-3-phosphocholine

KODdiA-PPC: 1-palmitoyl-2-(5'-keto-6'-octene-dioyl)-sn-glycero-3-phosphocholine

LA: Linoleic Acid

LAD: Left Anterior Descending (Coronary Artery)

LC-3: Light Chain 3

LCFA: Long Chain Fatty Acids

MCFA: Medium Chain Fatty Acids

MUFAs: Monounsaturated Fatty Acids

n-3 or n-6: Omega-3 or Omega-6

NF-κB: Nuclear factor *kappa*-light-chain-enhancer of activated *B*

ω: Omega

PAzPC: palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine

PGPC: 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine

PLA_2: Phospholipase A_2

PONPC: 1-palmitoyl-2-(9'-oxo-nonanoyl)-sn-glycero-3-phosphocholine

POVPC: 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine

PUFAs: Polyunsaturated Fatty Acids

rTFAs: Ruminant (Natural) Trans Fats

SCFA: Short Chain Fatty Acids

SDG: Secoisolariciresinol Diglucoside

SFA: Saturated Fatty Acids

SSO: Sulfo-*N*-succimidyl-palmitate

STZ: Streptozotocin

TFAs: Trans Fatty Acids

TNF: Tumor Necrosis Factor

VA: Vaccenic Acid

VLCFA: Very Long Chain Fatty Acids

CHAPTER 1: LITERATURE REVIEW

SECTION 1: FATTY ACIDS

I) Fatty Acids

i) Structure and Composition

Fatty acids are carboxylic acids attached to an aliphatic (or long chain) of carbons [3]. This distinct structure identifies fatty acids from other organic compounds such as hormones, neurotransmitters and vitamins and antigens. Depending on the length of the aliphatic chain, the fatty acid can be identified in different ways. Short chain fatty acids (SCFAs) are identified as fatty acids with aliphatic tails less than six carbons in length. Medium (MCFAs) and long chain fatty acids (LCFAs) have aliphatic tails which are six to twelve carbons in length and thirteen to twenty one carbons in length, respectively. Very long chain fatty acids (VLCFAs) contain aliphatic tails which are more than twenty two carbons in length [4].

ii) Saturated vs. Unsaturated Fatty Acids

Carbon-carbon chain interactions in fatty acids can be found in two major forms: saturated and unsaturated. The term saturation denotes the number of double bonds found in the aliphatic chain [4]. Saturated fatty acids (SFAs) are 'saturated' with hydrogen. Therefore, no double bonds can exist. Of interest, the aliphatic chain will also be linear in SFAs. The aliphatic chain is packed with hydrogen bonds; this creates an increase in Van Der Waals forces. These

forces also determine a higher melting point in saturated fats than unsaturated fats. As a result, saturated fats are more likely to be found in a solid state at room temperature [3].

Unsaturated fatty acids have distinct double bonds in the aliphatic tails. These double bonds create kinks within the tail which allow for the fatty acids to bend into different conformations. This difference in conformation causes many of these fats to be found in liquid states in room temperature. Double bond creation will also interfere with the number of hydrogens occupying either side of the double bond; there are two major confirmations of unsaturated fatty acids: *cis* and *trans*. The *cis* confirmation relates to hydrogen atoms being placed on the same side of the double bond found in an unsaturated fatty acid [4]. This conformation results in a rigidity of the aliphatic tail and does not allow for conformational shifts within the carbon chain backbone. Kinks or bends result in the aliphatic backbone-a specific identifying factor of *cis* conformation. This differs in the case of the *trans* conformation. Here, the two hydrogen bonds are found on opposing sides of the double bond (Figure 1). Therefore, the kink which is found in the *cis* conformation is not apparent and the *trans* conformation backbone looks more similar to SFAs. It is also important to note the number of double bonds which are found within the aliphatic tail. Monounsaturated fatty acids (MUFAs) have one double bond in the carbon backbone. Polyunsaturated fatty acids (PUFAs) have more than one double bond in the carbon chain backbone. The placement of the double bond in PUFAs is also important for identifying different kinds of PUFAs. If the double bond is placed on the third from the last carbon on the carbon chain, as in α-linolenic acid (ALA), this is known as the omega-3 position (also denoted as n-3 or ϖ-3) (Figure 2). Conversely, if the first double bond is found on the sixth carbon, as in linoleic acid (LA) (Figure 2), this is known as omega-6 (also

denoted as n-6 or ω-6). These differences create different physiological and/or pathophysiological effects within the body[4].

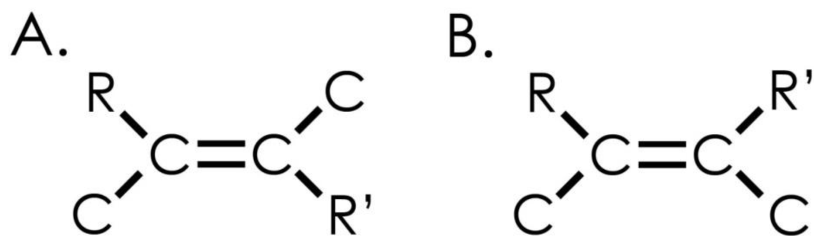


Figure 1: Difference in Trans (A) and Cis (B) Conformations in organic compounds

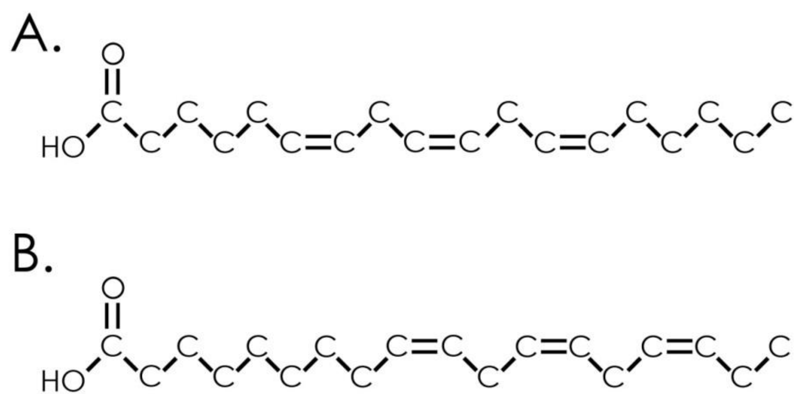


Figure 2: Structural difference between LA (A) and ALA (B)

Fatty acids can be obtained from dietary sources or biosynthesized within the body. Biosynthesis of fatty acids within the body is accomplished by freeing fatty acids from sugar. This process is carried out by particular enzymes known as fatty acid synthases (FAS). Briefly, with acetyl CoA being the input precursor, formation of manolyl CoA is carried out by a commitment step; malonate is then formed by adding CO_2 to acetyl CoA. Biotin is used as a cofactor for the enzyme acetyl CoA carboxylase. Elongation promptly follows this step using FAS. This is a complicated, multi-enzyme process. In the end, fatty acids can be synthesized by this process to support the metabolism and healthy function of cells [4].

iii) *Dietary Sources: An Overview*

Essential fatty acids are a designation to fatty acids which can only be found in dietary sources and required for adequate function of the human body. In short, these fatty acids cannot be made by the human body. It is important to note that there are two major sources of essential fatty acids-ALA and LA. ALA is an n-3 PUFA found in a variety of food items such as nuts and seeds. However, it is most prevalent in flaxseed. LA is an n-6 PUFA abundant in safflower, poppyseed and rapeseed oil [5]. These two fatty acids serve in a variety of cell maintenance functions (*see Introduction Section 3, V*). It is important to note that fatty acids (essential or nonessential) can be found in a variety of dietary sources. Depending upon the saturation, different fatty acids are found in different foods. For example, MUFAs are found in different nuts such as almonds and also found in avocados. PUFAs are generally found in fish, flaxseed and grape seed oils. SFAs are found in palm, coconut and cotton seed oils as well as chocolate products [6]. Trans fats (TFAs) are found in industrially produced foods such as margarines but also may be found naturally in dairy products. Each of these fatty acids has a variety of bioactive

effects within the body [7]. However, further research into some of these fatty acids to better elucidate their effects within the body and more specifically, their mechanism of action, will aid in the ongoing research within the field of nutraceuticals and functional foods [1, 8-12]. This is particularly relevant for both the TFA and PUFA categories.

II) Trans Fats

i) Dietary Sources

TFAs were originally thought to be a part of one particular category; industrially produced (iTFAs). However, TFAs are found in natural products as well. These are commonly known as ruminant trans fats or rTFAs [13].

ii) Industrially Produced Trans Fats

The creation of TFAs began within the invention of the process of hydrogenation, a process created by the Nobel Laureate Paul Sabietier in the 1890s. The process of hydrogenation requires the addition of hydrogen atoms to a particular organic compound. If scientists bubble hydrogen gas through vegetable oils in the presence of a nickel catalyst, this will successfully reduce and saturate the fatty acids within the oil. In this way, PUFAs can be converted to MUFAs or SFAs [14]. However, while Sabitier largely focused on the effects of hydrogenation in vapours, Wilhelm Norman took the procedure and was able to patent it in oils. In this way, he successfully caused liquid oils to become solids which lead to the creation of shortenings, butters and margarines [6]. Margarines are created by a process known as partial hydrogenation. During partial hydrogenation, some of the *cis* isomers found in the aliphatic chain become *trans* isomers

as the hydrogenation process leaves the double bond intact. Therefore, the aliphatic tail is no longer kinked causing distinct conformational changes. Partial hydrogenation is mainly used to remove unstable fatty acids. Mono- and polyunsaturated fatty acids are generally unstable. However, when partially hydrogenated, they are no longer unstable. In this way, one can increase the shelf life of partially hydrogenated products such as margarine. The process of partial hydrogenation is a hallmark identifier for iTFAs. Therefore, fried foods, fast foods, pastries, margarines, shortenings, cake mixes and of course, many frozen dinners and packaged foods will contain industrially produced trans fats. iTFAs are found primarily as elaidic acid (EA) (18:1 trans-9). Oleic acid (18:1) is a naturally occurring fatty acid found in many vegetable oils. Upon partial hydrogenation, it becomes elaidic acid and changes conformation. Although EA is the primary form of iTFAs; 18:2, 18:3 and 16:2 can also be found in *trans* form in industrially produced food products. North Americans consume between 5-10g/day of iTFAs daily which constitutes approximately 2-5% of total energy within our diets [9]. However, some studies suggest that some individuals consume up to 20g/day iTFAs [1] [15].

iii) <u>*Ruminant Trans Fats*</u>

rTFAs are often overlooked due to the abundance of literature focusing on the effects of industrially produced trans fats in different diseases. However, naturally produced trans fats make up 3-8% of dietary sources of trans fats. rTFAs are found in animals such as grass grazing sheep and cattle. Therefore, sheep and cattle meats as well as dairy products (cheese, milk and butter) contain rTFAs. The major contributor within the category of rTFAs is known as vaccenic acid. Vaccenic acid (VA) (18:1 trans-11) is derived from ruminant fats and constitutes 50-80% of all ruminant derived trans fats [15]. Conjugated linoleic acids (cLAs) consist of the rest of the

category. Briefly, cLAs are also found in ruminant products and are identified by two double bonds within the aliphatic chain that are separated by a single bond in between. Rumenic acid (c9t11-cLA) is an example of conjugated linoleic acids. VA on the other hand, consists of only one double bond. It is the isomer of oleic acid (18:1 t-11) and although similar to EA, the position of the double bond in VA (position 11 vs. position 9 in EA) plays an integral role in differences between the two fatty acids. VA is derived from incomplete biohydrogenation of the PUFAs, linoleic acid and linolenic acid, within the gut of the ruminant animals. Specialized gut microflora known as *Roseburia hominis A2-183T*, *Roseburia inulinivorans A2-192T* and *Ruminococcus obeum-like strain A2-162* have been identified to produce VA from these sources. As a result, this naturally produced trans-fat becomes present in ruminant derived foods. Although typically we consume only 2-9% of our total fatty acid content as rTFAs, the TRANSFAIR study approximates that up to 50% of all trans fats consumed in Mediterranean diets will be constituted as rTFAs [15]. It is therefore, imperative, that we understand the differences between iTFAs and rTFAs in terms of their detrimental or positive health benefits.

III) Trans Fatty Acids in Disease Models

i) Obesity, Cancer and Trans Fat Intake

Obesity is becoming an international epidemic. It is estimated that by 2015, 700 million people will suffer from obesity while an additional 2.3 billion people will be classified as overweight [14, 16]. Diet has a long standing relationship with obesity. Specifically, dietary fats have been linked to an increased or decreased risk of obesity development. Recently, TFAs have been the target of this epidemic. Obesity is dependent upon weight gain, increased adipose tissue

mass, inflammation and secondary factors such as type 2 diabetes. Clinically, it is not well understood if there is a clear correlation between weight gain and iTFA intake. However, studies in green monkeys have observed that a diet which was supplemented up to 8% of total energy as iTFA (18:1) increased weight gain compared to animals fed 8% of total energy as cis-MUFAs (18:1) [17]. This is particularly important since most weight gain was attributed to visceral (abdominal) fat, a major contributor to increased cardiovascular disease and insulin resistance associated with obesity and a major contributor to obesity. As discussed in *Section 3, IV* iTFAs induce an increase in pro-inflammatory markers such as tumor necrosis factor (TNF)-α. Conversely, emerging evidence suggests rTFAs decrease nuclear factor *kappa*-light-chain-enhancer of activated *B* (NF-κB) and TNF-α production suggesting a divergent effect of the two types of TFAs with respect to inflammation. Many animal studies also suggest that TFAs influence type 2 diabetes risk by increasing levels of triglycerides, decreasing glucose uptake in skeletal and cardiac tissue and lastly, decreasing hypothalamic insulin receptor expression [18]. This evidence further amplifies a non-beneficial role of iTFA in insulin resistance and obesity. However, data has not yet emerged on the role of rTFAs in insulin resistance. Therefore, further study in this area is necessary.

Cancer is one of the leading causes of death in North America. The Canadian Cancer Society reports that two out of five Canadians will develop cancer within their lifetime and one in four Canadians are expected to die from cancer [19]. Diet has been hypothesized to increase cancer risk [19] [20]. Cancer and inflammation have also been linked [21]. There is a clear correlation between iTFAs and inflammation (*See Introduction Section 3, IV, iii*), it is possible that iTFAs have the capacity to affect certain cancers. This may be particularly relevant to colon and prostate cancers. In epidemiological studies, four out of six studies found a positive

correlation between iTFA intake and prostate cancer [22] iTFA intake was also positively associated with colon and breast cancers [22]. However, the effect of rTFAs in cancer is still unclear. Four epidemiological studies [23] [22] [15] have examined the relationship of cLA or VA consumption with cancer risk. Of these four, three have identified a positive correlation between VA consumption and cancer risk [15] [22]. However, *in vitro* studies have demonstrated that VA may decrease tumor growth and metabolism [15]. Animal studies have further identified that VA may decrease tumor metabolism by inhibiting fatty acid uptake and decreasing cyclic-AMP (cAMP) as well as p44/42 c-Jun-N-terminal kinase (JNK) activity [24]. It is possible, therefore, that the positive results obtained from the epidemiological studies may have been influenced by an intake of other components within the diet besides rTFAs that may have enhanced the progression of the cancer. Taken together, these studies suggest that more evidence is required to confirm the role of VA in cancer progression. Similarly, cLA has shown a capacity to enhance [22] and decrease [22] tumor progression and cancer risk. Therefore, in different disease models, rTFAs and iTFAs have different effects on obesity or cancer risk. These results stress the importance of continued research on the effects of different TFAs in disease models.

IV) Polyunsaturated Fatty Acids

i) *Omega-6 and Omega-9 Fatty Acids*

LA is the shortest chain omega-6 fatty acid available from dietary sources. This fatty acid is essential and can be converted into arachidonic acid (AA) which has important biological functions including the synthesis of phospholipids or its use as a secondary messenger for key signaling pathways. There is evidence, however, suggesting that over-consumption of n-6

PUFAs may lead to increased inflammation within the body (*Section 3, V, iii)*. Unlike n-6 fatty acids, n-9 fatty acids are primarily found in foods such as olive oil, rapeseed oil, mustard seeds/oil and wallflower seeds. Omega-9 fatty acids are usually found in two forms, oleic acid (18:1 n-9) and erucic acid (22:1 n-9). Both of these fatty acids have important biological functions. For example oleic acid, found in olive oil, has been shown to decrease inflammation [6]. Erucic acid is affluent in colewart or kale. Early studies suggest no severe effects of eruicic acid on the heart however, some evidence may correlate high intake of euricic acid with complications within the heart. Therefore, euricic acid intake should be limited to 2% of total energy intake [25].

ii) *Omega-3 Fatty Acids*

Omega-3 fatty acids are found in a variety of sources. Marine oils contain two major n-3 fatty acids. Eicosapentaenoic acid (EPA) and docosahexanoeic acid (DHA) have been shown to have important anti-inflammatory and early developmental functions within the body [26]. Unlike ALA, DHA and EPA can be derived within the body through different enzymes known as desaturases. ALA is an essential fatty acid and can be a precursor for DHA and EPA. However, ALA is not completely converted to both EPA and DHA [27] (See Figure 3). ALA is a plant derived n-3 PUFA and can be found in walnuts, sunflower seed and flaxseed. ALA has important biological actions within the heart [10, 28]. However, a mechanism of action for its beneficial effects remains largely unknown.

There has been a great deal of interest in two distinct categories of PUFAs on cardiovascular disease, the n-6 and n-3 fatty acids [29]. The position of the double bond, either in the 3rd or 6th position will distinguish an n-6 fatty acid from an n-3 fatty acid. Within the cell, these ‘essential’ fatty acids are metabolized and converted into physiologically recognized compounds [30].

Short chain n-3 fatty acids like ALA can be converted into two longer chain derivatives (DHA and EPA), primarily by the 5- and 6- desaturase enzymes. DHA is derived from EPA by β-oxidation. n-6 fatty acids such as linoleic acid (LA) are converted into AA using the 5- and 6-desaturases similar to n-3 fatty acids. A higher ratio of n-6:n-3 intake in the diet will shift the desaturase action to favour the production of AA [30].

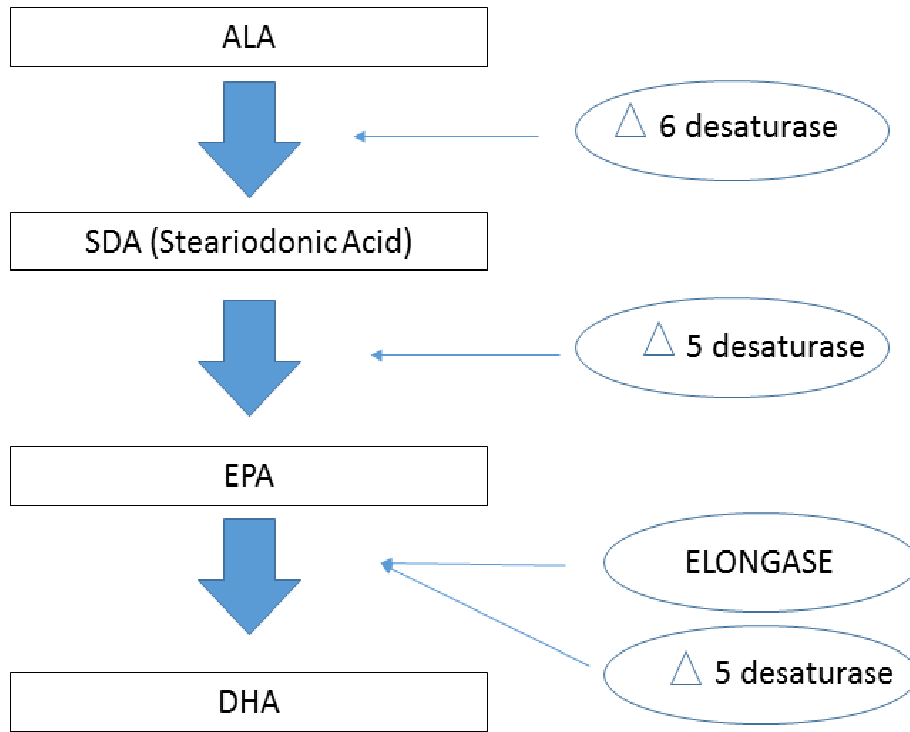


Figure 3: Metabolism of ALA into EPA and DHA by specific enzymes

V) Introduction to Flaxseed

Flaxseed (*Linum usitatissimum*) is one of the oldest crops in the world. It is a member of the genus *Linum* in the family Linaceae. It is a food and fibre crop that is grown in cooler regions of the world [11].

i) Omega-3 Content in Flaxseeds

Flaxseed contains one of the highest levels of the essential omega-3 fatty acid, α linolenic acid (ALA), than any other plant. Up to 57% of the seed is made up of omega-3 fatty acid under normal conditions. This can be increased to over 70% with genetic manipulation [2]. Once within the body, part of ALA can be converted into metabolic by-products known as DHA and EPA. However, the conversion is not 100% and ALA will also remain within the circulation upon ingestion. Desaturase enzymes play an important role in the conversion of ALA to DHA and EPA. ALA also has other by-products known as eicosanoids. Eicosanoids are metabolic derivatives of EPA, DHA and ALA and have been shown to affect cell viability, hypertension and inflammatory markers [31, 32]. Both n-3 and n-6 fatty acids can be converted into eicosanoids. Eicosanoids and their function are discussed further in the *Section 3, V*.

ii) Fibre Content in Flaxseed

Fibre is the other contributor to the effects of flaxseed in disease models. Fibre plays an important role in lowering plasma cholesterol levels [33]. The average recommended daily consumption for fibre is approximately 14 grams [33], and depending on your gender and age more or less fibre may be recommended ranging from 20-30 g/day [33]. Fibre can not only lower blood cholesterol, but is also important in boosting immunity and stool regularity [33]. About ¼

cup of flaxseed daily can contribute 7g of fibre per day. This amount will contribute ½ of the recommended daily intake of fibre [34].

iii) *Lignans*

Lignans are known to be polyphenolic, that is, a molecule containing two or more phenolic hydroxy groups [35]. Lignans are also phytoestrogens which are estrogen-like compounds and potent antioxidants [35]. Lignans are primarily found in plant products [35]. Crops such as barley, wheat and soy are examples of plants which have lignans [35]. Compared to other plant products, flaxseed contains some of the highest amounts of lignans [35]. The primary lignan found in flaxseed is known as secoisolariciresinol diglucoside (SDG) [34]. Within the body of mammals, lignans can be metabolized to the lignans pinoresinol, lariciresinol, secoisolariciresinol, matairesinol, hydroxymatairesinol, syringaresinol, sesamin, enterolactone and enterodiol [34]. It is still unclear of the exact physiological action of lignans; however, SDG decreases oxidative stress in streptozotocin (STZ)-induced diabetic rats [36], reduces tumor production in MCF-7 cells (breast cancer cell lines) [37] and decreases hypertension [38]. However, it is important to recognize that SDG does not enter the blood stream so the physiological importance of these data is questionable.

VI) Polyunsaturated Fatty Acids in Disease Models

i) Polyunsaturated Fatty acids in Obesity and Cancer

Although there is a clear association between fat intake and obesity [16], it is important to recognize that the type of fat ingested may influence obesity risk. This is particularly important

with respect to n-3 fatty acids. For example, n-3 fatty acids may have a protective effect in cardiovascular diseases (*See Introduction Section VII*). However, epidemiological studies remain unclear whether n-3 PUFA intake decreases weight gain and abdominal fat. Although some studies have indicated a decrease in body fat with up to 6 g/day EPA and DHA consumption [39], other large prospective health studies such as the Nurses' Health Study observe an increased prevalence of obesity with high fish intake and n-3 polyunsaturated fatty acid intake [40]. Taken together, these results cannot define a clear role of n-3 fatty acids in the prevalence of obesity; however, published data indicate a decrease in obesity risk with n-3 fatty acid consumption. As well, most studies have solely analyzed the effect of n-3 fatty acids EPA and DHA on weight gain.

Conversely, n-6 PUFA may be pro-adipogenic. Rat and mice work suggests that maternal intake of linoleic acid predisposes foetus exposure to arachidonic acid, the metabolic bi-product of LA. Increased exposure of the foetus to AA has been associated with increased adipocyte production suggesting increased maternal LA intake may predispose an individual to increased adipocyte production [41, 42]. Furthermore, studies conducted by Massiera et al. (2010) suggest that when rat diets were supplemented with 55% linoleic acid as a % of the total lipid fraction (19% total energy) for four generations, there was a progressive weight gain in each of four generations [43]. This evidence suggests that the early development of obesity may be correlated to an increased n-6 fatty acid consumption. Taken together, these studies suggest that n-3 and n-6 PUFAs have differential effects on obesity. However, more study is necessary to fully understand this phenomenon.

The western diet is characterized by a high intake of n-6 PUFAs and a lesser intake of n-3 PUFAs [43]. This imbalance of n-6:n-3 PUFAs has been suggested to increase the risk of

diseases such as cancer [44, 45]. Several clinical studies have suggested that the increased consumption of n-3 PUFAs will decrease breast cancer risk in several populations [46]. Specifically, two major studies, the Shanghai Women's Health Study and in a follow up to a pancreatic cancer study known as NIH-AARP, a significant negative interaction between marine derived n-3 fatty acids and breast cancer risk were observed [46]. Interestingly, the Shanghai Women's Study also observed that n-6 PUFA intake increased breast cancer risk. Cancer risk has also been correlated with inflammation. It is tempting to speculate that these results may be influenced by the effect of n-6 and n-3 PUFAs on inflammation (*see Introduction Section 3, V, iii)* In support of such a hypothesis, *in vitro* and *in vivo* evidence suggest that n-6 fatty acids may increase pro-inflammatory markers whereas n-3 fatty acids decrease these markers. These data lend further credence to the possibility that the n-6:n-3 ratio may influence cancer risk. However, several studies including the Multiethnic Cohort Trial observed no specific association between PUFA intake and breast cancer risk. Furthermore, clinical evidence has shown that flaxseed derived n-3 fatty acid ALA had no effect on colorectal cancer [46]. Paradoxically, *in vitro* studies have shown that ALA supplementation increases apoptosis in hepatoma cells. Furthermore, tumorigenesis induced in the nude mouse model implanted with MDA-MB 231 cells were successfully reduced with walnut supplementation (a significant source of ALA) [46]. Further research is clearly necessary to fully appreciate the effects of PUFAs in cancer. Therefore ongoing research within this area is still warranted.

SECTION 2: FATTY ACID UPTAKE AND METABOLISM

I) <u>Fatty Acid Uptake</u>

Dietary fats have long been associated with their effects on cellular metabolism and disease. Dietary fats currently make up to 30-40% of our diet [47]. Much of this fat is ingested as long chain fatty acids (LCFA). LCFA can be esterified into a triacylglycerol form. Key enzymes known as lingual or pancreatic lipases will metabolize and recognize LCFA and break LCFA into monoglycerols which can be recognized by intestinal cells (such as ileal and jejunal enterocytes). These monoglycerols and FFAs can become re-packaged into triacylglycerols, chylomicrons, or incorporated into other lipids or lipid soluble vitamins. These are secreted into the systemic circulation. Once released into the circulation, fatty acids can be stored within adipose tissue cells (also known as adipocytes). In order to be used for metabolism, FFAs are released by adipocytes using specific enzymes known as hormone sensitive lipases (HSL) or adipose tissue triacylglycerol lipases (ATGL) [47].

Fatty acid uptake across the plasma membrane relies on passive diffusion in parenchymal cells [48]. This is limited by plasma concentrations of the fatty acid and the rate of blood flow [47, 48]. In target tissue, however, a regulated mechanism is necessary to best suit the needs of the tissue. This is particularly relevant in the case of skeletal muscle and heart tissue. In these muscle tissues, the rate of metabolism is dictated by the amount of work done by the tissue [49]. For example, during exercise, more fatty acid is necessary for metabolism. However, in times of rest, this is no longer the case. Regulation of other metabolites, such as glucose, is facilitated by specific glucose transport proteins or GLUTs. GLUTs move to the plasma membrane when glucose is present and regulate glucose entry into the cell according to the needs of the cell or tissue. Recently, a similar pathway was proposed for fatty acid uptake into the cell [50]. However, a clear delineation of the direct mechanism of fatty acid uptake into the cell has only recently undergone investigation. In the following section, the mechanism of fatty acid uptake

and subsequent effects on cellular function and disease states will be considered together with an analysis of how different fatty acids affect target tissues such as the heart.

i) Mechanism of Fatty Acid Uptake into Cells

There are various mechanisms proposed for the uptake of fatty acids into the cell. Specific transport proteins have been identified that participate in the uptake of fatty acids into cells. However, it should be recognized that other modes of transportation have been identified including passive (simple) diffusion of the fatty acids into the cells.

ii) Fatty Acid Transport Proteins

Schaffer and Lodish (1994) [51] discovered a novel protein that enhanced fluorescently tagged fatty acid uptake. It was subsequently cloned and expressed in COS7 cells. These data were used to identify a 646 amino acid protein known as fatty acid transport protein or FATP [51]. FATP1 is an integral membrane protein with six membrane spanning regions. The discovery of FATP1 prompted other studies which have revealed the existence of five other FATPs which are known as FATP2, FATP3, FATP4, FATP5 and FATP6. These FATP isoforms are also integral membrane proteins and have unique tissue specific distribution patterns (Table 1) [47].

Table 1. The Tissue Distribution and Molecular Size of Proteins Identified that Participate in the Transport of Fatty Acid Molecules into Cells (Adapted from [47])

Fatty Acid Transport Protein (FATP)	Molecular Weight (kDa)	Tissue Distribution
FATP1	63	Adipose Tissue, Lung, Heart, Brain, Kidney, Skin
FATP2	63	Kidney, Liver, Intestine
FATP3	63	Lung, Liver, Testis, Skin
FATP4	63	Testis, Skin, Lung, Liver, Heart, Skeletal, Brain
FATP5	63	Liver
FATP6	63	Heart, Skeletal Muscle, Placenta, Testis, Adrenal Gland, Kidney, Bladder, Uterus, Skin
Plasma Membrane Fatty Acid Binding Protein (FABPpm)	40-43	Liver, Heart, Adipose Tissue, Intestine Placenta
Fatty Acid Translocase/CD36	88 (glycosylated) or 55 (unglycosylated)	Heart, intestine, skeletal muscle, adipose tissue,spleen, platelets, monocyte/macrophage, endothelium, epidermis, kidney, brain, liver
Caveolin-1	21-24	Ubiquitously expressed except in skeletal muscle and heart where caveolin-1 is the major form

Early studies in yeast models delineated a specific effect of FATPs in fatty acid uptake. Specifically, expression of FATP1, -4 and -2 were able to increase LCFA uptake into yeast [52]. Modest increases were observed with the expression of FATP3 and -5 and little uptake occurred with FATP-6 expression [52]. However, the tissue specific effects of FATPs were still not well understood. In cultured cell models, FATP4 was not observed and it was concluded that FATP4 did not exist in the plasma membrane [53]. Initially, FATP4 was not considered a fatty acid transport protein at all [53]. However, rat skeletal muscle expressed both FATP4 and FATP1 [54]. It was indicated that FATP4 is more effective than FATP1 in increasing fatty acid transport. In 3T3L1 cells, FATP1 knockdown decreased basal fatty acid uptake where FATP4 overexpression did not change basal fatty acid uptake [55]. Based on these examples, FATP expression is tissue and model specific. FATP function is also fatty acid specific. In HEK293 cells, FATP6 transported palmitate better than FATP1 or 4, whereas FATP4 facilitated oleate transport better than FATP1 or 6. In the heart, FATP6 has a relatively high expression pattern [47] However, it is best suited for short chain fatty acid (SCFA) transport [47]. Therefore, FATPs are both tissue and fatty acid specific.

iii) Plasma Membrane Fatty Acid Binding Protein (FABPpm)

Plasma membrane fatty acid binding proteins (FABPpm) have been identified in major tissues such as adipocytes, cardiomyocytes, liver cells and intestinal cells [56]. Fatty acid uptake was inhibited in adipocytes, liver cells and adipocytes using antibodies directed towards FABPpm, as well as in cardiac and skeletal muscle-derived giant vesicles. Fifty to seventy percent inhibition was observed in all of these tissues. However, increases in FABPpm protein content through overexpression only modestly increased fatty acid uptake [56]. Based on these

observations, it is clear that FABPpm is not solely responsible for fatty acid uptake and other mechanisms/transport proteins are involved.

iv) Fatty Acid Translocase (FAT)/CD36

Fatty acid translocase (FAT) was identified as an integral membrane protein identical to the leukocyte cluster of differentiation antigen (CD36) [54]. CD36 is also identified as a class B scavenger protein with multiple functions. These functions include binding to thrombospondin, oxidized LDL and anionic phospholipids [57, 58]. The identification of CD36 occurred through specific inhibition with sulfonyl-N-succidimide (SSO). This inhibition directly affected fatty acid uptake in rat adipocytes [59, 60]. CD36 has two transmembrane spanning regions, both NH2 and COOH termini and short segments within the cytoplasm [47]. CD36 is heavily glycosylated with ten predicted N-glycosylation sites in the large extracellular loop [61]. Extensive glycosylation increases the predicted mass of CD36 from 55 kDa to 88 kDa [62]. Other sites of potential modification of its activity include three phosphorylation sites, four palmitoylation sites and two ubiquitination sites at the COOH termini [63]. Caveolins may play an important role in targeting CD36 to the plasma membrane, however, glycosylation of CD36 has no effect on membrane expression [64]. Palmitolylation of the COOH termini may also result in CD36 recruitment to specific membrane microdomains [65]. CD36 is ubiquitously expressed in different tissues (Table 1). In adipocytes, CD36 binds LCFAs but not SCFAs [47]. CD36 has been implicated in VLCFA uptake in cultured cells and in the intestinal absorption of VLCFAs in mice [66]. Transfection of CD36 into fibroblasts augments fatty acid uptake [67]. CD36 has a variety of distinct effects in different cells including a specific role as a class B scavenger receptor [68].

The different glycosylation sites on CD36 may promote the different physiologic actions of CD36 [47].

Tissue specific effects have been observed in CD36 null mice. In this model, a reduction in basal fatty acid uptake with fatty acid analogs (5-(*p*-iodophenyl)-3-(*R*,*S*)-methyl pentadecanoic acid (BMIPP) and 15-(*p*-iodophenyl)pentadecanoic acid (IPPA)) were observed in heart, skeletal muscle and adipose tissue. However, little to no effects were observed in the liver [69]. This is not surprising as CD36 is absent or has little expression in this tissue. A decrease in palmitate uptake was observed in CD36 null skeletal muscle but not in CD36 null heart myocytes [49]. This may be as a result of FATP1 overexpression within the myocytes to compensate for CD36 deficits. CD36 null mice did, however, show a 25% decrease in fatty acid oxidation [49, 69]. It can be concluded on the basis of these studies that there is a different expression pattern for CD36 in different tissues and there are different downstream effects with the CD36 knockout model.

II) <u>Fatty Acid Metabolism</u>

i) Mechanisms

One of the major physiological roles of fatty acids in the body is to serve as a source of energy. The primary energy currency within the cell is adenosine triphosphate (ATP). Once fatty acids are taken up by the cell through transport proteins and/or passive diffusion, they can then be converted into energy [4]. This process is known as β oxidation. However, fatty acids also play a role in energy storage, phospholipid membrane formation and signaling pathways. Therefore, fatty acid metabolism can be catabolic with respect to energy and metabolite

generation or anabolic for creating biologically important molecules from dietary sources of fatty acids.

ii) β Oxidation

Once the fatty acid is taken up within the cell, a CoA group is added to the fatty acid using the enzyme fatty acid CoA synthase (FACS). This forms long chain acyl-CoA. Carnitine palmitoyltransferase 1 (CPT1) converts long chain acyl-CoA to long chain acylcarnitine. This allows the fatty acid moiety to be transported into the inner mitochondrial membrane via carnitine translocase. Inner membrane protein CPT2 then converts long chain acylcarnitine back to long chain acyl-CoA inside the mitochondria. The long chain acyl-CoA then enters the β oxidation pathway. Fatty acid β oxidation consists of breaking down the long chain acyl-CoA molecule into acetyl-CoA. Four main enzymes are used in the process to convert acyl-CoA to acetyl-CoA. The number of acetyl-CoA molecules is dependent upon the carbon chain length in the fatty acid. Acetyl-CoA can then enter the citric acid cycle and electron transport chain to yield ATP [4, 70].

iii) Fatty Acids and Cellular Function

Other than β oxidation, fatty acids can also be used in other key processes within the cell. Of note, polyunsaturated fatty acids are integrated into phospholipids within the cell membrane. Phospholipids (or glycerophospholipids) consist of a glycerol-3-phosphate molecule esterified at its carbon 1 (*sn*-1) and carbon 2 (*sn*-2) positions to non-polar fatty acids and at its phosphoryl group to a polar head group [71]. PUFAs are preferentially incorporated into the sn-2 position of phospholipids. On demand, specific PUFAs such as AA or EPA can be cleaved from the

phospholipid by phospholipase A_2 (PLA_2) [72]. Once cleaved, PUFAs such as EPA and AA can undergo enzymatic oxygenation and are transformed into short term lipid mediators [72]. Lipoxygenases and cyclooxygenases mediate this process. The by-products of this process include leukotrienes, prostanoids and expoxyPUFAs. Oxygenated PUFAs mediate different functions within the cell including cellular homeostasis, apoptosis and metabolic activity through activation of key transcription factors such as PPAR-γ [73]. Oxygenated PUFAs can also become potent bioactive models known as eicosanoid (Figure 4).

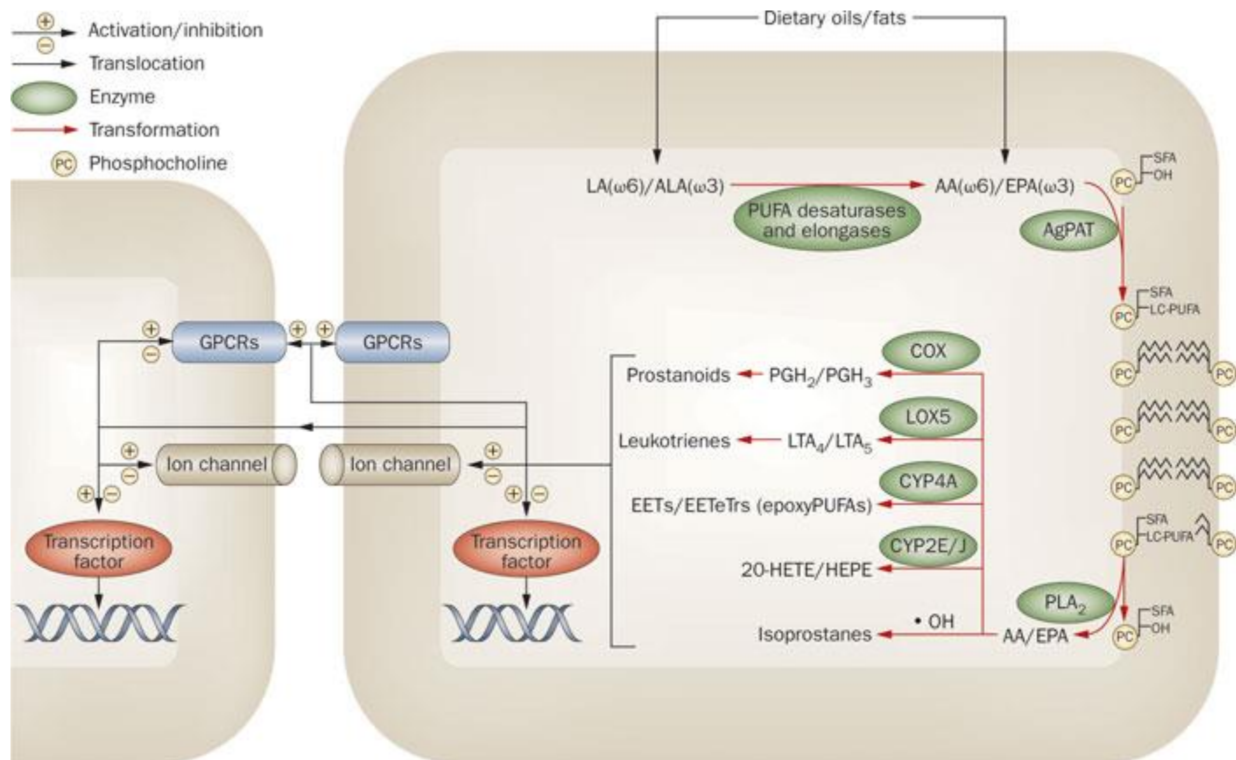


Figure 4: Long-chain PUFAs are incorporated into phospholipids and serve as oxygenated PUFA precursors. Reprinted with permission from Nature Publishing Group. Shapiro, H. *et al.* (2010) Effects of polyunsaturated fatty acid consumption in diabetic nephropathy. *Nat. Rev. Nephrol.* doi:10.1038/nrneph.2010.156

iv) Diseases and Fatty Acid Uptake

Diseases can either cause changes in fatty acid uptake or its oxidation, or, conversely, diseases can be caused by dysfunction of the fatty acid uptake/oxidation processes. For example, insulin resistance in the heart and skeletal muscle is associated with a reduced response to increased circulating free fatty acids [50]. This is a common occurrence in insulin resistant conditions such as obesity and type 2 diabetes [49, 50]. When the uptake of fatty acids exceeds the rate of β-oxidation, intramuscular lipids can accumulate. These can have subsequent deleterious effects on insulin action [49]. Several investigations have suggested that limiting fatty acid uptake pathways, such as through an inhibition of CD36, prevents lipid accumulation resulting from insulin resistance or non-alcoholic fatty liver disease (NAFLD) [74]. Conversely, CD36 plays an important role in the selection of the energy source in skeletal muscle cells, specifically during the adaptation of exercise performance to fatty acid oxidation. Taken together, these results suggest that while CD36 knock-out may play a beneficial role in limiting disease progression (such as type 2 diabetes) it is still important for basal metabolic function. Similarly, FATP1 deletion protects against high fat induced obesity in mice deleted of FATP1 expression [75]. Conversely, FATP1 expression is important in cold adaptation [76]. FATP1 knockout mice had difficulty with non-shivering thermogenesis since FATP1 is highly expressed within brown adipose tissue (BAT) [76]. With either FATP1 or CD36, it is important to understand the effects of knockout or overexpression under basal and disease conditions. Understanding the role of the proteins and pathways involved in fatty acid uptake and the role that changes in metabolism may play is particularly important in diseases of the heart. Analyzing fatty acid uptake and oxidation patterns are critical to a better understanding of the progression of cardiac diseases.

III) Role of Transport Proteins in the Heart

The transport proteins expressed within the heart are FATP1, 4, 6 and CD36 (Table 1). Caveolin-1 and FABPpm are also expressed in the heart (Table 1). Deletion of FATP1 has no effect on LCFA uptake within the heart under basal conditions [77] however, FATP1 is ubiquitously expressed and may have a role in trans-fat uptake within the heart. This has not been investigated. When cell lines transfected with human FATP4 were administered high bolus fatty acids to the media, FATP4 inhibitors had no significant effect of fatty acid uptake [77]. FATP4 may not have a significant role in LCFA uptake in most cell lines. FATP6 is highly expressed within the heart. FATP6 is localized in the sarcolemma of cardiomyocytes adjacent to small blood vessels [49, 77]. However, reports regarding the role of FATP6 remain conflicted. While some demonstrate that [49] FATP6 can participate in the increased LCFA uptake within the heart [77] when FATP6 is overexpressed, others suggest FATP6 only increases SCFA uptake [49]. Furthermore, FATP6 has been implicated in increased acyl-CoA synthase activity [49].

CD36 is also highly expressed within the heart [69]. CD36 has been implicated in LCFA uptake within the heart. Deletion of CD36 decreases fatty acid uptake and oxidation by 50-60% within the heart [49].

IV) Role of Fatty Acid Metabolism in the Heart

Fatty acid metabolism plays an important role in the heart. The continuous pumping action of the heart exhibits a high energy demand. ATP is the ultimate source of this energy. In order to generate ATP at a high rate to sustain cardiac contractility, almost all ATP (~95%) is derived from mitochondrial oxidative phosphorylation [49]. The remainder is derived from glycolysis within the tricarboxylic acid (TCA) cycle. β oxidation within the heart is under a complex

control. The factors which affect the cycle include the supply of fatty acid, the presence of competing substrates, the energy demand of the heart and the supply of oxygen to the heart [49]. Fatty acids can be recognized by the heart as free fatty acids (FFA), triacylglycerol (TAG) or very low density lipoproteins (VLDL). In the case of VLDL, the enzyme lipoprotein lipase (LPL) can hydrolyze TAG and VLDL within cardiomyocytes. Regulation of LPL plays a significant role in fatty acid metabolism within the heart. Increased LPL translocation to the plasma membrane has been correlated with increased β oxidation [78]. Mitochondrial uptake of fatty acids is regulated by carnitine palmitoyltransferase (CPT1). The enzyme CPT1 catalyzes and converts long chain acyl-CoA into long chain acyl-carnitine. This facilitates uptake into the mitochondria and will have distinct effects on rates of β oxidation. Changes in fatty acid metabolism within the heart have been implicated in a variety of complex cardiovascular diseases.

V) Role of Fatty Acids in Heart Disease

Changes in different proteins involved in metabolism and changes within substrate utilization may occur depending on the type of cardiovascular disease. During cardiac hypertrophy, LCFA uptake is decreased and slowly replaced by glucose utilization. Long term use of agents such as sulfo-*N*-succimidyl-palmitate (SSO) that inhibit FA uptake through CD36 increase the risk of cardiac hypertrophy in rats [69]. In contrast, the diabetic heart switches nearly completely to fatty acid utilization while glucose utilization is severely decreased [79]. In animal models of type 2 diabetes, LCFA supplemented diets increased the plasma content of CD36 but not FABPpm in cardiomyocytes. Treatment with the inhibitor SSO decreased LCFA uptake and the subsequent accumulation of lipids in the heart. CD36, therefore, plays an important role in

increasing LCFA uptake into the heart and increasing lipid accumulation when β oxidation rates do not meet the energy needs of the heart [49]. CD36 inhibition is also associated with an increased incidence of stroke and increased left ventricular mass. [80]. CD36 may also have an involvement in atherosclerosis since CD36 is a class B scavenger protein and associated with macrophage infiltration. CD36 inhibition also leads to complications in the heart such as development of hypertrophy [81]. Taken together, these studies suggest a clear role of CD36 in cardiovascular disease progression.

Functional changes in the β oxidation biochemical pathway have also been observed in disease states. For example, decreased β oxidation is observed in models of heart failure [82]. Using a canine model of tachycardia, a progressive fall in β oxidation was observed [49]. Decreases of β oxidation enzymes were also observed in models of coronary artery ligation or aortic constriction. In either model, transcriptional defects of β oxidation enzymes were observed. After 6 months of coronary artery ligation in rats, there was a progressive decrease in cardiac palmitate oxidation [83]. Some of these results correlated with distinct changes in the fatty acid oxidation pathway. Specifically, CPT2 activity was down regulated in dogs with end stage tachycardia [49]. Researchers hypothesized that PPAR-α may be a key transcription factor that regulates enzyme production for β oxidation. Targeting the PPAR-ligands is currently an area of research hypothesized to improve metabolic changes that occur during heart failure. Specifically, activation of PPAR-α/γ correlates with improved β oxidation in both adipose tissue and skeletal muscle [84]. Nicotinic acid is another current strategy that could improve shifts in metabolism during heart disease. Nicotinic acid decreases fatty acid lipolysis in the adipose tissue, therefore, decreasing FFA availability within the heart. Inhibitors of CPTs are also being investigated to improve cardiac function. In the 1970s, the CPT1 inhibitor prehexiline was

prescribed to decrease angina however, negative effects were observed within the liver. Prehexiline continues to show anti-ischemic effects by correcting imbalances of glucose vs. fatty acid metabolism in the heart. However, due to its toxicity in the liver, it is not prescribed today. Therapies that limit fatty acid uptake and improve fatty acid vs. glucose metabolism are currently being investigated [49].

VI) Metabolism of PUFAs and TFAs in the Heart

To date, little is known or understood with respect to PUFA or TFA uptake. PUFAs can directly incorporate into phospholipids to become cleaved by PLA_2 and subsequently used in cellular processes. PUFAs may use a transport protein or cross into the cell by passive diffusion. However, to date, studies have not investigated this process in detail to identify if there are specific fatty acid binding or transport proteins for specific PUFAs. Similarly, studies suggest direct incorporation of TFAs into phospholipids [85]. Decreases of β oxidation enzymes were also observed in models of coronary artery ligation or aortic constriction [83]. In either model, transcriptional defects of β oxidation enzymes were observed in how they are taken up by the cell. Furthermore, no studies have looked at differential effects of rTFAs and iTFAs with respect to use of cellular transport proteins. This area of research clearly needs to be investigated.

SECTION 3: CARDIOVASCULAR DISEASE

I) Overview and Statistics

The heart is a four chambered, pumping organ found in the middle mediastitium, posterior to the sternum; between the second and sixth costal cartilages. The heart is oblique to the thorax

and points with its apex left of the midline of the sternum [86]. A human heart weighs approximately 250-350g and is approximately 12 cm long and 8 cm wide. The heart is primarily responsible for pumping oxygenated blood throughout the body. Although a relatively simple concept, proper function of the heart is vital for life. Vessels that bring blood to and from the heart are known as veins and arteries, respectively. Arteries and veins also supply blood to the heart itself to ensure proper function [86].

Cardiovascular disease is a term that broadly defines diseases which affect the heart and vessels surrounding the heart. Cardiovascular diseases (CVD) lead to compromises in heart function including inadequate function of the pump and decreasing blood supply to surrounding organs. CVD is becoming an increasing problem within the United States and Canada. In 2008, it was estimated that 29% of all deaths in Canada were attributed to CVD. Recent statistics also suggest that 1.3 million Canadians suffer from CVD over the age of 12 [87]. CVD is quickly becoming a global epidemic. The World Health Organization (WHO) reports that CVD is the number one killer globally [88]. In 2008, it was estimated that 17.8 million people died from CVD around the world [88]. It is, therefore, essential that researchers and medical practitioners continue the search for preventative and treatment strategies for this significant disease.

II) Risk Factors

CVD can be characterized by two major risk factors: modifiable and non-modifiable. Non-modifiable risk factors include genetic predisposition to CVD and family history [89]. Hypertrophic cardiomyopathy (HCM) is an excellent example of a genetic predisposition to CVD. In hypertrophic cardiomyopathy, the myocardium is thickened creating disproportioned chambers. Most prominently, HCM affects the left ventricle. When HCM is present in the

absence of risk factors such as aortic stenosis or hypertension, it is thought to have a genetic pre-disposition [90]. Disruptions in sarcomeric proteins create a basis for genetic predisposition to HCM [90]. These genes include β-myosin heavy genes (such as MYH7) and α tropomyosin genes (TPM1). Genetic basis for other CVDs such as dilated cardiomyopathy and arrhythmias are currently under investigation [90]. Sex, age, race and being post-menopausal are also non-modifiable risk factors for CVD [90].

CVD can also be caused by modifiable risk factors. These are associated with lifestyle choices. These include but are not limited to increased smoking, high cholesterol (specifically high LDL vs. HDL), hypertension, obesity, physical inactivity, diabetes and stress. Diet has also been associated with heart disease. This is particularly relevant to the types of fat consumed within a diet including saturated fats, MUFAs, PUFAs and trans fats. Risk of CVD and consumption of trans fats or PUFAs will be discussed in the following sections.

III) Diseases

CVD disease encapsulates a wide range of different pathologies. In the following section, we will discuss major diseases found within CVD, risk factors and the molecular mechanisms responsible for CVD.

i) Atherosclerosis

Atherosclerosis is a sub-type of arteriosclerosis. Arteriosclerosis is the term used to describe thickening or hardening of the arteries. This includes general stiffening of the arteries as an adult begins to age. The hardening of the artery is derived from factors such as cholesterol, calcium,

fibrin or cellular waste products [91]. Atherosclerosis can be attributed to risk factors such as high cholesterol and triglycerides, smoking and high blood pressure.

The development of atherosclerosis is still incompletely understood. To date, atherosclerosis is dependent on low density lipoprotein (LDL) accumulation and inflammatory responses within the vessel wall. Atherogenesis describes the progression of atheromatous plaques. Atheromatous plaques develop from fatty streaks within the vessels. Age contributes to increased fatty streaks within the arteries. However, early atherogenesis develops when monocytes adhere to the endothelium and migrate to the sub-endothelial space within the vessel. This migration increases monocyte-derived macrophage activation. LDL can then invade the endothelium and ultimately the vascular wall where it becomes oxidized further contributing to increased inflammatory response and an increase in the size of the atheroma. An increased blood pressure or infections can contribute to injuring the endothelial layer of vessels [92]. These injuries can become sites of inflammation and atheroma development. Over time, as atheromas grow they can harden, or calcify. On their own, atheromas may not contribute to the increased risk of CVD. However, if the atheromas grow to eventually block or hinder blood flow within the arteries of the heart or throughout the body, severe complications can occur. These include heart attacks, strokes and organ failure. Furthermore, if the atheroma becomes dislodged, it can travel throughout the body and block blood flow. This often occurs within the brain to induce a stroke.

Atherosclerosis is a complicated, serious disease with a variety of risk factors. Although the exact mechanism is unclear, decreasing risk factors such as smoking and cholesterol levels have been associated with a decreased risk of atherosclerosis progression and its subsequent complications [91, 93].

ii) Ischemic Heart Disease

Ischemic heart disease (IHD) refers to a condition in which the substrate supply is inadequate to meet the demands of the tissue. For example, blood flow may decrease while metabolic demands remain normal or, alternatively, blood flow may be normal while metabolic demands are increased. Ischemic heart disease, therefore, can be defined in two major ways: 1) blood flow can be reduced (low flow ischemia), or 2) blood flow can be completely inhibited (global ischemia) [94]. Reperfusion injury can result when oxygen, metabolites and/or blood flow is returned to a previously ischemic region. Reperfusion causes an increase in inflammatory cascades which increase cell oncosis and apoptosis. As a result, reperfusion injury may be a major determinant of infarct size associated with a myocardial infarction [95].

a. Development of Ischemic Heart Disease

Ischemic heart disease involves three major phenomena: hypoxia and accumulation of metabolites and ions. If ischemic conditions become persistent, there is a possibility of myocardial damage and scar formation within the heart. Ischemia induces major contractile changes within the heart including a decrease or cessation of contractile function [92]. Associated with the contractile dysfunction, changes in the electrical activity of the heart occur during ischemic conditions. Ischemia can decrease resting membrane potential, action potential (AP) amplitude, velocity and upstroke of AP and decrease the plateau phase of the AP [92]. These changes can lead to arrhythmias or cardiomyocyte death. Inhibition of the sodium pump by the excess hydrogen that accumulates during ischemia is a major mechanism that leads to changes in action potentials. Metabolic changes also occur at the level of the cardiomyocyte. These include decreased glycogenesis, inhibition of glycolysis, and inhibition of β oxidation of

fatty acids and decreased translocation of ATP from the mitochondria to the cytoplasm [96]. These metabolic changes during ischemia play an important role in the damage to the myocardium.

b. Models of Ischemic Heart Disease

Currently, there are a few models of ischemic heart disease. Atherosclerotic plaque development causes long term or permanent ischemia. Vascular contractile spasms or exercise can cause brief periods of ischemia. In cellular models, cells can be made to be devoid of nutrients, flow can be reduced (if the cells are undergoing perfusion), hypoxia induced and they can be challenged in an acidic environment to mimic the ischemic environment *in vivo*. It is important to recognize that ischemia corresponds to a change in both metabolic demands and blood flow [97]. Therefore, hypoxia alone does not substitute for conditions of ischemia [98]. In animal models, ligation of a major coronary artery (such as the left anterior descending artery (LAD)) can also mimic conditions of long term/permanent ischemia [99].

iii) Myocardial Infarctions

a. Development of Myocardial Infarctions

Myocardial infarctions (MI) are commonly referred to as heart attacks. The pathology of heart attacks is contingent upon a lack of blood flow to the heart. In these cases, the heart does not receive enough oxygen to support its metabolic activity and the heart muscle becomes necrotic. This can then lead to further complications within the heart including arrhythmias, bradycardia, pulmonary edema, septal defects and ventricular aneurysms. MIs generally occur as a result of atherosclerotic lesion development within coronary arteries in the heart. When lesion

development severely impedes blood flow, MIs can result. Blood clots (thrombi) are another common mechanism that increases the risk of heart attacks. MIs are defined as necrotic tissue (scar) development within the heart [97]. Cardiomyocyte cell death is a hallmark feature of myocardial infarcts. The border zone or immediate lateral area of affected tissue may also contain cardiomyocytes with alterations in metabolism and increased rates of cell death. Hallmark features of an infarct include left ventricular disturbances and rhythm disturbances. Fibroblast infiltration within the area of infarct is also an important feature post-MI [100]. This may create stiffening and hardening of the myocardium. This is as a result of increased collagen deposition within the area. This hardening is commonly known as fibrosis and is one of the major events in cardiac remodeling post-MI [101]. In order to assess infarct regions, scientists currently use a major marker of fibrosis (increased collagen deposition as detected by masson's trichrome staining) to quantify area and percentage of infarcts.

b. Models of Myocardial Infarctions

At the cellular level, specifically cardiomyocytes, the model of myocardial infarction should mimic those of ischemic heart disease [98]. *Ex vivo* models can include cardiac global ischemia by aortic ligation during Langendorff perfusion [102]. *In vivo*, LAD ligation is the most common method to induce an infarct and mimic the effects of coronary artery disease in humans [99].

iv) Cardiomyocyte Alterations during Ischemia/Reperfusion Injury

During ischemia/reperfusion injury, cardiomyocytes undergo rapid changes which are directly related to viability. Some of these changes occur as ion changes and oxidative stress [103, 104]. Other changes can be categorized as 1) accidental or 2) programmed cell death [105].

Accidental cell death is not reversible whereas programmed cell death has the opportunity to become reversed at specific stages. Accidental cell death is also termed oncosis. Programmed cell death is known as apoptosis [105]. Here these two mechanisms will be defined, their role in disease and how they affect cardiomyocyte cell death during ischemia/reperfusion injury. We will also examine other changes which occur within the cardiomyocyte including oxidized phospholipid content and changes in myokine (or cardiokine) production during both ischemia and ischemia/reperfusion injury.

a. Ion Changes

The buildup of protons due to anaerobic glycolysis is a major contributor to calcium build up and loss of cardiomyocyte contractility and viability during ischemia. Due to an excess of intracellular hydrogen ions (H+) within the cell, the Na/H exchanger begins to extrude at a rapid rate intracellular hydrogen in exchange for extracellular sodium [104]. Furthermore, the loss of ATP will cause deactivation of ATPases including the Na/K ATPase, ATP-dependant Ca^{2+} uptake and Ca^{2+} excretion; these result in excessive Ca^{2+} buildup within the cell [106]. These changes are followed by changes in other key proteases such as calpain which causes a fragile contractile structure and hypercontracture. Contraction band necrosis and initiation of apoptotic signaling pathways (*see Introduction Section IV iii*) can be followed by these changes. Should ischemia become persistent, these changes can cause increased injury and cell death [107]. During reperfusion, there is a prompt restoration of the ions that were depleted or altered during ischemia. Rapid normalization of pH will cause an extreme hydrogen ion gradient across the plasma membrane. Changes in Na/H exchange will be observed and a buildup of intracellular Na will occur. This buildup will cause a reversal of the Na/Ca exchanger (i.e. instead of calcium

extrusion, extracellular calcium will enter the cell in exchange for excess intracellular Na to exit). This will cause a buildup of calcium within the cell and cause calcium overload. Xanthine oxidase is also activated with calcium dependent proteases and the intermitochondrial respiratory chain. These changes can lead to increased oxidative stress and risk of cell death. Together, these findings suggest that both ischemia alone and ischemia/reperfusion increase the risk of ion changes that will ultimately lead to changes in cardiomyocyte contractility and viability [108].

b. *Oxidative Stress*

Activation of calcium derived proteases and the mitochondrial respiratory chain causes a sudden recovery of aerobic metabolism which, in turn, causes increases in the formation of reactive oxygen species (ROS) [107]. ROS are defined as chemically reactive molecules containing oxygen [109]. In the mitochondria, ROS are produced as a normal product of cellular metabolism. Superoxide is the major ROS found in the mitochondria. Generally, superoxide is converted into hydrogen peroxide by superoxide dimustase (SOD). This hydrogen peroxide by-product then becomes converted into water and oxygen by catalase.

Oxidative stress is particularly relevant in ischemia/reperfusion injury. During ischemia/reperfusion injury, there is a surplus of ROS produced which causes excessive hydroxyl radical production. These very unstable products have a high potential to cause cellular damage through destruction of enzymes, cellular channels or organelles. The production of these unstable compounds can also be spread across cardiomyocytes through gap junctions [107]. Targeting ROS production through anti-oxidant treatments may be a strategy to reduce ischemia/reperfusion injury.

c. Oncosis

Oncosis is a term for ischemic cell death [110]. Oncosis is defined as a swelling of the cell and cellular components [110]. This swelling leads to lethal injury. Swelling results from failing ionic pumps within the plasma membrane during stress conditions such as ischemia [104]. This will result in significant changes in intracellular ion content and the cell will begin to swell and will eventually burst if left unchecked. Necrosis is defined as a post mortem term that describes dispersion of intracellular content to the extracellular environment upon cell rupture [110]. Necrosis, following oncosis, leads to dispersion of organelles that are no longer functional. This phenomenon can lead to increased cell death in the surrounding area as potentially toxic material may be released in this process [111]. Oncosis is not a programmed process. Oncosis/necrosis can be observed in a variety of pathological phenomena including frost-bite, gangrene and ischemia/reperfusion injury [111]. A myocardial infarction can increase the rate of oncosis within cardiomyocytes [112]. Oncosis is an unregulated process therefore increased ROS formation can occur [105]. If these become dispersed, antioxidant treatments will need to be used to decrease the rates of cell death in surrounding myocytes [95]. Visualization of cell blebbing during oncosis may be a defining factor to identify the presence of cellular oncosis [105]. Due to the un-structured method of death, to date, no markers can be used to directly quantify oncotic cell death.

d. Apoptosis

Apoptosis is programmed cell death. Apoptosis comes from the Greek term for “falling leaves” [4]. There are many different kinds of cell death, each working through different mechanisms. A major difference between oncosis and apoptosis is the morphological appearance

of the cell death which occurs. During oncosis, necrosis occurs when the cell bursts causing spillage of non-viable organelles into the extracellular space. In contrast, cells shrink during programmed cell death and the organelle contents remain viable. These contents can be neatly packaged and can become engulfed by neighbouring cells or macrophages by phagocytosis. The steps involved in apoptosis include: 1) extracellular or intracellular signals to promote cell death; 2) collapse of the cytoskeleton to promote cell shrinkage; 3) disassembly of the nuclear envelope, and 4) DNA fragmentation [4]. Apoptosis is dependent on proteolytic cleavage of key proteins known as caspases. Caspases are cysteine-dependent, aspartate-specific proteases [113]. There are two types of caspases: initiator caspases 2, 8, 9 and 10, and effector caspases 3, 6 and 7. Active effector caspases are activated by proteolytic cleavage and carry out cell death by proteolytically degrading host proteins [113]. This helps to carry out the cell death program. There are also two major modes of apoptosis: intrinsic and extrinsic [114]. The extrinsic pathway is activated by pro-apoptotic receptors on the cell surface. These are known as pro-apoptotic ligands [115]. Binding of the death inducing signaling complex (DISC) to the pro-apoptotic ligands is a key regulator of extrinsic apoptosis [115]. Alternatively, intrinsic apoptosis is regulated by mitochondrial parameters. In this pathway, pro-apoptotic proteins are released by the mitochondria and initiate the cell death cascade [115]. However, the mitochondria also contain anti-apoptotic proteins. These pro- and anti-apoptotic proteins are part of the B-cell lymphoma-2 (Bcl-2) family. The balance of pro- and anti-apoptotic proteins determines the existence of apoptosis within a cell [116]. Anti-apoptotic proteins within this family include B-cell lymphoma extra-large (Bcl-XL). This protein can actively antagonize pro-apoptotic Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (BAK) by binding to their active BH3 domains [117]. This antagonism can be relieved by pro-apoptotic proteins which

bind to Bcl-XL [117]. Apoptosis can also be mediated by BH-3 only pro-apoptotic proteins. Examples include BCL2L11 (BIM), Bcl associated death promoter (BAD), NAD(P)H oxidase (NOX) and p53 upregulator modulator of apoptosis (PUMA) [115]. During cellular stress, increases in these BH-3 proteins are a key factor leading to intrinsic apoptosis [117].

Apoptosis plays an integral role in developmental processes. In mouse paws, apoptosis is used to create individual digits from spade-like structures within the embryonic stages. Similarly, apoptosis is used in transitions such as the disappearance of tails when a tadpole transitions into a frog. Within humans, apoptosis is also important in maintaining cell numbers and ensuring that cells that may be toxic are safely disposed. Changes in proteins controlling cell division can create issues such as cancer. In these instances, upregulation of apoptotic proteins reduces the risk of tumor development [4]. However, upregulation of apoptotic proteins may be detrimental to terminally differentiated cells, such as adult cardiomyocytes. Doxorubicin is a common agent used in cancer treatment. Doxorubicin is an anthracycline antibiotic which is used in cancer chemotherapy. It is used to treat a wide variety of cancers including lung, breast, ovarian and leukemia. It primarily reduces cancer progression by targeting cell death in monocytes and macrophages. Its adverse side effect includes an up regulation of apoptosis in cardiomyocytes. In this way, doxorubicin is known as cardiotoxic [118]. As well, hearts which have undergone injury from a myocardial infarction may also be susceptible to increased rates of apoptosis within cardiomyocytes - particularly within the border zones of an infarction [101]. In this way, apoptosis plays a significant role in cardiotoxicity. This is particularly relevant to cardiomyocytes, since these cells are terminally differentiated and play a substantial role in heart contractility. Currently, no therapies are available which specifically target rates of apoptosis

within the cardiomyocytes. However, natural products and myokines may play an important role in decreasing rates of apoptosis specifically within cardiomyocytes.

e. Autophagy

Autophagy comes from the Greek terms *auto* "self" and *phagein* "eat" [119]. Autophagy is a catabolic process which entails cell degradation through formation of lysosomes [119]. This is particularly relevant when there are dysfunctional organelles present within the cell. Regulated autophagy promotes the safe degradation or recycling of cellular components. Cellular components can be targeted through autophagosome production. Once the component is engulfed, it is fused to a lysosome to promote safe degradation or recycling. Autophagy can promote cell survival or cell death depending on the degree of damage which occurs within the cell. There are three forms of autophagy, microautophagy, macroautophagy and chaperone-mediated autophagy [120]. Macroautophagy is the major form of autophagy which occurs within the cell [121]. Macroautophagy occurs when the cell needs to remove damaged organelles or unused proteins. Macroautophagy is initiated by formation of an autophagosome-a double membrane around cytoplasmic substrates [122]. During canonical starvation induced pathways, phosphoinositide 3 kinase (PI3K) autophagy related gene (ATG)-6 (also known as beclin-1) are important regulators of autophagosome production [123]. Non-canonical induction of autophagy increases ATG-6 and PI3K. Up regulation of these initial proteins creates a cascade effect where other autophagy related proteins such as ATG-4, -12, -5 and -16 also become up regulated. ATG-5 plays an important role in elongation of the isolated membrane. These proteins play a key role in the formation of the autophagosome and tagging/engulfing proteins of interest. Once ATG-5 becomes a complex with other proteins such ATG-12, a mature autophagosome will develop.

Conversion of LC-3I to LC-3II is an important step in finalizing autophagosome development since LC-3II is recruited to the autophagosome [124]. Once this occurs, the autophagosome travels through the cell and fuses to a lysosome. The acidic contents of the lysosome spill into the autophagosome. The protein of interest then becomes recycles/degraded.

During microautophagy, the lysosome itself engulfs cytoplasmic material. Conversely, during chaperone mediated autophagy, a chaperone known as hsc-70 (heat shock chaperone-8) finds a recognition site on the protein that needs to be degraded/recycled and brings it directly to the lysosome [125]. Chaperone mediated autophagy is selective vs. micro- and macro-autophagy which may target several proteins or an entire region of the cell that may need to be degraded/recycled. Autophagy plays an important role in several cellular functions. These include cellular starvation. Here, amino acids that are released by protein degradation can be used by the cell in times of starvation or nutrient necessity. ATG-7 is specifically up regulated during times of starvation. Autophagy also plays an important role in infection by removing intracellular pathogens. Cellular damage as a result of aging will also up regulate autophagy. All of these cellular functions may be related to a subsequent up regulation of apoptotic proteins to cause cell death if the damage is irreversible. In this way, apoptotic cells protect surrounding cells from dispersion of toxic materials (a phenomenon observed in oncosis) [126].

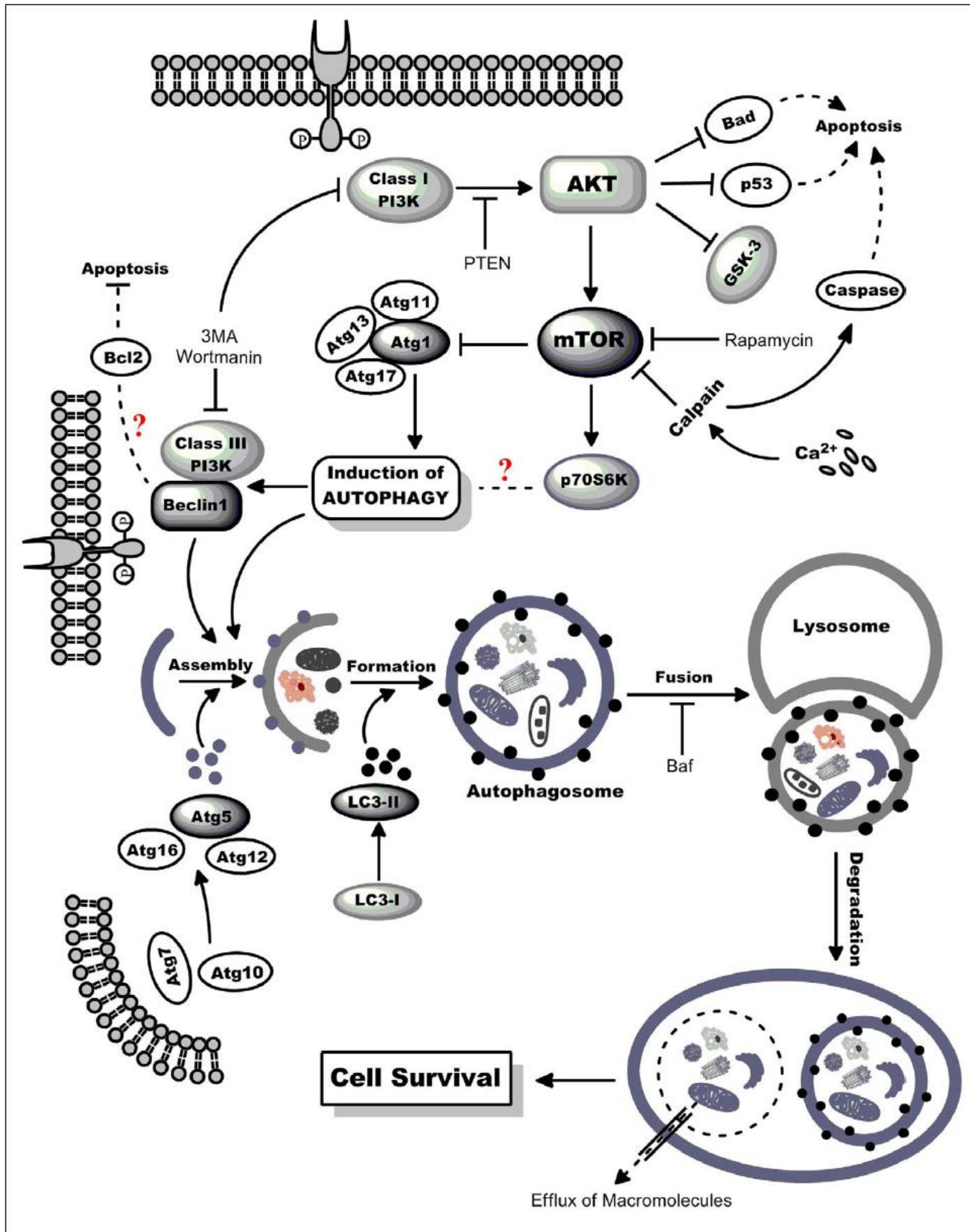


Figure 5: Schematic model of the molecular machinery and signaling pathways involved in autophagy. Reprinted from with Permission from SAGE Publisher. J Cardiovasc Pharmacol Ther. 2010 Sep;15 (3):220-30. doi: 10.1177/1074248410370327.

Autophagy may be a key regulator of cellular processes related to heart disease. Atg 5 knock-down in mice leads to a cardiomyopathy characterized by contractile dysfunction, ventricular remodeling and heart failure [127]. This is particularly evident in the context of ischemia/reperfusion injury. Changes in ion channels cause a buildup of calcium within cells during ischemia/reperfusion injury. These changes as well as increases in oxidative stress directly influence rates of autophagy. Furthermore, increased transcription of certain regulatory proteins such as Bnip3 during ischemia/reperfusion influence rates of autophagy, although the mechanism of action remains unclear. Therefore, it is not surprising that autophagy is up regulated in ischemia/reperfusion conditions.

The question remains whether or not autophagy is cardioprotective. *In vitro* studies reveal that beclin directly influences the anti-apoptotic protein Bcl-2. In this way, beclin may confer cardioprotective effects by decreasing rates of apoptosis [127]. However, beclin-1 inhibition also decreases rates of apoptosis in other models of ischemia-reperfusion injury. This paradoxical evidence confirms that the involvement of beclin-1 activity in cardioprotection remains inconclusive. Furthermore, Matsui and colleagues [128] confirm that beclin-1 and autophagosome formation at the time of reperfusion injury will depend on the duration and severity of the ischemic challenge. Therefore, unlike apoptosis, a definitive role for autophagy in ischemia/reperfusion is not clear in myocytes. However, it is well understood that autophagy is associated with apoptotic proteins including pro-apoptotic caspase-3 or anti-apoptotic Bcl-2 [127]. It is important to recognize that an upregulation in apoptotic markers may, therefore, be associated with an up regulation of key proteins in the autophagy pathway.

f. Oxidized Phospholipids

Similar to reactive oxygen species, oxygen derived free radicals (ODFR) increases the likelihood of oxidative stress and changes in cellular viability. ODFR can oxidize cellular phospholipids. Phospholipid oxidation causes increases in ODFR. Oxidation of phospholipids can create ODFR by converting phospholipids into bioactive compounds. These oxidized phospholipids (OxPL) have become important indicators of cell stress [129]. Phosphatidylcholine (PC) is the major phospholipid component of biological membranes and the best described group of OxPL are the oxidized phosphatidylcholines (OxPC). The bioactive properties of OxPC are compound, tissue, and cell specific. For instance, 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC) blocks lipopolysaccharide (LPS)-induced intracellular inflammatory signaling by acting on LPS binding-protein and CD14 [130] in human umbilical vein endothelial cells [131], but acts in a pro-inflammatory manner within mouse lung macrophages by inducing IL-6 production [132]. OxPC molecules like POVPC have been closely linked with atherosclerosis progression [133]. These OxPL compounds are also pro-apoptotic to bone marrow-derived macrophages [134], rat oligodendrocytes [135] and vascular smooth muscle cells [136]. POVPC and PGPC (1-palmitoyl-2-glutaryl phosphatidylcholine) can activate acidic sphingomyelinases, caspase-3 and mitogen activated protein kinase signaling as initial apoptotic stress responses in smooth muscle cells [136]. These findings have been supported by DNA fragmentation and flow cytometry [137]. Although it is well known that ischemia/reperfusion injury increases oxidative stress, the effect of ischemia/reperfusion on the oxidation of phospholipids has not been investigated. Based on these findings, we hypothesize that ischemia and ischemia/reperfusion injury will induce an increase in oxidized phospholipids within cardiomyocytes.

g. Cytokine/Myokine/Cardiokine Changes

Inflammation plays another important role in the progression of cardiovascular disease that is particularly relevant in ischemia/reperfusion injury. This inflammatory challenge to the heart involves adipose tissue. Adipose tissue was originally thought to be a simple storage depot. However, adipose tissue is a dynamic and complex endocrine organ which produces a host of cytokines known as adipokines. These adipokines can be pro-inflammatory or anti-inflammatory. Pro-inflammatory adipokines tumor necrosis factor α (TNF-α), interleukins 1, -6, -10, -12, nuclear factor kappa light chain enhancer activated B (NF-κB) and leptin are secreted from adipose tissue [138]. Conversely, certain adipokines such as adiponectin are anti-inflammatory. Interestingly, adipokines can also be secreted by target tissues such as skeletal muscle [139]. These are known as myokines. Similarly, cardiokines are derived from heart muscle. Cardiomyocytes contain TNF-α, adiponectin and leptin [140]. The effects of these cardiokines is still under investigation; however, pro-inflammatory cytokines such as TNF-α increase cardiomyocyte cell death through activation of apoptotic pathways [141]. However, not all pro-inflammatory cytokines increase cell death. Leptin, for example, decreases cardiotoxicity in the heart [142]. However, anti-inflammatory adipokines remain cardioprotective in nature. Adiponectin decreases cardiomyocyte apoptosis associated with hypoxia/reoxygenation [143], improves metabolism [144] and plays an important role in decreasing the extracellular remodeling associated with cardiovascular disease [145]. Adiponectin primarily exists in its 30 kDa full length form which can oligomerize in circulation to form high molecular weight, mid molecular weight and low molecular weight adiponectin. Furthermore, the globular head can be cleaved to form globular adiponectin. Adiponectin can have differential effects depending on its

form and currently these forms are being investigated within the whole body as well as secretion from cardiomyocytes [146].

IV) Trans Fats and Cardiovascular Disease [1, 12]

i) Atherosclerosis and Trans Fats

Fatty acids are incorporated into phospholipids in all cell membranes of the body [147, 148]. The fatty acid composition of the membrane can strongly influence its biophysical characteristics. Specifically, unsaturated and saturated fatty acids can act as potent regulators of membrane fluidity due to differential actions of phospholipids on cholesterol affinity and incorporation [149, 150]. This phenomenon can alter cellular activities such as the function of membrane proteins including membrane bound receptors [85]. It has been proposed that TFAs may alter cardiovascular integrity and function by incorporating into cell membranes and changing cellular membrane fluidity. Niu et al (2005) demonstrated that TFA-derived phospholipids had significantly higher membrane affinity for cholesterol than cis analogues. Phospholipid membranes that contained TFA exhibited a higher acyl chain packing order. This effect was also correlated with reduced G-protein coupled receptor activation [85]. Because membrane cholesterol levels and membrane receptors are involved in the regulation of cholesterol homeostasis, the elevation in membrane cholesterol content and the lower receptor activation induced by the presence of TFA in the membrane could represent the mechanism responsible for the elevation of LDL cholesterol in TFA supplemented diets [85]. Engelhard and colleagues [151] found an increase in membrane fluidity with TFA administration in choline supplemented membranes. The TFA elaidic acid increased calcium incorporation into the cell

[152] and decreased osmotic rupture of sheep and chicken erythrocytes in comparison to cis analogs [153]. Although there are clear differences between the effects of cis fatty acids and TFAs on membrane fluidity, studies have not investigated the effect of rTFAs on the membrane. These studies may be necessary since rTFAs have significantly different effects on atherosclerosis in animal studies. This difference was most prominently noted in a 2010 study by Bassett et al. [8]. Here, investigators observed a substantial decrease in atherosclerotic plaque lesions in LDLr-/- mice whose diets were substituted with both cholesterol and the ruminant trans fat, vaccenic acid. In contrast, diets supplemented with both cholesterol and elaidic acid (iTFA) induced accelerated atherosclerotic lesions compared to cholesterol-fed animals alone. This study concluded that there was a potential cardioprotective effect of ruminant trans fats compared to industrial trans fats in animals fed high cholesterol diets. In addition, although there are clear differences between the effects of cis fatty acids and TFAs on membrane fluidity, studies have not investigated the effect of rTFAs on the membrane. These studies may be necessary since, as previously discussed; rTFAs have significantly different physical characteristics and physiological effects on atherosclerosis in animal studies.

Trans fats have been implicated in endothelial dysfunction. Using fluorescence and mRNA measurements conducted by Bryk et al. (2011), an increase in vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1) expression was detected in human aortic endothelial cells (HAECs) after exposure to increasing doses of elaidic acid [154]. Elevated levels of E-selectin were detected in a randomized control trial of 50 men consuming elevated levels of TFAs [155]. Observational studies have also shown an increase in the expression of E-selectin, ICAM-1 and VCAM-1 [156]. These results are consistent with findings in *in vitro* work and animal studies.

ii) *<u>Trans Fats, LDL and Lipoproteins</u>*

There is a positive correlation between plasma LDL and atherosclerosis and/or CHD [157]. Although epidemiological evidence suggests that there is a positive association between TFA intake and elevated plasma LDL [158, 159] (as well as triglycerides [160]), a clear mechanism has not been established. In the human hepatoblastoma (HepG2) cell line, TFAs have been associated with an increased LDL:high density lipoprotein (HDL) ratio, an increased apolipoprotein B: apolipoprotein A (apoB:apoA) ratio and increased cholesterol content in both LDL and HDL particles in comparison to saturated fats [161]. All of these findings have in turn been associated with a higher risk of atherosclerosis and CHD. Similar findings were reported by Mitmesser and colleagues who suggested that TFAs altered the size and composition of apoB-100 containing lipoproteins [162]. These studies provide a basic mechanism whereby TFAs deposit cholesterol in arteries. However, it is important to recognize that these studies are primarily correlative and more concrete evidence is necessary. Furthermore, there is no distinction in these studies between rTFAs and iTFAs. These two types of TFAs are structurally different and, therefore, their biological effects may also be very different.

Epidemiological evidence has generated conflicting results with respect to an association of TFAs with serum lipid levels. In a meta-analysis of thirteen randomized controlled trials observing the effects of isocaloric replacement of polyunsaturated fatty acids (PUFAs), saturated fatty acids (SFAs) or monounsaturated fatty acids (MUFAs) with TFAs, a significant increase in low density lipoprotein cholesterol (LDL-C) levels, total cholesterol:high density lipoprotein cholesterol (HDL-C) ratio and the ratio of apo B:apo A was observed as well as a decrease in HDL-C levels [163]. Others have shown a decrease in LDL-C particle size with consumption of TFAs as opposed to unsaturated fatty acids [164]. It is important to recognize that these studies

investigated a particular isomer of TFAs associated with the hydrogenation of vegetable oils (such as the trans isomer of oleic acid), therefore, further investigation with a wider variety of TFAs may be necessary to fully understand the effect of TFAs on lipoproteins.

iii) Trans Fats in Cytokine and Adipokine Production

The binding of an agonist to a specific receptor protein which then affects a number of downstream signaling pathways is associated with the development of an inflammatory cascade which can then ultimately influence the progression of atherosclerosis, plaque rupture and cardiac failure and death [165, 166]. The up-regulation of certain inflammatory proteins is but one common but important example [166]. Adipose cells and the adipokines synthesized there are rapidly becoming recognized as important mediators of inflammatory pathways and associated recently with the atherosclerotic process. It is possible, therefore, that if TFAs are deposited within fat tissue, this change in fatty acid composition may influence adipokine synthesis, release, downstream signaling, and ultimately, inflammatory actions related to atherogenesis.

TFAs alter adipocyte size in *fa/fa* Zucker rats [167]. However, there were no significant effects on adipokine secretion and profile in these rats. The results were limited to the trans t-10, c12-conjugated linolenic acid (CLA) effects on adipocyte size and number in comparison to cis c-9, t-11-CLA enriched diets. Although this effect may be subject to the animal model used and the amount of TFA in the diet, this study does show an important effect of a specific isomer of TFAs. Obara et al. (2010) also analyzed the effect of TFA consumption in non-alcoholic fatty liver disease in mice [168]. They found an up-regulation of tumor necrosis fact (TNF)-α

expression in mice fed a high TFA supplemented diet. Up-regulation of TNF-α expression is associated with alterations in inflammation, endothelial function and cardiotoxicity [169].

Bryk et al. (2011) demonstrated an increase in NF-κB expression suggesting that there was an increase in the inflammatory response in human aortic endothelial cells (HAECs) after iTFA administration [40]. Inflammatory markers are a key risk factor for atherosclerosis [165, 166, 169] and this study identifies a potential mechanism whereby TFAs induce an atherogenic process. Similar results were obtained in HUVECs where a decrease in TNF-α and NF-κB with cLA administration was observed [170]. Again, studies on VA as well as EA are necessary to fully define the effects of TFAs on inflammatory reactions that can be generated by endothelial cells.

The mechanism whereby VA is protective in heart disease may involve anti-inflammatory action. Blewett and colleagues observed a decrease in inflammatory markers such as interleukins (IL)-6, IL-2 and TNF-α in obese JCR:LA-cp mice fed a diet supplemented with VA [171]. VA may share this anti-inflammatory action with other fatty acid species. Omega-3 fatty acids, for example, have an anti-inflammatory action [51]. An anti-inflammatory effect of omega-3 fatty acids and a pro-inflammatory effect of TFAs was reported in male Wistar rats that underwent coronary and femoral artery ligation [172]. The possibility exists that an inclusion of both omega-3 fatty acids in the diet with TFAs may negate the deleterious effects of iTFAs. Indeed, the atherogenic effects of dietary EA were recently prevented by inclusion of ALA in the diet [53], presumably through a complex interaction with the inflammatory/immune system.

As stated earlier, TFAs may also influence cardiovascular disease through an effect on HDL and LDL levels in animals. For example, iTFAs decrease the HDL:LDL ratio in rats fed a high TFA diet [173]. However, conflicting data have been reported that high TFA intake may

decrease total plasma cholesterol [174]. These results may be difficult to explain in view of the pro-atherogenic epidemiological and animal results identified above. Alternatively, these data suggest that TFAs may be stimulating atherosclerosis in a manner independent of cholesterol. This concept is supported by Bassett and co-workers who demonstrated that supplementation of the diet with EA will stimulate atherogenesis without an increase in plasma cholesterol [49]. Once again, the pro-inflammatory effects of the TFA diet are a logical alternative mechanism.

Adipokine regulation by TFAs has also been associated with the progression of atherosclerosis in animal models. Specifically, increased adiponectin levels have attenuated atherosclerosis progression [175]. Huang and colleagues showed that margarine-derived TFA diets fed to male Wistar rats decreased plasma levels of adiponectin, and increased resistin and leptin. TFA intake also affected adipocyte gene expression of peroxisome proliferator-activated receptors (PPARs), resistin and lipoprotein lipase (LPL) in rats [176]. Increases in tumor growth factor (TGF-β) expression may be another potential mechanism whereby TFAs are affecting atherosclerosis [177-179]. Once again, these results have focused upon iTFAs. Pathways affected by rTFAs are largely unknown in relation to atherogenesis.

In observational studies, TFAs have been associated with increased levels of specific inflammatory markers such as TNF-α and its receptors, IL-6, C-reactive protein (CRP) and NF-κB [171]. In a randomized cross-over trial of 50 healthy men, consumption of TFAs for five weeks increased plasma levels of IL-6 and CRP in comparison to consumption of oleic acid or carbohydrate intake [155]. However, Smit et al. (2011) reported that TFA consumption for 3 weeks did not severely affect markers of inflammation including tumor necrosis factor receptor-II (TNF-RII),TNF-α, CRP and IL-6 in participants consuming either cLA, iTFAs or oleic acid [180]. This randomized study was performed on 61 healthy adults. The amount of TFAs

consumed, the time-frame under investigation, the TFAs that were consumed and the characteristics of participants consuming the diets (i.e. males vs. females) may have participated in the conflicting results.

Many inflammatory markers are under genetic regulation by PPARs, liver X receptor and sterol regulatory element-binding protein-1 (SREBP-1) [181]. In animal studies, TFA consumption alters PPAR-gamma activity, resistin and lipoprotein lipase activity suggesting a potential mechanism whereby TFAs are exerting their effects [182]. However, identifying a distinction between isomers of TFAs in human studies is still necessary to fully understand how TFAs are exerting their effects on cytokine regulation and systemic inflammation.

V) Polyunsaturated Fatty Acids In Cardiovascular Diseases [1, 12]

i) Polyunsaturated Fatty Acids in Atherosclerosis

The relationship between fatty acids and the incidence of myocardial infarctions has been well established in the literature [8, 183-185]. For example, the first indication that PUFAs, specifically the n-3 PUFAs DHA and EPA, were considered beneficial was published by Dyerber et al.[186]. The authors showed that EPA inhibits platelet aggregation in humans preventing microthrombus formation which can lead to atherosclerotic formation [186]. Through the publication of this work, there have been many manuscripts showing corroborative evidence that a diet high in the n-3 PUFAs EPA and DHA can be cardioprotective [184, 187, 188]. Further work has extended the beneficial cardiovascular effects beyond EPA and DHA to also include other n-3 PUFAs like ALA. ALA is a structurally distinct n-3 fatty acid that is enriched in plants like flaxseed, walnuts and to a lesser extent in canola and hempseed. n-3 PUFAs are

less likely to create vessel lumen closure, such as atherosclerosis, therefore the myocardium is less likely to be subjected to an ischemic event and the subsequent scar formation. This would prevent an overall decrease in cardiac output [183, 184, 187, 189].

However, not all PUFAs nor all fatty acids are beneficial. A diet enriched in n-6 PUFAs has been associated with an increased incidence of myocardial infarctions. N-6 PUFAs are pro-atherogenic, pro-inflammatory and pro-thrombotic which can lead to vessel lumen closure more readily [190-193].

ii) *Polyunsaturated Fatty Acids in Ischemia/Reperfusion Injury*

The human myocardium consists of 4 to 5 billion cardiomyocytes, the primary contractile apparatus of the heart [194]. Loss of a significant portion of cardiomyocytes by apoptosis, autophagy or necrosis can create contractile dysfunction which can lead to cardiovascular remodelling and ultimately heart failure. Direct administration of EPA to neonatal cardiomyocytes protects against palmitate-induced apoptosis [195]. EPA administration also decreased caspase-3 activity and altered Bcl-2 and Bax activity, all of which were associated with palmitate administration. Cardiomyopathic hamsters exposed to an ALA rich diet had a lower mortality rate and better contractility. This finding was attributed to an anti-apoptotic effect [196].

N-6 fatty acids have also been analyzed for their effects on myocyte apoptosis [197]. Administration of 40 µM arachidonic acid (AA) directly to myocytes upregulated apoptotic pathways within the myocyte. AA increased the levels of intracellular calcium and sodium and this correlated with increased caspase-3 activity and cytochrome c release. The data suggest that AA increases the susceptibility of the myocyte to undergo apoptosis through a Ca^{2+}/Na^{+}

mediated action. This evidence also strongly supports the concept that the n-6:n-3 ratio can be an important determinant of cardiomyocyte viability.

Fatty acids have intracellular actions on ionic homeostasis within the cardiomyocyte that also play an important role in the genesis of ischemic myocardial injury. EPA has anti-arrhythmic effects presumably through suppression of L-type calcium channels in adult and neonatal rat cardiomyocytes [198]. Diets supplemented with cholesterol will induce a significant increase in arrhythmias during ischemic/reperfusion challenge [28]. When the diet was enriched with ALA, the incidence of arrhythmias during an ischemia/reperfusion challenge to the heart was significantly inhibited [28]. ALA administration also improved the calcium transients and contractility within the cardiomyocytes. These beneficial effects may have been achieved through an increase in sodium-calcium exchange [28]. Arrhythmias are also associated with increased TFA intake [199]. However, it is not yet clear if TFAs incorporate into the membrane of the cardiomyocyte and which membrane system is preferentially altered. This would have significant mechanistic effects with regard to the movement of ions within the cardiomyocyte and the generation (or inhibition) of arrhythmias.

The metabolic by-products of PUFAs may also affect cardiomyocyte viability and contractility, particularly under conditions of ischemia/reperfusion injury. The 17 (R), 18 (S)-epoxyeicosatetraenoic acid, which is derived from EPA, is important in cardiomyocyte contraction and decreases the incidence of arrhythmias [200]. Derivatives of AA, DHA and EPA can also be protective against ventricular fibrillation [201]. Cytosolic phospholipase A, which preferentially cleaves AA, has been shown to play a distinct role in ischemia/reperfusion injury, specifically in promoting apoptosis and subsequent tissue injury. Transgenic mice deleted in cPLA2 exhibit a decrease in tissue injury associated with ischemia/reperfusion as well as

decreased apoptosis as shown by TUNEL staining [202]. The decrease in the biosynthesis of LTB4 and TXB2 (biosynthesized products of AA) has an anti-inflammatory effect as well as a decrease in the incidence of cell death. Drug therapies directed towards cPLA2 reduction, therefore, may have cardioprotective effects. However, the protective effects of pioglitazone, an insulin-sensitizing thiazolidinedione, against a myocardial infarction were associated with an upregulation of cPLAα expression [203]. Other factors may have been involved in the cardioprotective actions of pioglitazone that may have over-ruled the negative effects of the upregulation of cPLAα expression. The effect of cPLA2 in cardiomyocyte viability, however, must still be considered controversial at the present time [203].

The Western diet today has been suggested to have a n-6:n-3 ratio of as high as 16:1 [43]. As a result, there is a distinct cellular shift in the amount of AA available for biosynthesis. AA can be further metabolized into epoxyeicosatrienoic acids (EETs). EETs display a variety of cellular effects within the heart including influencing L-type calcium channels and improving left ventricular function during post-ischemic recovery [204]. EETs can be further converted into dihydroxyeicosatrienoic acid (DHETs) by soluble epoxide hydrolase [205]. Soluble epoxide hydrolase inhibitors have anti-hypertensive and anti-inflammatory properties suggesting this pathway is important in cardioprotection [206]. AA can also be metabolized by CYP-ω-hydroxylase into 20-hydroxyeicosatetraenoic acid (20-HETE), a potent constrictor of coronary arteries. 20-HETE production is upregulated during ischemia/reperfusion injury making it an attractive target for drug therapy. Inhibitors of CYP-ω-hydrolase decrease infarct size [207]. Depending on the amount of n-6 and n-3 intake, there can be a shift in the primary types of eicosanoids that are biosynthesized since different enzymes may be preferentially upregulated. One of these enzymes includes COX-2 which has been shown to be involved in several

inflammatory pathways. PUFAs contained in fish oil can inhibit COX activity. Thus, the biosynthesis of certain eicosanoids and the upregulation of certain enzymatic pathways such as COX-2 will affect cellular viability.

iii) Polyunsaturated Fatty Acids, Cardiovascular Biomarkers and Adipokines

Cardiac biomarkers are commonly used to identify the involvement of fatty acids in myocardial injury [188, 199, 208]. A diet high in PUFAs can lead to the increased incorporation of PUFAs within neutrophils, macrophages and leukocyte membranes. All of these cells play an important role in the inflammatory process [209, 210]. Because inflammation plays such an important role in atherosclerotic heart disease, the production and release of inflammatory biomarkers can provide a reliable indication of the severity of atherosclerotic lesions. N-3 PUFAs have anti-inflammatory and anti-atherogenic effects while n-6 fatty acids favor pro-inflammatory conditions. Diets containing high amounts of the n-3 PUFAs, DHA and EPA, inhibited TNF-α, IL-1, IL-6, IL-8, and IL-12 production *in vitro* [211-213]. Cholesterol supplemented diets increased plasma IL-6, mac-3 and VCAM levels, all markers of inflammation. The cholesterol-enriched diet also induced significant atherogenesis as well. However, supplementation of the cholesterol-enriched diet with ALA from flaxseed decreased these same inflammatory biomarkers and inhibited the development of atherosclerotic lesions. PUFAs appear to have the capacity to down regulate the inflammatory process and, in turn, thereby provide anti-atherogenic properties [184]. Kalogeropoulos et al. [190] published one of the first studies to identify an association between serum PUFAs and biomarkers of cardiovascular disease in a clinical setting. They found that there was an inverse relationship between circulating levels of CRP, IL-6, and fibrinogen in relation to plasma levels of the

PUFAs, EPA and DHA. As PUFA levels decreased in the plasma, the inflammatory biomarkers increased [190]. This confirmed results from animal work. As atherosclerosis has been positively associated with increased inflammatory markers, the aforementioned study provided indirect evidence that PUFAs decrease inflammatory markers and in turn decrease atherosclerosis formation.

Together with altering the expression of cytokine and inflammatory markers, n-3 PUFAs also affect adipokine markers such as leptin and adiponectin. Leptin is an adipocytokine which was originally thought to be produced solely from white adipose tissue. However, leptin is now known to be secreted from target tissues such as bone marrow, skeletal muscle, stomach and heart. Leptin deficiency has been associated with difficulties in regulating satiety, causing an increase in inflammation and a higher risk of obesity. Conversely, obesity is associated with increased circulating leptin levels, causing leptin resistance and eventually increased difficulties regulating satiety. However, leptin will also affect macrophage infiltration and cytokine release. Increased circulating leptin levels have also been associated with increased hypertrophy in adult rat cardiomyocytes [214]. Therefore, it is important to regulate leptin levels. In mouse studies, n-3 fatty acid supplementation decreased circulating leptin levels [215]. EPA supplementation (1 g/kg) decreased circulating leptin in lean animals and decreased it in overweight rats [215]. Inclusion of EPA+DHA (3 g/day) in the diets of humans led to increased weight loss and decreased circulating leptin levels.

Adiponectin primarily exists in its 30 kDa full length form which can oligomerize in circulation to form high molecular weight, mid molecular weight and low molecular weight adiponectin. Furthermore, the globular head can be cleaved to form globular adiponectin. Within insulin resistant rats, n-3 fatty acid supplementation increased adiponectin levels [165].

Furthermore, n-3 (EPA 1.8 g/day) supplementation in humans increases circulating adiponectin levels within 3 months [216]. EPA also increased PPAR-gamma mRNA *in vitro* [217]. PPAR-gamma is a transcription factor responsible for adiponectin production [146]. Adiponectin also increased metabolic markers such as AMPK [144]. *In vitro* EPA supplementation (100-200 μM) increased phosphorylation of AMPK in 3T3-L1 adipocytes [218]. Taken together, evidence strongly suggest that the n-3 PUFA EPA directly influences adiponectin production in several models. However, fewer studies have investigated the correlation of DHA and ALA with adiponectin levels. Therefore, it is still unclear as to the direct role of these PUFAs in adiponectin production. Further study is necessary.

Table 2: Relationship of biomarkers with fatty acid intake from *in vitro* and clinical studies

[156, 174, 190, 211-213, 219-227]

	Fatty Acid				
		n-3 PUFA	*n-6 PUFA*	*iTFA*	*rTFA*
Biomarker	Troponin	↓	↑	↑	N/A
	CRP	↓	↓	↑	N/A
	IL-1	↓	↑	↑	N/A
	IL-6	↓	↓ or ↑	↑	↓
	IL-8	↓	↑	↑	N/A
	IL-12	↓	↑	↑	N/A
	TNF-α	↓	↑	↑	N/A
	siCAM-1	↓	↓	↑	N/A
	E-selectin	↓	↓	↑	N/A
	NF-κB	↓	N/A	↑	N/A
	PPAR-α	↓ or ↑	↓ or ↑	N/A	N/A
	PPAR-γ	↑	N/A	N/A	N/A

SECTION 4: CURRENT TREATMENT STRATEGIES FOR MYOCARDIAL INFARCTIONS

I) Primary Interventions

The PQRST wave pattern of the electrocardiogram (ECG) changes dramatically in the incidence of a myocardial infarction (MI) [92]. Treatment of myocardial infarction is contingent upon specific ECG characteristics. Specifically, ST-elevation myocardial infarction (STEMI) patients have a differential treatment regimen than non-ST elevation myocardial infarction (NSTEMI or non-STEMI) patients. STEMI is caused by the formation of blood clots in the coronary artery which significantly inhibits blood flow and energy delivery to the downstream myocardial tissue [228]. This resultant damage can be in the form of a loss of a large area of myocardial tissue and can extend deep into the myocardium. Non-STEMI does not extend as deeply into the cardiac muscle [228]. This may be as a result of different kinds of blood clots causing the heart attack, the size/severity of the clot as well as the extent of blood clotting proteins/platelet blood cells [229]. Patients presenting with STEMI generally receive percutaneous coronary interventions (PCI) or coronary artery bypass surgery (CABG) [230]. These interventions are administered quickly to restore oxygen supply and demand to the myocardium, relieve pain and lastly, prevent and treat any further complication. NSTEMI may present with a different intensity of angina and breathing difficulties [230]. As a result, a series of tests may be run and different sets of medications will be prescribed on the onset. However, both NSTEMI and STEMI can cause further complications and risk of heart failure [230].

Due to disruption to the myocardium during myocardial infarction, fibrillation is another common complication [231]. Up to 65% of all deaths as a result of MI occur within the first hour

[231]. However, 60% of these deaths can be prevented by the use of a defibrillation device [231]. Therefore, defibrillation techniques are also important primary intervention strategies. Administration of tissue-type plasminogen activator (t-PA), aspirin and heparin may be given to the patient by the paramedic within 90 minutes of the onset of symptoms [231]. This may play an integral role in patients surviving an MI.

II) <u>Post-Myocardial Infarction Treatment</u>

In-hospital and long term treatment strategies vary with respect to STEMI and NSTEMI patients. However, early treatment of MIs is designed to limit the extent of myocardial infarction, salvage jeopardized myocardium and re-canalize infarct-related arteries. β blockers are given intravenously 4 hours after the onset of pain and on a long term basis. β blockers reduce mortality related to Q-wave MI as long if administered early [230]. The severity and reduction of an MI related to unstable angina has also been reported with use of β blockers [230]. Aspirin is another common medication that is used in the treatment and also prevention of an MI [232]. It is currently recommended that 80 mg of aspirin be administered to patients suffering from unstable angina or to those who have suffered a previous MI [232]. Aspirin is chosen for this treatment because it has anti-thrombotic properties [232]. Angiotensin converting enzyme (ACE) inhibitors are also effective in reducing the risk of a future MI in patients who exhibit no evidence of hypotension. It is recommended that these drugs should be administered within the first 24 hours after symptoms of an MI [232]. Lastly, calcium channel blockers have been used to alleviate calcium entry into cells under prolonged stress [230]. However, calcium channel blockers may not be beneficial during acute MI and may be deleterious alone or when given with other medications [231]. However, calcium channel blockers do decrease the risk of fibrillation

[233] and other arrhythmias [234]. Ultimately, the use of anti-thrombotic and anti-platelet agents decrease the risk of future clots in patients who have suffered from an MI or experience angina.

III) Nutrition and Myocardial Infarction

Nutrition plays an important role in modulating the risk of incurring an MI. Within the Western diet, there is a prevalence of fried foods containing industrial trans fats [235]. Trans fats are a major risk factor for an MI (see Trans Fats and Cardiovascular Disease Section). Diets from other regions of the world or other countries can also modulate this risk. The Mediterranean diet contains a large portion of fats from olive oil (monounsaturated fats) and polyunsaturated fats (*See Section IV*). This diet decreases the risk of cardiovascular disease and complications [236]. Conversely, the South Asian diet has been shown to increase risk of cardiovascular disease [237]. Within Canada, South Asian immigrants are the most likely ethno-racial group to suffer from cardiovascular disease and diabetes [238]. This may be as a result of nine major risk factors including nutrition, increased apoB/apoA ratio, increased family history of diabetes and hypertension and psychosocial stress such as depression and stress from home and work [237]. Interestingly, the South Asian diet is primarily vegetarian. However, most of these foods are prepared and cooked. There is a lack of intake of fresh fruits and vegetables. Furthermore, food is sometimes prepared in clarified butter, known as ghee, which is made up of as much as 50% industrial trans-fat. This intake of trans fat may be responsible for the increased risk of heart disease in South Asians.

Diet may also play an important role in improving outcomes post-MI. The flaxseed derived polyunsaturated fatty acid α-linolenic acid (ALA) has anti-arrhythmic properties [28].

Furthermore, ALA from dietary flaxseed decreased apoptosis of cardiomyocytes that had been exposed to pro-apoptotic TNF-α [196]. However, very few studies have analyzed the direct effect of nutraceuticals in respect to a myocardial infarction. Specifically, how dietary interventions increase or decrease cardiomyocyte viability needs to be studied. Further investigation into the direct effect of nutraceuticals before and after an MI is also necessary. Identifying the mechanism of action for any effects of the nutraceuticals will also aid in better understanding how nutraceutical interventions can be used as both preventative and protective strategies when patients suffer from an MI.

CHAPTER 2: HYPOTHESES

1) Elaidic acid will promote direct detrimental effects on the heart muscle (*in vivo*) or cardiomyocytes (*in vitro*). Exposure of isolated cardiomyocytes to a suspension medium containing elaidic acid may exert direct detrimental effects (such as increases in cell death) during an ischemic/reperfusion challenge through an initiation of cell contractile dysfunction, injury and death. We hypothesize that elaidic acid may promote apoptosis and autophagy induced by the ischemic/reperfusion challenge.

2) Vaccenic acid will promote direct beneficial effects on the heart muscle (*in vivo*) or cardiomyocytes (*in vitro*). Exposure of isolated cardiomyocytes to a suspension medium containing vaccenic acid may exert direct beneficial effects during an ischemic/reperfusion challenge through an inhibition of cell injury and death. Specifically, we hypothesize that vaccenic acid may protect via an inhibition of apoptosis and autophagy induced by the ischemic/reperfusion challenge and preserve normal myocardial contractile function and Ca2+ homeostasis.

3) Exposure of isolated cardiomyocytes (*in vitro*) to a suspension medium containing ALA may exert direct beneficial effects during an ischemic/reperfusion challenge through an inhibition of cell injury and death. Specifically, we hypothesize that ALA may protect via an inhibition of apoptosis induced by the ischemic/reperfusion challenge.

CHAPTER 3: OBJECTIVES

OVERALL OBJECTIVE: To determine differential effects of fatty acids (ruminant trans fats, industrial trans fats or alpha linolenic acid) on cardiomyocyte viability acid during normoxic conditions or ischemia/reperfusion conditions

SPECIFIC OBJECTIVES:

1) To determine if dietary supplementation of VA or EA will increase uptake within the heart in LDLr-/- mice (*in vivo* model).

2) To determine if LDLr$^{-/-}$ mice fed elaidic acid will exhibit decreased cell viability within the heart. We expect that this will be correlated to an increase in autophagic and apoptotic markers.

3) To determine if LDLr-/- mice fed vaccenic acid will exhibit increased cell viability within the heart. Furthermore, we expect a decrease in pro-apoptotic and autophagic markers in the hearts of vaccenic trans-fat fed mice.

4) To determine if direct exposure of cardiomyocytes to VA or EA will increase intracellular uptake of the fatty acids (*in vitro* model).

5) To determine if direct exposure of cardiomyocytes to ALA will increase their intracellular uptake of the fatty acid (*in vitro* model).

6) To determine if short or long-term exposure of isolated cardiomyocytes to a suspension medium containing EA may exert direct detrimental effects through an augmentation of

cell injury and death. Specifically, we hypothesize that EA will induce apoptosis and autophagy.

7) To determine if short or long-term exposure of isolated cardiomyocytes to a suspension medium containing ALA may exert direct beneficial effects through an inhibition of cell injury and death. Specifically, we hypothesize that ALA will inhibit apoptosis and autophagy.

8) To determine if long term exposure of isolated cardiomyocytes to a suspension medium containing ALA or VA will increase myokine expression of adiponectin. We hypothesize this increase in adiponectin will decrease cellular apoptosis and autophagy.

9) To determine if short or long term exposure of cardiomyocytes to ALA, VA or EA will alter expression of adiponectin.

10) To determine if exposure of isolated cardiomyocytes to a suspension medium containing ALA may exert direct beneficial effects during an ischemic/reperfusion challenge through an inhibition of cell injury and death. Specifically, we hypothesize that ALA will protect via an inhibition of apoptosis and autophagy induced by the ischemic/reperfusion challenge and preserve myocardial contractile function and Ca^{2+} homeostasis.

11) To determine if exposure of isolated cardiomyocytes to a suspension medium containing EA will exert direct detrimental effects during an ischemic/reperfusion challenge through

an augmentation of cell contractile dysfunction, injury and death. We hypothesize that EA will promote apoptosis and autophagy induced by the ischemic/reperfusion challenge and impair myocardial contractile function and Ca^{2+} homeostasis.

12) To determine if exposure of isolated cardiomyocytes to a suspension medium containing VA will exert direct beneficial effects during an ischemic/reperfusion challenge through an inhibition of cell injury and death. Specifically, we hypothesize that VA will protect via an inhibition of apoptosis and autophagy induced by the ischemic/reperfusion challenge and preserve myocardial contractile function and Ca^{2+} homeostasis.

CHAPTER 4: MATERIALS AND METHODS

Section 1: Materials

α linolenic acid (ALA), vaccenic acid (VA) and elaidic acid (EA) were purchased from Nu-CHEK Preparations (Minnesota, MN, USA). Bovine serum albumin (BSA) was obtained from Sigma Aldrich (Oakville, ON, CA). M199, laminin and collagenase were obtained from Invitrogen (Burlington, ON, CA). Cell culture plates were received from VWR (Mississauga, CA). dUTP nick end labeling kit (TUNEL assay) was obtained from Novagen (Darmstadt, Germany).

Section 2: Techniques Used In All Results

a. Primary Cell Culture

Ventricular myocytes were isolated from 12-week old male Sprague-Dawley rats (250-300 g) as described previously [239]. In brief, an intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg) was used to anesthetize the animal. Hearts were excised and transferred to a Langendorff perfusion apparatus and perfused with calcium (Ca^{2+}) free buffer containing 90 mM NaCl, 10 mM KCl, 1.2 mM KH_2PO_4 (Sigma Aldrich), 5 mM $MgSO_4.7H_2O$ (Sigma Aldrich), 15 mM $NaHCO_3$ (Sigma Aldrich), 30 mM taurine and 20 mM glucose (Sigma Aldrich) for 10 min. The perfusion medium was then switched to Ca^{2+} free buffer containing collagenase (0.05%) and bovine serum albumin (BSA) (0.2%). After 30 minutes, ventricles were minced into pieces, incubated in a 37°C waterbath and separated into individual cardiomyocytes by gravitational centrifugation. Cardiomyocytes were then suspended in buffer containing Ca^{2+}. The supernatant was then replaced with Ca^{2+} buffers containing a higher concentration of calcium (150 μM). This step was repeated twice to increase the

extracellular Ca^{2+} concentration to 500 μM and then to 1.2 mM. Cells were finally re-suspended in medium-199 (M199) and transferred to laminin coated culture dishes.

b. Fatty Acid Administration

After 1 hour of incubation in a CO_2 incubator (5% CO_2 and 95% O_2), fatty acid medium is added. The existing M199 medium was replaced with medium containing the fatty acids of interest. Fatty acids were stored in ethanol in stock solution of 1 M in -20^{0}C. When needed, the fatty acids were reconstituted at a final concentration of 400 μM conjugated to 1% fatty acid free BSA solution in M199. The cells were incubated for 0-48 hours and the medium replaced every 24 hours.

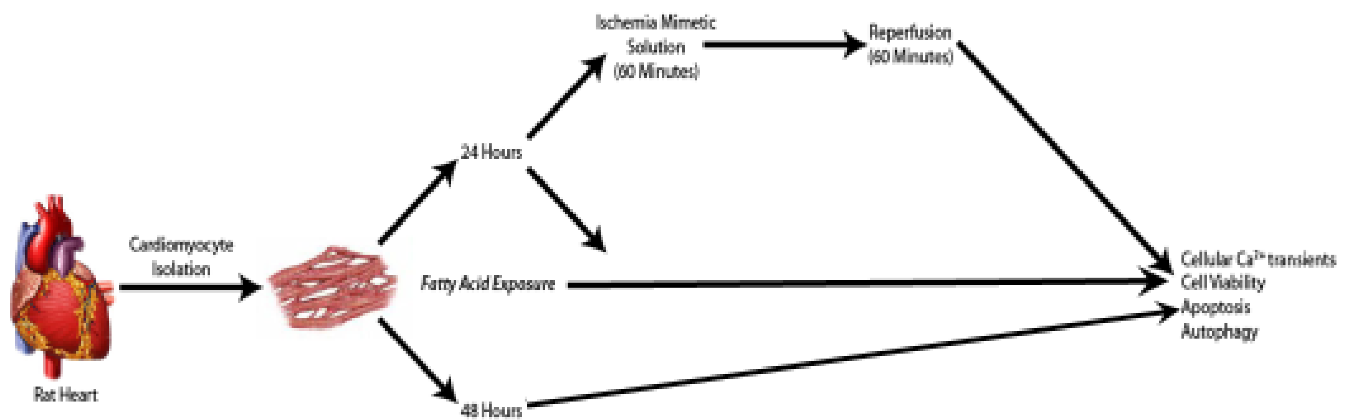


Figure 6: Conditions for Cardiomyocytes in Parts 2-5

c. Immunoblotting

Western blotting was utilized to visualize Bcl-2 associated protein X (Bax) (Cell Signaling, Boston, MA, USA), Bcl-2 (Cell Signaling), beclin (Cell Signaling), caspase-3 (Cell Signaling), Light Chain 3 (LC-3I/II) (Cell Signaling), adiponectin (Roche, Missisauga, ON, CA), CD36 (Abcam), Fatty Acid Transport Protein 1 (Santa Cruz) and total actin (Cell Signaling). Attached cardiomyocytes were lysed with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, and 1 mM EGTA, Protease Inhibitor Cocktail with 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM benzamidine (pH 7.5) and collected for Western blot analysis. Samples (30 μg of total protein/lane) in Laemmli sample buffer were resolved on 10-15% SDS-PAGE. Proteins were transferred to nitrocellulose membrane in a wet transfer apparatus for 2 hours at 4°C at 120 volts. Membranes were probed with primary antibodies at a concentration of 1:1000-1:3000 overnight. The signal from horseradish peroxidase-conjugated secondary antibody was developed using West Pico chemiluminescence substrate (Pierce, Rockford, IL). The signal was collected in a Bio-Rad detection system and quantified by densitometry analysis using Quantity One software (Bio-Rad, Mississauga, ON, CA).

d. Immunofluorescence

Cells on cover slips were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 2% milk-0.1% Triton X-100 in PBS. Cleaved caspase-3 primary antibody (1:100 dilution) was followed by Alexa 488-conjugated goat anti-rabbit secondary antibody (1:700 dilution; Molecular Probes, Burlington, ON, CA). CD36 (1:100 dilution, Abcam) was used with Alexa 488-conjugated goat anti-rabbit secondary (1:700 dilution;

Molecular Probes, Burlington, ON, CA). Cells were mounted on glass slides using mounting medium with DAPI (Vectashield; Vector Laboratories, Burlington, ON, CA). All images were collected with a Nikon Eclipse TE2000S fluorescent microscope. All ischemia/reperfusion experiments were conducted within a 10 mm area of cells plated on 25mm coverslips.

Section 3: Techniques Used in Parts 1 and 2

a. Heart Tissue Collection

Tissues were collected from studies performed by Bassett (2010) [8]. Hearts were flash frozen in liquid nitrogen and stored in -80°C. Study consisted of forty female C57BL/6J LDL receptor-deficient mice (Jackson Laboratory). Mice were 5–7 wk old and randomly assigned, following a 1-wk acclimatization period, to experimental diet groups of 5 mice each. The four experimental diets consisted of a base of a delipidated nonpurified 5P00 Prolab RMH 3000 diet from LabDiet (Richmond) with the fat content replaced by 1 of 4 fat sources: 4% regular fat (pork/soy) (RG); 4% manufactured partially hydrogenated vegetable shortening providing 1.5% TFA, mainly in the form of elaidic TFA (ES); 15% regular butter (RB) providing 0.3% naturally present TFA (RB); and 15% butter providing 1.5% naturally occurring TFA, mainly in the form of vaccenic TFA (VB). Butter production described in Bassett 2010 [8].

b. Heart Tissue Preparation for Western Blot Analysis

Frozen hearts were homogenized using a mortar and pestle and liquid nitrogen. The homogenates were lysed with RIPA buffer (50 mM Tris (Sigma Aldrich, St.Louis MO, USA), 150 mM sodium chloride (NaCl) (Sigma Aldrich), 1% Triton X-100, 0.5% sodium deoxycholate (SDS) (Sigma Aldrich), 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich), and 1 mM ethylene glycol tetraacetic acid EGTA (Sigma Aldrich), pH 7.5, with 1 mM

phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, and a protease inhibitor cocktail) and centrifuged at 14,000 *g* for 15 min at 4°C to remove cellular debris. Aliquots of lysates were analyzed by SDS-PAGE electrophoresis, and proteins were transferred onto nitrocellulose membranes using a wet transfer protocol.

Section 4: Techniques Used in Part 1 only

a. Fatty Acid Analysis (Tissue)

The samples were analyzed on a Varian GC/MS/MS instrument equipped with a CP-3800 GC, CP-8400 autosampler and a Flame Ionization Detector (FID). Lipids were extracted from plasma (100 μL) and a 1-g sample of ground diet using chloroform:methanol (2:1, v:v) [240]. Briefly, the extracted lipids (stored in 100 μL of dichloromethane containing the internal standard, C 11:0) were first esterified using 1 mL of 0.5 mol/L methanolic NaOH at 90°C for 10 min under an atmosphere of nitrogen. After a brief cooling period, 1 mL of 14% BF_3-methanol was added and the contents heated as described above. The reaction was terminated upon the addition of 1 mL distilled water and the fatty acid methyl esters (FAME) extracted into 300 μL hexane. Completion of the derivatization reaction was verified using TLC. FAME were then analyzed using GC coupled with flame-ionization detection. The separation methodology is based upon the 150°C GC temperature. FAME were analyzed against an authentic standard, GLC 469A, with the addition of eicosapentaenoic acid (Nu-Chek, Prep).

b. Preparation of Cardiomyocytes for Fatty Acid Analysis

Cells were scraped from 6 well plates in 1 ml of phosphate buffer saline (PBS). Cells were centrifuged at 5000 rpm for 5 min and PBS removed. The cells were then reconstituted in 4 ml 2:1 (v/v) chloroform:methanol. Mixture was covered in aluminum foil and placed in the

refrigerator overnight. 145 mM NaCl was then added to the mixture. The mixture was vortexed. Mixture was centrifuged at 5000 rpm for 5 min. The chloroform layer was isolated and added to a pre-weighed tube. Solvent was removed with nitrogen. Then 0.5 ml of CH2Cl2 was added to the dried extract to solubilize the lipids. This was then stored under N2 (g) at -80°C until ready to derivatize.

c. Fatty Acid Analysis in Cardiomyocytes

The samples were analyzed on a Varian GC/MS/MS instrument equipped with a CP-3800 GC, CP-8400 autosampler and a Flame Ionization Detector (FID). 1 uL was injected onto a 100m x 0.25mm Select for FAME capillary column with a split ratio of 5. Helium was used as the carrier gas at a constant flow of 1.4ml/min. The oven temperature was held at 45 degrees C for 4 minutes, then increased to 175C at a rate of 13.0 C/min. It was held for 41 minutes before ramping up to 215C at a rate of 0.8 C/min and held for 20 minutes. The final temperature of 230C was reached after ramping at 20 C/min and holding for 2 minutes for a total run time of 113.75 minutes. The temperature of the injector and FID were both held at 250C. An internal standard of C11:0 was used and the standard reference was GLC469, both purchased through Nu-Chek Prep, Inc.

Section 5: Techniques Used in Parts 2-5

a. Cell Viability Assays

Cell viability was assessed as previously described [98]. Cell morphology was used as another index of cellular integrity. Cardiomyocytes were visually assessed and counted on the full microscopic field with a ×10 lens. Cardiomyocytes were judged to be healthy if they had a normal rectangular rod shape, whereas damaged cells were subjectively determined by the

presence of surface blebbing, rounded cell edges, or complete balling up of the cell. Furthermore, ethidium homodimer staining of cell nuclei was used as another method of assessing membrane integrity. This was carried out at the end of the experimental intervention using the Live/Dead assay kit (Molecular Probes, Eugene, OR, USA). These data were a measure of live vs. dead cells.

b. dUTP Nick End Labeling (TUNEL Assay)

The TUNEL assay was performed as described previously [241]. Briefly, cells were fixed with 4% paraformaldehyde for 15 minutes. Cells were washed with 1x TBS then permeabilized with 2 mg/ml proteinase K for 5 minutes. Cells were washed then exposed to 5x TdT Equilibration Buffer for 30 minutes. Cells were then exposed to TdT labeling reaction mixture for 1.5 hours in a 37°C incubator. Cells were then washed again 3x for 1 minute with 1x TBS and mounted on a glass coverslip using Fluorescein FragEL Mounting Media. The total cell population was visualized using a filter for DAPI (330-380 nm). Labeled nuclei were visualized using a standard fluoroscein filter (465-496 nm).

Section 6: Techniques Used in Parts 4 and 5

a. Cell Perfusion Conditions

Cardiomyocytes were perfused as previously described in detail [23] with a control Tyrode buffer (TB) for 60 minutes or ischemic buffer for 60 minutes and paced at 0.5Hz. The TB buffer is composed of (in mM): 140 NaCl, 6 KCl, 1 $MgCl_2$, 1.25 $CaCl_2$, 6 HEPES (pH 7.4), and 10 d-glucose at 37°C and bubbled with 100% oxygen. The ischemic buffer was composed of

(in mM): 140 NaCl, 8 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 6 HEPES (pH 6.0) and bubbled with 100% nitrogen. In some studies, the ischemia was followed by a 60 minute period of reperfusion with the control TB solution at a rate of 1 ml/min. In some conditions, cells were not paced and placed in a hypoxic chamber with ischemia TB buffer for 1 hour of ischemia followed by oxygenation conditions (control TB buffer) for 1 hour to mimic ischemic and ischemia/reperfusion conditions.

b. Calcium Transient Measurements

The Ca^{2+} sensitive dye fura-2 AM was used as an intracellular indicator of Ca^{2+}. Labelled cardiomyocytes were placed in a Leiden chamber mounted on a Nikon Diaphot microscope. Cardiomyocytes were perfused with a control Tyrode buffer (TB) for 60 minutes or ischemic buffer for 60 minutes and paced at 0.5 Hz. The TB buffer is composed of (in mM): 140 NaCl, 6 KCl, 1 $MgCl_2$, 1.25 $CaCl_2$, 6 HEPES (pH 7.4), and 10 d-glucose at 37°C and bubbled with 100% oxygen. The ischemic buffer was composed of 140 mM NaCl, 8 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 6 mM HEPES (pH 6.0). In some studies, the ischemia was followed by a 60 minute period of reperfusion with the control TB solution at a rate of 1 ml/min. Cells were excited at 340 and 380 nm with an emission of 505nm.

c. Oxidized Phospholipid Extraction

Cell phospholipids were extracted using a modified protocol for 6-well plates [242]. Each well was scraped into 500 µL of methanol/acetic acid (3% v/v)/butylated hydrotoluene (BHT) (0.01% w/v). Two wells were pooled in a 10 mL glass centrifuge tube on ice and capped under nitrogen gas. Samples were spiked with five internal standards for each class (10 ng 1, 2-dinonanoyl-sn-glycero-3-phosphocholine, DNPC; 100 ng 1, 2-diheptadecanoyl-sn-glycero-3-

phosphoethanolamine, PE 34:0; 100 ng 1,2-dipalmitoyl-sn-glycero-3-phospho-L-serine, PS 32:0; 100ng 1-heptadecanoyl-2-tetradecenoyl-sn-glycero-3-phospho-(1'-myo-inositol), PI 31:1; 100ng 1,2-dimyristoyl-sn-glycero-3-phospho-(1'-rac-glycerol), PG 28:0, Avanti Polar Lipids Inc., Alabaster, AL, USA) to allow for quantitation. The wash steps were with 2 mL of hexane/BHT under nitrogen gas, with vortexing, and centrifuging at 3500 rpm for 5 minutes at 4˚C. The hexane/BHT wash was repeated three times. The final wash is followed by application of 2 mL of chloroform/BHT and 750 µL of PBS before centrifugation as above. The lower organic layer was collected in a new glass tube for evaporation to a lipid film using a nitrogen evaporator. Lipid films were reconstituted into 250 µL of chloroform/methanol (2:1 v/v) for storage at -80˚C in glass autosampler vials.

d. High Performance Liquid Chromatography

The lipid extract was run to analyze non-oxidized phospholipids using a Shimadzu SIL-20 AC HT autosampler (Shimadzu, Columbia, MD, USA) to inject 10 µL into an Ascentis® Si HPLC column (15cmx1mm, 3µm; Supelco Analytical, Bellefonte, PA, USA). The normal-phase solvent system consisted of a mobile phase Solvent A (chloroform/methanol/ammonium hydroxide, 80:19.5:0.5 v/v/v) and Solvent B (chloroform/methanol/water/ammonium hydroxide, 60:34:5.5:0.5 v/v/v/v). Two Shimadzu LC-20 AD pumps regulated the portions of the two mobile phases by the following time program; Solvent B was initially at 0% and was uniformly increased to 100% by 14.00 min; at 24.10 min Solvent B was rapidly reduced to 0% of the total mobile phase until the run was stopped at 30.10min. The flow rate was held at 70µl/min for the duration of the run.

For OxPC analysis, a 100 µL portion of the lipid extract was reconstituted into the reverse-phase solvent system (acetonitrile/isopropanol/water, 65:30:5 v/v/v). The autosampler injected 30µL into an Ascentis® Express C18 HPLC column (15cmx2.1mm, 2.7 µm; Supelco Analytical) held at 45˚C by a Shimadzu CTO-20 AC column oven. Mobile phase consisted of Solvent C (acetonitrile/water, 60:40 v/v) and Solvent D, (isopropanol/acetonitrile, 90:10, v/v) containing 10mM ammonium formate and 0.1% formic acid. Solvent D was initially at 32% until 4.00 min when it was increased to 45%; at 5.00 min to 52%; at 8.00 min to 58%; at 11.00 min to 66%; at 14.00 min to 70%; at 18.00 min to 75%; at 21.00 min to 97% before decreasing Solvent D to 32% at 25.10, until 30.10 min when the run was stopped. The flow rate was held at 260 µl/min for the duration of the run. The auto sampler tray was held at 4˚C throughout all analyses.

e. Electrospray Ionization Tandem Mass Spectrometry

A 4000 QTRAP triple quadrupole linear ion trap mass spectrometer system with a Turbo V electrospray ion source (AB Sciex, Framingham, MA, USA) was linked to the eluate from the HPLC column for mass spectrometry. The PC-specific detection, for both unoxidized and OxPC, was carried out in positive ion mode by multiple reaction monitoring (MRM) using PC- specific product ion (184.3 m/z, Da) which corresponds to the PC head group fragment as previously described[243, 244]. The parameters for positive electrospray ionization were voltage = 5500V, ion source temp = 500˚C, curtain gas = 26psi, nebulizer gas = 40psi, heater gas = 30psi. The MRM settings were as follows: declustering potential (DP) = 125V, entrance potential (EP) = 10V, collision energy (CE) = 53V, cell exit potential (CEP) = 9V and dwell time (DT) = 50msec.

Other unoxidized phospholipids were detected using MRM negative ion mode with PE-specific ion = 139.8Da, PI-specific ion = 241.1Da, and PG-specific ion = 170.9Da. Fragment loss of an 87Da fragment from the precursor ion was used for PS detection. The parameters for negative electrospray ionization were voltage = -4500V, ion source temp = 500˚C, curtain gas = 20psi, nebulizer gas = 30psi, heater gas = 30psi. The MRM settings were species specific; PI: DP = -80V, EP = -10V, CE = -60V, CEP = -20V, DT = 100msec; PS: DP = -85V, EP = -10V, CE = -40V, CEP = -15V, DT = 100msec; PG: DP = -80V, EP = -10V, CE = -50V, CEP = -30V, DT = 100msec; PE: DP = -80V, EP = -10V, CE = -70V, CEP = -25V, DT = 100msec.

Section 7: Statistics

Data are expressed as means ± SEM. The data were analyzed by a Student's *t*-test or a one-way ANOVA. A Student Newman-Keul post hoc test was used to determine statistical difference after the ANOVA. Statistical significance was set at a $p < 0.05$.

RESULTS

SECTION 1: Uptake of VA, EA Acid and ALA Acid into the Heart

i) Quantification of Trans Fatty Acid Uptake in Heart Tissue

Gas chromatography was employed to measure fatty acid uptake within heart tissue. $LDLr^{-/-}$ mice were exposed to a diet containing either regular diet (4% pork/soy or RL), low industrial trans-fat elaidic acid (1.5% elaidic acid or LT), high industrial trans-fat (3% elaidic acid or HT), butter group (BR or 0.5% trans-fat), ruminant (natural) trans-fat vaccenic acid (1.5% vaccenic acid or BT) for 14 weeks as described by Bassett et al. (2010). Hearts were excised and flash frozen in liquid nitrogen until analyzed for fatty acid content. A statistically significant increase in elaidic acid uptake was observed in both the LT and HT groups. Vaccenic acid uptake was also significantly increased in the BT group. Significant but smaller increases were observed in the LT and HT group with respect to vaccenic acid content (Figure 7).

ii) Mechanism of Trans Fatty Acid Uptake in Heart Tissue

A mechanism has not been identified for trans-fatty uptake into heart tissue. It has been suggested that fatty acid transport proteins are involved in fatty acid uptake into the heart. To measure the possibility that trans-fat uptake occurs through fatty acid transport proteins, we measured fatty acid transport protein content in $LDLr^{-/-}$ hearts that were exposed to dietary vaccenic acid or elaidic acid for 14 weeks. FATP4 and CD36 have been implicated in LCFA uptake in the heart [69], therefore we measured the content of these proteins within the hearts. No significant changes in the expression of these fatty acid transport proteins (CD36 and FATP4) were observed as a function of either the vaccenic acid or elaidic acid dietary

interventions. It is important to recognize that RG served as a control for LT and BR served as a control for BT (Figure 8A and B).

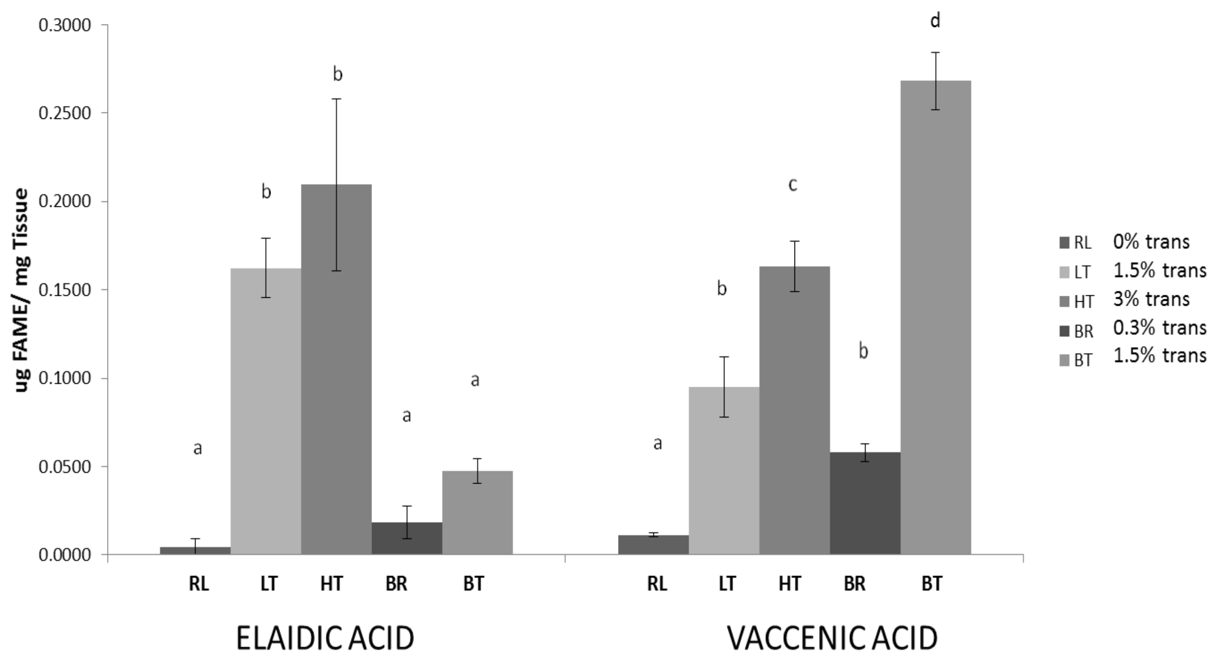
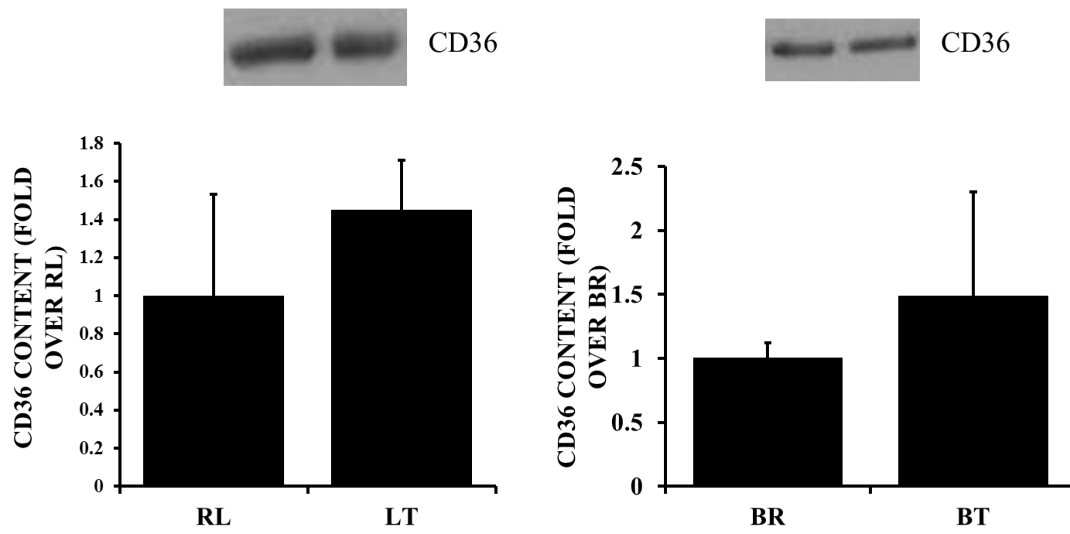


Figure 7: **Fatty Acid Uptake in Hearts after Dietary Intervention for 14 weeks**. LDLr$^{-/-}$ mice were exposed to a diet supplemented with RL=regular diet, LT=1.5% elaidic acid (EA) supplemented diet, HT= 3.0% EA, BR=butter group, BT=1.5% vaccenic acid (VA) supplemented diet for 14 weeks. Hearts were extracted and prepared for gas chromatrography measurements. Intracellular fatty acid content of VA or EA was measured. Values represent mean ± standard error mean (SEM). n=4. Means with different letters (*a, b, c, d*) are significantly different. Significance was achieved with $p<0.05$.

iii) Quantification of Trans Fatty Acid Uptake in Cardiomyocytes

Fatty acid uptake within cardiomyocytes was measured after cardiomyocytes were exposed to a 1% BSA solution containing 50 μM or 400 μM of fatty acid for 6 or 24 hours. Elaidic acid uptake was significantly increased in cells that were exposed to both 50 μM and 400 μM at all-time points. Vaccenic acid uptake was also increased in cells that were exposed to both 50 μM and 400 μM at all-time points (Figure 9).

A



B

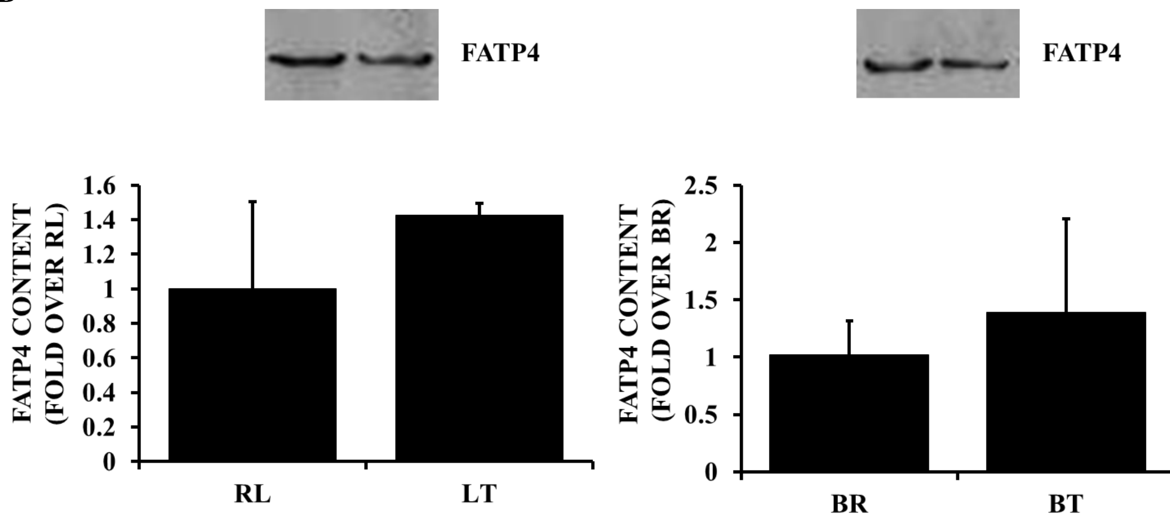


Figure 8: **Expression of Fatty Acid Transport Proteins with 14 weeks Dietary Intervention.** $LDLr^{-/-}$ mice were exposed to a diet supplemented with RL=regular diet, LT=1.5% EA supplemented diet, BR=butter group (0.3% VA), BT=1.5% VA supplemented diet for 14 weeks. Hearts were then extracted and whole heart homogenates were prepared and run on western blots. Expression of fatty acid transport proteins was measured. A) Cluster of Differentiation 36 (CD36) expression measured by densitometry of western blot and; B) Fatty Acid Transport Protein (FATP4) expression was measured by densitometry of western blot. No significant differences were observed. Values represent mean ± SEM. n=3.

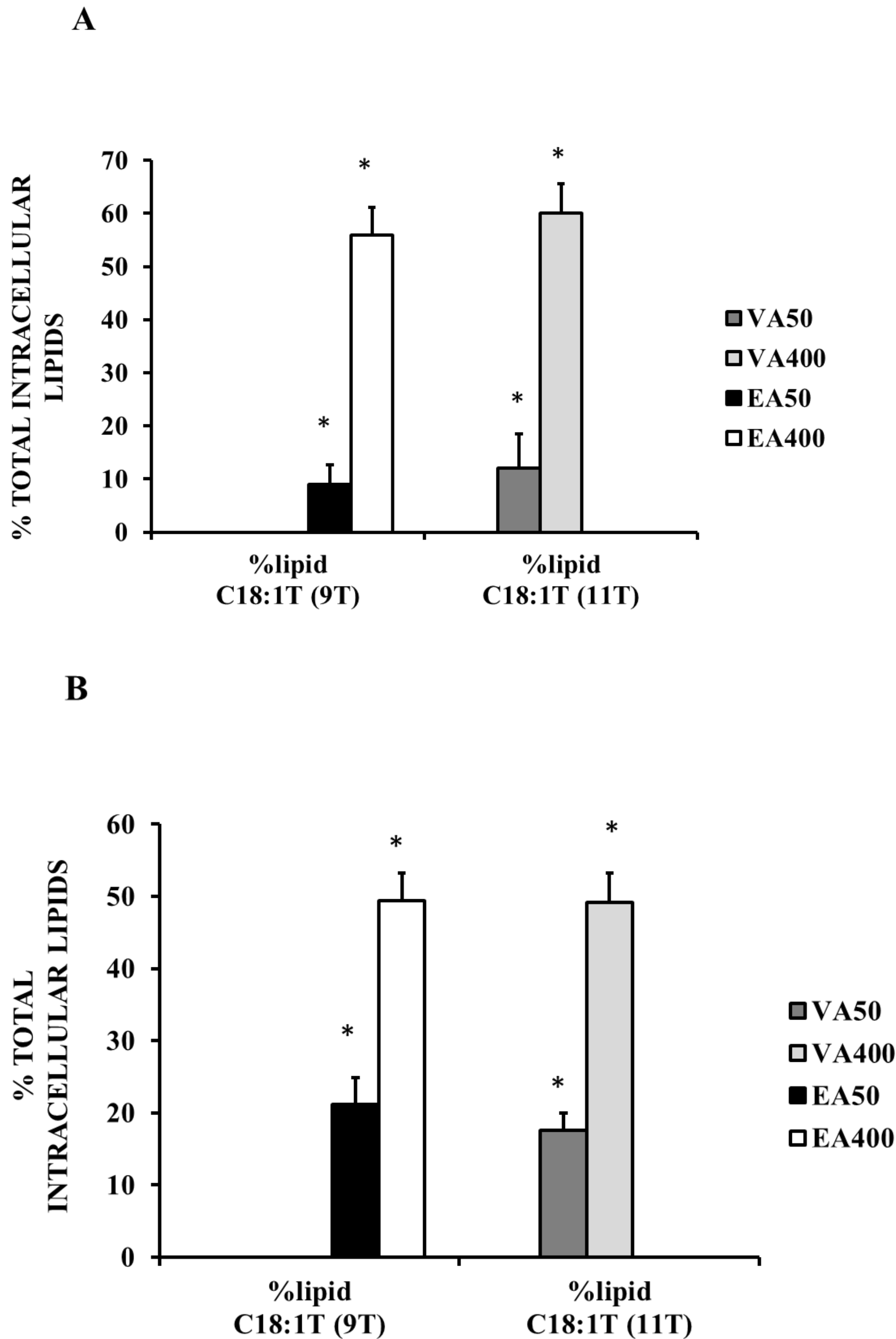


Figure 9: **Fatty Acid Uptake in Hearts after Direct Supplementation to Cardiomyocytes**. Cardiomyocytes were exposed to 50 or 400 µM EA or VA by direct supplementation to the media. Briefly, after 6 or 24 hours cells were extracted and then prepared for gas chromatography (GC). Intracellular fatty acid content was measured by GC. Representative graphs demonstrate intracellular VA or EA content after A) 6 hours and B) 24 hours.*$p<0.05$ compared to CON. Values represent mean ± SEM. n=4.

iv) Mechanism of Trans Fatty Acid Uptake in Cardiomyocytes

iv) Mechanism of Trans Fatty Acid Uptake in Cardiomyocytes

A direct mechanism of action has not been implicated for trans-fatty uptake into heart tissue. It has been suggested that fatty acid transport proteins are involved in fatty acid uptake into the heart. We measured fatty acid transport protein in cardiomyocytes after exposure to vaccenic acid or elaidic acid. FATP4, FATP1 and CD36 have been implicated in LCFA uptake [47], therefore, the expression of these proteins within the hearts was measured. Cardiomyocytes were exposed to 50 μM or 400 μM of either VA or EA for 24 and 48 hours. We did not detect any FATP4 within the cardiomyocytes with western blot analysis (Data not shown). We also observed no significant changes in the expression of these fatty acid transport proteins such as CD36 (Figure 10A and B) or FATP4 with either 50 or 400 μM pre-treatment (Figure 10C and D).

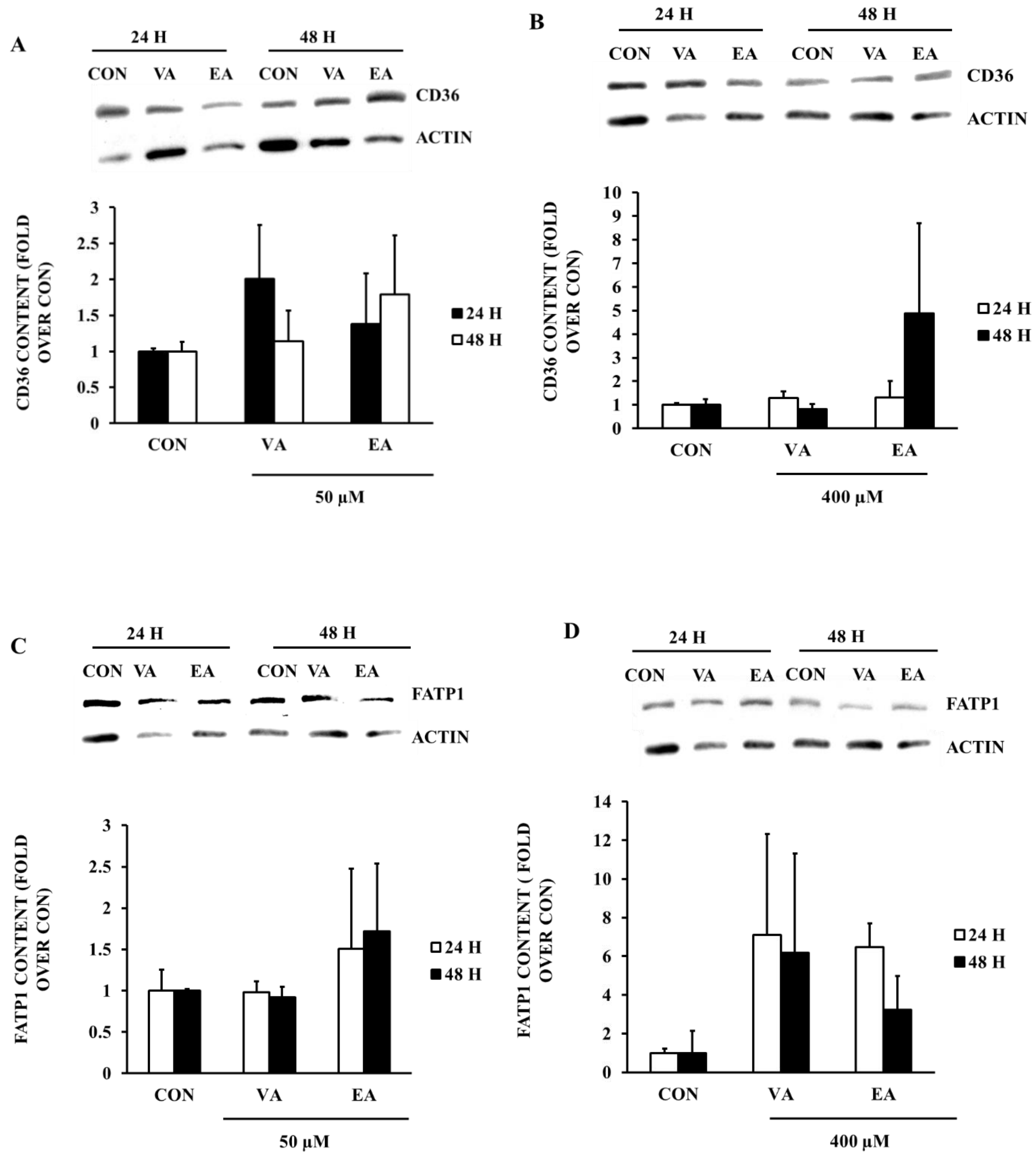
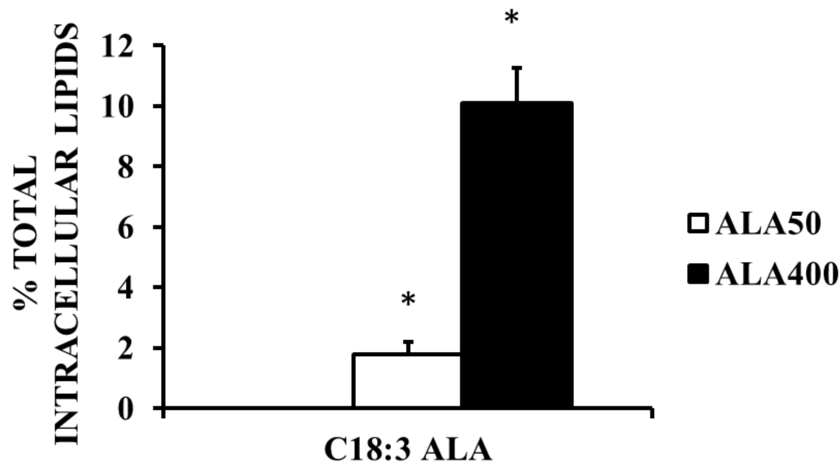


Figure 10: **Expression of Fatty Acid Transport Proteins.** Cells were exposed to fatty acids for 24 or 48 hours. Cell extracts were then prepared and run on western blots. Antibodies against fatty acid transport proteins (CD36 and FATP1) were used and densitometry was employed to quantify relative amounts. CD36 Expression in cardiomyocytes was measured after exposure to A) 50 µM and B) 400 µM of fatty acids (VA or EA). Fatty Acid Transport Protein 1 (FATP1) expression was also measured in cardiomyocytes pre-treated with C) 50 µM and D) 400 µM. Anti-actin (total) antibodies were also employed and served as a loading control. No significant differences were observed with any treatment groups after 24 or 48 H. Values represent mean ± SEM. n=3.

v) ALA Uptake in Cardiomyocytes

Cardiomyocytes were exposed to 1% BSA solution containing 50 µM or 400 µM of ALA. Control conditions were composed of 1% BSA with an equivalent amount of ethanol. After 6 or 24 hours, cardiomyocytes were measured for their content of fatty acids. ALA content was significantly increased in cells that were exposed to both 50 µM and 400 µM at all-time points. (Figure 11A and 11B).

A



B

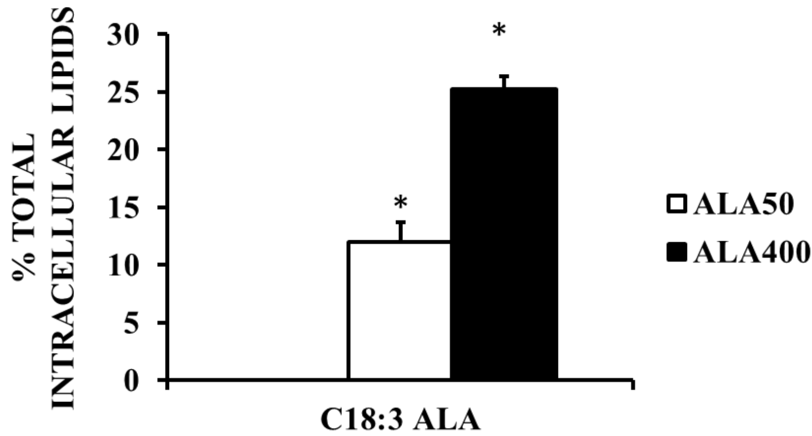


Figure 11: **ALA Fatty Acid Uptake in Cardiomyocytes**. **Cardiomyocytes**. Cardiomyocytes were exposed to 50 or 400 μM alpha linolenic acid (ALA) by direct supplementation to the media. Briefly, after 6 or 24 hours cells were extracted and then prepared for gas chromatography. Intracellular fatty acid content was measured. Here, measurements of intracellular ALA content was measured and represented in a bar graph. Representative graphs show intracellular ALA content of cardiomyocytes exposed to 50 or 400 μM of ALA for A) 6 hours and B) 24 hours. *$p<0.05$ compared to CON. Values represent mean ± SEM. n=4.

vi) Quantification of ALA Incorporation into Phosphatidylcholine

It was hypothesized that ALA pre-treatment of the cardiomyocytes for 24 hours would result in a significant increase in ALA incorporation into the cellular pool of phosphatidylcholine. Phospholipid molecules that had 36 carbons with 2 unsaturation positions (36:2) and with 3 unsaturation positions (36:3) were analyzed to determine the change in distribution from the major class (18:2) to the class that contained ALA (18:3). Using LC/MS, ALA incorporation into phosphatidylcholine was measured. A significant increase in the ALA content within cardiomyocyte phosphatidylcholine was observed after exposure of the cells to ALA in the medium (Figure 12).

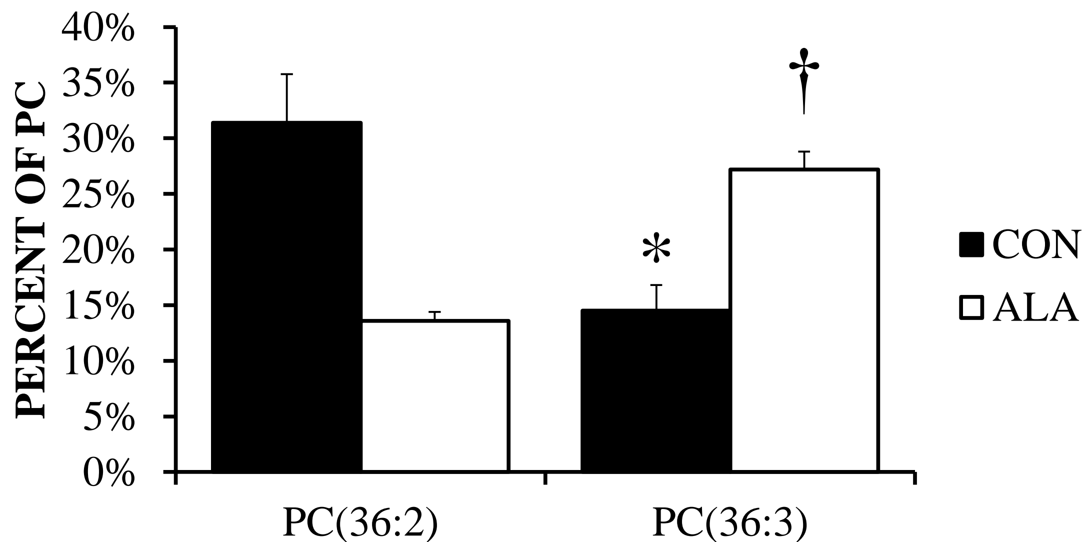
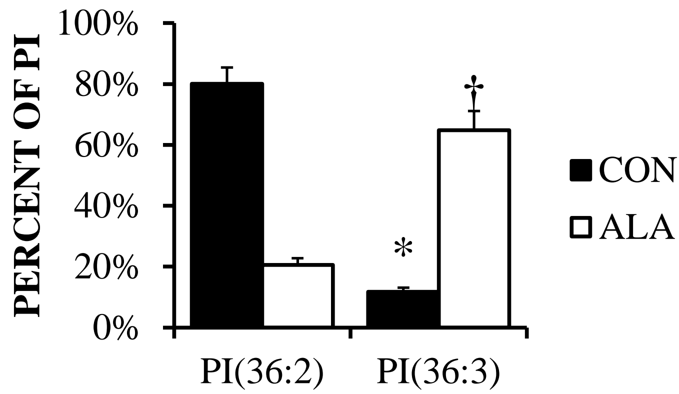


Figure 12: **Phosphatidylcholine Content in Cardiomyocytes after 24 H of ALA Pre-Treatment.** Cardiomyocytes were exposed to 400 μM ALA by direct supplementation to the media. Briefly, after 24 hours cells were extracted and then prepared for liquid chromatography/mass spectrometry (LC/MS). Incorporation of ALA into phosphotidylcholine was measured. Cells treated with ALA incorporated the ALA into cellular phospholipids with a concomitant decrease in other fatty acid components. Phosphatidylcholine is the largest class of phospholipids found within cells (*$p<0.05$ compared to CON (36:2); †$p<0.05$ compared to ALA (36:2) in the 36:2 and 36:3 phospholipid species). Values represent mean ± SEM. N=4.

vii) ALA Incorporation into Other Phospholipid Classes

Phosphatidylcholine represents the major class of phospholipids present within the cell. It is estimated that phosphatidylcholine represents up to 70% of the total phospholipids within the cell [133]. However, ALA administration for 24 hours to cardiomyocytes increased the incorporation of ALA into other phospholipid classes including phosphatidylinositol (Figure 13A), phosphatidylserine (Figure 13B), phosphatidylglycerol (Figure 13C) and phosphatidylethanolamine (Figure 13D). We observe the largest increase in incorporation within PI and PS, where the lowest incorporation was observed in PG and PE (Figure 13).

A



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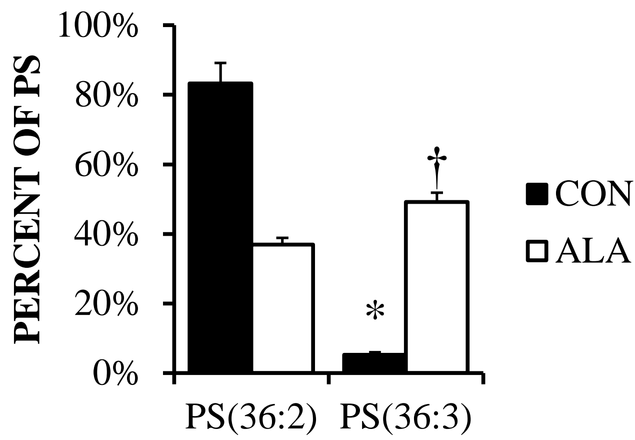

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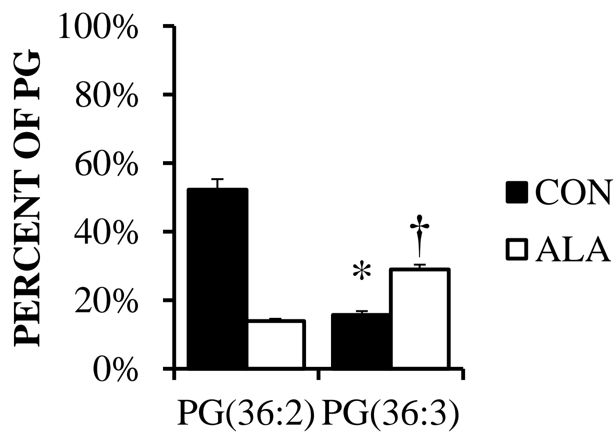

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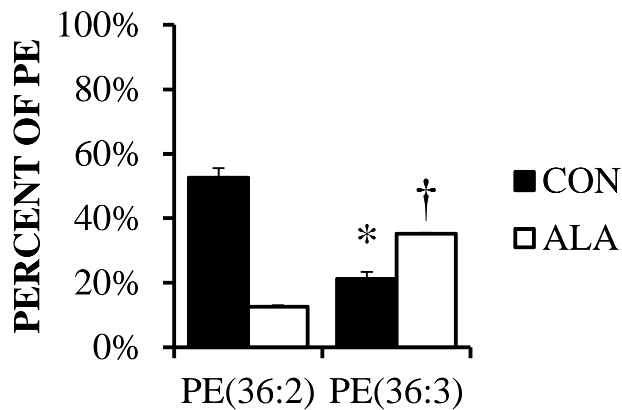


Figure 13: **Phospholipid Content in Cardiomyocytes after 24 hour Exposure to a Medium Supplemented with 400 µM ALA.** Cardiomyocytes were exposed to 400 µM ALA by direct supplementation to the media. Briefly, after 24 hours cells were extracted and then prepared for liquid chromatography/mass spectrometry (LC/MS). Incorporation of ALA into phospholipids was measured. Cells treated with ALA incorporated the ALA into cell phospholipids with a concomitant decrease in other fatty acid components. A: phosphatidylinositol (PI), B: phosphatidylserine (PS), C: phosphatidylglycerol (PG), and D: phosphatidylethanolamine (PE) were all significantly changed (*p<0.05 compared to CON (36:2); †p<0.05 compared to ALA (36:2) in the 36:2 and 36:3 phospholipid species. Values represent mean ± SEM. n=4.

Section 2: Effect of VA or EA on Adiponectin, and Markers of Apoptosis and Autophagy

i) Caspase-3 Activity within LDLr$^{-/-}$ Mice Heart Tissue After Dietary Supplementation with VA or EA for 14 weeks

Apoptosis, or programmed cell death, is an important mechanism of cell death. The effects of VA and EA on apoptosis were studied. Caspase-3 is a major effector protein that can induce DNA fragmentation. Cleavage of caspase-3 activates this protein and will ultimately cause downstream effects. Using western blot analysis, caspase-3 activity was measured in heart extracts from LDLr-/- mice fed a diet supplemented with either 1.5% VA or EA for 14 weeks. No significant changes were observed in caspase-3 activity in heart homogenates upon comparison of the RL (regular diet) and LT (elaidic acid diet) group as well as when comparing the BR (butter group-serves as a control for vaccenic acid group) and BT (vaccenic acid group) (Figure 14).

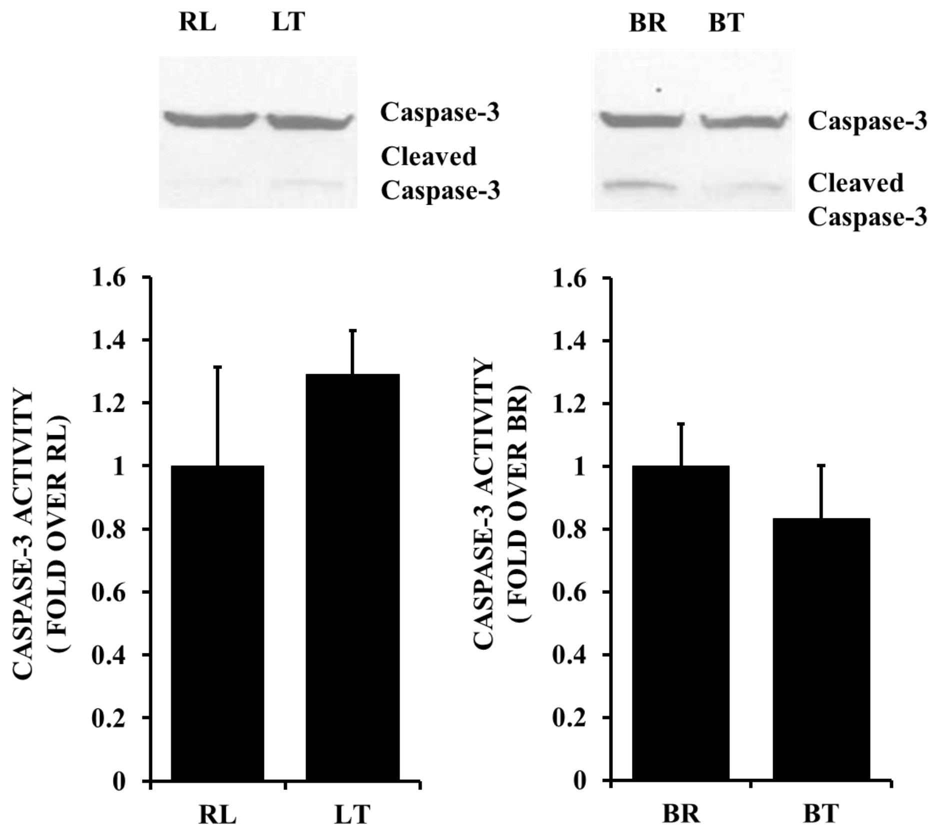


Figure 14: **Caspase-3 Activity as a Function of Dietary Trans Fatty Acid Intervention for 14 Weeks**. Mice were fed a regular diet (RL), or a diet supplemented with 1.5% elaidic acid (LT), or a diet containing butter with 0.3% vaccenic acid (BR) or with 1.5% vaccenic acid (BT). Hearts were excised from animals and stored in -80 degrees centigrade. Representative image of whole heart homogenates were run on western blot. Antibodies against caspase-3 (both cleaved and total forms) were used to visualize western blot results. Values represent mean ± SEM. n=3.

ii) Measurement of Autophagy Proteins within Heart Tissue from LDLr$^{-/-}$ Mice Supplemented with VA or EA Acid in their Diets for 14 weeks

Cell death may also be induced by autophagic mechanisms. Two biomarkers of autophagy, beclin-1 and light chain 3 (LC3) were studied. To confirm that autophagy was induced, LC3 expression (examined as LC3II/LC3I expression ratio) was measured. Using western blot analysis, beclin and LC-3 activity was measured in heart extracts from LDLr$^{-/-}$ mice fed a diet supplemented with either 1.5% VA or EA for 14 weeks. As per western blot analysis, no significant changes were observed in either beclin content or LC3II/LC3I ratio upon comparison of the RL (regular diet) and LT (elaidic acid diet) group as well as the BR (butter control for vaccenic acid group) and BT (butter + vaccenic acid group) (Figure 15).

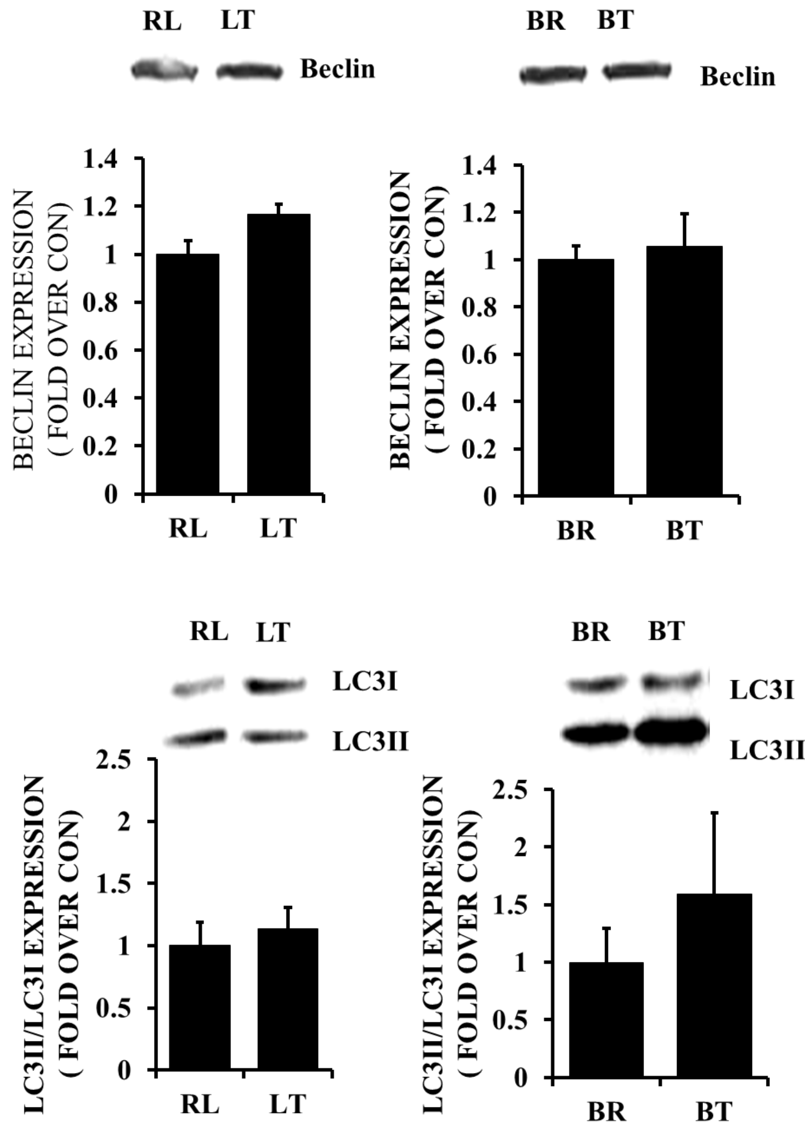


Figure 15: **Cardiac Autophagic Markers as a Function of Dietary Trans Fatty Acid Intervention for 14 Weeks.** Mice were fed a regular diet (RL), or a diet supplemented with 1.5% elaidic acid (LT), or a diet containing butter with 0.3% vaccenic acid (BR) or with 1.5% vaccenic acid (BT). Hearts were excised from animals and stored in -80 degrees centigrade. Representative image of whole heart homogenates were run on western blot. Antibodies against A) Total beclin and B) LC-3 (both I and II) were used to visualize western blot results. Values represent mean ± SEM. No significant values were recorded. n=3

iii) Quantification of Cell Death in Cardiomyocytes Exposed to VA or EA for 24 or 48 H

Cell death can be quantified by visualization of the cell morphology. Morphological changes including rounding up of the cell are key indicators of cardiomyocyte cell death. These have been used previously to measure cell death [98, 106]. This method was used to quantify cell death in cardiomyocytes exposed to two concentrations of EA or VA (50 or 400 μM) for 24 or 48 hours (Figure 16). Twenty-four hours of exposure of myocytes to 50 μM of VA or EA increased the number of dead cells vs. live cells (Figure 16). After exposure of cardiomyocytes to 400 μM VA or EA, cell death increased after 48 hours in comparison to control condition (Figure 16). All other conditions elicited effects that remained comparable to control.

iv) Measurement of Caspase-3 Activity in Cardiomyocytes Exposed to VA or EA (50 or 400 μM) for 24 or 48 H

Apoptosis, or programmed cell death, is a major mechanism to determine cell death [4]. The effects of VA and EA on apoptosis were studied. Caspase-3 is a major effector protein that leads to DNA fragmentation [113]. Cleavage of caspase-3 activates this protein and will ultimately cause downstream effects [113]. Using western blot analysis, caspase-3 activity was measured in cardiomyocytes that were exposed to two concentration of VA or EA (50 or 400 μM) for 24 or 48 hours. Caspase-3 activity remained comparable to control conditions after exposure of cells to a 50 μM concentration of all fatty acids and at both the 24 and 48 hour time points (Figure 17). With 400 μM pre-treatment, both VA and EA increased caspase-3 activity at 48 hours (Figure 17). Representative images have been cropped and altered. Original images contained two lanes of ALA which are represented in Figure 22.

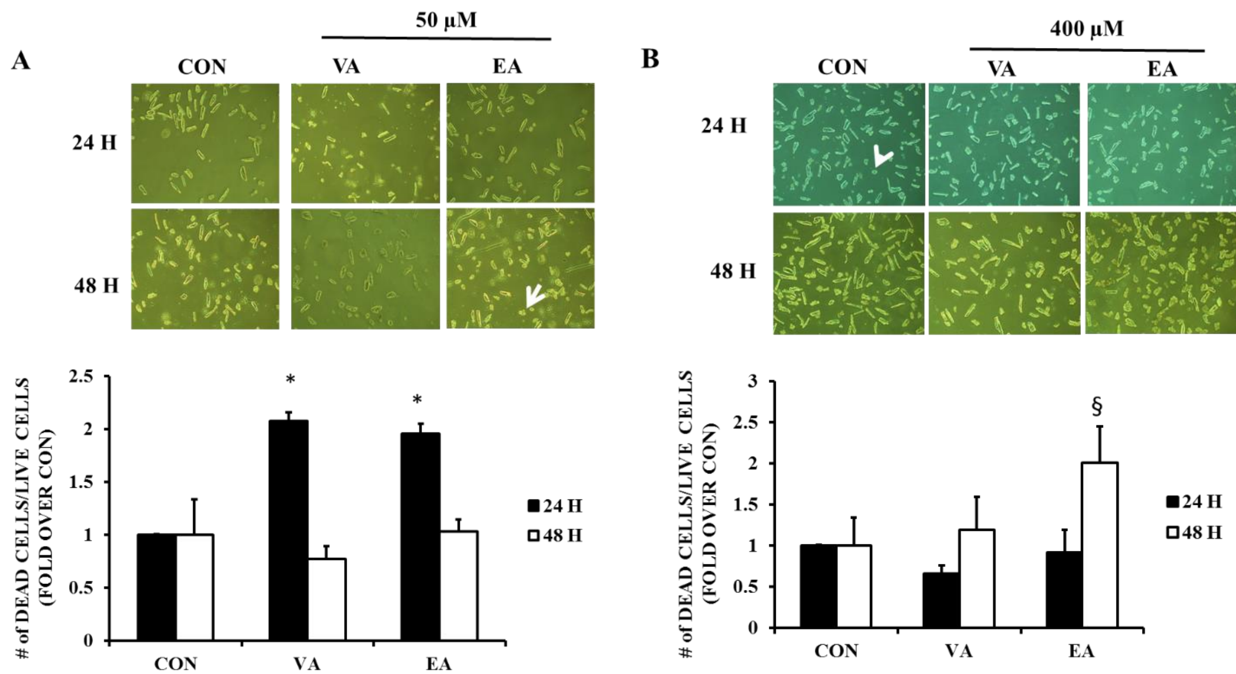


Figure 16: **Cardiomyocyte Viability After Exposure to Media Supplemented with EA or VA Fatty Acids**. *A:* Microscopic images of cardiomyocytes after 24 and 48 hour pre-treatment with fatty acid (VA or EA) and Graphical Representation of Number of Dead Cells vs. the Number of Live Cells as determined by morphological visualization. A) 50 µM pre-treatment and B) 400 µM pre-treatment *$p<0.05$ compared to CON 24 H and §$p<0.05$ compared to CON 48 H. Values represent mean ± SEM. n=5-6

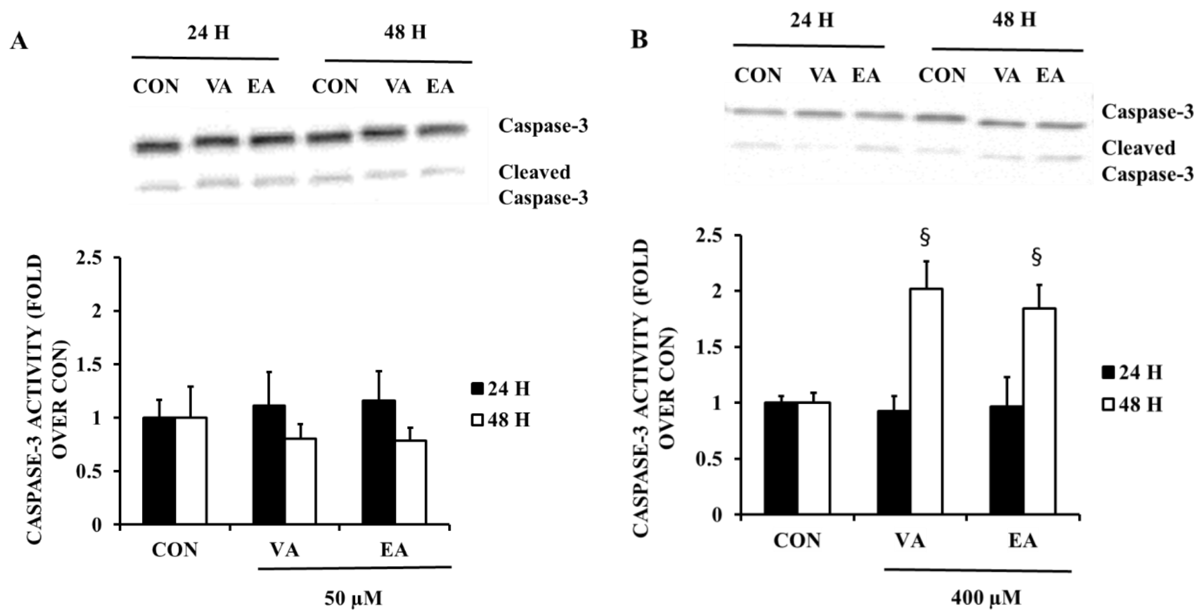


Figure 17: **Caspase-3 Activation in Cardiomyocytes as a Function of Trans Fatty Acid Exposure for 24 or 48 H.** Cells were exposed to fatty acids for 24 or 48 hours. Cell extracts were then prepared and run on western blots. Antibodies against fatty cleaved caspase-3 were used and densitometry was employed to quantify relative amounts. Caspase-3 activity in cardiomyocytes was measured after exposure to A) 50 µM and B) 400 µM of fatty acids (VA or EA).*$p<0.05$ compared to CON 24 H and §$p<0.05$ compared to CON 48 H. Values represent mean ± SEM. n=3.

v) *Bax/Bcl-2 Ratio in Cardiomyocytes exposed to VA or EA (50 or 400 µM) for 24 or 48 H*

The molecular mechanisms responsible for the apoptosis-induced changes in cell death were investigated further by measuring Bax and Bcl-2 expression. Bax is a pro-apoptotic protein which induces apoptosis through the intrinsic pathway. This includes release from the mitochondria. However, the mitochondria also releases anti-apoptotic proteins such as Bcl-2. Changes in the ratio of the expression of these two proteins are frequently used as a marker of apoptosis [245]. No significant changes in Bax/BCL-2 ratio were observed with 50 µM pre-treatment of VA or EA for 24 hours. However, a significant increase in the Bax/Bcl-2 ratio was observed after 48 hours exposure to 400 µM EA (Figure 18). Exposure to VA induced a significant decrease on Bax/Bcl-2 ratio at 400 µM pre-treatment after 48 hours (Figure 18). Representative images have been cropped and altered. Original images contained two lanes of ALA which were cropped out.

vi) Changes in Autophagy Related Proteins in Cardiomyocytes after Exposure to VA or EA (50 or 400 µM) for 24 or 48 H

Cell death may also be induced by autophagic mechanisms. Two biomarkers of autophagy, beclin-1 and LC-3 were studied. LC-3 expression (examined as LC3II/LC3I expression ratio) was measured. Using western blot analysis, beclin and LC-3 activity was measured in cardiomyocytes exposed to VA or EA (50 or 400 uM) for 24 or 48 hours (Figure 19). At 50 µM pre-treatment, beclin-1 activity did not significantly change from control after either 24 or 48 hours with pre-treatment of VA or EA (Figure 19A). At 400 µM, beclin-1 activity was not significantly different after treatment of VA or EA after 24 hours of exposure. However, beclin expression decreased after exposure of cells to 400 µM EA for 48 hours (Figure 19B). VA at a 400 µM concentration did significantly alter beclin expression at 48 hours (Figure 19B).

LC3 expression (examined as LC3II/LC3I expression ratio) remained similar in both EA and VA treatments after 24 hours supplementation with either 50 or 400 μM fatty acid. However, LC3 expression significantly increased in cardiomyocytes after 48 hours of exposure to 50 or 400 μM EA (Figure 19C and D). VA did not significantly alter LC3 expression at 48 hours with either the 50 or 400 μM pre-treatment (Figure 19C and D). Representative images have been cropped and altered. Original images contained two lanes of ALA which are represented in Figure 23.

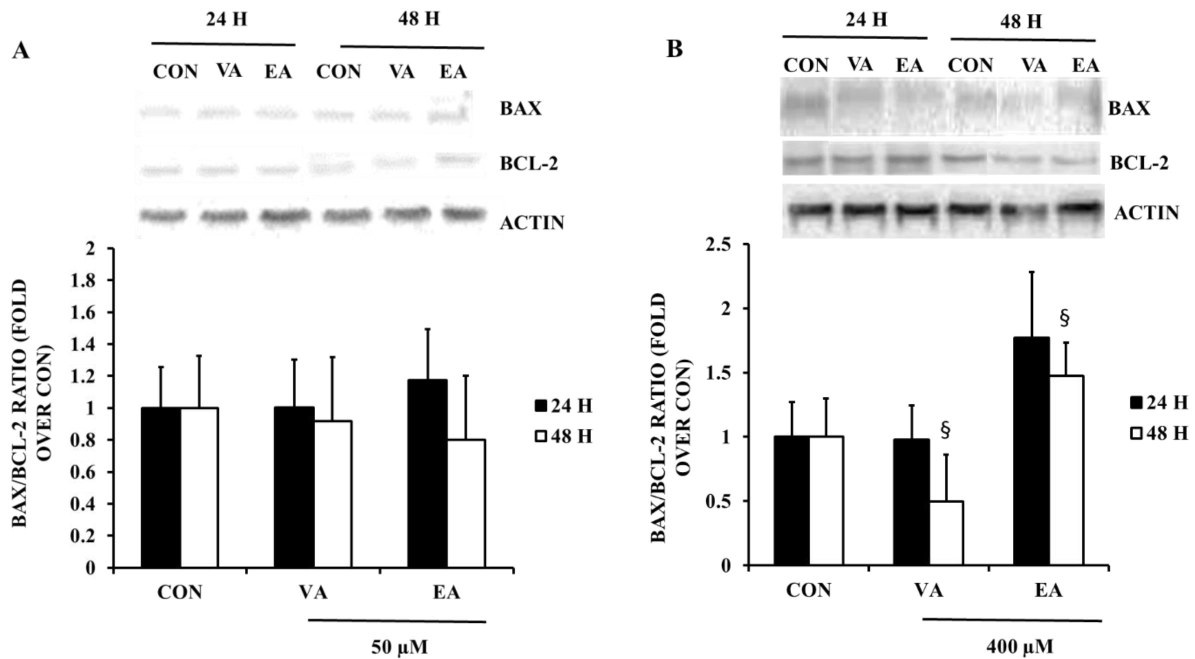


Figure 18: **Apoptotic Markers as a Function of Trans Fatty Acid Pre-Treatment for 24 or 48 H.** Cells were exposed to fatty acids for 24 or 48 hours. Cell extracts were then prepared and run on western blots. Antibodies against fatty Bax and Bcl-2 were used and densitometry was employed to quantify relative amounts. Bax/Bcl-2 ratio in cardiomyocytes was measured after exposure to A) 50 µM and B) 400 µM of fatty acids (VA or EA). Anti-actin (total) antibodies were also employed and served as a loading control.*$p<0.05$ compared to CON 24 H and §$p<0.05$ compared to CON 48 H. Values represent mean ± SEM. n=3.

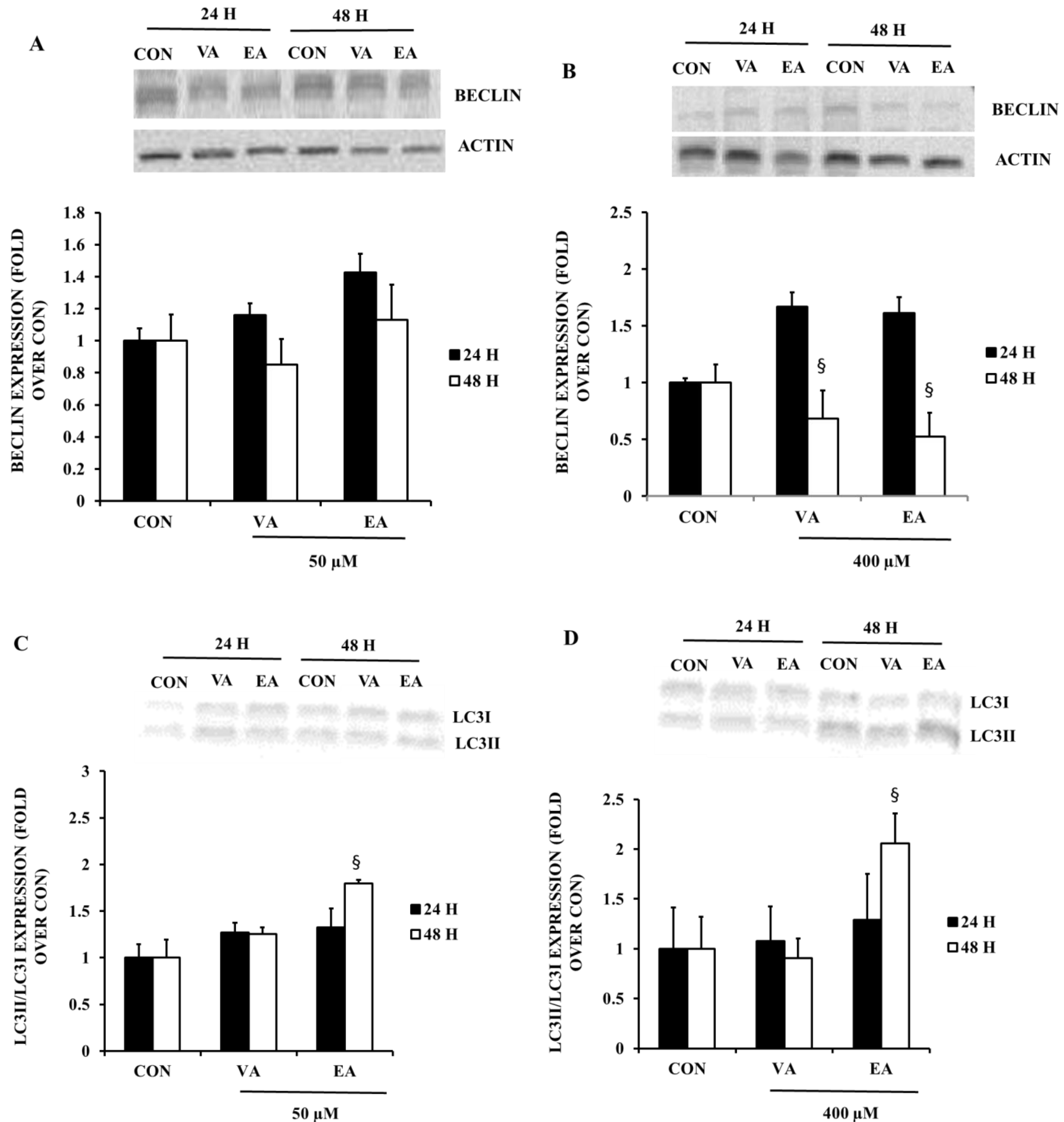


Figure 19. **Expression of Autophagic Markers after 24 or 48 H of Exposure to Different Trans Fatty Acids.** Cells were exposed to fatty acids for 24 or 48 hours. Cell extracts were then prepared and run on western blots. Antibodies against beclin (A and B) as well as LC-3 (C and D) were used and densitometry was employed to quantify relative amounts. Beclin content in cardiomyocytes was measured after exposure to A) 50 μM and B) 400 μM of fatty acids (VA or EA). LC-3 activity (as a function of LC-3II/LC-3I expression) was measured after exposure to C) 50 μM and D) 400 μM of fatty acids (VA or EA). Anti-actin (total) antibodies were also employed and served as a loading control.*$p<0.05$ compared to CON 24 H and §$p<0.05$ compared to CON 48 H. Values represent mean ± SEM. n=3.

vii) Expression of the Myokine Marker, Adiponectin, in Cardiomyocytes Treated with VA or EA (50 or 400 µM) for 24 or 48 H

Adiponectin is a 30 kDa protein that is secreted by tissues such as the heart, skeletal muscle and adipose tissue [146]. Adiponectin production by cardiomyocytes was studied under control conditions or in the presence of media supplementation with VA or EA. Cardiomyocytes were exposed to two concentrations of fatty acids (50 and 400 µM) for two different durations (24 and 48 hours) (Figure 20). No significant changes were observed in comparison to control with 50 µM VA administration for 24 or 48 hours (Figure 20A). However, 50 µM EA supplementation decreased the adiponectin content within cardiomyocytes over 48 hours of exposure (Figure 20A). We found that 48 hour exposure to VA or EA at 400 µM decreased adiponectin content (Figure 20B). Representative images have been cropped and altered. Original images contained two lanes of ALA which are represented in Figure 24.

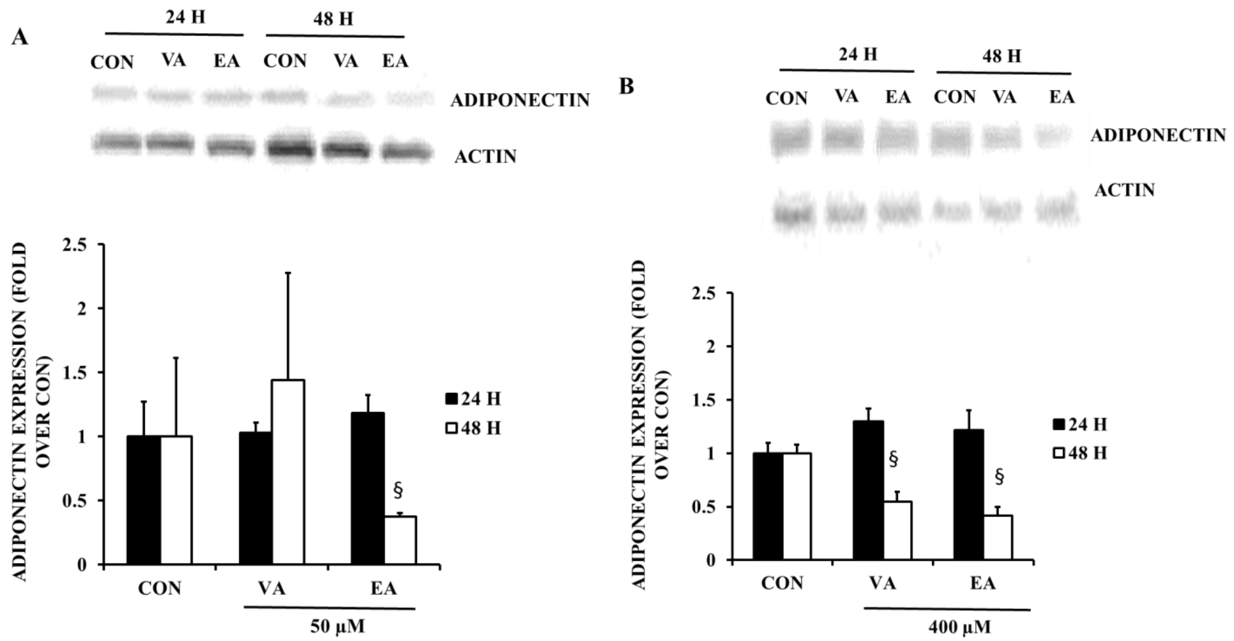


Figure 20: **Expression of Adiponectin in Isolated Cardiomyocytes after 24 or 48 H Trans Fatty Acid Incubation**. Cells were exposed to fatty acids for 24 or 48 hours. Cell extracts were then prepared and run on western blots. Antibodies against fatty adiponectin were used and densitometry was employed to quantify relative amounts. Adiponectin content in cardiomyocytes was measured after exposure to A) 50 µM and B) 400 µM of fatty acids (VA or EA). Anti-actin (total) antibodies were also employed and served as a loading control.*$p<0.05$ compared to CON 24 H and §$p<0.05$ compared to CON 48 H. Values represent mean ± SEM. n=4.

Section 3: Effect of ALA on Adiponectin, and Markers of Apoptosis and Autophagy in Cardiomyocytes

i) Quantification of Cell Death in Cardiomyocytes Exposed to ALA for 24 or 48 H

Cell death can be quantified by visualization of the cell morphology. Morphological changes including rounding up of the cell are key indicators of cardiomyocyte cell death. These have been used previously to measure cell death [98]. This method was used to quantify cell death in cardiomyocytes exposed to two concentrations of ALA (50 or 400 μM) for 24 or 48 hours (Figure 21). Twenty-four hours of exposure of myocytes to 50 μM of did not change the number of dead cells vs. live cells (Figure 21A). However, 48 hours of exposure to 50 μM decreased number of dead cells vs. number of lives cells. Similarly, exposure of cardiomyocytes to 400 μM ALA, cell death decreased after 48 hours in comparison to control condition (Figure 21B).

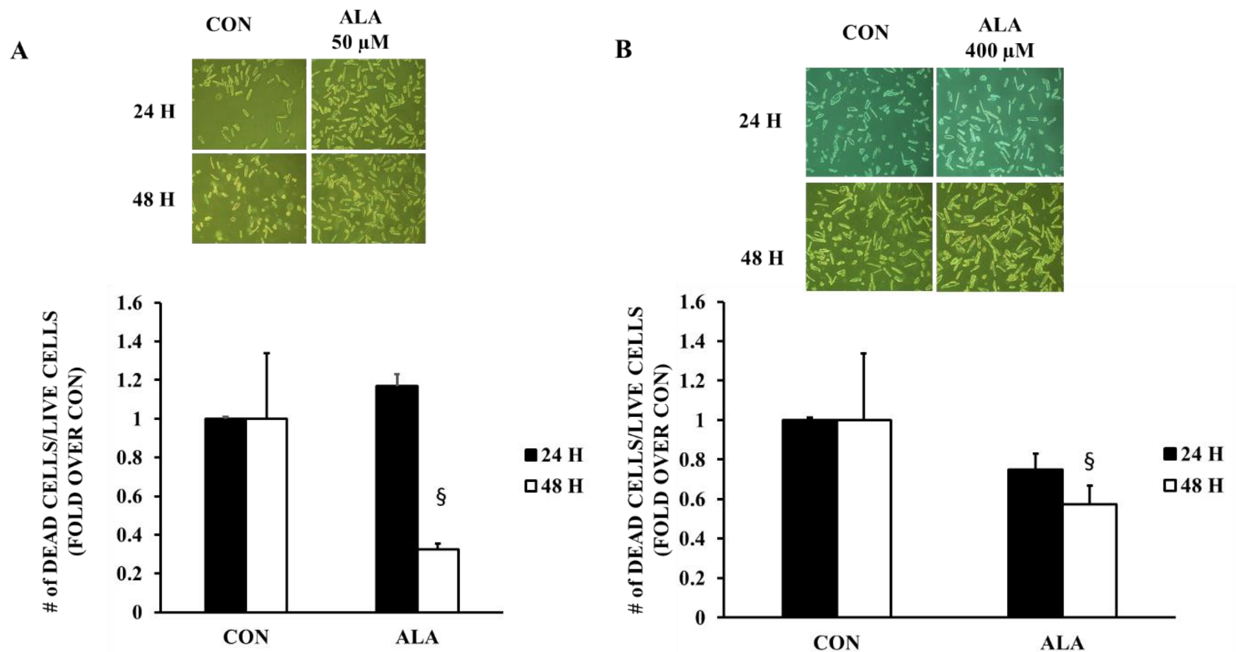


Figure 21: **Cardiomyocyte Viability After Exposure to Media Supplemented with ALA**. *A:* Microscopic images of cardiomyocytes after 24 and 48 hour pre-treatment with fatty acid (VA or EA) and Graphical Representation of Number of Dead Cells vs. the Number of Live Cells as determined by morphological visualization. A) 50 μM pre-treatment and B) 400 μM pre-treatment $*p<0.05$ compared to CON 24 H and §$p<0.05$ compared to CON 48 H. Values represent mean $\pm$ SEM. n=5-6

ii) Measurement of Caspase-3 Activity in Cardiomyocytes Exposed to ALA (50 or 400 μM) for 24 or 48 H

Apoptosis, or programmed cell death, is a major mechanism to determine cell death. The effects of ALA on apoptosis was studied. Caspase-3 is a major effector protein that leads to DNA fragmentation. Cleavage of caspase-3 activates this protein and will ultimately cause downstream effects [113]. Using western blot analysis, caspase-3 activity was measured in cardiomyocytes that were exposed to two concentration of ALA (50 or 400 μM) for 24 or 48 hours. Caspase-3 activity remained comparable to control conditions after exposure of cells to a 50 μM concentration of all fatty acids and at both the 24 and 48 hour time points (Figure 22A). With 400 μM pre-treatment, ALA pre-treatment did not change caspase-3 activity after 24 or 48 hours of exposure (Figure 22B). Representative images have been cropped and altered. Original images contained lanes of VA and EA which are represented in Figure 17.

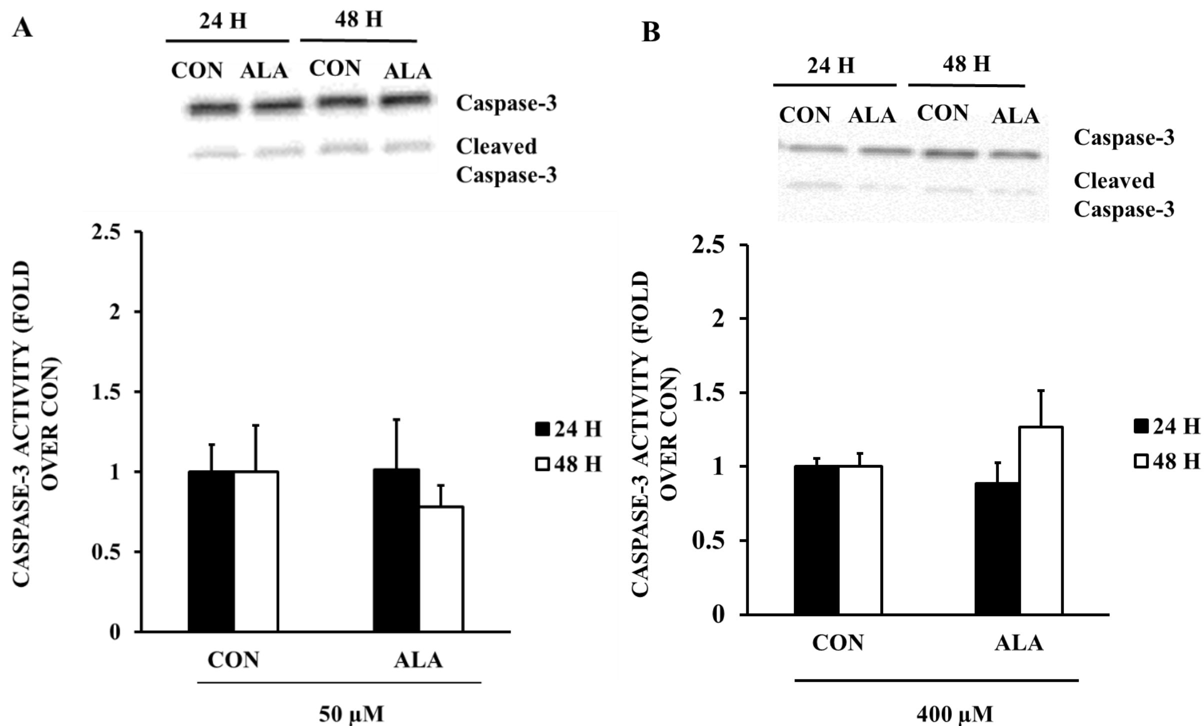


Figure 22: **Caspase-3 Activation in Cardiomyocytes as a Function of ALA Exposure for 24 or 48.** Cells were exposed to ALA for 24 or 48 hours. Cell extracts were then prepared and run on western blots. Antibodies against fatty cleaved caspase-3 were used and densitometry was employed to quantify relative amounts. Caspase-3 activity in cardiomyocytes was measured after exposure to A) 50 µM and B) 400 µM of ALA.*$p<0.05$ compared to CON 24 H and §$p<0.05$ compared to CON 48 H. Values represent mean ± SEM. n=3.

iii) Changes in Autophagy Related Proteins in Cardiomyocytes after Exposure to ALA (50 or 400 μM) for 24 or 48 H

Cell death may also be induced by autophagic mechanisms. Two biomarkers of autophagy, beclin-1 and LC-3 were studied. LC-3 expression (examined as LC3II/LC3I expression ratio) was measured. Using western blot analysis, beclin and LC-3 activity was measured in cardiomyocytes exposed to ALA (50 or 400 μM) for 24 or 48 hours (Figure 23). At 50 μM, beclin-1 activity was not significantly different after ALA pre-treatment for 24 or 48 hours (Figure 23A). At 400 μM pre-treatment, beclin-1 activity significantly changed with 24 hour pre-treatment of ALA (Figure 23B). To confirm that the increases observed with ALA pre-treatment at 400 μM for 24 hours were related to autophagy we examined LC3 activity. LC3 expression (examined as LC3II/LC3I expression ratio) remained similar in with ALA pre-treatment after 24 hours or 48 hours of supplementation with either 50 or 400 μM fatty acid (Figure 23C and D). Representative images have been cropped and altered. Original images contained lanes of VA or EA which are represented in Figure 19.

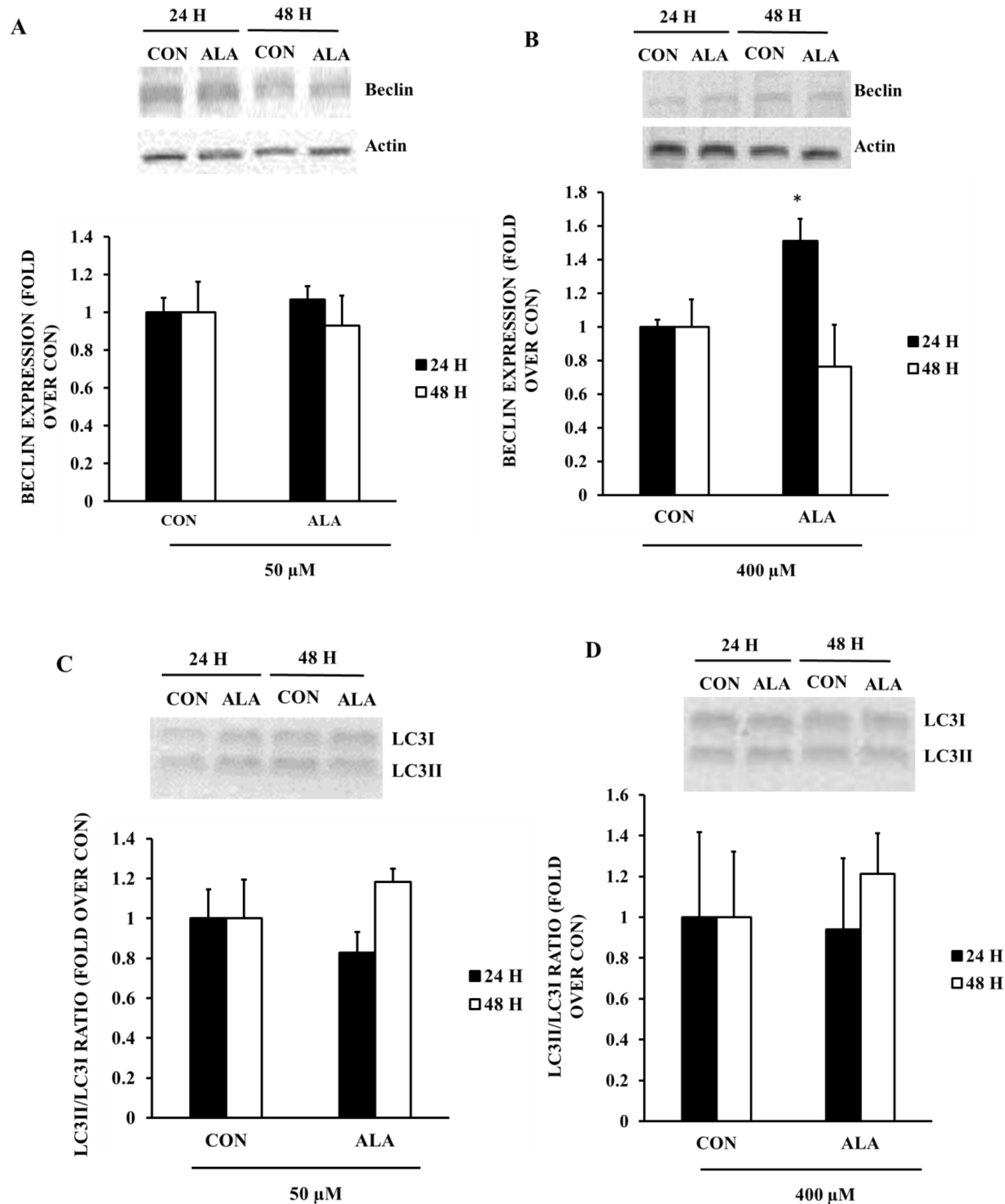


Figure 23: **Expression of Autophagic Markers After 24 or 48 H of Exposure to ALA.** Cells were exposed to fatty acids for 24 or 48 hours. Cell extracts were then prepared and run on western blots. Antibodies against beclin (A and B) as well as LC-3 (C and D) were used and densitometry was employed to quantify relative amounts. Beclin content in cardiomyocytes was measured after exposure to A) 50 µM and B) 400 µM of fatty acids (ALA). LC-3 activity (as a function of LC-3II/LC-3I expression) was measured after exposure to C) 50 µM and D) 400 µM of fatty acids (ALA). Anti-actin (total) antibodies were also employed and served as a loading control.*$p<0.05$ compared to CON 24 H and §$p<0.05$ compared to CON 48 H. Values represent mean ± SEM. n=3.

iv) Expression of the Myokine Marker, Adiponectin, in Cardiomyocytes Treated with ALA (50 or 400 μM) for 24 or 48 H

Adiponectin is a 30 kDa protein that is secreted by tissues such as the heart, skeletal muscle and adipose tissue [146]. Adiponectin production by cardiomyocytes was studied under control conditions or in the presence of media supplementation with ALA. Cardiomyocytes were exposed to two concentrations of fatty acids (50 and 400 μM) for two different durations (24 and 48 hours) (Figure 24). No significant changes were observed in comparison to control with 50 μM or 400 μM administration of ALA for 24 or 48 hours (Figure 24A and B). Representative images have been cropped and altered. Original images contained lanes of VA and EA which are represented in Figure 20.

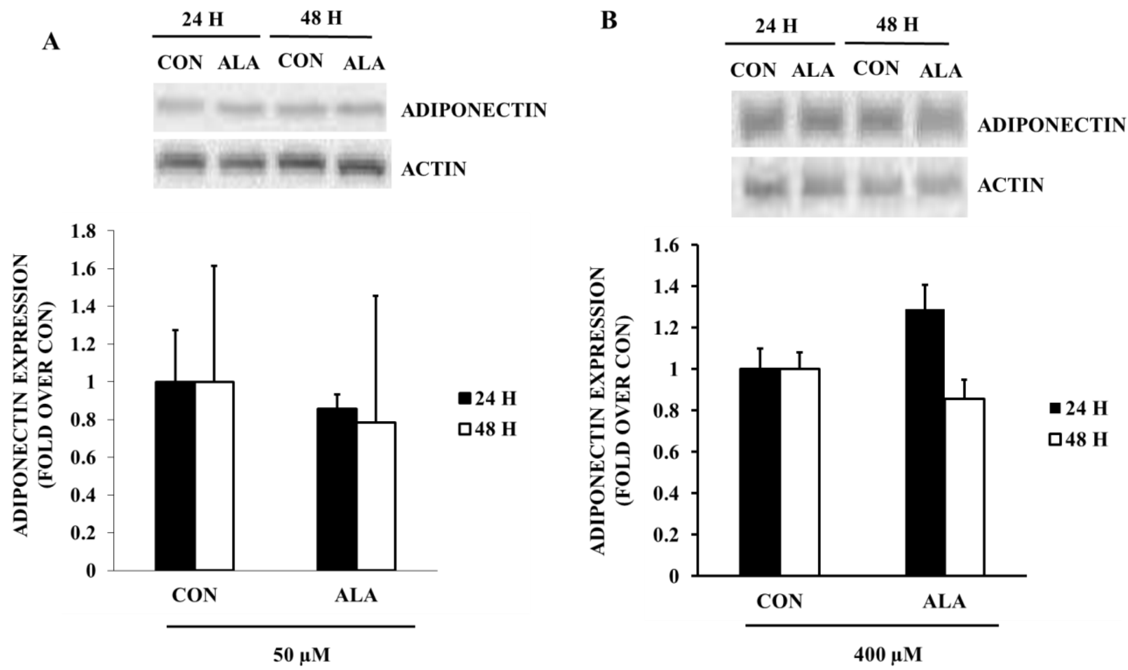


Figure 24: **Expression of Adiponectin in Isolated Cardiomyocytes after 24 or 48 H Exposure to ALA**. Cells were exposed to fatty acids for 24 or 48 hours. Cell extracts were then prepared and run on western blots. Antibodies against fatty adiponectin were used and densitometry was employed to quantify relative amounts. Adiponectin content in cardiomyocytes was measured after exposure to A) 50 µM and B) 400 µM of fatty acids (ALA). Anti-actin (total) antibodies were also employed and served as a loading control.*$p<0.05$ compared to CON 24 H and §$p<0.05$ compared to CON 48 H. Values represent mean ± SEM. n=4.

Section 4: Differential Effects of Trans Fats on Cardiomyocyte Viability under Ischemic (ISCH) or Ischemia/Reperfusion (IR) Conditions

i) Measurement of cardiomyocyte viability after exposure to ISCH or IR alone or after Pre-Treatment of Cells with 400 μM VA or EA for 24 H

In order to better understand the role of these fatty acids during cellular stress, cardiomyocytes were challenged to either ischemia alone ISCH or ischemia/reperfusion conditions (IR). Cardiomyocytes were exposed to 60 minutes of ischemic-mimetic buffer to simulate ISCH). During ischemia/reperfusion injury the cells were exposed to ischemic mimetic buffer for 60 minutes followed by control buffer for 60 minutes (IR). Prior to exposure to ISCH or IR, the cells were immersed in trans fatty acids (400 μM VA or EA) for 24 hours. Cells were subsequently analyzed for viability. EA did not increase number of dead/live cells present on the plates after ISCH or IR (Figure 25). Conversely, a significant decrease in the number of dead/live cells was observed when cells were exposed to ISCH 24 hours pre-treatment with VA (Figure 25).

ii) DNA Fragmentation in Cardiomyocytes exposed to ISCH or IR alone or after Pre-Treatment with 400 μM VA or EA for 24 H

The type of cell death and the potential mechanism for this action during ISCH or IR was studied further. DNA fragmentation is a well characterized end point of apoptosis and was analyzed using the dUTP nick end labeling assay (TUNEL assay). Exposure of cardiomyocytes to EA for 24 hours prior to ISCH increased the number of TUNEL positive cells compared to

control (Figure 26). Exposure to VA did not change the number of TUNEL positive cells compared to control (Figure 26).

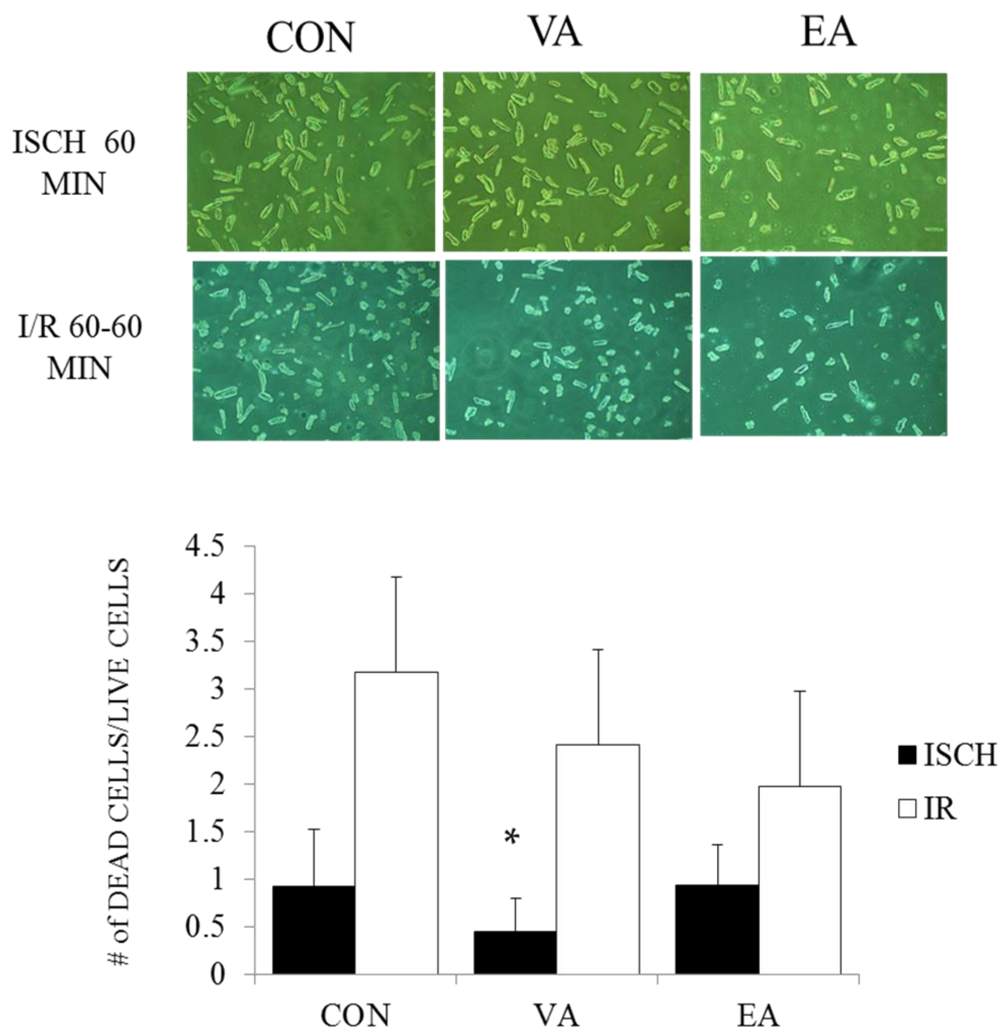


Figure 25: **Cell Viability as a Function of 400 µM Pre-Treatment with Trans Fatty Acid followed by ISCH or IR.** Cells were exposed to 400 µM fatty acid for 24 H prior to ISCH. *Top Panel*: Representative microscopic images of cells after 60 minutes ischemia (ISCH) or 60 minutes of ischemia and 60 minutes of reperfusion (IR). *Bottom Graph:* Graphical Representation of Live/Dead cell data. * $p<0.05$ compared to CON ISCH. Values represent mean $\pm$ SEM. n=6.

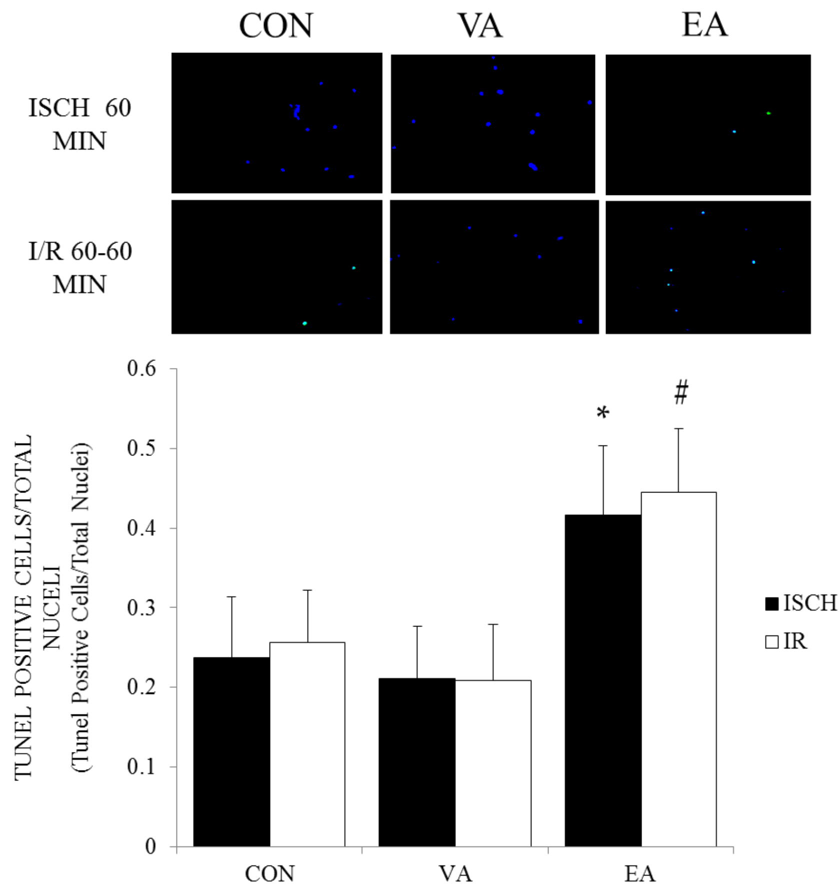


Figure 26: **TUNEL Data in Cardiomyocytes after Pre-Treatment with EA or VA Fatty Acid and ISCH or IR**. Cells were exposed to 400 uM fatty acid for 24 H. *Top Panel*: Representative microscopic images of cells after 60 minutes ischemia (ISCH) or 60 minutes of ischemia and 60 minutes of reperfusion (IR). Blue denotes total nuclei (DAPI) staining and green denotes TUNEL positive cells. *Bottom Graph*: Total Nuclei vs. TUNEL positive cells are calculated in this graph. *$p<0.05$ compared to CON ISH and #$p<0.05$ compared to CON IR. Values represent mean ± SEM. n=6.

iii) Caspase-3 Activity in Cardiomyocytes exposed to ISCH or IR alone or after Pre-Treatment of 400 μM VA or EA for 24 H

Apoptosis, or programmed cell death, is an important mechanism for cell death that is particularly relevant during ischemia/reperfusion injury. The effects of VA and EA on apoptosis during both ISCH and IR were studied. Caspase-3 is a major effector protein that leads to DNA fragmentation. Therefore, the levels of cleaved caspase-3 in cardiomyocytes was studied in these conditions. The levels of cleaved caspase-3 in cardiomyocytes increased after EA pre-treatment during both ISCH and IR challenge (Figure 27). Exposure of cells to VA did not significantly alter caspase-3 expression in cells compared to control (Figure 27).

iv) Changes in Calcium Transients in Cardiomyocytes exposed to ISCH or IR alone or after Pre-Treatment of 400 μM VA or EA for 24 H

Changes in intracellular calcium have been implicated with changes in cardiomyocyte viability during ISCH or IR [104]. The effects of fatty acid pre-treatment on calcium transients and diastolic calcium during ISCH and IR were studied. As shown in Figure 28, none of the fatty acid pre-treatment regimes affected the calcium transients during IR (Figure 28A). Pre-treatment with EA trended towards an increased resting calcium during IR compared to control conditions and VA cells trended below control levels during IR (Figure 28B). However, none of these responses demonstrated a statistically significant difference from each other.

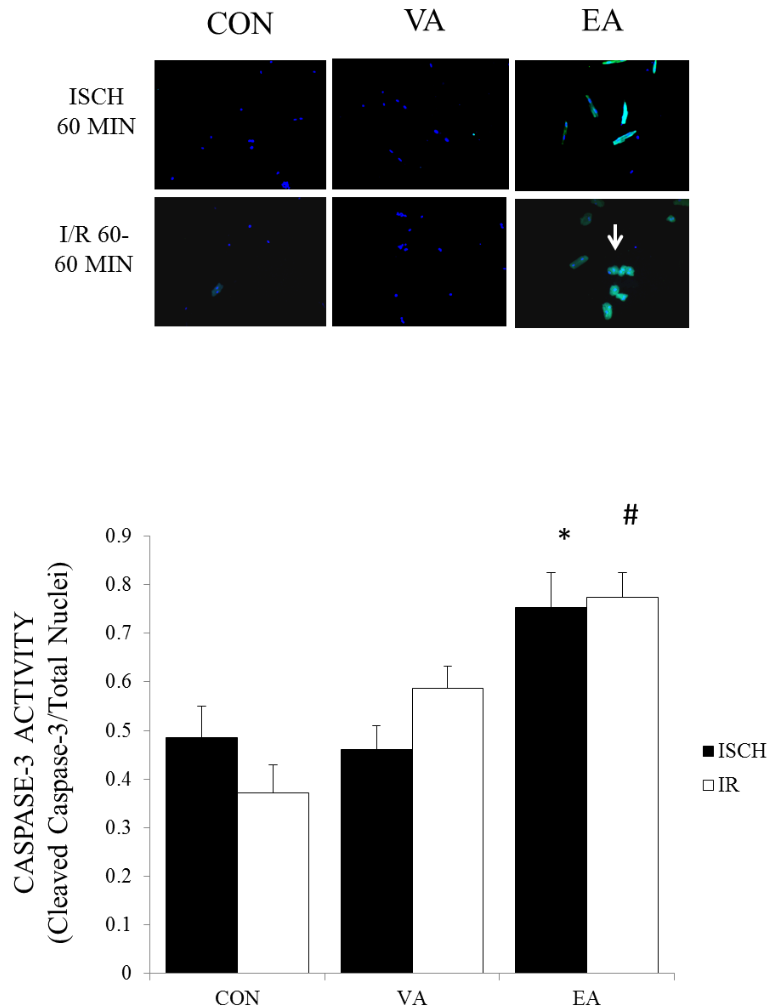
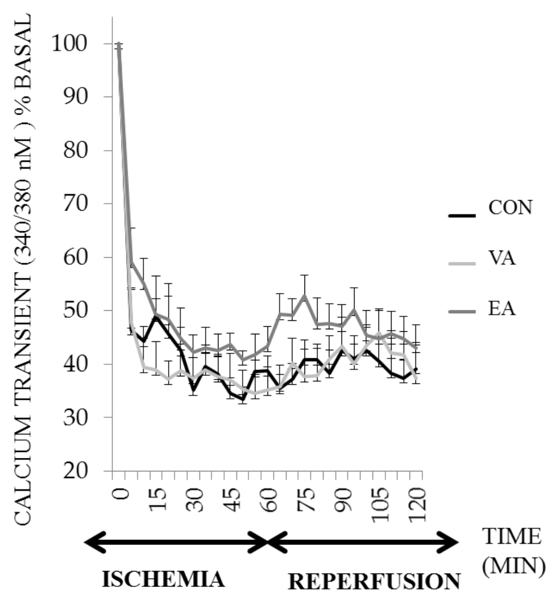


Figure 27: **Cleaved Caspase-3 Detection by Immunofluorescence under ISCH or IR Conditions with VA or EA Pre-Treatment for 24 H**. Cleaved caspase-3 antibody (1:100) was used in this assay to detect cleaved caspase-3 positive cells when cells are exposed to ISCH or IR conditions after 24 H of pre-treatment with 400 µM fatty acid. Green Denotes Caspase-3 positive cells and Blue Denotes Nuclei. * $p<0.05$ compared to CON ISH and # $p<0.05$ compared to CON IR. Values represent mean $\pm$ SEM. n=5-6.

A



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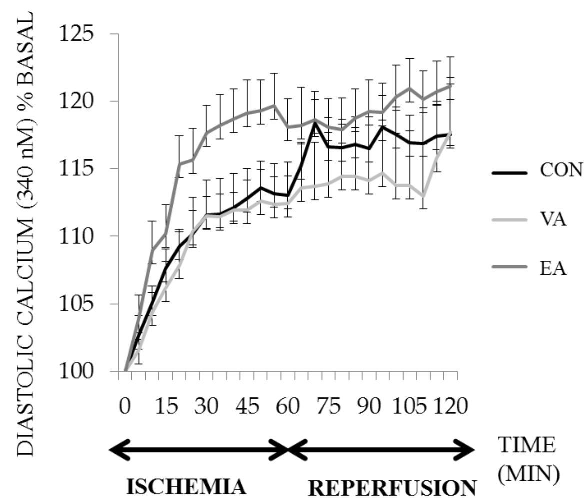


Figure 28: **Calcium Transients and Intracellular Diastolic Calcium in Cardiomyocytes as a Function of Exposure to Trans Fatty Acid.** Cells were treated with 400 μM fatty acid 24 H prior to induction of ischemia/reperfusion conditions. *Top Graph:* Calcium transients were expressed as a ratio of 340/380 nm with the Ca^{2+} sensitive dye FURA2AM. Red Arrow denotes induction of reperfusion. *Bottom Graph:* Diastolic Calcium measurement with FURA-2AM. Values represent mean ± SEM. n=12-16.

v) Oxidized Phospholipid Content in Cardiomyocytes after Pre-Treatment with 400 μM VA or EA for 24 H

Oxidized phospholipids may be associated with cell death [246]. During non-ischemic control conditions, there was a significant decrease in oxidized phospholipids in cardiomyocytes after 24 hours of VA pre-treatment in comparison to control (Figure 29). There was a significant reduction in the raw mass quantitation of six OxPC species identified after 24 hours of exposure to vaccenic acid (6.2±1.52 ng/mL) but not elaidic acid (10.0±0.17 ng/mL) compared to control (12.7±1.14 ng/mL, $p < 0.05$). The OxPC species, 1-palmitoyl-2-(5'-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), 1-palmitoyl-2-(5'-keto-6'-octene-dioyl)-sn-glycero-3-phosphocholine (KOdiA-PPC), and 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (PAzPC) had significantly reduced masses after 24 hour VA administration (Figure 29).

vi) Oxidized Phospholipid Content in Cardiomyocytes Exposed to ISCH or IR

Oxygen derived free radicals may be a mechanism of cell death when cardiomyocytes are exposed to stress conditions such as ischemia/reperfusion injury [247]. Exposure of phospholipids to free radicals can create bioactive compounds in the form of oxidized phospholipids. Phosphatidylcholine (PC) is the most abundant phospholipid present within the cell, therefore, it is relevant to investigate oxidized PC species (oxPC) as a potential influence on cellular viability. POVPC and PGPC was significantly increased under ISCH whereas PGPC was increased by IR only (Figure 30). The other OxPC species were not significantly different than control values (data not shown).

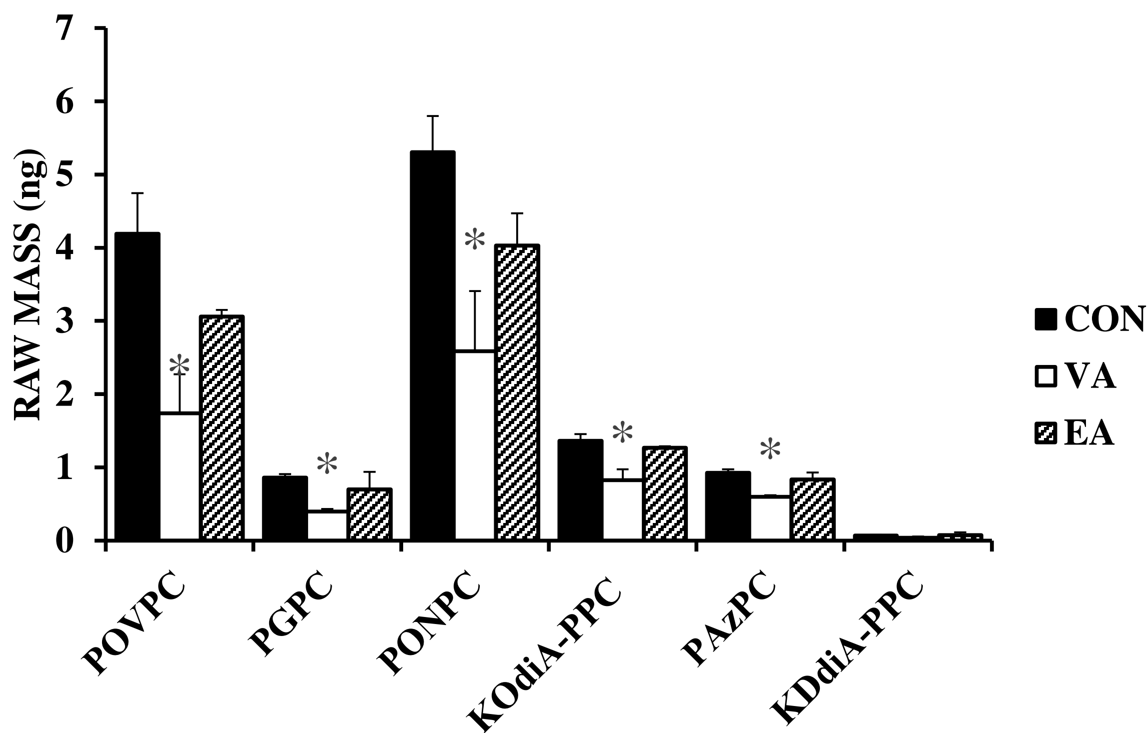


Figure 29: **Oxidized Phospholipid Content in Cells Treated with VA or EA for 24 H.** Cells were treated with 400 µM of VA or EA for 24 hours. Oxidized phospholipid content was measured using LC/MS. Six oxPC's were identified and raw mass was measured. Significant values were expressed as *$p<0.05$ compared to CON conditions. Values represent mean ± SEM. n=3.

vii) Oxidized Phospholipid Content in Cardiomyocytes exposed to ISCH or IR alone or after Pre-Treatment of 400 μM VA or EA for 24 H

To further explore if VA or EA could change oxidized phospholipid levels during ISCH or IR, we measured oxPC content after ISCH or IR with cells that were pre-treated with 400 uM EA or VA for 24 hours. During ISCH there was a marked increase in PGPC and kDdiA-PPC content when the cells were pre-treated with EA (Figure 31A). Although VA trended to an increased PGPC content compared to ISCH alone, this increase was not statistically significant. During IR, a significant increase in PGPC content was observed, however, with EA pre-treatment, a small but significant decrease in PGPC was detected. No change in PGPC content with VA pre-treatment was observed (Figure 31B).

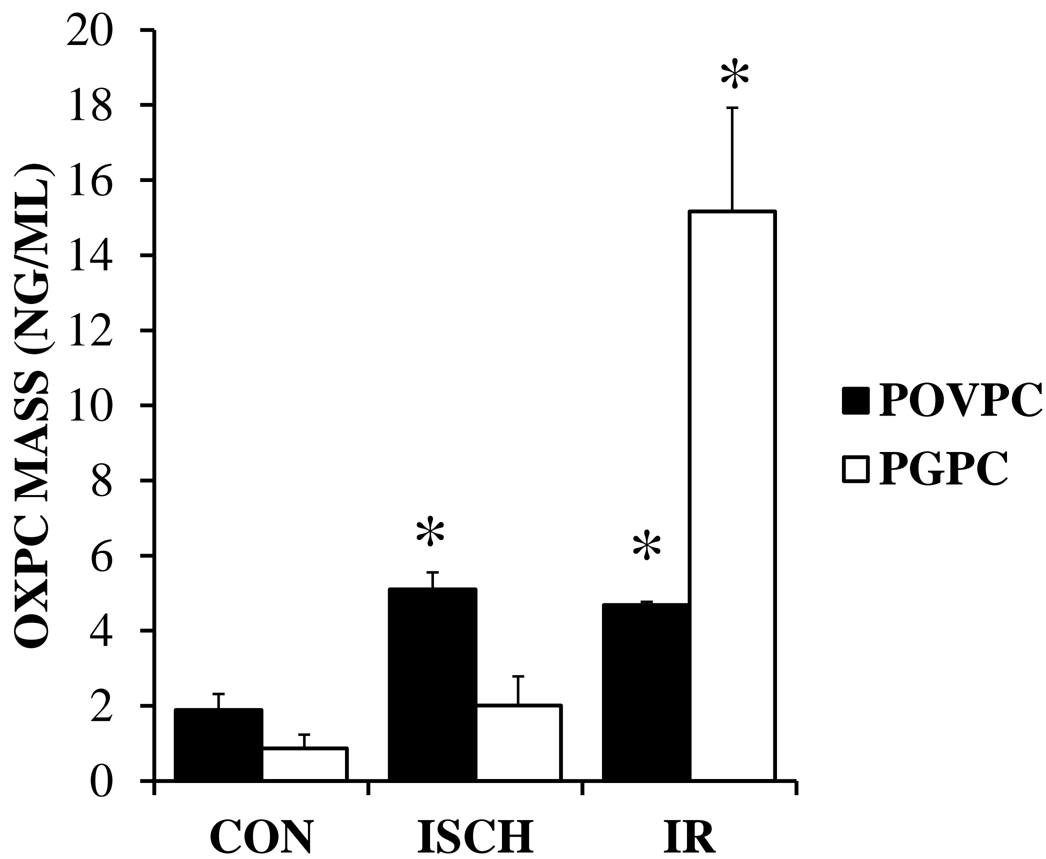
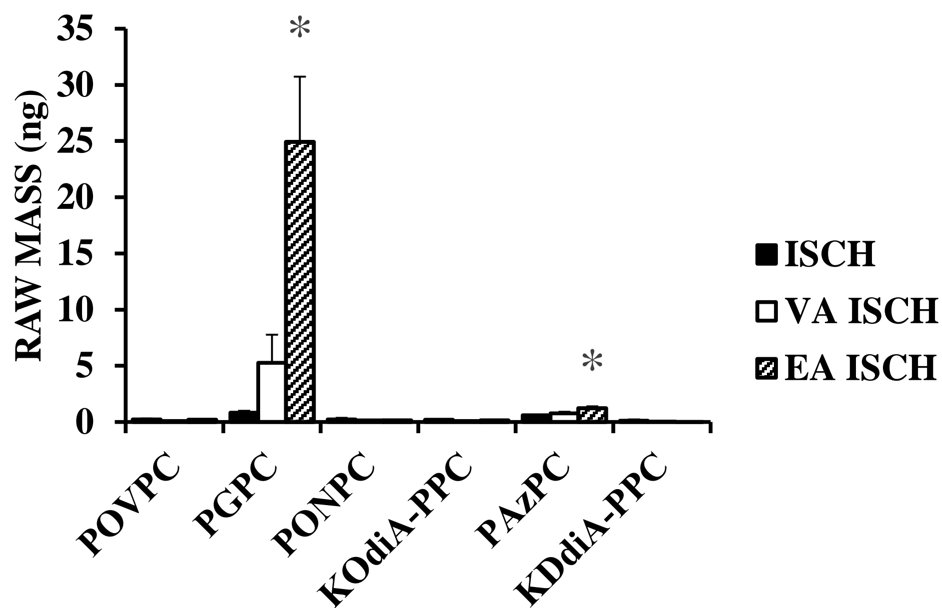


Figure 30: **Oxidized Phospholipid Content in Cardiomyocytes undergoing ISCH or IR.** Oxidized phospholipid content was measured using LC/MS after cardiomyocytes underwent ISCH or IR. Significant values were expressed as *p<0.05 compared to CON conditions. Values represent mean ± SEM. n=3*p<0.05 compared to CON conditions. Values represent mean ± standard error mean (SEM). N=3

A



B

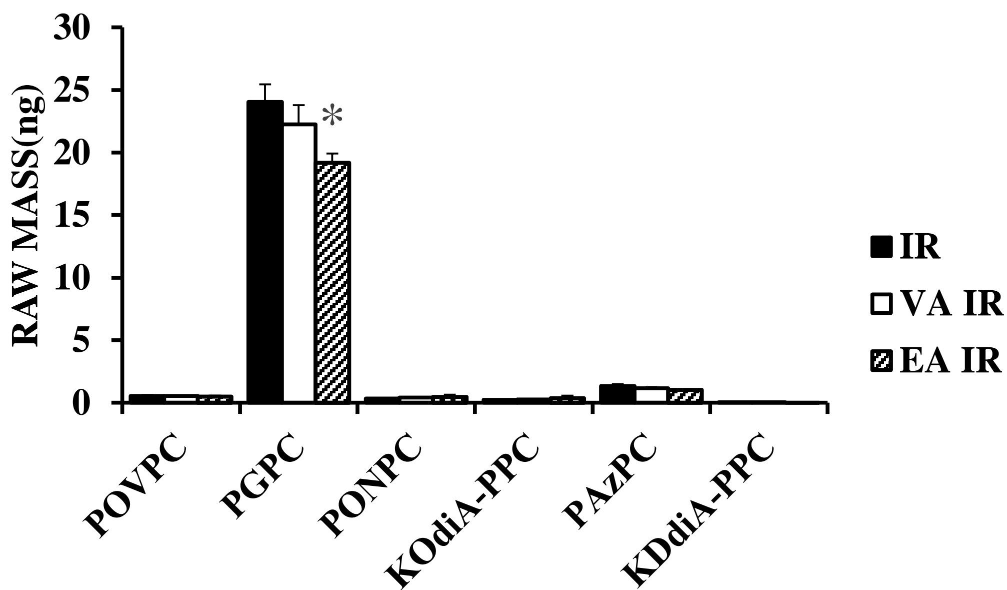


Figure 31: **Changes in OxPC Content as a Function Of VA or EA Pre-Treatment and ISCH or IR**. A: OxPC content after VA or EA pre-treatment for 24 hours (400 μM) followed by ISCH. B: OxPC content after VA or EA pre-treatment for 24 hours (400 μM) followed by IR. *p<0.05 compared to CON conditions. Values represent mean ± SEM. n=3.

Section 5: Direct Effects of ALA on Cardiomyocyte Cellular Viability under Ischemic (ISCH) or Ischemia/Reperfusion (IR) Conditions

i) Cardiomyocyte Viability after Exposure to ISCH or IR alone or after Pre-Treatment of 400 µM ALA for 24 H

Adult rat cardiomyocytes were exposed to 400 µM ALA for 24 hours. Isolated adult cardiomyocytes were then challenged with 60 minutes of simulated ischemia (ISCH) and, in some cases, 60 minutes of ischemia reperfusion (IR). Cells were subsequently analyzed for viability (Figure 32). Under control conditions, ISCH induced a significant decrease in cell viability as shown by morphological analyses (Figure 32). Post-ischemic reperfusion did not induce further decreases in cell viability (Figure 32). If cells were pre-treated with ALA, there was a strong cardioprotective effect as shown by a significant increase in the ratio of live to dead cells during ISCH or IR (Figure 32).

ii) Measurement of DNA Fragmentation through TUNEL Assay of Cardiomyocytes Exposed to ISCH or IR alone or after Pre-Treatment of 400 µM ALA for 24 H

The potential cellular mechanisms responsible for these effects were studied. DNA fragmentation is an end-stage indicator of apoptosis [114]. The dUTP Nick Labeling (TUNEL) assay is a well-established method to measure DNA fragmentation. Cells exposed to ISCH or IR displayed a significant increase in dUTP Nick Labeling as shown by the TUNEL staining procedure (Figure 33). Cardiomyocytes pre-incubated with ALA for 24 hours exhibited significantly fewer TUNEL positive cells during ISCH or IR (Figure 33).

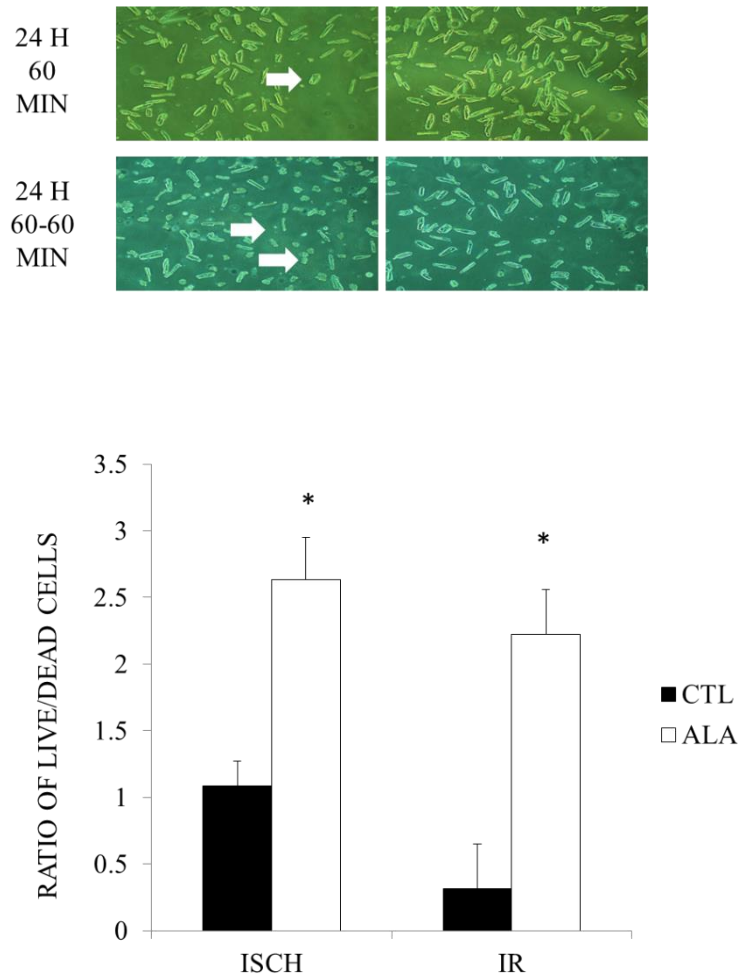


Figure 32: Live/Dead Cell Data with Pre-Treatment of ALA under ISCH or IR Conditions. *Upper Panel:* Microscopic images of live cardiomyocytes after 24 pre-treatment of fatty acid (ALA) and undergoing either control (CTL), ischemic (ISCH) or ischemia reperfusion (IR) conditions. *Lower Panel:* Graphical Representation of Number of Dead Cells vs. the Number of Live Cells. *$p<0.05$ compared to CON 24 H. White arrows denote dead cell morphology. Values represent mean ± SEM. n=6-8.

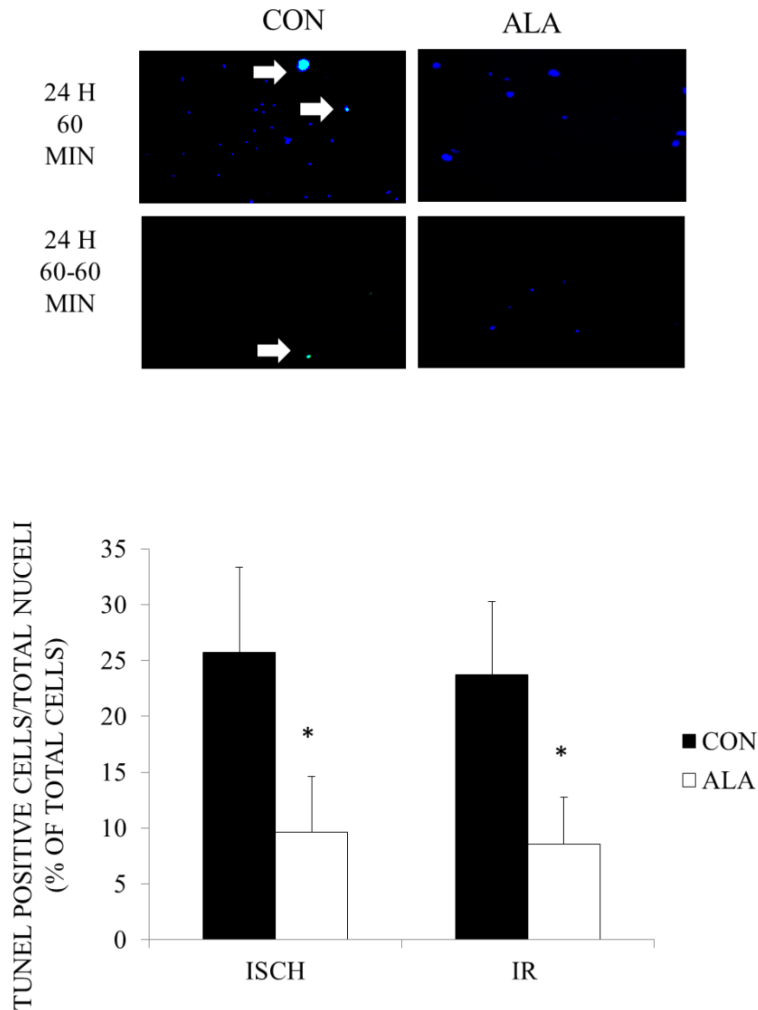


Figure 33: DNA Fragmentation Influenced By Pre-Treatment of ALA For 24 H under ISCH or IR Conditions. TUNEL positive cells vs. total nuclei with 400 μM ALA pre-treatment for 24 hours. Blue denotes total nuclei (DAPI staining) where green denotes fragmented DNA. *$p<0.05$ compared to CON 24 H. White arrows denote TUNEL positive. Values represent mean ± SEM. n=5.

iii) Caspase-3 Activity in Cardiomyocytes exposed to ISCH or IR alone or after Pre-Treatment with 400 μM ALA for 24 H

The molecular mechanisms responsible for the apoptosis-induced changes in cell death were investigated further by measuring caspase-3 activation. Caspase-3 activation, as shown by caspase-3 cleavage, is recognized as a marker of apoptosis [115]. TUNEL positive cells were observed during ISCH and IR, therefore, caspase-3 activity was measured in these conditions using immunofluorescence. Cleaved capase-3 was observed during ISCH and IR in control cells (Figure 34). In both the ISCH and IR conditions, decreased cleaved caspase-3 content was observed after ALA pre-treatment (Figure 34).

iv) Changes in Calcium Transients in Cardiomyocytes exposed to ISCH or IR alone or after Pre-Treatment with 400 μM ALA for 24 H

The involvement of intracellular Ca^{2+} overload was also studied as a potential mechanism for cardiomyocyte damage and death during ISCH and IR. Under ISCH conditions, Ca^{2+} transients decreased substantially (Figure 35). This did not recover during IR. ALA pre-treatment of the cells did not induce a protective effect (Figure 35). As expected, diastolic Ca^{2+} rose during ISCH and IR (Figure 35). However, although diastolic Ca^{2+} levels trended towards lower resting levels in cells pre-treated with ALA (Figure 35), this did not achieve statistical significance.

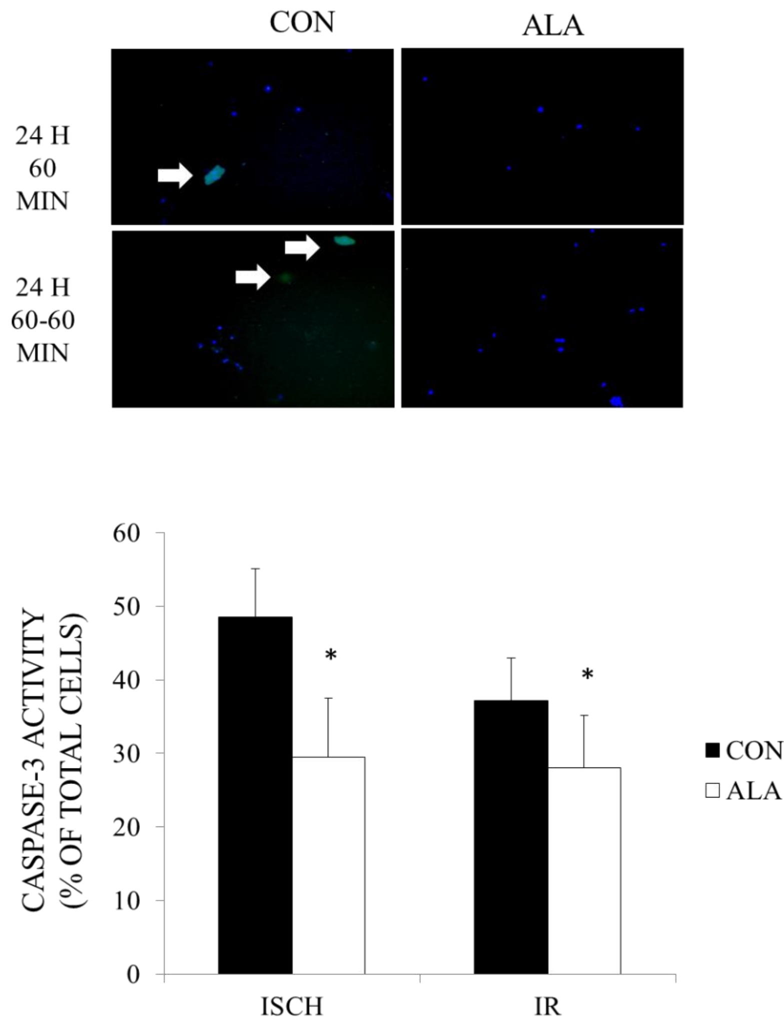


Figure 34: Detection of Cleaved Caspase-3 by Immunofluorescence under ISCH or IR Conditions. Cleaved caspase-3 antibody (1:100) was used in this assay to detect cleaved caspase-3 positive cells when cells were exposed to ISCH or IR conditions after 24 hours of pre-treatment with 400 μM fatty acid. Green denotes Caspase-3 positive cells and blue denotes nuclei. * $p<0.05$ compared to CON 24 hours. White arrows denote cleaved caspase-3 positive cells. Values represent mean ± SEM. n=6.

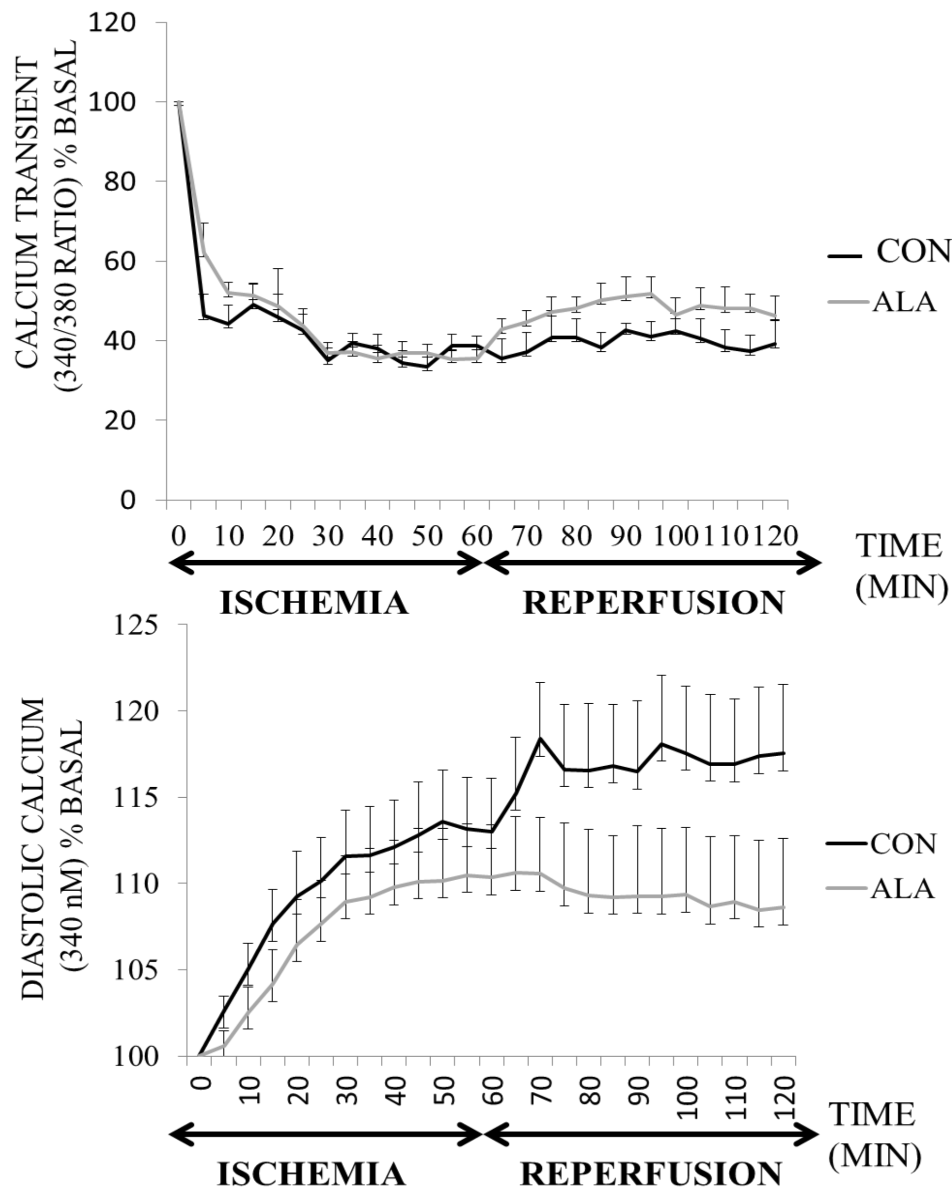


Figure 35: Calcium Transients and Intracellular Diastolic Calcium after 24 H of ALA pre-treatment under IR Conditions. Cells were treated with 400 μM ALA for 24 hours prior to induction of ISCH and IR conditions. *Top Graph:* Calcium transient measurement as a ratio of 340/380 nm wavelength with Fura2AM. Bottom Graph: Diastolic Calcium Measurements as a measure of 340 nM. Values represent mean ± SEM. n=12-16.

v) Oxidized Phospholipid Content in Cardiomyocytes exposed to ISCH or IR alone or after Pre-Treatment of 400 μM ALA for 24 H

Currently, it is only possible to reliably identify OxPC compounds. It is not possible to accurately identify other OxPL species [244]. The present data, therefore, is restricted to OxPC species alone. POVPC and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) were the only OxPC species that were significantly increased under control ISCH and IR conditions (Figure 30). Both POVPC and PGPC levels in the cardiomyocytes were significantly reduced several-fold after ALA pre-treatment under ISCH and IR conditions (Figure 36 A-C).

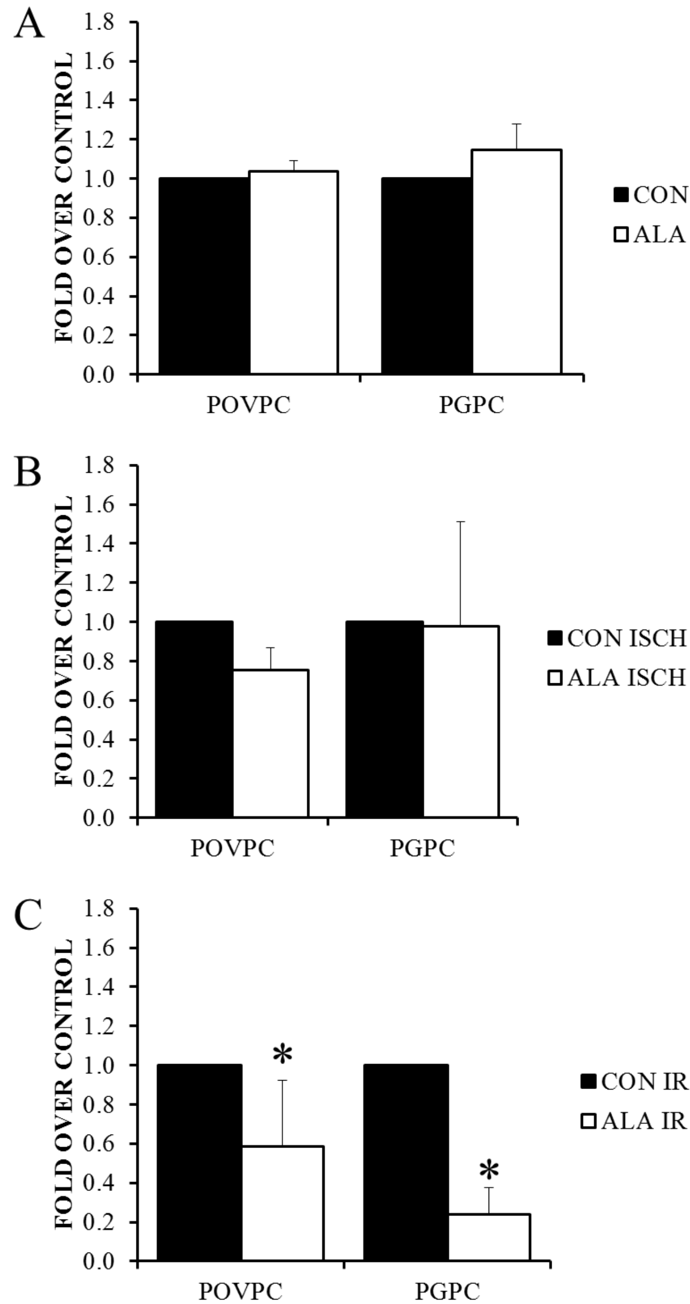


Figure 36: Reduction Of POVPC And PGPC Contents due to 24 H of ALA Pre-Treatment under ISCH or IR Conditions. A: OxPC content after ALA pre-treatment for 24 hours (400 μM) followed by ISCH. B: OxPC content after ALA pre-treatment for 24 hours (400 μM) followed by ISCH. C: OxPC content after ALA pre-treatment for 24 hours (400 μM) followed by IR .*p<0.05 compared to CON conditions. Values represent mean ± SEM. n=3 *p<0.05 compared to CON. Values represent mean ± standard error mean (SEM). N=3

DISCUSSION

Section 1: Uptake of VA, EA and ALA Acid into the Heart

LDLr$^{-/-}$ mice who were fed a diet supplemented with 1.5% of either VA or EA for 14 weeks exhibited plasma levels of TFAs of approximately 0.03 mg/ml or 0.0106 mM [9]. The average intake of TFAs can range from 1-29g/day with an average intake of 2.5g/day [1]. The present study reflects a moderate concentration of approximately 0.125 mg/ml (low concentration) or 1 mg/ml (high concentration) of TFAs over a relatively short range of time (24 and 48 H). EA and VA were taken up within the heart. Changes in fatty acids at the level of the heart were observed as a function of a long term dietary intervention or by direct supplementation to cardiomyocytes in M199 media. To our knowledge, this is the first account demonstrating trans fats, specifically VA and EA, are taken up by the heart through dietary interventions. The heart is composed of a variety of cell types, therefore, this increase in trans fat content in the heart may be a result of uptake into any number of different cell types. To ensure cardiomyocyte specific uptake of either VA or EA, we administered VA or EA directly to isolated cardiomyocytes. For the first time, time and dose dependent uptake of trans fats, specifically VA and EA was observed. Trans fat uptake was as early as 6 hours and this was maintained for up to 24 hours.

The mechanism of action responsible for the uptake of these trans fats into the cardiomyocytes is not clear. The role of fatty acid transport proteins in VA and EA uptake was investigated. CD36, FATP4 and FATP1 have been implicated in LCFA uptake within the heart [47, 54, 69, 81]. These proteins, therefore, represented logical starting points for our mechanistic studies. After 14 weeks of dietary intervention with either VA or EA, no changes in FATP4 or

CD36 expression within the heart were observed despite significant increases in tissue VA and EA content. Consistent with these findings, no changes in cardiomyocyte FATP4, CD36 or FATP1 expression were detected despite increase in VA and EA uptake in these cells. Thus, we can conclude that an increase in the expression levels of these fatty acid transport proteins is not necessary for a stimulation of trans fat uptake into cardiomyocytes. Alternatively, translocation of fatty acid transport proteins have been implicated in changing fatty acid uptake in cells [54, 81] Further study into the potential for trans fats to induce a movement of fatty acid transport proteins from the sarcolemmal membrane into the cytoplasm is necessary to accurately assess the mechanism for trans fat uptake into cardiomyocytes.

Previous studies have shown that New Zealand rabbits or $LDLr^{-/-}$ mice fed a diet supplemented with 10% ground (milled) flaxseed would incur plasma levels of ALA of approximately 1-3 mg/ml or 0.359-1.077 mM [184, 187]. Humans who ingested 30 g flaxseed for 1-12 months exhibited plasma levels of ALA of 0.6 mg/ml or 0.22 mM [248]. The present study employed levels of ALA that would reflect a low (0.125 mg/ml) concentration or a moderate (1 mg/ml) concentration of ALA based upon those studies. This flooding of ALA into the circulation would be expected to induce cellular mechanisms to induce uptake of the ALA into the tissue. In the present study, the uptake of ALA into the cardiomyocytes occurred in a dose and time dependent manner. As discussed previously, specific fatty acid transport proteins such as CD36 or FATP1 may have been involved in ALA uptake. The data in the present study do not support an increase in the expression of these fatty acid transport proteins as being involved in the uptake of ALA into the cardiomyocyte. Translocation studies of CD36 within the cardiomyocyte may be involved in the uptake of ALA into cardiomyocytes, as is the case for

skeletal myocytes . More research is necessary to conclusively establish the mechanism of action for ALA uptake into cardiomyocytes.

Once within the cell, ALA can be stored and metabolized in various forms. Long chain PUFAs will become preferentially incorporated in the sn-2 position of different cell types [72]. These mechanisms have been established with other PUFAs like arachidonic acid and EPA in cell types such as kidney cells [72]. Once incorporated into the phospholipids, PUFAs can become cleaved by specific enzymes such as PLA_2 [72]. Once cleaved, they can be released inside the cell where they are metabolized by specific enzymes such as lipoxygenases or cycloxygenases to become bioactive eicosanoids. Although this pathway has been well established in other cell types, this concept has not been assessed for ALA in cardiomyocytes.

Once within the cardiomyocyte, ALA can also be incorporated into membrane phospholipids. ALA incorporation into phospholipids was assessed in isolated cardiomyocytes in the present study after 24 hours of media supplementation. This is the first observation of ALA incorporation into phospholipids after direct supplementation to cardiomyocytes. Through alteration of the phospholipid bilayer, ALA may promote its effects on downstream signaling pathways by altering the function of membrane bound receptors [85]. Furthermore, ALA may be released into the cell by enzymes such as PLA_2 where it can have specific effects on transcription factors such as PPAR-γ [72]. These effects may promote different cellular activities such as metabolism or increasing cellular viability [249]. As discussed previously, ALA incorporation into phospholipids may alter the effects of oxygen derived free radicals, as shown by the decrease in oxidized phospholipid content during cellular stress. This represents the first demonstration of a direct effect of ALA on cardiomyocyte viability through decreasing oxidized phospholipid content. It is also the first report that ISCH and IR increases oxidized phospholipids

within the cell and that ALA pre-treatment prior to the induction of ISCH or IR decreases the oxidized phospholipid content. This may represent the mechanism whereby the direct beneficial effects of ALA on cardiomyocyte viability is achieved through decreases in oxidized phospholipids during stressful conditions.

Section 2 and 3: Effect of VA, EA and ALA on the Expression of Adiponectin and Markers of Apoptosis and Autophagy

EA, VA and ALA can induce direct effects on cardiomyocyte viability after extended exposure to the cells. The effects of the fatty acids differed depending upon not only the class (omega-3 PUFA versus trans fatty acid) but also even within the same general class (trans fat: EA versus VA). Generally, EA promoted whereas VA and ALA protected against cell death. The mechanism whereby EA promoted cell death when exposed to cardiomyocytes involved, in part, a stimulation of apoptosis through an activation of the caspase-3 and Bax/Bcl-2 pathways. Exposure of cardiomyocytes to 400 μM EA simultaneously induced autophagy as well as apoptosis. EA induced a marked increase in autophagy as shown through an increase in LC-3 and a decrease of beclin after 48 H of exposure of EA to cardiomyocytes. ALA had no effect on caspase-3 activity, Bax/Bcl-2 activity or markers of autophagy (beclin and LC-3). The effects of VA were more similar to those of ALA than the effects of EA. VA did not have a significant effect on cell death or autophagic markers with either 50 or 400 μM supplementation after 24 hours. However, the beneficial effects of VA may disappear with longer exposure times or at higher concentrations. After 48 hours, 400 μM VA pre-treatment increased caspase-3 cleavage similar to EA. Similarly, a decreased adiponectin production with both EA and VA pre-treatment was observed after 48 hours at 400 μM. This suggests a non-beneficial role of VA or EA after

long term treatment at a 400 μM concentration. However, differences in the Bax/Bcl-2 ratio were observed with VA and EA pre-treatment at 400 μM for 48 hours. These differences may reflect a potential beneficial effect of VA on intrinsic apoptosis.

Increased trans-fat consumption has been correlated with an increased risk in cardiovascular disease [1, 8, 9, 199, 250, 251]. Trans fats increase atherosclerotic risk [9]. Trans fats derived from partially hydrogenated food products (industrially produced trans fats) exhibit decreased HDL levels, increased LDL levels and increased cardiovascular risk [199]. Perhaps unfortunately, all trans fats have been lumped together as having deleterious cardiovascular effects. Work by Ghavami et al. (2012) [245] demonstrated that a 400 μM concentration of both EA and VA increased apoptosis and autophagy within cardiac fibroblasts - an important regulator in wound healing and extracellular matrix remodeling within the heart. In control conditions, with longer term exposure (48 hours), we observed similar results with VA or EA supplementation, particularly with the 400 μM concentration. However, shorter term exposure with VA or EA had no significant effects on any parameters of apoptosis, autophagy or myokine expression studied. Long term intake of fatty acids may induce more detrimental effects on the heart than than shorter term intake.

Although currently controversial [252, 253], an important cardioprotective role of omega-3 fatty acids has been demonstrated in cardiovascular disease [10]. Dietary flaxseed decreases atherosclerotic risk [10], lowers blood pressure in hypertensive patients [254] and protects the hearts of cardiomyopathic hamsters from apoptotic cell death [196]. In all of these studies, the mechanism was proposed to be through the rich content of ALA in flaxseed. ALA is protective in other pathologies as well. For example, a cardioprotective action of ALA was observed when cells were exposed to the cardiotoxic agent doxorubicin [255]. In the present study, ALA

inhibited apoptosis in cardiomyocytes. Paradoxically, ALA [256, 257] increased the rate of apoptosis within cancer cell lines. Thus, it would appear to be contradictory that that while ALA decreases apoptosis within cardiomyocytes, it conversely increases apoptosis within cancer cells. However, the omega-3 fatty acids EPA, DHA [258, 259] have similar opposing effects on apoptosis in cardiac and cancer cells. In addition, adiponectin has shown similar conflicting actions in heart and cancer models. Adiponectin is anti-apoptotic in cardiomyocytes [260] but is pro-apoptotic in cancer cell lines [261, 262]. It can be concluded that the beneficial actions of ALA, therefore, depend upon the cell type examined and the pathology under study as well.

Section 4: Differential Effects of Trans Fats on Cardiomyocyte Viability under Ischemia (ISCH) or Ischemia/Reperfusion (IR) Conditions

Although no significant changes in cell death with VA or EA were observed under non-ischemic conditions after 24 hours of administration with 400 μM, a significant decrease in cell death with VA supplementation was demonstrated for the first time when cells were exposed to the stress parameters ISCH or IR. These results are contrasted with a significant increase in cell death with EA pre-treatment during ISCH and IR conditions. Furthermore, the cell death associated with EA supplementation was associated with an increase in the apoptotic markers caspase-3 activity and DNA fragmentation. Similar increases in these apoptotic markers were not observed with VA supplementation. We conclude from these observations that an important differential effect of VA and EA on cell death exists when cells are exposed to ISCH or IR.

The oxidized phospholipid content within the cardiomyocytes may explain these beneficial and detrimental effects. Oxidation of phospholipids may play an influential role in cell signaling pathways and apoptosis [246]. Oxidized phospholipids are related to cardiovascular

pathology [263]. They influence gene expression and have been toxic to multiple types of cells [264]. Oxidized phospholipids in apoB-100 lipoproteins may increase the inflammatory response [265]. Further, the elevated OxPL/apoB content was associated with metabolic syndrome, the risk of stroke, femoral and carotid disease and myocardial infarctions [265]. OxPC are also accurate markers of oxidative stress [263]. Addition of oxygen molecules to cardiolipin have been associated with increased cell death during ischemia-reperfusion injury [266]. These results together indicate an important role of phospholipid oxidation in cardiovascular disease. The phospholipid oxidation products identified in the present study have all been correlated with increased cell death in other models [136]. Two of these OxPC species, POVPC and PGPC, can induce apoptotic signaling within vascular smooth muscle cells [267]. The decrease in these oxidative phospholipids with VA pre-treatment in the present study, therefore, is novel and noteworthy. This decrease in oxidized phospholipid content correlated with the decreased cell death observed with cardiomyocytes pre-treated with VA prior to ISCH and IR. The decrease in phospholipid oxidation, therefore, may be a potential mechanism of action for the cardioprotective effects of VA.

Increased TFA consumption has been associated with an increased risk for cardiovascular disease [1, 8, 9, 199, 250, 251]. This increased risk of cardiovascular disease has been attributed to an increase in atherosclerotic vascular disease. Trans fats derived from partially hydrogenated food products (industrially produced trans fats) exhibit decreased HDL levels, increased LDL levels, atherogenesis and increased cardiovascular risk [199]. This has led to increased legislation regarding the use of trans fats in our foods and ultimately resulted in decreased availability of trans fats within our diet [268]. However, this may not be optimal because, as was the case in atherogenesis [8], VA was cardioprotective in our study. Our study has shown that,

unlike EA, VA does not have a pro-apoptotic or pro-autophagic effect in response to a stress such as ISCH or IR. However, an increase in apoptosis and autophagy under non-ischemic conditions have been reported in fibroblasts with similar concentrations of VA [245]. Thus, our results may be cell specific. It will be important in future studies of other cell types to include cell stress conditions to reveal additional actions of the TFAs.

Section 5: Direct Effects of ALA on Cardiomyocyte Cellular Viability under Ischemic (ISCH) or Ischemia/Reperfusion (IR) Conditions

Our data demonstrates, for the first time, a direct protective effect of ALA on cardiomyocytes undergoing ISCH and/or IR challenge. This appears to be through an inhibition of apoptosis induced by ISCH and IR. Mechanistically, this cardioprotective effect of ALA is achieved through an inhibition of caspase-3 cleavage and DNA fragmentation. Changes in resting intracellular Ca^{2+} did not participate in the anti-apoptotic cardioprotective action of ALA during ISCH and IR challenge. The concentration of ALA employed in these studies (400 μM) and the extended exposure time (24 hours) of cells to ALA prior to the ischemic insult were designed to approximate dietary conditions found *in vivo* [269]. Indeed, these cardioprotective effects of ALA agree well with the beneficial actions of dietary ALA observed in epidemiological trials [270, 271] and other cardiac ISCH studies [272] as well as the anti-apoptotic effects [273, 274].

The present study also reports, for the first time, changes within the OxPL content of adult rat cardiomyocytes as a function of ISCH and IR. This is also the first study to show that the OxPC that is generated within the ischemic cardiomyocyte may be associated with apoptosis in these cells. Phosphatidylcholines represent the majority of the phospholipids within these

cells, and as a result we focused on PC oxidation products [275]. ISCH and IR challenge induced important changes in the OxPC profile within cardiomyocytes. The short chain OxPL products POVPC and PGPC were elevated after ISCH and IR insult to the cardiomyocytes and may play a role in the induction of the cardiac apoptosis observed here. The two compounds have been reported to induce apoptosis in smooth muscle cells, macrophages, oligodendrocytes, and endothelial cells [134-136, 276]. POVPC and PGPC can activate acidic sphingomyelinases, caspase-3 and mitogen activated protein kinase signaling as initial apoptotic stress responses in smooth muscle cells [136]. These findings have been supported by DNA fragmentation and flow cytometry [137]. These pro-apoptotic actions of POVPC and PGPC can now be extended to cardiomyocytes during ISCH and IR challenge.

The observation that ALA pre-treatment reduces POVPC and PGPC compared to control during ISCH and IR is important from a number of perspectives. It is the first demonstration that any lipid molecule can inhibit the generation of OxPL. It is also the first demonstration that ALA specifically can inhibit OxPC generation. Previously, antioxidants have been shown to reduce LDL oxidation, during which OxPL can be generated. However, a direct mechanism to explain the decreases associated with OxPL reduction and antioxidant application has not been shown [277-280]. The present study demonstrates that ALA pre-treatment of cardiomyocytes inhibits the generation of the pro-apoptotic POVPC and PGPC species by 42% and 77%, respectively, during IR conditions. As observed in other models, this effect may be associated with a subsequent decrease in apoptotic activity [136, 281]. This was likely a result of the incorporation of ALA into the cardiomyocyte membrane phospholipid pool. It has previously been established that 18:0 and 18:2 fatty acids are the primary fatty acid components in the rat heart, represented as 36:2[282]. ALA pre-treatment led to a significant incorporation of the ALA fatty acid (as

18:3) into the phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, and phosphatidylethanolamine phospholipid pool, represented as 36:3. As would be expected, after ALA treatment, the 36:2 pool was significantly decreased and the 36:3 pool was significantly increased demonstrating the successful incorporation of ALA into membrane phospholipids within the cardiomyocytes. This may act as the basis for the physiological benefits of ALA within cardiac cells and its cardioprotective effects.

A trend in resting and calcium transients was observed with ALA supplementation during both ISCH and IR conditions. We observe a decrease in resting calcium and calcium transients in both of these conditions but significance was not achieved. ALA does not have a significant effect on calcium transients or ion channels. Although clinical[283] and *in vivo* data[274, 284] have suggested an anti-arrhythmic action of omega-3 fatty acids, the results remain controversial. Recent clinical data suggest that marine derived omega-3 fatty acids have no effect on deaths from arrhythmias in groups fed 1g omega-3 fatty acids/day vs. placebo controlled groups [285]. However, there is a distinct role of ALA on sodium/calcium exchanger function in cardiomyocytes [284]. Therefore, further study is necessary to fully appreciate the role of ALA on calcium influx and efflux within the cell.

In summary, the presence of the iTFA EA in isolated cardiomyocytes resulted in the generation of OxPL in the form of POVPC and PGPC during ISCH and IR. These oxPC species would, in turn, be expected to induce the apoptotic cell death observed in the present study. This effect on cardiomyocytes is in stark contrast to the effects observed with the rTFA, VA. VA was cardioprotective, possibly through its capacity to lower the content of oxPC in the cell. Pre-treatment of cardiomyocytes with ALA and the resultant incorporation of ALA into the phospholipid pool mitigated the apoptotic stress response within cardiomyocytes. Although

currently controversial [286, 287], these data add to a body of evidence supporting an important cardioprotective role of omega-3 fatty acids in cardiovascular disease [269, 283, 288]. ALA is a source of omega-3 fatty acid that is structurally different than the omega-3 species found in marine products. The provision of ALA through supplementation of the diet with flaxseed decreases atherosclerotic risk [269, 289], inhibits arrhythmogenesis [283, 284], lowers blood pressure in hypertensive patients [254] and protects the hearts of cardiomyopathic hamsters from apoptotic cell death [288]. In all of these studies, the mechanism was proposed to be through the rich content of ALA in the flaxseed. Direct provision of ALA was also cardioprotective in cells exposed to the cardiotoxic agent doxorubicin [274]. Our data suggest that the long term delivery of ALA (directly or via dietary supplementation) prior to myocardial ISCH and IR challenge represents a compelling therapeutic treatment strategy that warrants further study.

CONCLUSIONS

1) Dietary supplementation with the TFAs EA and VA will result in a significant deposition within the heart. The EA and VA were taken up by isolated cardiomyocytes in a dose and time dependent manner.
2) The fatty acids ALA, EA and VA have a differential effect on key signaling pathways within the cardiomyocyte, specifically in regards to cellular viability pathways such as apoptosis, autophagy and myokine regulation.
3) Exposure of cardiomyocytes to EA (50 and 400 μM) elicited a decrease in cell viability, a decrease in the beneficial myokine adiponectin and an increase in autophagic and apoptotic pathways. EA at high concentrations was cardiotoxic.
4) Exposure of cardiomyocytes to VA (50 and 400 μM) elicited variable effects on cell viability, myokine adiponectin expression and biomarkers for the autophagic and apoptotic pathways under control conditions.
5) When cardiomyocytes were exposed to simulated ischemia/reperfusion injury, VA pre-treatment decreased cell death. VA was not protective through an effect on apoptosis.
6) VA decreased oxidized phospholipid content under basal conditions.
7) EA increased oxidized phospholipid content under ischemic conditions.
8) Oxidized phospholipid content may be a novel mechanism of action whereby cellular function is altered under stressful conditions.
9) ALA was taken up by isolated cardiomyocytes in a dose and time dependent manner.
10) ALA (50 and 400 μM) inhibited cell death under control cell culture conditions but did not alter other signaling molecules.

11) Under ischemia/reperfusion conditions, ALA was cardioprotective and decreased caspase-3 activation, DNA fragmentation and cell death.

12) ALA decreased oxidized phospholipid content during both ISCH and IR. This is a novel mechanism of action whereby ALA may be improving cellular viability during stressful conditions.

FUTURE STUDIES

1) Establish a mechanism of action whereby TFAs and ALA are taken up by the cell. Our initial data suggest that CD36 or FATP1/4 may not be involved in TFA uptake since expression patterns do not change with either direct supplementation or dietary intake. However, the subcellular location of the fatty acid transport protein may be an important measure to better establish whether or not it is involved in TFA uptake. Similarly, ALA uptake may also be investigated in this manner through increased fatty acid transport.
2) Determine if supplementation of ALA with EA may decrease the detrimental effects of EA on cardiomyocytes. These studies may be followed by dietary supplementation of these fatty acids to see how they affect cardiomyocyte viability and heart function *in vivo*.
3) This study primarily focused on pre-treatment of fatty acids prior to ISCH or IR. However, it may be useful in the future to examine the effects of these fatty acids on cardiomyocyte viability and function if administered directly to cardiomyocytes during or after ISCH.

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