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The effect of alpha-linolenic acid (ALA) on endothelial cell expression of connexin43

Benjamin Pichette

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GRAND VALLEY STATE UNIVERSITY

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Acknowledgements

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Abstract

Atherosclerosis is the leading cause of cardiovascular diseases like myocardial infarctions and strokes. The formation of these atherosclerotic plaques occurs through localized inflammatory responses in blood vessels. Epidemiological evidence suggests omega-3 fatty acids (FAs) decrease vascular inflammation. The omega-3 FAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) reduce inflammation through pathways that may include the free fatty acid-4 (FFA-4) receptor. While EPA and DHA are well studied, less is known about the essential omega-3 FA from which they are synthesized: alpha-linolenic acid (ALA). Thus, this study will investigate whether ALA can activate the FFA-4 receptor and thereby contribute to anti-inflammatory responses in the endothelium. Although the FFA-4 receptor is a G-protein coupled receptor, its activation reduces the nuclear transcription factor NF-κB thereby decreasing the synthesis and release of inflammatory cytokines. The resulting decrease in inflammatory cytokines is expected to decrease expression of connexin-43 (Cx43). Cx43 is a gap junction protein whose endothelial cell expression is increased during inflammation contributing to an unhealthy endothelium. Treatment of endothelial cells with 30 μM ALA is hypothesized to decrease the expression of Cx43 through the activation of the FFA-4 receptor. Cultured mouse endothelial cells (bEnd.3) were treated with physiological ALA concentrations (30 μM) for 48 hours. Whole-cell protein was isolated from treated and controlled endothelial cells at 1.5, 3, 6, 12, 24 and 48 hours and expression of Cx43 determined. Over the 48 hours of ALA exposure, Cx43 expression did not change (ratio of Cx43 expression in ALA treated/time-control was not different from 1: $p>0.05$). Increasing ALA to 100 μM did nothing to affect Cx43 expression in cells at 12 – 48 hours ($p>0.05$). While this suggests ALA is not involved in omega-3 FA dependent anti-inflammatory responses in the endothelium, these results could be affected by

ALA activating the pro-inflammatory FFA-1 receptor. However, treatment of cells with 10 μM GW-9508 (selective FFA-1 receptor agonist) did not affect Cx43 expression at 48 hours ($p>0.05$). Thus, treatment of bEnd.3 cells with ALA did not alter expression of Cx43. These results suggest ALA may not contribute to the anti-inflammatory influence omega-3 FAs exert on endothelial cells.

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Abbreviations

FAs: Fatty Acids

ALA: Alpha-linolenic Acid

EPA: Eicosapentaenoic acid

DHA: Docosahexaenoic acid

FFA (1-4): Free fatty acid receptors

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

Cx: Connexin

bEnd.3: Brain endothelial cells (cultured endothelial cells)

CVD: Cardiovascular disease

MI: Myocardial infarction

LDL: Low density lipoproteins

oxLDL: oxidized low density lipoproteins

PPARs: Peroxisome proliferating activating receptors

HNF-4α: Hepatocyte nuclear factor-4 alpha

IL: Interleukin (6 or 1 Beta)

TNF-α: Tumor necrosis factor alpha

ATCC: American Tissue Culture Collection

EC: Endothelial cells

SMC: Smooth muscle cells

TLR-4: Toll like receptor 4

AA: Arachidonic acid

VCAM: Vascular cell adhesion molecule

LPS: Lipopolysaccharides

TAB1: TAK1 Binding Protein

TAK1: Transforming growth factor-beta-activated kinase

HG-DMEM: High glucose Dulbecco's modified eagle's medium

FBS: Fetal Bovine Serum

PD: Calcium free phosphate buffered saline

TV: Trypsin versene

GW-9508: Free fatty acid 1 receptor agonist

PBS: Phosphate buffered saline

PVDF: Polyvinylidene fluoride membrane

TBS: Tris-buffered saline

TTBS: Tween 20 in Tris-buffered saline

NFDM: Non-fat dry milk

Ab: Antibody

HRP: Horseradish peroxidase

SEM: Standard error of the mean

COX: Cyclooxygenase

LOX: lipoxygenase

PG: Prostaglandins (E or F)

Chapter 1: Introduction

Cardiovascular disease (CVD) is the leading cause of deaths worldwide accounting for over 17.7 million (WHO, 2015) and costing the world economy nearly $475 billion (Ahmad et al., 2018 and Adkins et al., 2010). A majority of these deaths are due to heart attacks and strokes (WHO, 2015), which are caused by atherosclerotic changes in the vascular wall. Atherosclerotic changes are characterized by a stiffening and narrowing of the arteries due to a chronic, low grade inflammatory response (Gu et al., 1998 and Libby, 2002). This inflammatory response is initiated by damage to the endothelium allowing circulating low density lipoproteins (LDLs) to migrate into the underlying smooth muscle layer (Stoll et al., 2006). These LDLs are oxidized and ingested by macrophages releasing inflammatory cytokines to recruit other immune cells (Stoll et al., 2006). Those cytokines also turn contractile smooth muscle cells into secretory cells releasing the collagen which drives the inward growth of the plaque. The macrophages in the center of the plaque continue to ingest LDL molecules until they become foam cells and die. This results in the formation of a necrotic core in the plaque that weakens it (along with matrix metalloproteinases released by immune cells) causing plaques to become unstable. The shearing forces of the blood break the plaque free from the vessel wall and occlude smaller arteries of the coronary and cerebral circulation (Stoll et al., 2006, Calder, 2013, Kris-Etherton, 2002, and Kwak et al., 2002). The exposed necrotic core is rife for platelet aggregation resulting in thrombus formation. This thrombus has the potential to be sheared free by the blood to occlude a larger vessel in comparison to the vessel occluded by the plaque, further resulting in a heart attack or a stroke. While some individuals develop clinically relevant complications from this process, these plaques form in all humans at different rates that are impacted by risk factors. Thus, we all have the potential for the development of CVDs.

One of the initial steps in atherosclerotic plaque development involves LDL particles migrating into the smooth muscle layer. LDL particles circulate in the blood transporting lipids in a membrane bound structure (with proteins embedded in the phospholipid membrane). One type of lipid within the LDL particle is a triacylglycerol which is composed of 3 fatty acids (FA) and a glycerol molecule. Those FAs are released by lipoprotein lipases in the vessel wall (Mead et al., 2002) and become free FAs. FAs are composed of 3 types of carbon-carbon bonds: trans or cis (stereoisomers), saturated (no double bonds) or unsaturated (1 or more double bonds). While consumption of trans and saturated FAs increases the risk for developing CVDs (Pietinen et al., 1997 and Wang et al., 2017), unsaturated FAs (specifically Omega-3s) decrease the risk for developing CVDs (Wang et al., 2017, Schmidt, 1997, Nair et al., 1997, William-Bey et al., 2014, and Rodriguez-leyva et al., 2010). There are 3 omega-3 FAs proposed to decrease the risk of CVD: alpha-linolenic acid (ALA, 18 carbon), eicosapentaenoic acid (EPA: 20 carbon), and docosahexaenoic acid (DHA, 22 carbon). Their anti-inflammatory properties are mediated by a number of pathways including reduced production of inflammatory cytokines (Mohebi-Nejad et al., 2014), reduced expression of endothelial cell and platelet adhesion molecules (Mozaffarian et al., 2011), decreased acute phase reactants (Adkins et al., 2010), and the ability to bind to nuclear receptors including peroxisome proliferator activating receptors (PPARs: Adkins et al, 2010) and hepatocyte nuclear factor-4 alpha (HNF-4a: Adkins et al., 2010). More recently, the discovery of G-protein coupled free FA receptors (FFAs), which can also act as nuclear receptors, has spurred interest in their role of impacting CVDs.

The FFA receptor superfamily includes four members: FFA-1 through FFA-4 (Oh et al., 2014). The FFA-2 and FFA-3 receptors are stimulated by short chain FAs (carbon backbone length of 2-6 carbons: propionate, butyrate, acetate, etc.) and are expressed exclusively within

entero-endocrine cells (Nøhr et al., 2013). The FFA-1 and FFA-4 receptors are stimulated by long chain FAs (carbon backbone of 12 to 22 carbons: palmitic, oleic, etc.) including both saturated and unsaturated FAs like the omega-3s (Nøhr et al., 2013). Expression of the FFA-1 receptor has only been observed in the human pancreas and intestinal entero-endocrine cells (Ichimura et al., 2014). The FFA-1 receptor activation increases secretion of intestinal hormones (Ichimura et al., 2014) and increases inflammation, however, the mechanism of action has yet to be determined (Cheshmehkani et al., 2015 and Mena et al., 2016). In contrast, the FFA-4 receptor is ubiquitously expressed throughout the body (Cheshmehkani et al., 2015, Oh et al., 2014, and Ichimura, et al, 2014). The FFA-4 receptor can be stimulated by the omega-3 FAs and the receptor mechanism of action, promoting an anti-inflammatory response, has been studied and illustrated (Denis et al., 2017, Oh et al., 2014, Williams-Bey et al., 2014 and Cheshmehkani et al., 2015). Once the FFA-4 receptor is activated, it interrupts the signaling for the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) translocation into the nucleus (Oh et al., 2014, Cheshmehkani et al., 2015, Denis et al., 2017, and Nøhr et al., 2013). NF-κB is a transcription factor responsible for the production of pro-inflammatory cytokines including interleukin-6 (IL-6), IL-1β, and TNF-α (Williams-Bey et al., 2014). Inhibition of NF-κB translocation by the FFA-4 receptor would reduce inflammation by decreasing pro-inflammatory cytokine release.

Exposure of the endothelium to inflammatory cytokines affects cell to cell communication by altering gap junction protein expression (Brisset et al., 2009). Gap junctions are composed of a connexon (6 connexin (Cx) proteins) that forms a hemi-channel in the cell membrane. When these connexons from neighboring cells come into contact, they form a complete channel for communication of signals including the exchange of metabolites, ions, and

other messenger molecules between cells (Brisset et al., 2009 and Kwak et al., 2002). Each connexin isoform has a unique permeability to signaling molecules and, thus, a different message sent. There are three Cx isoforms in endothelial cells including Cx37, Cx40, and Cx43 (Kwak et al., 2002). Healthy endothelial cells express many Cx37 and Cx40 (Brisset et al., 2009), however, Cx43 is mainly restricted to smooth muscle cells (Kwak et al., 2002). When inflammation is present within the endothelium and there is an increased expression of NF-κB, not only are inflammatory cytokines released, but NF-κB has been shown to increase the expression of Cx43 on the cell surface as well. NF-κB translocation into the nucleus has been found to bind directly to the promoter of the Cx43 gene (Alonso et al., 2010). As NF-κB is a pro-inflammatory transcription factor, associated with regions of inflammation in the endothelium (i.e. atherosclerotic plaques), and is responsible for the increased expression of Cx43 on endothelial cells, Cx43 can be used as an indicator of inflammation and poor cardiovascular cell health (Brisset et al., 2009, Kwak et al., 2002, and Puebla et al., 2017)

ALA is the essential omega-3 FA (Rodriguez-Leyva et al., 2010) and therefore must be consumed in the diet (Kris-Etherton et al., 2002). In the liver, ALA is exposed to desaturase and elongase enzymes, which adds carbon atoms to ALA resulting in the synthesis of EPA and DHA (Calder, 2013). ALA binds to the FFA-4 receptor in entero-endocrine cells and stimulates the secretion of cholecystokinin and glucagon like peptide 1 (Hirasawa et al., 2005). If this receptor is expressed in the endothelium, ALA should activate the FFA-4 receptor and stimulate an anti-inflammatory response within the endothelium. Since the FFA-4 receptor is expressed in many different cells including bEnd.3 mouse cultured endothelial cells (Rogers and Kurjiaka: Personal communication), treating these cells with physiological concentrations of ALA should initiate an anti-inflammatory response through FFA-4 receptor activation. If activation of the FFA-4

receptor generates an anti-inflammatory response in the endothelium via inhibition of NF-κB translocation into the nucleus, we would expect the expression of Cx43 to also decrease. We hypothesize that treating cultured endothelial cells (bEnd.3) with ALA will decrease Cx43 expression as an indication of their anti-inflammatory response, thus increasing endothelial cell health. As the duration of FFA-4 receptor activation required to alter Cx43 expression has yet to be determined, we will evaluate the response of bEnd.3 cells to 30 μM ALA from 1.5 to 48 hours of treatment.

Purpose:

Cardiovascular disease is one of the leading causes of death worldwide. Many of these diseases are the results of the development of atherosclerotic plaques within the arterial walls due to the presence of a chronic low grade inflammatory response. Omega-3 FA consumption has long been reported to decrease the risk of CVDs. This appears to be due in part to their ability to decrease inflammation in general as well as within atherosclerotic plaques. The purpose of this research is to determine whether this anti-inflammatory response in the endothelium can be mediated by the plant-based essential omega-3 FA, ALA. This evidence would support efforts to reduce atherosclerotic plaques. If ALA can also decrease inflammation within the endothelium, there may be another method for the prevention and/or treatment of atherosclerosis and subsequent CVD.

Scope:

This experiment is being completed on a mouse endothelial cell culture (cell line bEnd.3) purchased from American Tissue Culture Collection (ATCC).

Assumptions:

Vascular endothelial cells are usually quiescent (not in the cell cycle), only proliferating in response to damage/inflammation. However, bEnd.3 cells are immortalized mouse endothelial cells that are proliferating in an uncontrolled manner. Even the removal of serum does not stop this proliferation. With that said, these cells do express the FFA-4 receptor and the downstream signaling pathway. Therefore, these endothelial cells should respond to physiological concentration of ligand (ALA) by altering Cx43 expression.

Hypothesis:

Omega-3 FAs, such as EPA and DHA, stimulate anti-inflammatory responses upon activation of the FFA-4 receptor (Williams-Bey et al., 2014, Oh et al., 2014, and Hirasawa et al., 2005). ALA is the essential omega-3 FA and has been shown to activate the FFA-4 receptor, however, not within the endothelium. If the FFA-4 receptor is present within the endothelium, ALA should render it activated, decrease NF-κB translocation into the nucleus, and decrease the expression of Cx43 on endothelial cells, thereby reduce inflammation. Therefore, ALA stimulation on bEnd.3 endothelial cells is hypothesized to activate the FFA-4 receptor and decrease the expression of Cx43, thereby decreasing inflammation and increasing endothelial cell health.

Significance:

Due to the millions of people around the world impacted by CVDs and their economic consequence, developing a better understanding of the mechanisms by which omega-3 FAs reduce inflammation, and thereby CVDs, is very important. Results from this study may present additional avenues for drug development in the area of the FFA-4 receptor agonist to take advantage of the downstream consequence of its activation. At the same time, they provide

people with reasons to adjust their diet to take advantage of the positive effects of the omega-3 FAs on their cardiovascular health.

Chapter 2: Review of Literature

Cardiovascular Disease*:* CVD continues as the leading cause of death in the United States accounting for 36% of those recorded (WHO, 2015). Most CVDs result from blockage of coronary and cerebral arteries by atherosclerotic plaques leading to myocardial infarctions and strokes (respectively). The resulting impact of these CVDs on the US economy is substantial accounting for an estimated $475 billion ((Ahmad, et al., 2018 and Adkins et al, 2010). The extent of the economic costs to the US make it essential we understand the factors impacting the development and progression of these plaques.

Atherosclerosis: Atherosclerotic plaques develop (Figure 1) as a localized inflammatory response in the arterial wall (Nair et al., 1997). These plaques start as fatty streaks generated by damage to the overlying endothelium (Stoll et al., 2006). Circulating LDL particles begin to accumulate in these damaged sites and become oxidized (oxLDL), stimulating the release of inflammatory cytokines from endothelial cells (EC: Adkins et al, 2010) including interleukin 6 (IL-6), IL-1β, and TNF-α (Williams-Bey et al., 2014). These inflammatory cytokines stimulate EC expression of adhesion molecules including P-selectin and vascular cell adhesion molecules (VCAMs), which recruit monocytes to the site of damage (Calder, 2004). There, monocytes differentiate into macrophages that absorb the accumulating oxLDL to the extent that they turn into foam cells. These foam cells then die leaving the core of the plaque to become necrotic (Stoll et al., 2006). At the same time, more cytokines are released to recruit additional leukocytes, (e.g., T cells) which stimulate the overlying smooth muscle cell (SMC) to migrate inward, proliferate, and secrete collagen to form the fibrous cap of the plaque (Libby, 2002). These recruited T-cells act on the plaque by inhibiting SMC collagen secretion and stimulate the release of additional cytokines, collagenases, and matrix metalloproteinases that destabilize the

plaque (Libby, 2002 and Stoll et al., 2006). These unstable plaques are at risk of rupturing, releasing the overlying cap, and occluding small vessels downstream. The rupture of the fibrous cap exposes the necrotic core which causes platelet aggregation and thereby thrombus formation (Stoll et al., 2006). The thrombus can grow large enough that when it breaks free, a larger vessel, compared to the vessel occluded by the plaque, will become occluded. This occlusion poses the potential for causing a myocardial infarction or stroke (Kris-Etherton, 2002 and Eritsland et al., 1996), potentially leading to death.

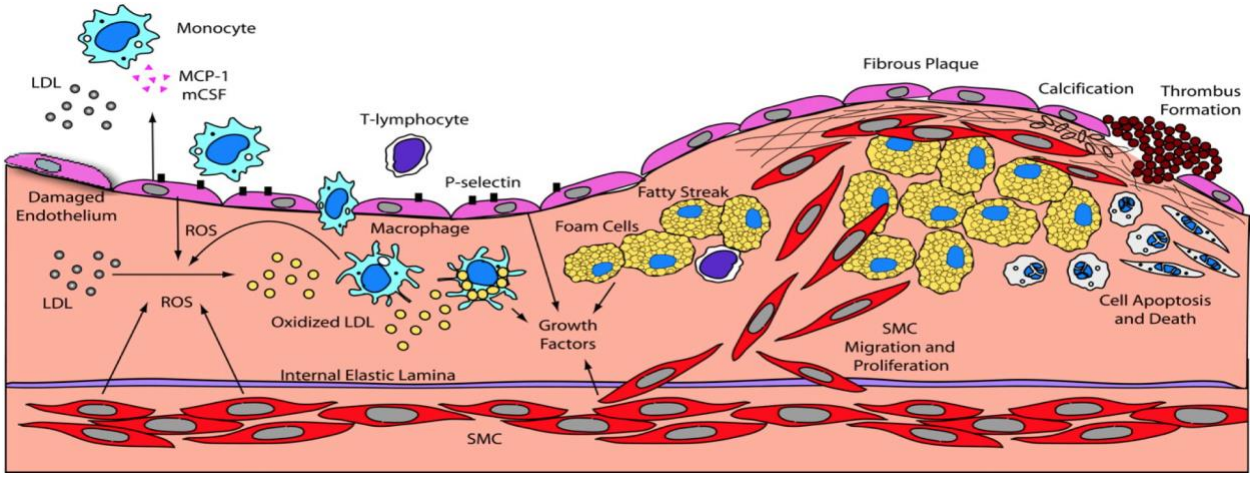

Figure 1: The Development of atherosclerosis. Atherosclerosis begins with the damaged endothelium from an external source and ends with potential thrombus formation**.** Note that the thrombus being formed in this image is due to a previous event where the unstable plaque broke free. The thrombus being formed has the potential to break free due to hemodynamic forces and induce a fatal MI or stroke (Madamanchi, 2004).

Dietary Fatty Acids: Plaque development within an artery is a dynamic process involving multiple factors. One of the most important influences on this process is diet, specifically, the consumption of dietary fats. Epidemiological evidence dating back to the 1970's links free FAs to plaque development (Dyerberg, 1978). FAs provide many diverse functions from energy storage to being a signaling molecule (prostaglandin and leukotriene synthesis: Egert et al., 2007). FAs are composed of a hydrocarbon chain with a hydrophobic methyl group on one end (omega end) and a hydrophilic carboxyl group at the opposite end (delta end: Schmidt, 1997).

FAs are defined by the extent and type of double bond(s) along its length from saturated (no carbon double bond) to unsaturated (at least one double bond). Unsaturated FAs are further divided by the type of double bond: a cis-unsaturated double bond adds a kink along its length whereas trans-unsaturated FAs have undergone partial hydrogenation, which straightens the FA (Figure 2: Lichtenstein, 2016). This partial hydrogenation process allows trans-FAs to pack tightly together and will remain in the body for an extended period of time as it is not readily available for energy production (Mozaffarian et al., 2007). Not only are these trans-FAs a poor source for energy, but they also bind to the toll like receptor 4 (TLR-4) to stimulate inflammation (Harper et al., 2001).

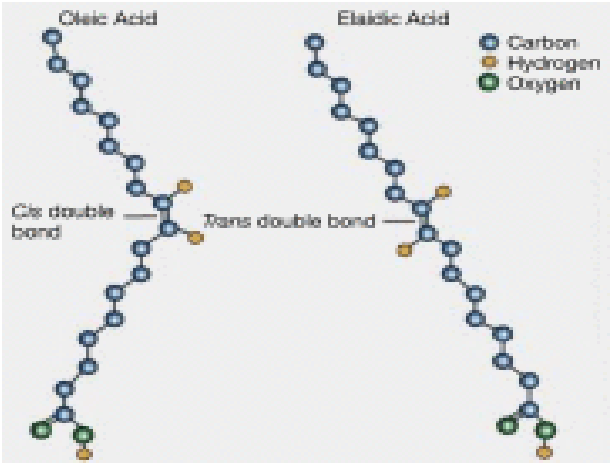

Figure 2: Trans-FA structure in comparison to a cis-FA structure. Note the kinked formation associated with the double bond in the cis-FA structure (Left: Oleic acid). The trans-FA (Right: Elaidic acid) has a double bond in the trans configuration rendering it linear (Lichtenstein, 2016)

Polyunsaturated FAs are further distinguished by the location of the first cis double bond: omega-3 (double bond on the third carbon from omega end) and omega-6 (double bond located on the sixth carbon: Roche, 1999). Alpha-linolenic acid (ALA) is the essential omega-3 FA and is the precursor for eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). This synthesis involves desaturase and elongase enzymes (Figure 3: (Pereira, 2004 and Calder, 2013). Linoleic acid is the essential omega-6 FA converted by the same desaturase and elongase enzymes (Pereira et al., 2004) into arachidonic acid (AA: figure 3). Arachidonic acid is a precursor for prostaglandin synthesis (Pereira et al., 2004 and Calder, 2013), which would influence inflammation. The body's response to these FAs is affected by the degree of

unsaturation (mono vs polyunsaturated: Roche, 1999), the first carbon unsaturated (omega-3 vs omega-6: Pischon, 2003) and its stereoisomeric structure (cis- or trans-unsaturated: Roche, 1999). Depending on these factors which influence FA structure within our diet, inflammation can be altered within our vessels leading to an increase or decrease in the progression of atherosclerosis.

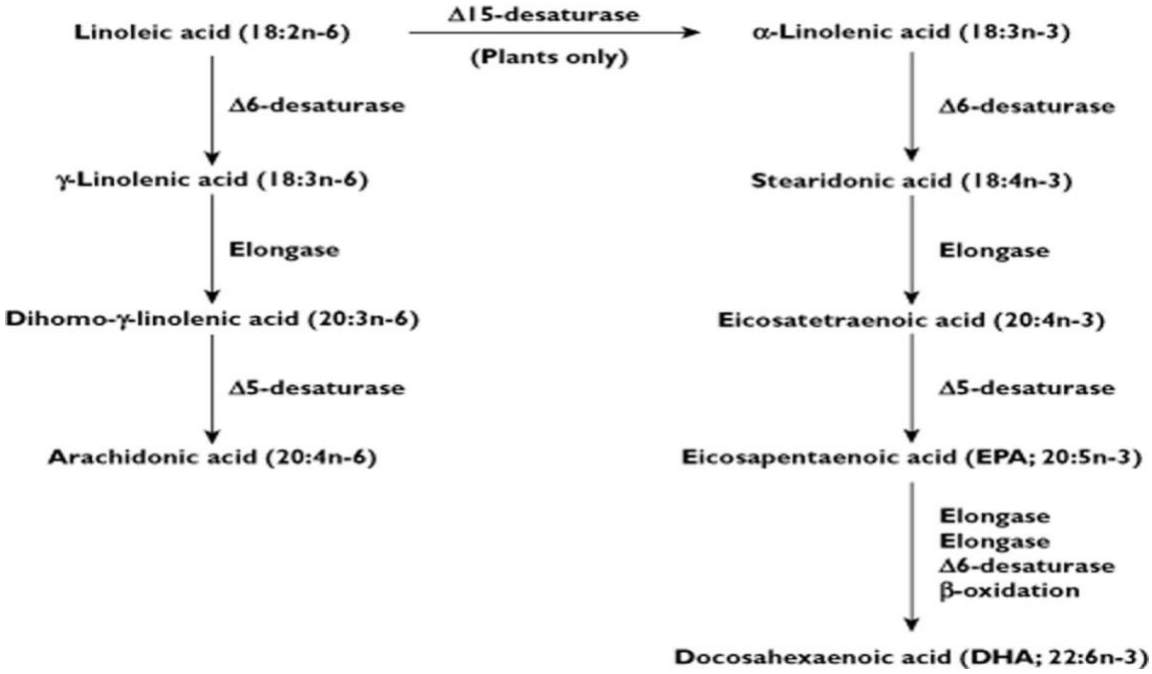

Figure 3: The conversion of omega-6 and omega-3 FAs in the body: The enzymes involved in both the conversion of omega-3's and omega-6's are the same, suggesting competition in conversion of FAs. Note the inflammatory effects of the omega 6 FA contrast that of the omega-3 FA (Calder, 2013).

Omega-3 Fatty Acids: The anti-inflammatory effects of omega-3 FAs were first suggested by studies on Greenland Eskimos (Dyerberg, 1978). Despite their high fat diet, including seal meat and whale blubber, incidence of myocardial infarctions and strokes were much lower in Eskimos than expected (Dyerberg, 1978). This was later attributed to their higher omega-3 FA diet (fish oils: EPA and DHA). Consumption of these omega-3 FAs markedly decreased triglycerides within their circulation by 34% (Dyerberg, 1978). Similar reductions in CVD risk in Japan and

the Netherlands (regions with high omega-3 consumption) supported the positive effects of omega-3's on human health (independent of activity and other risk factors: Yano et al., 1988 and Kromhout et al., 1985). This has spurred an effort to understand how omega-3 FAs reduce CVD risk. To date, omega-3 FAs have been shown to reduce inflammation by decreasing leukocyte chemo-attraction (Sperling et al., 1993), inflammatory cytokine expression (TNF-α, IL-1B and IL-6: Calder, 2013), EC adhesion molecule expression (Collie-Duguid et al., 1996), platelet adhesion, and platelet aggregation (Calder, 2004). Specifically, the reductions in platelet aggregation and adhesion increase the stability of late stage plaques (Calder, 2004). These reductions in inflammatory mediators are thought to occur via inhibition of the NF-κB cell signaling pathway (Calder, 2013).

Inflammation via NF-κB: NF-κB is primarily an inflammatory signaling molecule, which translocates into the nucleus and increases the expression of pro-inflammatory genes. These genes result in the increased production of cytokines, chemokines, cell surface adhesion molecules, and gap junction proteins, further increasing inflammation (Williams-Bey et al., 2014, Oh et al., 2014 and Denis et al., 2017). NF-κB can be activated by reactive oxygen species, TNFα, lipopolysaccharides (LPS), and saturated FAs (Williams-Bey et al., 2014). Specifically, LPS and saturated FAs bind to the toll like receptor 4 (TLR-4) to elicit a pro-inflammatory response (Williams-Bey et al., 2014). Thus, any changes in inflammatory responses are likely to involve alterations in the NF-κB cell signaling pathway.

Free Fatty Acid Receptors: The mechanism by which omega-3 FAs (or any other anti-inflammatory mediators) reduce NF-κB signaling could involve reductions in TLR-4 activation or activation of another signaling pathway. While saturated FAs and LPS bind to TLR-4, omega-3 FAs do not antagonize this physical interaction (Oh et al., 2014). Rather, they reduce

NF-κB by binding to free FA receptors (FFA). Four isoforms of these de-orphaned receptors were identified [FFA-1 (GPR40), FFA-2 (GPR43), FFA-3 (GPR41), and FFA-4 (GPR120)]. Each FFA receptor is coupled to a different signaling pathway: some have anti and others pro-inflammatory effects (Williams-Bey et al., 2014 and Cheshmehkani et al., 2015). FFA-2 and FFA-3 receptors bind to short chained FA (fewer than 6 carbons: Ichimura et al., 2014), whereas the FFA-1 and FFA-4 receptors bind to long chained FA (more than 12 carbons: refer to figure 4: Ichimura et al., 2014), including all omega-3 FAs (Figure 4: Ichimura et al., 2014 and Cheshmehkani et al., 2015). Although omega-3 FAs bind to FFA-1 and FFA-4 receptors, the FFA-1 receptor is only expressed in the pancreas and entero-endocrine cells, contrasting the broader expression of the FFA-4 receptor in tissues including the lungs, macrophages, intestines, and endothelial cells (Ichimura et al., 2014, Oh et al., 2014, and Nøhr et al., 2013). The FFA-4 receptor was shown to reduce inflammation (Oh et al., 2014 and Williams-Bey et al., 2014) by interrupting NF-κB translocation (Cheshmehkani et al., 2015).

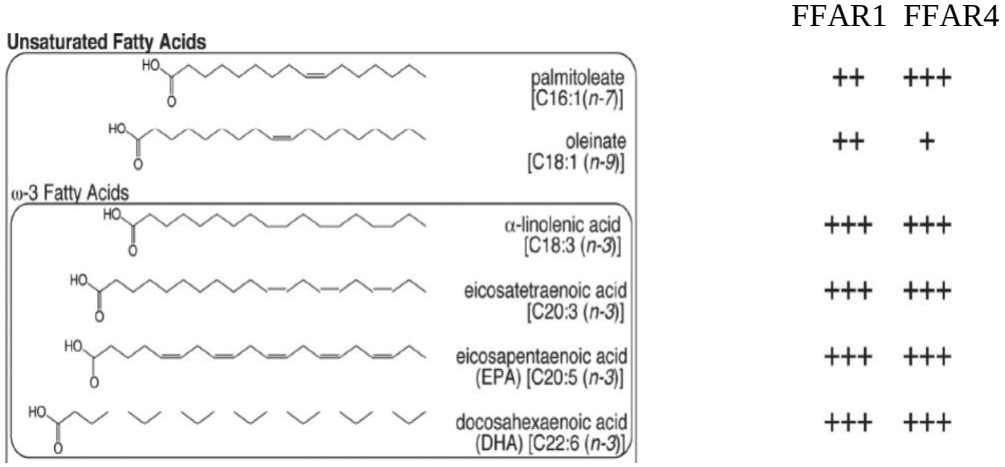

Figure 4: Unsaturated FA structure and its affinity to bind to FFA-1/4 (Ichimura et al., 2014). All the omega-3 FAs show the ability to bind to both the FFA-4 as well as the FFA-1. The FFA-receoptors shown on the right coorelate with the FA of interest directly to the left. A plus sign (+) indicates the affinity of the FA to the FFA-receptor. A single (+) is indicative of a slight affinity to the receptor while three (+)'s is indicative of having high affinity. Note the location of the receptors vary throughout the body and could produce different effects.

Free Fatty Acid 4 Receptor: The FFA-4 receptor has potential to reduce NF-κB signaling in many tissue types (i.e., macrophages, heart, lungs, GI tract, and vasculature: Cheshmehkani et al., 2015) due to its diverse expression. The mechanism of action involves the activated FFA-4 receptor binding to β-arrestin-2 which internalizes and inhibits TAB1 activation (an adaptor protein for the pro-inflammatory kinase TAK-1: Denis et al., 2017). TAK-1 is thereby prevented from initiating NF-κB translocation (Figure 5: Oh et al., 2014, 2014, Williams-Bey et al., 2014, and Cheshmehkani et al., 2015). Thus, activation of the FFA-4 receptor reduces pro-inflammatory cytokine production (Williams-Bey et al., 2014) via inhibition of NF-κB translocation. Although this response was demonstrated in macrophages (Williams-Bey et al., 2014 and Hirasawa et al., 2005), a similar signaling response has yet to be reported in endothelial cells.

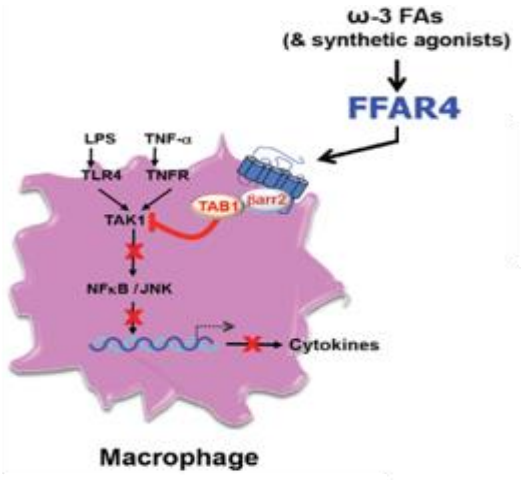

Figure 5: FFA-4 receptor mechanism of action when stimulated. Stimulation may occur by omega-3 FAs or synthetic agonist. Normal TLR-4 activation and NF-kB translocation is inhibited (Oh et al., 2014)

Endothelial cell NF-κB is activated by many factors including bacterial and viral infections (Pahl, 1999), LPS activation of TLR-4 (Williams-Bey et al., 2014), and oscillating shear stress (Denis et al., 2017). Increases in endothelial cell NF-κB activation increases the release of cytokines and chemokines (Pahl, 1999), alters cell surface protein expression (including VCAMs: Oh et al., 2014), and alters vascular connexin expression (Brisset et al., 2009).

***Connexins*:** Connexin (Cx) proteins are embedded in the cell membrane as hexamers to form connexon hemi-channels (Brisset et al., 2009) that couple with hemi-channels in neighboring cells allowing cell-to-cell communication. The permeability and gating of these channels is

determined by the specific connexins that comprise the channels. (Figure 6: Kumar et al., 1996). Of the 21 Cx isoforms expressed in mice (Brisset et al., 2009), 3 main Cxs are expressed in the vascular wall: Cx37, Cx40 and Cx43 (Puebla et al. 2017).

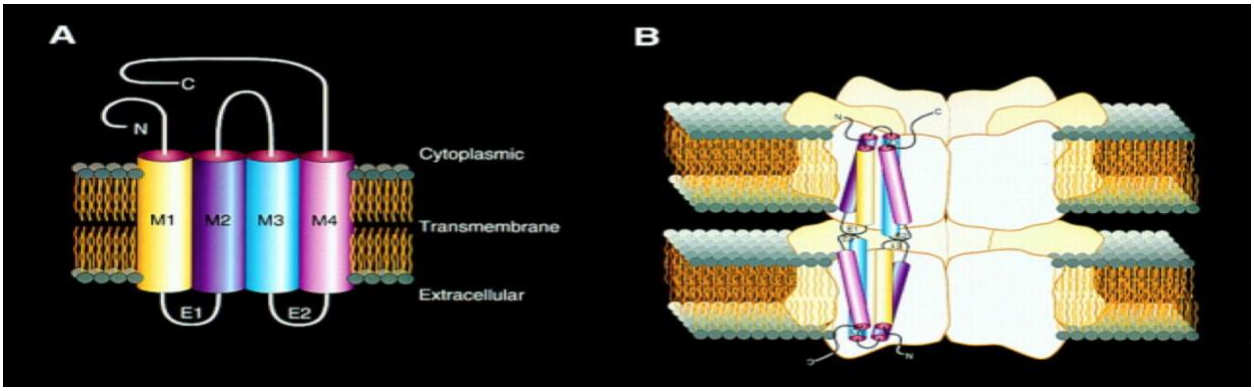

Figure 6: Connexin (Cx) topological molecular models (A): Topological model of a connexin illustrating the 4 trans-membrane domains with 2 extracellular loops (E1 and E2) influencing hemi-channel docking, located extracellularly. (B) Gap junction model: 6 individual Cx proteins arranged in an oligomeric arrangement to form a hemi-channel coming into contact with a neighboring cell. The adjacent cells dock to one another via the extracellular loops and form a communicatory pore with varying selectivity; a gap junction (Kumar et al., 1996).

Healthy endothelial cells are preferentially coupled by Cx37 and Cx40 (Brisset et al., 2009, Kwak et al., 2002, and Denis et al., 2017). Cx43, in normal physiological conditions, is restricted to expression in healthy smooth muscle cells and not on the endothelium (Kwak et al., 2002). However, within the presence of NF-κB along with the inflammatory cytokines released, Cx43 expression lining the endothelium is increased at the shoulders of the developing plaque and at sites of oscillating shear stress (Figure 7: Brisset et al., 2009). NF-κB translocation into the nucleus has been found to bind directly to the promoter of the Cx43 gene and increase its expression (Alonso et al., 2010). Since NF-κB is expressed within an inflammatory environment (Williams-Bey et al. 2014 and Pahl, 1999) and increases the expression of Cx43 (Alonso et al., 2010) within the endothelium, indicating inflammation and plaque development, Cx43 protein expression will be used as a marker and assayed as an indication of inflammation within the vessel wall.

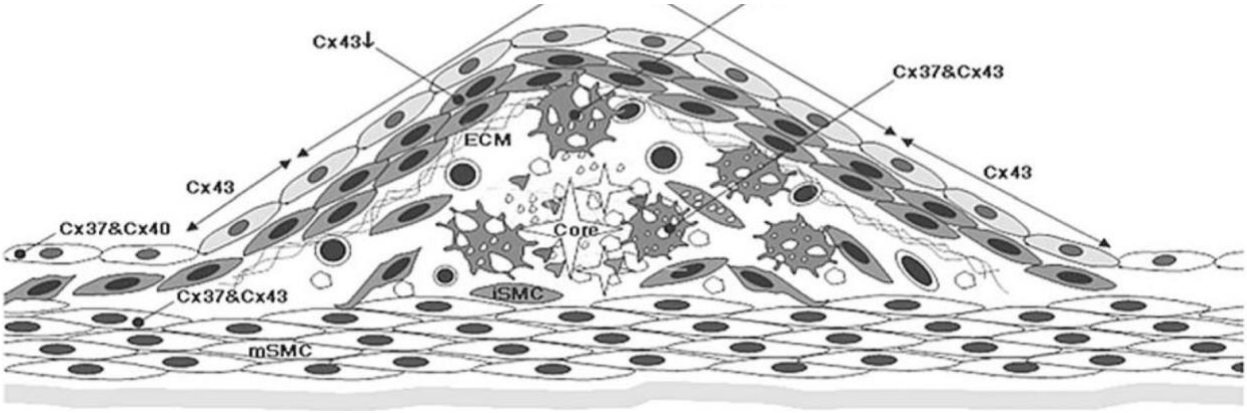

Figure 7: An advanced atheroma and Cx expression: The lesion underlying the endothelium consists of the fibrous cap and the necrotic core. The fibrous cap is composed of SMC and collagen while the necrotic core is filled with foam cells, migrating SMC, LDL particles, and debris. The endothelium covering the lesion is composed of increased expression of Cx43 on the shoulders and a decreased expression of Cx37 and Cx40 due to inflammatory cytokines (Brisset et al., 2009).

Alpha-Linolenic Acid: ALA is the essential omega-3 FA and is considered an important component of many "superfoods" including chia, flax, and hemp seeds (Egert et al., 2007). Previous studies established that fish oil omega-3's (EPA and DHA) are anti-inflammatory. However, it has yet to be determined if ALA elicits anti-inflammatory properties the same as the fish oils. While ALA has been shown to decrease CVD prevalence (Rodriguez et al., 2010) , the mechanism by which this occurs has yet to be determined. Through previous studies, among all FAs, the FFA-4 receptor is most potently activated by ALA within the gastrointestinal tract (Hirasawa et al., 2005).

Cultured endothelial cells express more Cx43 than they do *in vivo* (Alonso et al., 2010 and Kwak, 2003). This suggests that these cultured cells are in a chronically inflamed state with elevated activation of NF-κB genes driving the elevation in Cx43 expression. Since cultured endothelial cells express the FFA-4 receptor (Rogers & Kurjiaka: personal communication), activation of the FFA-4 receptor upon binding of ALA should reduce NF-κB gene activation (Oh et al., 2014 and Williams-Bey et al., 2014) and thereby decrease Cx43 expression. Thus, we will evaluate the impact of the FFA-4 receptor activation on endothelial cell expression of Cx43

when treated with ALA. Stimulation of 30 μM ALA on bEnd.3 endothelial cells is hypothesize to decrease Cx43 expression. Previous data from other omega-3 FA experiments suggest the change in Cx43 expression would occur at 12-24 hours after ALA treatment is initiated. Since ALA response can be different from other omega-3 FAs, expression was evaluated at 1.5 – 48 hours after treatment.

Chapter 3: Methodology:

Cell Culture: Cultured mouse endothelial cells (bEnd.3) were purchased from ATCC (American Tissue Culture Collection) and maintained (and grown) in high glucose Dulbecco's modified eagle's medium (HG-DMEM: Thermo Scientific D6429) supplemented with 10% fetal bovine serum (FBS: Atlanta Biologicals) and antibiotics (streptomycin and penicillin: Sigma: P4333). Cells were grown on 100 mm plates in an incubator at 100% humidity, 5% CO_2, and 38°C. New media was added every 2-6 days, depending on cell confluency, to provide cells with adequate nutrients and energy for proliferation. To split cells into a plate where they can continue to proliferate, the media was aspirated and replaced with 10 mL of a calcium free phosphate buffered saline (PD in mM: 137 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 Na_2HPO_4). PD was then aspirated and replaced with 3 mL of 0.25% Trypsin and 0.1% EDTA in PD (TV: Thermo Scientific: 2520056). The TV was aspirated and plates placed into the incubator. After the cells had lifted from the plate, the activity of the TV was stopped by adding 10% FBS HG-DMEM and cells from multiple plates were collected in a sterile tube (50 mL centrifuge tube). After thoroughly mixing the cells, they were evenly distributed into 13 plates. Cells were allowed to proliferate in the incubator for 48 hours before being treated with ALA (see experiments below). In later experiments, cells that were collected were then counted with a hemocytometer (10 μl of mixed solution pipetted into the hemocytometer). 800,000 cells were added to each plate.

Endothelial cell response to 30 μM ALA: First, a stock ALA solution of 30 mM was prepared by adding ALA (Nu-Chek: U-62A) to 100% ethanol. A 1:1000 dilution of stock ALA was added to 10% FBS HG-DMEM for a 30 μM ALA solution. The media was aspirated from 12 plates and 6 of those treated with the 30 μM ALA media and another 6 with 0.1% ethanol in the media (controls). Protein was isolated immediately from the 13th plate (time zero control: method for

protein isolation described below). Thereafter, protein was isolated from a treatment and control plate after 1.5, 3, 6, 12, 24, and 48 hours.

Endothelial cell response to 100 μM ALA: A stock ALA solution of 100 mM was prepared by adding the ALA to 100% ethanol. A 1:1000 dilution of stock ALA was added to 10% FBS HG-DMEM for a 100 μM ALA solution. The media was aspirated from 7 plates and 3 of those treated with the 100 μM ALA media and another 3 with 0.1% ethanol in the media (controls). Protein was isolated immediately from the 7th plate (time zero control). Thereafter, protein was isolated from a treatment and control plate after 12, 24 and 48 hours.

Endothelial cell response to 10 μM GW-9508 (FFA-1 receptor agonist): A stock agonist (FFA-1 receptor agonist) solution of 10 mM was prepared by adding GW-9508 (Tocris: 2649) to 100% ethanol. A 1:1000 dilution of stock GW-9508 was added to 10% FBS HG-DMEM for a 10 μM solution. The media was aspirated from 3 plates and 1 of those plates treated with the 10 μM GW-9508 media and another plate treated with 0.1% ethanol in the media (control). Protein was isolated immediately from the 3rd plate (time zero control). Thereafter, protein was isolated from a treatment and control plate after 48 hours.

Protein Isolation/Concentration: Cells were first washed with ice cold phosphate buffered saline (PBS in mM: 137 NaCl, 2.7 KCl, 1.7 Na_2HPO_4, 6.5 NaH_2PO_4, 1 $CaCl_2$, and 0.5 $MgCl_2$) to remove secreted proteins. The cells were scraped from the surface of the plate and pipetted into a 1.5 mL centrifuge tube and centrifuged (Eppendorf 5424 R, 7500 rpm, at 4°C for 5 minutes) to form a cell pellet. The supernatant was aspirated and about 30 μL (volume dependent on the size of the pellet) of Laemmli sample buffer (32.9 mM Tris HCl, 1.1% SDS, 13% glycerol, 0.01% g bromophenol blue, pH of 6.8) was added. The tube was then sonicated on ice for 30 seconds (Misonix XL-2000 series) before the membrane fragments were pelleted by the final

centrifugation (15000 rpm at 4°C for 10 minutes). Those samples were then stored in the freezer at -20°C. Protein concentration was determined using the BCA assay (Thermo Scientific: 23225) and following the manufacturer's protocol. Each protein sample was diluted with sample buffer to make a 2 μg/μL solution. β mercaptoethanol was applied (10% sample volume) to reduce the proteins and the samples were boiled at 100°C for 5 min (Fisher Scientific: Dry Bath Incubator) before being placed in the freezer (-20°C).

Western Blot Analysis of Cx43 Expression:

Gel Electrophoresis: Running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3) was first prepared from a 20X stock solution. The antioxidant (Na Bisulfite) was added to the Running buffer for a 5 mM solution. Two 4-20% gradient SDS-polyacrylamide gel (Sigma: PCG2016**)** were placed in a gel electrophoresis cassette and the cassette placed within the tank. Running buffer with 5 mM Na Bisulfite was added to the inside of the cassette and the 17 wells thoroughly rinsed. Equal volumes of the 2 μg/μL sample (5 μL for 10 μg/well) were pipetted into each well along with a pre-stained, low molecular weight ladder (Thermo Scientific: PI 26616). Running buffer was added to the tank and the gel ran at 200 V (180 mA: E-C Apparatus Corporation: EC570-90) for 30-45 minutes to ensure protein separation.

Protein transfer: Transfer buffer (25 mM Tris base, 192 mM glycine, and 20% methanol) was first prepared from a 20X stock solution. A piece of Polyvinylidene fluoride membrane (PVDF: Advanta: L-08008-001) was activated in methanol (Sigma: M3641). Filter paper (Whatman) was trimmed to a size that fit the transfer apparatus. A sandwich was prepared with a mesh, two pieces of filter paper, gel with protein, PVDF, two pieces of filter paper, and the final mesh. This sandwich was carefully generated ensuring no air bubbles would block current flow. The sandwich was placed into the transfer tank and the tank placed into an ice-cold water

bath. Protein was transferred for 4 hours at 100 V (180 mA). Once the transfer was complete, the membrane was placed into a small box (protein side up) and Tween 20 in Tris buffered saline (TTBS: 0.5% Tween 20, 25 mM Tris, 150 mM NaCl, pH 7.2) added before the box was placed into the refrigerator (4°C).

Probe membrane for Cx43: Non-fat dry milk (NFDM: Bio-Rad 170-6404) was added with TTBS to generating the Blocking solution (5% NFDM-TTBS). The box, holding the membrane, was placed on a rocker (Boekel Scientific: Rocker II model 260350) at room temperature and the blocking solution was added (7 mL per membrane). After 2 hours, the membrane was washed with TTBS before the Primary Ab solution [1% NFDM-TTBS with Cx43 Ab (Sigma: C6219) at a 1:2000 ratio: 7 mL per membrane] was added. After 2 hours, the membrane was washed with TTBS and the Secondary Ab solution [1% NFDM-TTBS with a biotinylated goat anti-rabbit IgG (Invitrogen: A16100) at a 1:3500 ratio: 7 mL per membrane] was applied. After 2 hours, the membrane was washed with TTBS and the streptavidin HRP solution [1% NFDM-TTBS solution with streptavidin HRP (Thermo Scientific: PI 21130) at a 1:4667 ratio: 7 mL per membrane] was added. After 2 hours, the membrane was washed with TTBS followed by TBS and stored at 4°C until imaged.

Chemiluminescence: The membrane was treated with the chemiluminescent substrate (Advansta: K12045) as indicated by the manufacturer and the membrane was imaged (UVP EC3 Imaging System). Pictures were taken at 20 second intervals and a summative image generated. The camera was stopped when the image showed signs of saturation. The image with the greatest intensity signal (without saturation) was selected and converted into a JPG file and analyzed with ImageJ (freeware). A background subtracted signal was determined by subtracting the intensity of a background image (close to the Cx43 band) from the 43 kDa band

itself. The background subtracted signals for the ALA treated cells were normalized by the background subtracted control samples isolated at the same time. In addition, background subtracted control samples were normalized to the background subtracted control sample at time zero. A ratio of 1 indicates the expression of Cx43 was not altered by the treatment.

Statistical Analysis: The normalized data are reported as means and standard error of the means (SEM). To address whether Cx43 expression was altered, Cx43 expression from each sample was compared to 1 with a single sample t test. With use of the single sample t test, we can evaluate if there is a change in expression of the treated group as well as the control group. Significant differences between samples was set at $P<0.05$.

Chapter 4: Results

ALA is an omega-3 FA which binds to the FFA-4 receptor (Hirasawa et al., 2005) to stimulate an anti-inflammatory response (Oh et al., 2014, and Cheshmehkani et al., 2015). As previous work in the lab showed the FFA-4 receptor was expressed on bEnd.3 cells (Rogers and Kurjiaka: personal commmunication), we expect physiological concentration of ALA (30 μM) to decrease bEnd.3 cell expression of Cx43. Representative results from a single experiment are presented in Figure 8. The intensity of the Cx43 signal for treated samples (+) was divided by the control sample isolated at the same time (-). For this experiment, the ratios of Cx43 expression was close to 1 suggesting no change in expression. Summary data in Figure 9a (n=6) support lack of change in Cx43 expression from 1.5 – 48 hours after treatment with 30 μM ALA ($p>0.05$). However, the SEM values indicate substantial variability between experiments.

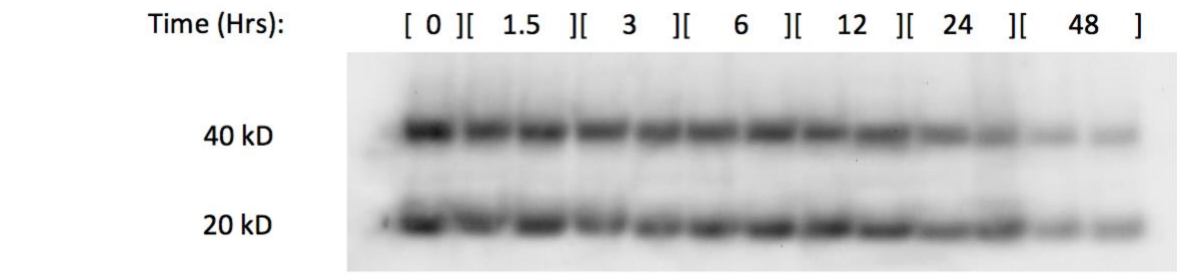

Figure 8: Western blot representative of Cx43 expression: 30 μM stimulation. The Cx43 signal from cells treated with ALA (+) was normalized to the Cx43 signal from control cells treated with 0.1% ethanol and isolated at the same time (-). Changes in Cx 43 expression are indicated by a difference from 1.0 (Cx43 Expression (TRT)). The values for each time were close to 1. A comparison was also generated for controls to determine whether Cx43 expression changed over the 48-hour period (Cx43 Expression (CNTL)). The control Cx43 signal at each time point was normalized to time 0 (protein sample isolated as the experiment started). The controls show a reduction in Cx43 expression across the course of this experiment (Cx43 expression (CNT))

To determine whether Cx43 expression was decreased across the 48-hour experiment, Cx43 signal at each time was divided by the Cx43 signal from the sample isolated at the beginning of the experiment (time zero). Interestingly, the values below 1 suggest the expression of Cx43 decreased over the 48-hour experiment. This trend was supported by the summary data (Figure 9b). While not significant due to the variability in the data ($p>0.05$), there was a trend towards a decrease in Cx43 expression across the course of the experiment. Thus, the time controls were an essential part of this experiment.

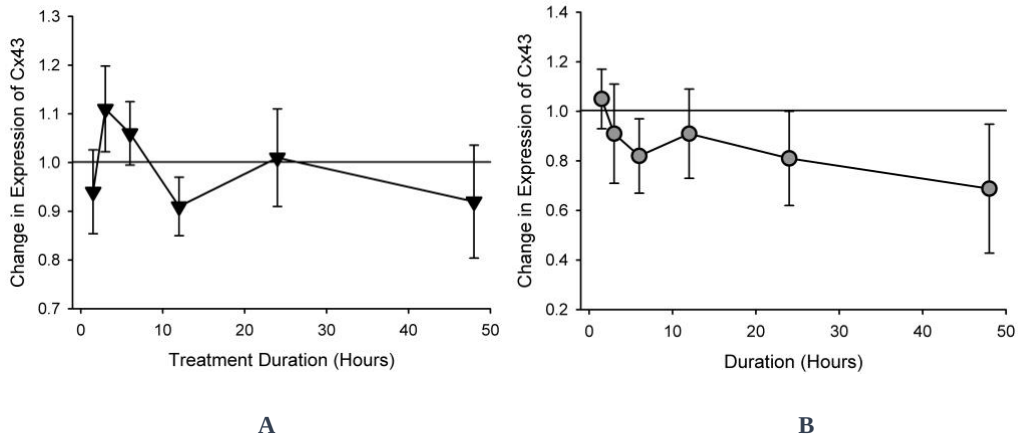

Figure 9: Summary of Cx43 expression after 30μM ALA stimulation and controls. **(A)** Cx43 expression is the ratio of ALA treated to control: change is indicated by a difference from 1. While there were small changes in Cx43 expression (elevated at 3 hours and reduced at 12 and 48 hours), none of them reached significance ($p>0.05$). However, the relatively high SEM values means there was substantial variability. **(B)** Cx43 expression in controls is the ratio of time control to time 0. While not significant ($p>0.05$), there was a trend towards reduced Cx43 expression across the 48-hour experiment.

As the data (Figure 9a) argue physiological concentrations of ALA did not affect Cx43 expression, the concentration of ALA was increased to 100 μM. As previous work in the lab on other omega-3 FAs suggested the reductions in Cx43 expression would occur after 12 hours, the experiments evaluated Cx43 expression at 12, 24, and 48 hours. Representative results (Figure 10) suggest 100 μM ALA may be decreasing Cx43 expression at 48 hours (0.86 in comparison to 1.0: a decrease in Cx43 expression). These data show similar reductions in Cx43 expression in control cells (0.77 in comparison to 1.0 at 48 hours: a decrease in Cx43 expression). However,

summary data (n=7) indicated Cx43 expression was not altered by 100 μM ALA (Figure 11a) and there was a similar trend towards a reduction in Cx43 expression in control experiments (Figure 11b).

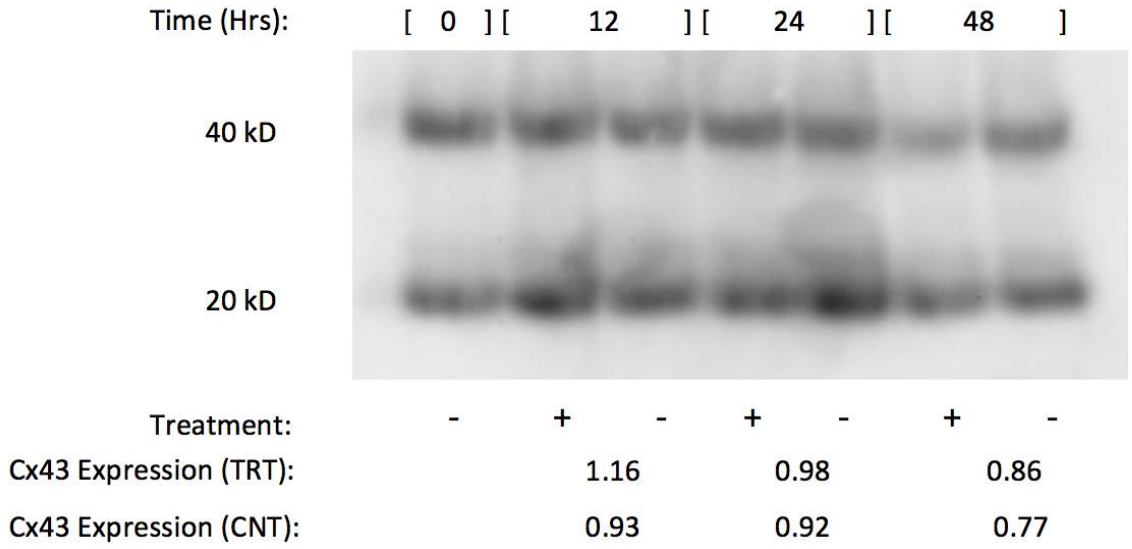

Figure 10: Western blot representative of Cx43 expression: 100 μM stimulation. Cells treated with ALA (+) were compared to cells from the control (-) group at the same time point. Changes in Cx43 expression were indicated by a difference from 1 (Cx43 Expression (TRT)). The values for each time were close to 1, indicating little change in expression of Cx43 among all time points. Cx43 expression in control experiments showed a modest trend in decreased expression as time progressed (Cx43 Expression (CNT)).

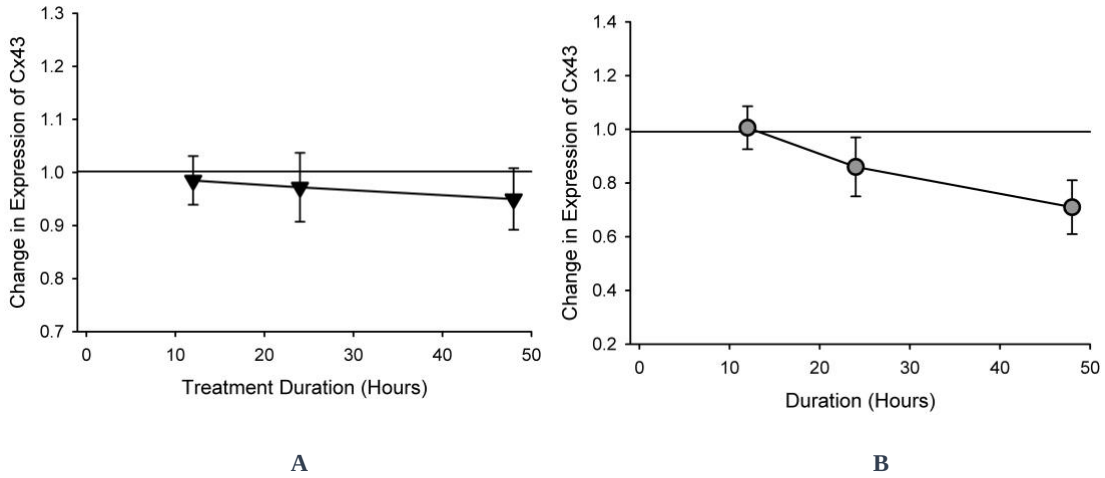

Figure 11: Summary of Cx43 expression after 100 μM ALA stimulation and controls. **(A)** With respect to cells treated with higher ALA concentration, the ratios were not different from 1 ($p>0.05$) which argues Cx43 expression did not change. **(B)** Cx43 expression from controls were evaluated to understand whether Cx43 expression changed during the experiment. While not significant, the data show a trend towards decreased Cx43 expression (especially at 48 hours).

As our original hypothesis regarding the impact of ALA on Cx43 expression was not supported, we wanted to address a reason why our hypothesis was not supported by the data collected. Previous work suggested ALA can bind to both the FFA-1 and FFA-4 receptors (Ichimura et al., 2014). Although the FFA-4 receptor is present on bEnd.3 cells (Rogers and Kurjiaka: personal communication), there is no evidence of FFA-1 receptor presence in the bEnd.3 cells. Although previous work argues FFA-1 receptor expression is localized to the intestines and pancreas (Hirasawa et al., 2005 and Ichimura et al., 2014), it is unclear whether this receptor could be expressed in other tissues, including bEnd.3 endothelial cells. Thus, we evaluated whether FFA-1 receptor responses could be generated in bEnd.3 cells by treating them with an FFA-1 receptor agonist GW-9508 (10 μM). While GW-9508 can bind to the FFA-4 receptor, its affinity for FFA-1 receptor is 100X greater with an EC50 of 7. A representative experiment of Cx43 expression after GW-9508 stimulation after 48 hours is shown in Figure 12. There was no difference in Cx43 expression of the control group compared to the treated group (1.09 in comparison to 1.0: no change in Cx43 expression), indicating ALA is not activating the pro-inflammatory FFA-1 receptor. A summary of Cx43 expression in response to GW-9508 (48 hour stimulation) is shown in Figure 13 (n=8). Among all trials, Cx43 expression remained unchanged.

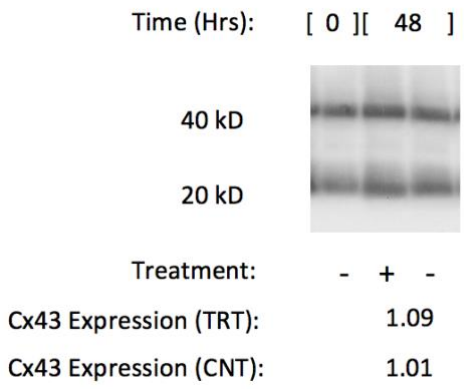

Figure 12: Western blot representative of Cx43 expression after GW-9508 stimulation. Cells treated with GW-9508 (+) were compared to cells from the control (-) group at 48 hours. Neither the treated (TRT) nor the controls (CNT) appeared to have a change in Cx43 expression (close to 1).

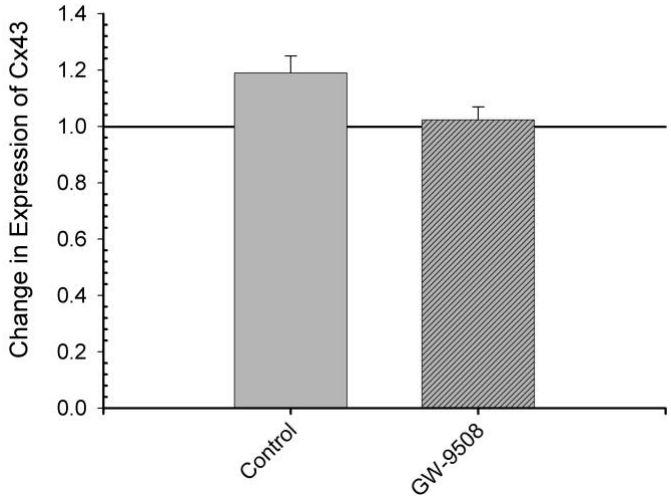

Figure 13: Summary of Cx43 expression after 10 μM GW-9508 stimulation. GW-9508 is the FFA-1 receptor agonist**.** Change in expression of Cx43 is shown by being above or below the line representing 1. There was no significant change ($p>0.05$) in Cx43 expression in response to GW-9508.

Chapter 5: Discussion

As previous studies have shown omega-3 FAs (specifically EPA and DHA) bind to the FFA-4 receptor to reduce inflammation (Williams-Bey et al., 2014 and Oh et al., 2014), we investigated the impact of ALA on cultured endothelial cell. When bEnd.3 cells were treated with 30 μM ALA, expression of Cx43 was unaffected (Figs 1 and 2). When ALA concentration was increased to 100 μM, no change in Cx43 expression was observed out to 48 hours. Since the FFA-4 receptor was present on bEnd.3 cells, the co-expression of the FFA-1 receptor might counter the anti-inflammatory responses to ALA. However, stimulation of the FFA-1 receptor agonist GW-9508 (10 μM) did not alter Cx43 expression at 48 hrs. Thus, the omega-3 FA ALA does not appear to affect endothelial cells expression of Cx43. The interpretation of these results for endothelial cell function will be addressed below.

Variability:

Cultured endothelial cells provide a convenient tool to evaluate the impact of specific molecules on endothelial cell function. However, as with every other technique, there are potential confounding influences that impact the outcome of an experiment. The data from figures 3 and 5 suggest a strong confounding influence: the effect of cell density on Cx43 expression (confluency). The expression of Cx43 increased initially and decreased through the duration of the experiment (especially at 24-48 hours). While these changes were not significant, they point to the importance of matching the cell density between treated and time control samples. If the control plates were not of a similar density (confluency) as the treated plates, they would not adequately control for the effect of time on Cx43 expression. The 30 μM ALA experiments were not performed in a way that controlled for this possibility (cells were not counted). For the 100 μM ALA experiments, cells were plated evenly at 800,000 cells per plate

and allowed to grow for 48 hours. The reduction in SEM for the control data (Figure 11b) support the assertion that our techniques contributed to the variability in 30 μM ALA data. However, the reduction in variability did not affect the outcome of the experiment as 100 μM ALA did not affect Cx43 expression (Figure 11a). The mechanism by which cell density impacts Cx43 expression is an avenue of investigation that would be interesting to address in the future.

The quantification of Cx43 expression relies on the equal concentration of protein in each well of the gel (i.e. from each sample). An evaluation of a constitutively expressed control protein was not included in these experiments for financial reasons (cost of the antibodies). Thus, small errors in pipetting for the BCA assay or the preparation of samples for Western blot (2 μg/μL) could have affected the results of our experiments in ways we cannot account for. In addition, the samples would have been concentrated if the lid was not sealed during the boiling of the samples. Thus, future experiments should evaluate a constitutive expressed protein (i.e. tubulin) to allow correction of any equal protein loading errors.

Impact of ALA on endothelial cells:

While endothelial cells have many energy sources, ALA could also contribute to energy production via beta oxidation. This use in energy production would decrease the ALA available to bind to the FFA-4 receptor. However, endothelial cells do not seem to rely on FAs to provide the energy they require (Terramani et al., 2000 and Sawada et al., 2017). FAs also have the capacity to enter the cell via a FA transport protein (Sawada et al., 2017). Alternatively, endothelial lipases could assist the incorporation of ALA into the cell membrane (Kratky et al., 2005). Once in the membrane, ALA alters membrane fluidity which can affect ion channel gating (Cordero-Morales et al., 2018) and gap junction communication (Puebla et al., 2017).

These alterations in channel function and cell-cell communication could be beneficial to the cell. However, once in the membrane, ALA could become a substrate for a cyclooxygenase (COX) or lipoxygenase (LOX) enzyme which convert FAs to prostaglandins (PG) or leukotrienes, respectively (Ding et al., 2003). Interestingly, bEnd.3 cells express the COX-2 enzyme responsible for the synthesis of PGE2 from arachidonic acid (Lin et al., 2014). The expression of COX 2 in these cells is upregulated by lipopolysaccharides (Chuang et al., 2014) that can stimulate an inflammatory response. The increase in ALA in these cells could become a direct COX-2 substrate and produce PGE3. Prostaglandins themselves can alter Cx43 expression (Qin et al., 2016): PGE2 increases Cx43 expression (Yun et al., 2012) while PGF2 decreases Cx43 expression (Xu et al., 2014). Thus, the lack of change in Cx43 in the bEnd.3 cells could reflect the combined effects of ALA on the FFA-4 receptor along with ALAs conversion to prostaglandins.

While the preponderance of epidemiological evidence argues omega-3 FA have anti-inflammatory effects (Adkins et al., 2010, Collie-Duguid et al., 1996, and Kris-Etherton et al., 2002), individual studies addressing the mechanisms by which these effects are mediated have not always support this conclusion. In the case of the FFA-4 receptor, some have argued they do not mediate anti-inflammatory responses (Pærregaard et al., 2016). The FFA-4 receptor is a G-protein coupled receptor that usually turns off very quickly once activated. Previous work from the lab argued any anti-inflammatory effect from the omega-3 FAs occurred 12-48 hours after the responses were initiated (Sundblad and Kurjiaka and Pageau and Kurjiaka: personal communications). These data do not fit the time course expected for a G-protein coupled response. Rather, they appear more like a prostaglandin response. The synthesis of prostaglandin from ALA would require enough time to increase to a concentration that would

alter Cx43 expression. In addition, the PPAR-alpha, PPAR-gamma, and/or PPAR-beta/delta pathway, could be involved in the response to omega-3 FAs (Ringseis et al., 2010 and Adkins et al., 2010). PPAR-alpha, PPAR-gamma, and PPAR-beta/delta are ligand activated transcription factors that are important regulators for lipoprotein metabolism and inflammation (Ringseis et al., 2010). Poly-unsaturated FAs, including omega-3 FAs, along with prostaglandins activate these PPAR receptors and elicit an anti-inflammatory, anti-atherogenic response (Marx, 2004). Whether the PPAR receptor is expressed (and more importantly activated) in the bEnd.3 cell has yet to be addressed.

Inflammation is a disease where the cytokines released alters the expression of many cell surface proteins and transcription factors. Cx43 gap junction protein is a cell surface protein altered by NF-κB. However, Cx43 expression can also be altered by factors unrelated to inflammation (i.e. cell confluency, oxidative stress (Madamanchi, 2004), and hypoxia (Wu et al., 2013)). As the only protein evaluated in this study was Cx43, assessment of additional endothelial cell markers of inflammation such as VCAM or direct assessment of NF-κB would strengthen the outcome of future experiments.

Summary:

The data collected in the experiments above suggest there is no anti-inflammatory effect of ALA on endothelial cells. While this is not what was expected, this study should not reduce our ALA consumption. There are several confounding variables that have made the study of FAs very challenging (prostaglandin synthesis, PPAR activation, energy storage, etc.). Based upon the study design, the possibility that ALA might still be anti-inflammatory cannot be eliminated (evaluation of different markers of inflammation). This result should redouble our efforts to understand the role of FAs in human health and disease. The FFA-4 receptor may still

be part of the anti-inflammatory response in the vasculature, and body in general, as there is much interest in the use of omega-3 FA to impact other inflammatory disease processes.

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