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## Effects of Eicosapentaenoic Acid (EPA) on Mouse Endothelial Cell Health

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Effects of Eicosapentaenoic Acid (EPA) on Mouse Endothelial Cell Health

Spencer Christian Pageau

A Thesis Submitted to the Graduate Faculty of

GRAND VALLEY STATE UNIVERSITY

In

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## Abstract

Cardiovascular disease is the number one killer of adults in the US (~800,000 lives every year: 1 in 3 deaths). Evidence suggests omega-3 fatty acids like eicosapentaenoic acid (EPA), found in fish oils, reduce the risk of developing cardiovascular diseases. Many of these diseases are caused by atherosclerotic plaque development in vessels due to endothelial cell damage initiating inflammatory responses. Recent work argues omega-3 fatty acids stimulate anti-inflammatory responses by binding to Free Fatty Acid (FFA) receptors. Inflammatory responses in endothelial cells alter the expression of many proteins that affect the function of those cells. In particular, gap junction protein (connexin: Cx) expression is affected by inflammatory signals with Cx43 increasing by NF- $\kappa$ B signaling. This study will evaluate the impact of EPA on endothelial cell expression of Cx43. We hypothesize EPA treatment will decrease endothelial cell expression of Cx43 via the FFA-4 receptor. Mouse endothelial cells (bEnd.3) were treated with 30  $\mu$ M EPA for up to 48 hours, with protein isolated from a control (0.1% ethanol) and EPA treated cells at specific times. The expression of Cx43 was evaluated via Western Blot. The Cx43 signal in the EPA treated samples was normalized to the Cx43 signal in control samples isolated at the same time (a ratio of 1 indicates no change). EPA decreased ( $p < 0.05$ ) endothelial cell expression of Cx43 after 48 hours. However, addition of AH-7614 (FFA-4 receptor antagonist) to EPA treated cells did not alter the EPA induced decrease ( $p < 0.05$ ) in Cx43 expression at 48 hours. More importantly, blocking the FFA-4 receptor for 48 hours decreased ( $p < 0.05$ ) Cx43 expression, suggesting something in the cell culture media caused basal FFA-4 receptor stimulation. Treatment with the FFA-4 agonist (TUG-891) for 48 hours caused a non-significant increase in Cx43 expression. Thus, the reduction in expression of Cx43 at 48 hours did not involve the FFA-4 receptor. In fact, the response of endothelial cells to FFA-4 receptor activation is consistent with increased inflammation (i.e.

increased Cx43 expression). EPA has an anti-inflammatory effect on mouse endothelial cells at 48 hours that is not mediated by the FFA-4 receptor.

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## **Abbreviations**

VCAM: Vascular Cell Adhesion Molecule

MCP-1: Monocyte Chemoattractant Protein-1

M-CSF: Macrophage-Colony Stimulating Factor

TGF: Transforming Growth Factor

MMP: Matrix Metalloproteinase

IL: Interleukin

LDL: Low-density Lipoprotein

EPA: Eicosapentaenoic acid

GLP: Glucagon-like Peptide

Cx: Connexin

HDL: High-density Lipoprotein

DHA: Docosahexaenoic acid

TNF: Tumor Necrosis Factor

TLR: Toll-like Receptor

FFA: Free Fatty Acid

TTBS: Tween 20 Tris-Buffered Saline

COX: Cyclooxygenase

TV: Trypsin Versene

PD: Calcium-free Phosphate Buffered Saline

PBS: Phosphate-buffered Saline

SMC: Smooth Muscle Cells

IFN: Interferon



## **Chapter 1: Introduction**

Cardiovascular disease accounts for 1 in every 3 deaths in the United States; more than 830,000 each year (American Heart Association, 2018). The economic burden resulting from the millions of people whose lives are impacted by cardiovascular disease and stroke weighs heavy on the country, costing the United States an estimated \$300 billion (American Heart Association, 2018). The leading cause of these cardiovascular diseases is atherosclerotic plaques, which are characterized by lipid deposits in the intima of arteries. The growth of these plaques is initiated by damage to the endothelium and continues as a localized chronic inflammatory response. These plaques can grow large enough to occlude arteries. Established risk factors for the development of plaques include unhealthy blood cholesterol (elevated LDL and/or reduced HDL), hypertension, smoking, diabetes, insulin resistance, being overweight, inactivity, diet, age and family history (NIH, 2017). Many of these risk factors are interrelated (e.g., diet, unhealthy cholesterol, obesity, insulin resistance, and inactivity) and modifiable.

Atherosclerotic plaques commonly develop in areas of disturbed flow (e.g., vessel branches and curvatures) where altered shearing forces cause endothelial cells to become poorly aligned, have a high turnover, and are under oxidative stress (Ando & Yamamoto, 2009). These oscillating shearing forces alter endothelial cell expression of proteins resulting in a more permeable endothelium which allows lipids and leukocytes to invade the arterial intima. For example, cell adhesion molecules (CAMs) expressed on endothelial cells recruit leukocytes (e.g., monocytes and T lymphocytes) to the site of inflammation (Cybulsky & Gimbrone, 1991). Circulating monocytes are slowed by their interaction with endothelial CAMs so they can follow the chemotactic factor (monocyte chemoattractant protein-1: MCP-1) by diapedesing into the vascular intima and beginning to form atherosclerotic plaques (Ylä-Herttuala et al., 1991). Once

they exit the vessel, monocytes mature into intimal macrophages expressing scavenger receptors through the action of macrophage colony-stimulating factor (M-CSF: Clinton et al., 1992). These macrophages, along with T cells, form the basis of the immune response in the plaque. Growth factors and cytokines released from these cells stimulate the migration of smooth muscle cells (SMC) into the intima and their subsequent dedifferentiation into collagen secreting cells causing the growth of the atherosclerotic lesion into the vessel lumen and potentially blocking blood flow (Newby & Zaltsman, 1999; Allahverdian et al., 2018).

While the overgrowth of plaques can occlude vessels and cause ischemic events, a bigger concern is the potential of plaque rupture. Ruptured plaques enter the flow stream and are carried downstream to occlude blood flow. The remaining plaque is comprised of the necrotic core which is a potent initiator of platelet aggregation and thereby thrombus formation inside the vessel. The resulting (larger) thrombus is weakly attached to the vessel and can break free to occlude a larger vessel (Fuster et al., 1990). The likelihood of a plaque rupture is a function of the strength of the fibrous cap (Loree et al., 1992) which is affected by the extent of collagen secreted by the SMC into the plaque. Infiltration of T-lymphocytes into the plaque can inhibit basal collagen secretion by releasing IFN- $\gamma$  (Amento et al., 1991). These cells also release IL-1 and CD40 ligand which increases the release of matrix metalloproteinases (MMPs) that degrade the collagen cap (Sukhova et al., 1999; Horton, Libby, & Schonbeck, 2001).

In healthy vessels, laminar flow and the resulting stable shearing forces stimulate endothelial cells production of nitric oxide (Gimbrone et al., 2000). In addition to its vasodilatory effect, nitric oxide is atheroprotective reducing the activation of NF- $\kappa$ B signaling in the endothelium (De Caterina et al., 1995). The activation of NF- $\kappa$ B signaling induces the expression of the proteins of inflammation including CAMs (De Caterina et al., 1995),

inflammatory cytokines (Williams-Bey et al., 2014) and gap junction proteins (Alonso et al., 2010), all of which further the local inflammation.

Atherosclerotic plaque development is affected by the migration of LDL particles into the intima where they are ingested by macrophages. LDLs transport lipids (e.g., cholesterol and triacylglycerol) from the liver to cells of the body with LDL receptors (Cox & García-Palmieri, 1990). In macrophages, scavenger receptors absorb LDL particles that were oxidized by their increased concentration in the intima and the length of time they remain in the intima (Parthasarathy et al., 2010). The sheer number of ox-LDL absorbed by macrophages overwhelms the pathways responsible for metabolizing these particles (Nayer & Ortega, 2014) causing them to accumulate, converting the macrophage into a foam cell that dies to form the necrotic core of the plaque (Rafieian-Kopaei et al., 2014). LDL particles transport lipid soluble molecules like triacylglycerol to cells of the body. Triacylglycerol molecules function primarily as an energy storage molecule with 3 fatty acids (FAs) bound to a glycerol molecule. Lipoprotein lipases in the endothelium can act on LDL particles releasing FAs into the bloodstream (Mead & Ramji, 2002) making them available as energy substrates. However, recent work suggests FA can communicate signals by binding to receptors on cells of the body. The particular signal communicated by these FAs can be atherogenic or atheroprotective, depending upon the receptor activated. For example, trans-unsaturated FA bind to the toll like receptor-4 (TLR) and stimulate a local inflammatory response (Huang et al., 2012). Thus, the structure of the FA determines the receptor activated and thereby the nature of the signal being communicated.

The structure of FAs is classified by the number of carbon double bonds and their orientation. Saturated FAs have no double bonds, and increases in their consumption are linked to elevated risk of cardiovascular diseases (Dayton et al., 1969; Leren, 1970; Willett, 2012;

American Heart Association Nutrition Committee et al., 2006). Unsaturated FAs have one or more (mono vs poly) double bonds which are orientated in either a cis or trans configuration. Trans unsaturated FAs are uncommon as they require high temperatures to be synthesized (Stender, Astrup, & Dyerberg, 2008). As mentioned above, trans unsaturated FA (as well as saturated FAs) appear proatherogenic by activating the TLR-4 receptor (Huang et al., 2012). Although the receptor responsible has yet to be determined, omega 6 polyunsaturated FA also appear pro-atherogenic (Simopoulos, 2008). The cis and omega-3 unsaturated FAs (namely Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA)) appear anti-atherogenic as evidenced by their ability to reduce the risk for cardiovascular diseases (Simopoulos, 2008; Hirafuji et al., 2003; Harris, Park, & Isley, 2003).

We chose to focus on the omega-3 FAs as increased consumption reduces the risk for cardiovascular diseases by decreasing inflammatory mediators, thereby improving endothelial cell function (Adkins & Kelley, 2010; Kromhout et al., 2012). More specifically, omega-3 FA can down-regulate NF- $\kappa$ B and alter gene expression during inflammation and cardiovascular disease (Adkins & Kelley, 2010; de Winther et al., 2005). EPA also inhibits the activation of Rho-kinase, which up-regulates pro-inflammatory signaling molecules and decreases nitric oxide synthase activity in the endothelium (Gao et al., 2011; Takemoto et al., 2002; Yada et al., 2005). Recent evidence suggests the anti-inflammatory effect of omega-3 FA may derive from their binding to recently the de-orphaned free fatty acid (FFA) receptors. In particular, the FFA-4 receptor in macrophages and adipocytes decreases inflammatory mediators like JNK and NF- $\kappa$ B (Oh et al., 2010). EPA stimulates anti-inflammatory responses in other tissues of the body including the pancreas and liver (Kantha, 1987; Scorletti & Byrne, 2013; Flachs, Rossmeisl, & Kopecky, 2014). Other omega-3 FAs (DHA) inhibit NF- $\kappa$ B signaling thereby decreasing

inflammation through binding to the FFA-4 receptor (Williams-Bey et al., 2014). Thus, evidence suggests the anti-inflammatory effects of the omega-3 FA EPA are mediated by their binding to the FFA-4 receptor to decrease NF- $\kappa$ B signaling.

Inflammatory responses alter protein expression in the endothelium through an increase in NF- $\kappa$ B signaling (among others) which increases the expression of inflammatory cytokines (Pahl, 1999), CAM proteins involved in leukocyte recruitment (Oh & Walenta, 2014) and vascular connexins (Brisset, Isakson, & Kwak, 2009). Endothelial cells express 3 different connexin (Cx) proteins: Cx37, Cx40, and Cx43 (Haefliger, Nicod, & Meda, 2004). These proteins form hexameric hemichannels in the cell membrane that couple neighboring cells by forming gap junction channels. Both types of channels (hemi and gap junction) play a role in the function of the vasculature. Gap junctions allow ions, second messengers, and small metabolites to be diffused between cells (Nielsen et al., 2012). The expression of these Cxs is affected by the health of the endothelium: Cx37 and 40 are highly expressed undamaged regions whereas Cx43 expression is elevated in regions of damage (Gabriels & Paul, 1998). Further, the expression of these vascular Cxs is altered in atherosclerotic plaques as Cx37 and 40 expression are reduced in advanced plaques and whereas Cx43 is elevated at the shoulder of plaques (Kwak et al., 2002).

Changes in the expression of vascular Cxs affects plaque development by altering the recruitment of leukocytes: Cx37 increases recruitment (Wong et al., 2006) whereas Cx40 reduces recruitment (Chadjichristos et al., 2010). At the same time, decreasing Cx43 expression reduces atherosclerotic lesion formation (Wong, et al, 2003; Kwak et al., 2003). The pro-inflammatory nature of Cx43 is supported by work showing NF- $\kappa$ B binds to the Cx43 promotor to increase Cx43 expression (Alonso et al., 2010). Since EPA can bind to the FFA-4 receptor (which is

expressed on the endothelium: Rogers & Kurjiaka, 2017), the resulting disruption of NF- $\kappa$ B signaling should decrease Cx43 expression.

## **Purpose**

Cardiovascular disease is a leading cause of death around the world, but especially in the United States and other developed countries. Consumption of omega-3 fatty acids, often found in fish oils, exhibit anti-inflammatory effects that reduce the risk for cardiovascular diseases. The recently de-orphaned FFA-4 receptor has been proposed to explain the anti-inflammatory responses to EPA. However, direct evidence that EPA can reduce inflammation in vascular cells is lacking. Thus, this research project could help determine whether the anti-inflammatory effects of EPA are mediated by the FFA-4 receptor by evaluating changes in expression of Cx43.

## **Scope**

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

## **Assumptions**

Vascular endothelial cells are quiescent, only proliferating in response to disturbance of the vasculature, such as inflammation. bEnd.3 cells are an immortalized mouse brain endothelial cell. As these cells are continuously dividing, they are no longer acting like healthy endothelial cells and may be expressing some basal level of inflammation in culture. We will evaluate the responses of bEnd.3 cells because they express the FFA-4 receptor (Rogers & Kurjiaka, 2017). The data collected from this experiment can then be used to address whether similar responses are observed in human endothelial cells.

## **Hypothesis**

We hypothesize treatment with 30  $\mu$ M EPA for up to 48 hours will reduce Cx43 expression in endothelial cells through activation of the FFA-4 receptor.

### **Significance**

Cardiovascular disease is the number one cause of death around the world. The economic consequence of this on developing countries (as well as developed countries) demonstrates the importance of developing additional tools to counter the huge financial impact of this disease. While EPA is being used to treat cardiovascular diseases (and other inflammatory diseases: (Swanson, Block, & Mousa, 2012),) determining the receptor responsible for the anti-inflammatory effects provides another target for drug treatment (e.g., FFA-4 receptor agonist). In addition, it provides supportive evidence for changing cultural norms and increases the consumption of healthy FA over the unhealthy ones.

## **Chapter 2: Review of Literature**

### **Epidemiology**

Cardiovascular disease is the number one killer of both men and women in the United States and many other areas around the world. In addition to taking the lives of hundreds of thousands of individuals, the economic burden weighs heavily on the world. In 2009, coronary heart disease and stroke alone brought with it an estimated indirect and direct economic cost of \$234 billion. Due to these extensive effects on society, it is important to study risk factors that both progress and inhibit cardiovascular diseases (Ford et al., 2009).

### **Atherosclerosis**

Atherosclerosis is one of the main causes of cardiovascular disease. The initiating step in the formation of atherosclerotic plaques is endothelial cell damage. Chemoattractants are released by macrophages to bring leukocytes to the site of damage and various adhesion molecules are expressed (Paulus et al., 2011). Selectins are particularly involved in the integrating of rolling leukocytes into the endothelium (Mcever, 1997). Additionally, VCAMs play a part in the adhesion of leukocytes to the endothelium (Rao et al., 2007). These molecules also take part in intracellular signaling that lead to a change in the shape of the endothelium that allow for leukocyte extravasation (Cook-Mills, Marchese, & Abdala-Valencia, 2011). These monocytes, now in the arterial intima, propagate, mature, and ingest lipids to form foam cells (Libby, 2012). Foam cells also have an affinity for oxidized LDL that enters the intima through an endothelial lesion, which leads to the recruitment of additional monocytes (Cohen, Myerscough, & Thompson, 2014). Smooth muscle cells then migrate into the intima and propagate around the foam cells, eventually releasing extracellular matrix proteins, including collagen, that participate in the formation of the fibrous cap of the atherosclerotic plaque. As



these plaques progress, foam cells die and release lipids that form the core of the plaque (Libby, 2012). The release of these lipids and presence of these dead foam cells recruits additional leukocytes, primarily T-cells. These cells release that enzymes degrade the extracellular matrix proteins and make plaques unstable. Unstable plaques are at an increased risk of losing their fibrous caps which travel downstream to occlude a vessel (Libby, 2012). The loss of the fibrous caps also initiates platelet to aggregation and the formation of a thrombus. That thrombus is weakly linked to the blood vessel wall and can therefore break free and occlude tissue blood flow. (Hansson, 2005; Morel, Burnier, & Kwak, 2009).

### **Role of Lipids**

Lipids play an important role in cardiovascular disease. There exists strong epidemiological evidence supporting high density lipoprotein (HDL) as “good cholesterol” that supports cardiovascular health and low density lipoprotein (LDL) as “bad cholesterol” that diminishes cardiovascular health (Goldbourt, Yaari, & Medalie, 1997; Wilson, Abbott, & Castelli, 1988; Zhao et al., 2016). As mentioned above, LDL enters the intima through damaged endothelium and binds to macrophages to form foam cells. HDL works to prevent atherosclerosis by removing LDL from foam cells and limiting the inflammatory cascade (Bandeali & Farmer, 2012).

Another lipid that plays a role in cardiovascular health are very low-density lipoproteins (VLDL). Secreted by the liver, VLDL is poor in cholesterol but rich in triglycerides, which a lipid used for energy storage in the blood and is associated with cardiovascular disease at high levels (Nordestgaard & Varbo, 2014). Lipoprotein lipase, secreted by adipose tissue and skeletal muscle, converts the triglycerides in VLDL to free fatty acids that circulate freely through the blood and can have a myriad of effects (Barter, 2005).

## **Fatty Acids**

### **Inflammatory effects**

Free fatty acids circulating in the blood can have both pro-inflammatory and anti-inflammatory effects on cardiovascular endothelium. Saturated fatty acids are well documented as being pro-inflammatory (Siri-Tarino et al., 2010; Jakobsen et al., 2009). These substances can stimulate inflammation via a number of endothelial cell signaling pathways: diacylglycerol and ceramide, NF- $\kappa$ B, protein kinase-C, and mitogen-activated kinases, and via the expression of proteins involved in the recruitment of inflammatory mediating immune cells such as macrophages, neutrophils, and dendritic cells to different body tissues including white adipose tissue and muscle (Kennedy et al., 2009). Monounsaturated and polyunsaturated fats are well documented as having protective cardiovascular effects (Kelly & Sabaté, 2006; Siscovick et al., 2017). Both types of fats are associated with increasing HDL levels and lowering LDL levels (Rudel, Parks, & Sawyer, 1995). Population studies have also shown that a higher intake of polyunsaturated fats is associated with a lower risk of cardiovascular disease (Kushi et al., 1985). While the data largely suggests polyunsaturated fatty acids are protective, they could potentially have harmful effects, such as hemorrhagic activity and oxidative potential (Serini et al., 2011).

The most common anti-inflammatory polyunsaturated are omega-3 polyunsaturated fatty acids, commonly found in fish oils. The two most common omega-3 polyunsaturated fatty acids are docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA). These fatty acids combat inflammation in a number of ways, including inhibiting leukocyte chemotaxis, adhesion molecule expression and leukocyte binding, eicosanoid production (such as prostaglandins and leukotrienes), inflammatory mediators (such as IL-6, IL-8 and TNF- $\alpha$ ), and T-cell reactivity (Calder, 2013). While palmitic and linoleic acids increased mRNA expression of inflammatory

mediators, such as TNF- $\alpha$  and IL-6, in adipocytes, oleic and docosahexaenoic acid (DHA) had the opposite effect (Rodriguez-Pacheco et al., 2016). The inflammatory responses seen by saturated fatty acids, such as palmitic acid, are mediated by TLR4 receptors located on the endothelial layer (Huang et al., 2012).

Lower serum EPA is associated with a greater risk of cardiovascular disease. Additionally, a number of studies have shown that EPA has a multitude of benefits to human cardiovascular health, including lowering triglycerides and anti-inflammatory effects (Baum & Hamm, 2012). EPA has also been linked to lowering plasma triacylglycerol and blood pressure, cardiac cell ion flux, as well as influencing serum markers such as triglycerides and HDL-cholesterol (Anderson & Ma, 2009). EPA has been shown to reduce symptoms of chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease, as well as other diseases such as cardiovascular disease (Magee et al., 2012).

### **Free Fatty Acid Receptors**

Free fatty acid receptors (FFAR) are members of the G protein-coupled receptor family and are involved in a variety of tissues and disease responses (Hara et al., 2014). Based on the length of EPA, it would be expected to bind to the FFA-1 receptor (GPR40) and/or the FFA-4 receptor (GPR120) receptor (D. C.-H. Lin et al., 2012). It appears that EPA is a ligand for a particular FFA, FFA-4 receptor, that is involved in its anti-inflammatory effects (Oh et al., 2010, p. 12).

The FFA-4 receptor signaling cascade likely inhibits inflammation by taking components from the inflammatory cascade, thereby halting its continuation (Oh & Walenta, 2014). When the GPR120 gene was silenced in both obese and non-obese subjects, the effects of a number of

fatty acids on inflammatory mediator expression was significantly annulled (Rodriguez-Pacheco et al., 2016). Therefore, this particular receptor will be utilized in determining the effect of EPA.

FFA-4 receptor is expressed in numerous tissues throughout the body, including the heart. FFA-4 receptor can go in one of two ways when omega-3 fatty acids are bound:  $G_{\alpha q}$  or  $\beta$ -arrestin-2. By using the  $G_{\alpha q}$  pathway, intracellular calcium ion concentration is increased without effecting the level of cyclic AMP (Oh & Walenta, 2014). In the  $\beta$ -arrestin-2 pathway, FFA-4 receptor forms a complex with  $\beta$ -arrestin-2 and is internalized. This complex associates with TGF- $\beta$  activated kinase 1 (TAK1) binding protein (TAB1). TAB1 is an adaptor for TAK1 that can help signal for inflammatory mediators to go to a certain area. Therefore, this signaling is inhibited by the FFA-4 receptor complex (Oh & Walenta, 2014).

### **Connexins**

Intercellular communication is important in the process of inflammation, and one method of communication between cells is through the use of gap junctions. These gap junctions form channels between cells that allow for communication (Söhl & Willecke, 2004). Gap junctions are formed when transmembrane subunit protein called connexins come together. Two of the main connexin proteins associated with cardiovascular endothelium are Cx37 and Cx43 (Johnson & Nerem, 2004).

Connexin 37 appears on healthy endothelial cells, but not in cells with advanced atherosclerotic plaques (Morel et al., 2009). Connexin 37 expression was diminished when presented with TNF- $\alpha$  (Reifen et al., 2015). Therefore, inflammatory cytokines associated with atherosclerotic plaques interfere with connexin expression.(Morel et al., 2009) In mice with the Cx37 gene deleted that were fed high cholesterol diets, atherosclerotic plaque development was significantly accelerated (Wong et al., 2006). Additionally, connexin 37 gene polymorphism

resulted in an increased risk of subclinical atherosclerotic plaques. Connexin 37 seems to have a significant role in the maintenance and protection of cardiovascular endothelium.

Connexin 43 is found at arterial branching sites and other areas that are common sites of formation of atherosclerotic plaques (Gabriels & Paul, 1998). In mice fed a cholesterol rich diet, connexin 43 expression increased in intimal smooth muscle cells in early atherosclerotic lesions, as well as in the endothelium in the shoulder region of atherosclerotic lesions (Kwak et al., 2002). It has been hypothesized that connexin 43 enhance endothelial dysfunction and proliferate leukocyte migration and proliferation (Morel et al., 2009). Additional data shows that the deletion of the connexin 43 gene results in benefits in regards to atherosclerotic lesion progression and composition (Morel et al., 2008). The relation of connexin 43 to endothelial damage, the first step in the progression of atherosclerotic plaques, makes it a good marker for the measurement of endothelial cell health.

### **Chapter 3 Methodology**

**Cell culture** Mouse endothelial cells (bEnd.3: ATCC) were cultured in 100 mm plates using high glucose Dulbecco's minimal essential media (HG-DMEM: Thermo Scientific D6429) supplemented with 10% fetal bovine serum (Atlanta Biologicals) to drive their proliferation. These cells were maintained in an incubator at 38°C with 5% CO<sub>2</sub> and 100% humidity. Cells were prepared for the time course experiments below once 6 plates were confluent. After aspirating the HG-DMEM, calcium was removed with 10 mL of calcium-free phosphate-buffered saline solution (PD in mM: 137 NaCl, 2.7 KCl, 1.8 KH<sub>2</sub>PO<sub>4</sub>, 10 Na<sub>2</sub>HPO<sub>4</sub>). The PD was aspirated and replaced with 3 mL of 0.25% Trypsin and 0.1% EDTA in PD (TV: Thermo Scientific: 2520056) and the plate tipped to ensure all cells were exposed. After aspirating the TV, plates were placed into the incubator where they remained until the cells lifted (~5 minutes). The cells from these plates were combined and evenly distributed into 13 plates. Those plates were placed into the incubator until confluent (2-3 days).

**Endothelial response to 30 μM EPA:** First, a stock EPA solution of 30 mM was prepared by adding EPA (Enzo Scientific: BML-FA001) to 100% ethanol. The 10% FBS HG-DMEM was removed from 13 confluent bEnd.3 cells and replaced in 6 with 10% FBS HG-DMEM containing 30 μM EPA (1:1000 dilution of 30 mM stock). The other six plates were treated with the control 0.1% ethanol in 10% FBS HG-DMEM.

**Protein isolation:** Once 12 plates were prepared, protein was isolated from the thirteenth plate. The plate was placed on ice and the cells washed with ice cold phosphate buffered saline (PBS in mM: 137 NaCl, 2.7 KCl, 1.7 Na<sub>2</sub>HPO<sub>4</sub>, 6.5 NaH<sub>2</sub>PO<sub>4</sub>, 1 CaCl<sub>2</sub>, and 0.5 CaCl<sub>2</sub>). After 3 washes, the cells were scraped from the plate and pelleted in a centrifuge (7500rpm for 5 minutes; Eppendorf Centrifuge 5424 R). Laemmli sample buffer (32.9 mM Tris HCl, 1.1% SDS, 13%

glycerol, 0.01% g bromophenol blue, pH of 6.8) was added to the pellet and the sample sonicated for 30 seconds on ice (Misonix XL-2000 Series). The membrane fragments were pelleted (15000 rpm for 10 minutes) and the sample stored at -20 °C. At 1.5, 3, 6, 12, 24, and 48 hours, protein was isolated using the technique outlined above from two plates (one control and one EPA treated).

**Protein preparation:** Once protein was isolated from all 13 plates, protein concentration for each sample was determined using the BCA assay (Thermo Scientific: 23225) following the manufacturers' directions. Once determined the protein concentration in each sample was diluted with sample buffer to 2 µg/µL and beta mercaptoethanol added (10% of sample volume). Samples were then boiled at 100 °C for ~5 minutes (Fisher Scientific: Dry Bath Incubator) and placed in the freezer (-20 °C) until enough samples were obtained for the next step (~5-7 days).

**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 was placed within the tank. Running buffer with 5 mM Na Bisulfite was added to the inside of the cassette and the 17 wells were 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 (leading band at or near the end of the gel).

**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) until it was probed the following day.

***Probe membrane for Cx43:*** Non-fat dry milk (NFDM: Bio-Rad 170-6404) was added with TTBS to generate 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 it was imaged the following day.

***Quantification:*** Chemiluminescence was used to detect HRP. An image was obtained by treating the membrane with 3 mL of both WesternBright ECL and Peroxide (Advanta: K12045) using



the UVP EC3 Imaging System. ImageJ software was used to quantify the signal. Cx43 signal for the EPA treated sample at each time point was normalized to the control sample isolated at the same time to determine whether EPA altered Cx43 expression.

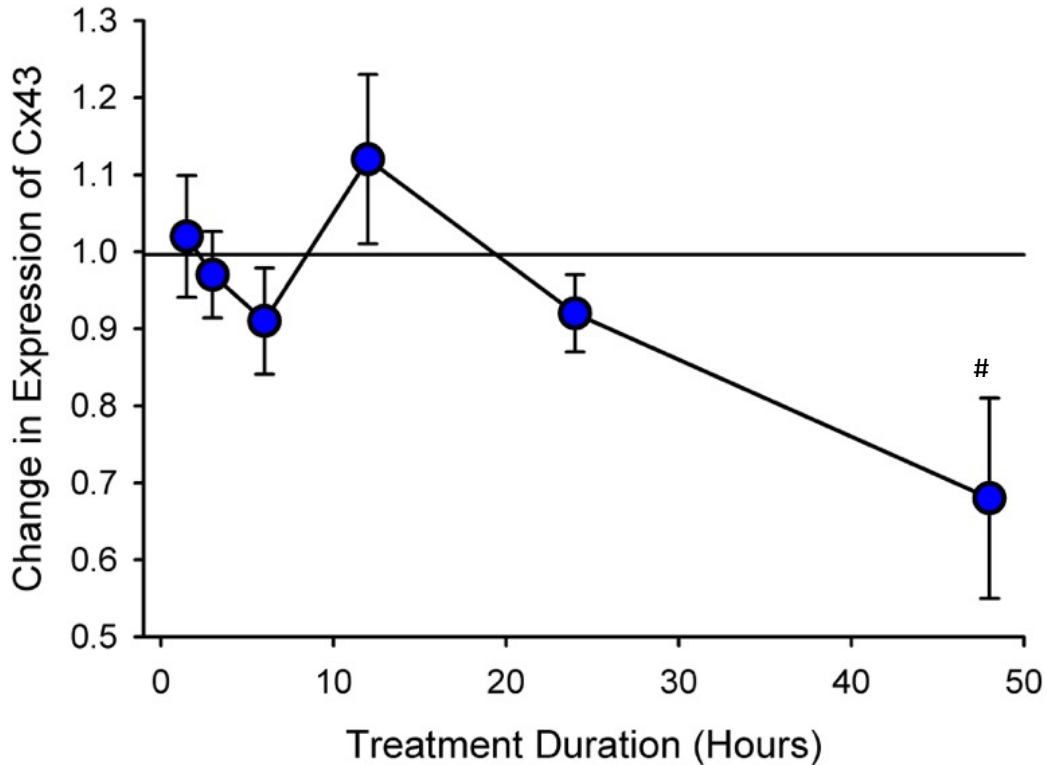
***Impact of FFA-4 receptor inhibition (AH-7614 - 5  $\mu$ M) on EPA response:*** The previous experiment determined the duration of EPA treatment that maximally decreased Cx43. For this experiment, 4 plates were used. Media from two plates were replaced with media containing an FFA-4 receptor blocker (AH-7614: Tocris 5256). After one hour, the media with blocker was removed from both plates and replaced with blocker in one and blocker with 30  $\mu$ M EPA in the other. The third plate was treated with 30  $\mu$ M EPA and the fourth treated with ethanol. At the time determined by the first experiment, protein was isolated from each of these plates and run in a gel as described above.

***Determine the impact of FFA-4 receptor stimulation on Cx43 expression:*** In order to evaluate the role of the FFA-4 receptor in the regulation of Cx43 expression, endothelial cells were treated with an FFA-4 receptor agonist (TUG-891: 10  $\mu$ M). Two plates of bEnd.3 cells were prepared as outlined above. At the start of the experiment, the media in one plate was replaced with media containing TUG-891 (5 mM stock in ethanol) while media in the second plate was replaced with media containing 0.2% ethanol. At the same time point where the maximal change in Cx43 expression was observed, protein was isolated from these plates and the expression of Cx43 evaluated as outlined above.

***Statistics:*** In order to determine whether EPA altered Cx43 expression in the time course experiments, a ratio of the Cx43 signal intensity (EPA treated / 0.1% ethanol control) was calculated at each isolation time. After compiling data across multiple experiments, an unpaired t-test was employed to compare each time to the 1.5 hour (the first sample with EPA treated and

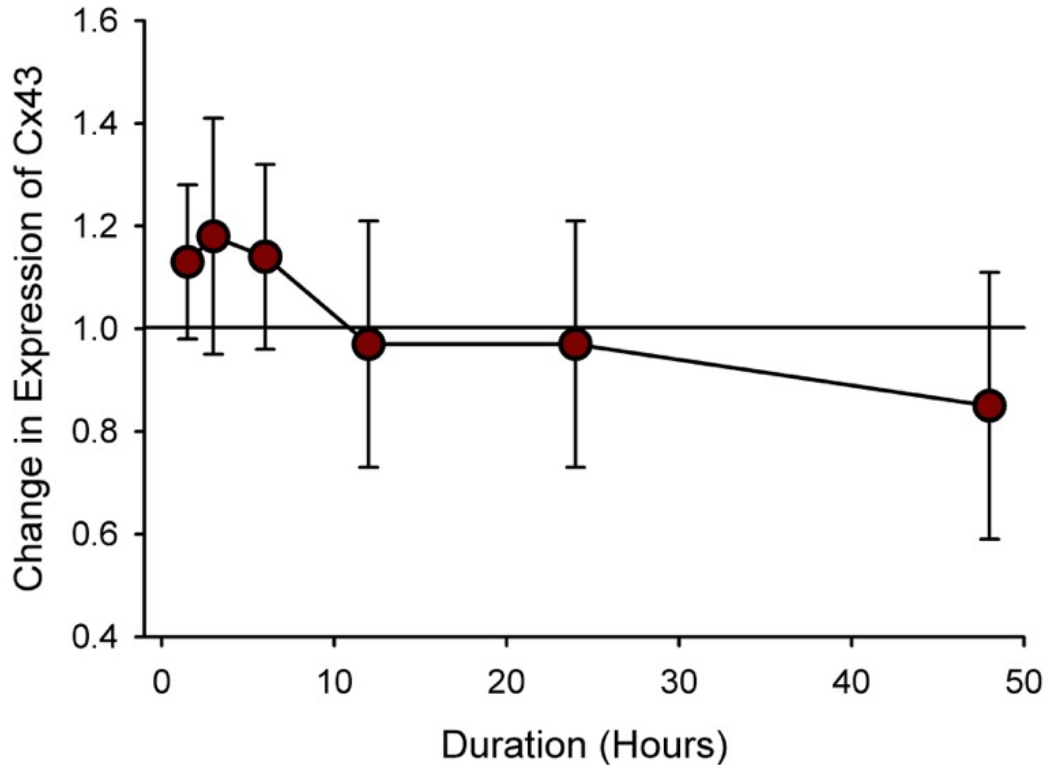
control values). The Bonferroni adjustment was employed to determine a family wide significance at the  $p < 0.05$ . At the same time, the change in control Cx43 expression was also evaluated by dividing control values at each time by the bEnd.3 cells never treated with EPA or ethanol (control at each time/control at time point 0). A single sample t-test (two tailed) was employed at each time to determine whether the average ratios were different from one ( $p < 0.05$ : a ratio different from 1 indicates a change in expression). The same single sample t-test was employed for samples treated with EPA, AH-7614 and EPA/AH-7614 (Cx43 signal was divided by the 48 hr time control). Data are repeated as mean  $\pm$  SEM.





**Figure 2: Cx43 expression of bEnd.3 cells treated with 30  $\mu$ M EPA.** Values are the ratio of Cx43 signal (treatment/time control) mean  $\pm$  SEM. Note the decrease in expression of Cx43 at 48 hours of EPA exposure. # - significantly different from 1.5 hour exposure ( $p \leq 0.05$ ,  $n=5$ ).

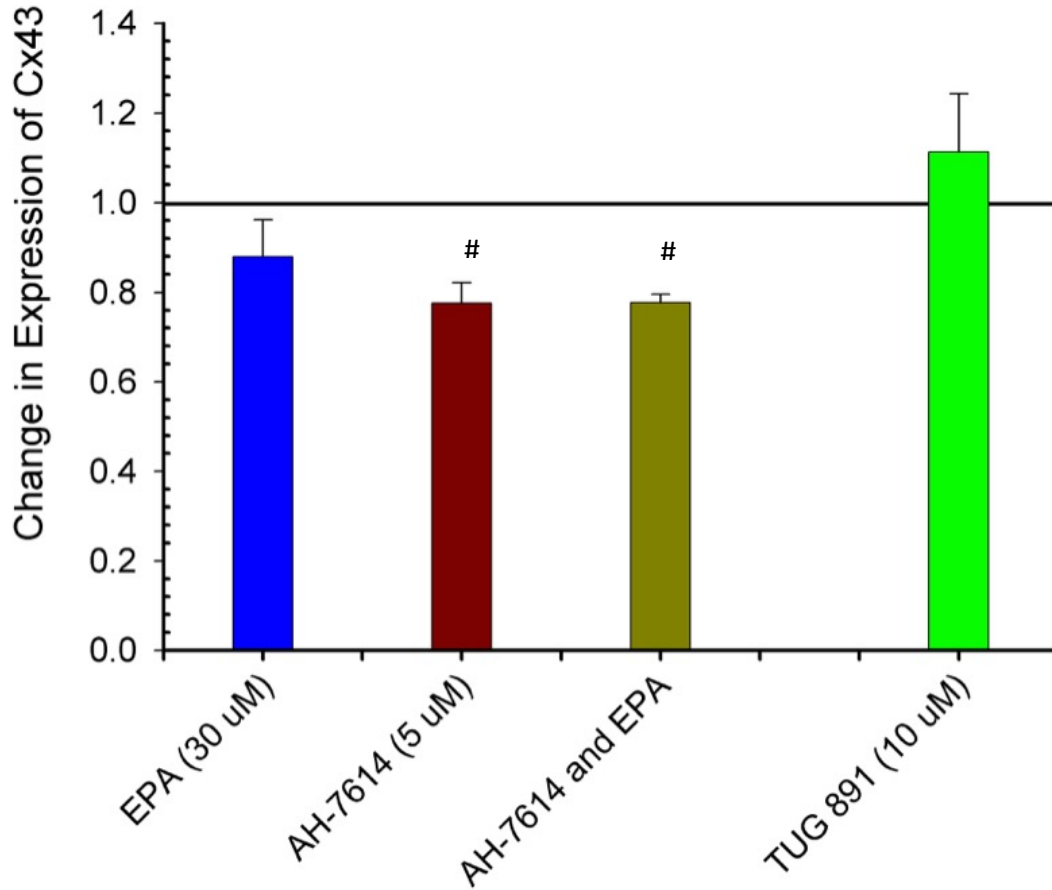
The representative data in Figure 1 also suggested Cx43 expression was altered in controls with a slight elevation up to 6 hours and a reduction at 24 and 48 hours (Control Cx43). When the data were summarized for all 5 experiments (Figure 3), there are no significant changes in Cx43 expression. However, there is a trend towards an increase in Cx43 expression up to 6 hours and a reduction at 48 hours. This reinforces the importance of the time controls and the normalization of Cx43 expression in EPA treated samples to the controls isolated at the same time.



**Figure 3: Cx43 expression of bEnd.3 cell time controls.** Values are the ratio of Cx43 signal in the controls (time control/time 0) mean  $\pm$  SEM. Note the trend in Cx43 expression change with exposure time: slight elevation (1.5 – 6 hours) to a reduction at 48 hours (n=5).

When the FFA-4 receptor antagonist (AH-7614: 5  $\mu$ M) was evaluated, 48 hour exposure to both EPA and FFA-4 receptor antagonist reduced Cx43 expression ( $p < 0.05$ : Figure 4). As this is similar to the outcome of Figure 1 and 2, it suggests the FFA-4 receptor antagonist had no impact on the EPA-induced reduction in Cx43 expression. However, the FFA-4 receptor antagonist alone decreased Cx43 expression ( $p < 0.05$ ) which suggests the 10% FBS HG-DMEM media contained enough fatty acids (or some other ligand for the receptor) to stimulate the FFA-4 receptor under resting conditions (before application of EPA). If inhibition of the FFA-4 decreases Cx43 expression, activation of the FFA-4 receptor in endothelial cells should elevate Cx43 expression. While not consistent with the original hypothesis, we decided to test this new

hypothesis by treating endothelial cells with an FFA-4 agonist (TUG-891: 10  $\mu$ M). After 48 hour exposure to the FFA-4 receptor agonist, Cx43 expression was not significantly altered. Interestingly, EPA alone did not significantly reduce Cx43 expression as was observed in earlier experiments (Figure 2).



**Figure 4: Impact of the FFA-4 receptor antagonist (AH 7614: 5  $\mu$ M, n=6) and agonist (TUG 891: 10  $\mu$ M, n=7) on Cx43 expression.** Values are the mean  $\pm$  SEM of the ratio of Cx43 signal (treatment/48 hour control). Concomitant treatment of bEnd.3 cells with EPA and an FFA-4 receptor antagonist decreased Cx43 expression ( $p < 0.05$ ). Interestingly, blocking the FFA-4 receptor alone also reduced Cx43 expression suggesting Cx43 expression is increased by stimulation of the FFA-4 receptor. When treated with an agonist (TUG 891), endothelial cell expression was slightly increased.

## **Chapter 5: Discussion and Conclusions**

These data show treatment of mouse endothelial cells with EPA for 48 hours decreased Cx43 expression. However, this reduction in Cx43 expression was not mediated by the FFA-4 receptor as originally hypothesized. More importantly, activation of this receptor may have the opposite effect of increasing Cx43 expression (decreasing endothelial cell health). Thus, while EPA has a positive effect on endothelial cells as evidenced by the decrease in Cx43 expression after 48 hours, that effect was not mediated through activation of the FFA-4 receptor. The remainder of this discussion will address some of the issues encountered in the experiments (and their interpretation) as well as proposing pathways that may be responsible for the EPA induced reduction in endothelial cell Cx43 expression.

One potential issue with the interpretation of the data is the lack of independent verification of equal loading of protein in each well of the gel (each sample included exactly 10 µg of protein). Therefore, small errors in pipetting during the BCA assay, preparing sample for the gel (pipetting and boiling) and loading them into the gel would not be evident in the final Cx43 signal. While we did not evaluate a constitutively expressed protein, Ponceau S was used to provide a general indication of equal loading for the data in Figure 4 (few differences were observed in the intensity of the Ponceau S stain between bands). Subsequent experiments should identify an endothelial cell protein that is constitutively expressed (not affected by inflammation or cell proliferation which are stimulated by EPA) to account for potential errors in protein loading.

The evidence from Figure 3 suggests Cx43 expression is decreased by confluency. This is consistent with other data recently collected in the lab (Pichette, 2018) and reinforces the importance of the experimental design that has a control for each time tested. However, while

cells were counted (800K in a 100 mm plate) for the first 3 time course experiments, the remaining 2 experiments were not similarly performed. Cells were equally plated into the 13 plates without an indication of the number of cells added to each plate. Thus, differences in cell density between control and EPA treated plates due to a lack of counting could have impacted the results. In addition, the point in time where those experiment were started (48 hours after plating) varied as we were more interested in having enough cells. Thus, the plates were likely more confluent when the experiments were started which could affect the outcome. In addition, EPA has been shown to stimulate cell proliferation. If that is the case in these endothelial cells, then the decrease in Cx43 expression in the EPA treated group could be due to the greater cell density after 48 hours (the more confluent cells would have a greater reduction in Cx43 expression but to differences in confluency). Thus, subsequent experiment should take into account the potential effect of cell density on Cx43 expression in their design.

The changes in expression of Cx43 observed across the 48 hours experiment (Figure 2: slight decrease early, slight increase at 12 hours and progressive decrease thereafter) suggests multiple pathways may be involved in endothelial cell responses to EPA. The rapid initial decrease in Cx43 expression is consistent with the action of a G-protein-coupled receptor. Thus, EPA may decrease Cx43 expression through the FFA-4 receptor, just at an earlier time than when we evaluated the impact of the FFA-4 receptor. After 24 hours, the decrease in Cx43 expression could be driven by prostaglandin synthesis. Fatty acids are the substrate for prostaglandin synthesis (Smith, DeWitt, & Garavito, 2000). The pro-inflammatory prostaglandins derive from the conversion of arachidonic acid in the cell to PGE2 by COX2 (the rate-limiting step; (Smith et al., 2000)). More importantly, PGE3 is produced by the conversion of EPA by COX2 (Hansen, 1983). Thus, EPA may provide the substrate for COX2, which is



expressed in bEnd.3 cells (C.-C. Lin et al., 2013; Syeda et al., 2006; Hsieh et al., 2008). This could increase the production of the anti-inflammatory PGE3, potentially resulting in a decrease in Cx43 expression.

In summary, the data argue the FFA-4 receptor is not responsible for the EPA-induced decrease in Cx43 expression. Whether the FFA-4 receptor is responsible for the Cx43 responses at other times along those 48 hours remains to be determined in future work. The presence of COX-2 enzymes in these cells presents a reasonable alternative to the FFA-4 receptor. Thus, increasing EPA appears to decrease the inflammatory response in the endothelium that may impact the initiation and/or growth of atherosclerotic plaques in these vessels.

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