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Does EPA Cause A Decrease in Inflammation of bEnd.3 Cells Through FFAR4?

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Does EPA Cause a Decrease in Inflammation of bEnd.3 Cells Through FFAR4?

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2 Abstract

Atherosclerosis is an inflammatory disease initiated by low and oscillatory shear stress on the endothelium. The inflammatory process recruits leukocytes to the vessel wall by expression of the adhesion molecule VCAM-1. Activation of the NF-κB inflammatory signaling pathway is responsible for the increase in VCM-1 expression. Omega 3 FAs, such as EPA, reduce the risk of atherosclerosis by decreasing this inflammatory response. The pathway by which omega 3 FAs is proposed to inhibit inflammation includes activating FFAR4 to decrease NF-κB activation thereby reducing expression of adhesion molecules. We hypothesized that treatment of endothelial cells with 30 μM EPA would decrease inflammation via activating FFAR4. We evaluated the changes in 2 signaling molecules (IκB and pIKK) and VCAM-1 expression in bEnd.3 cells. Endothelial cells were treated with 30 μM EPA for 24 hours and showed an early decrease in inflammation (0.5 hours; based upon inflammatory signaling molecules) followed by an increase in inflammation after 24 hours. To evaluate if FFAR4 was mediating this response, bEnd.3 cells were treated with 10 μM TUG-891. Treatment with the FFAR4 agonist decreased pIKK at 24 hours suggesting a decrease in the inflammatory response, however, VCAM-1 expression was not changed. The addition of 10 μM of the FFAR4 antagonist AH-7614, increased inflammation (1.5 hours) which was followed by a decrease (24 hours). This response mirrored that of EPA treatment suggesting that AH-7614 is acting as an agonist to FFAR4 (or is affecting inflammation through another pathway). Further experiments are required to understand the role of FFAR4 in mediating the anti-inflammatory response to EPA.

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5 List of Abbreviations

bEnd.3: Mouse Brain Endothelial

cAMP: Cyclic Adenosine Monophosphate

COX2: Cyclooxygenase 2

$cPLA_2$: Cytosolic Phospholipase A_2

Cx43: Connexin 43

DAG: Diacylglycerol

DHA: Docosahexaenoic Acid

DMSO: Dimethyl Sulfoxide

EPA: Eicosapentaenoic Acid

Erg-1: Early Growth Response Protein 1

ERK1/2: Extracellular Regulated Kinase 1/2

FAK: Focal Adhesion Kinase

FAs: Fatty Acids

FBS: Fetal Bovine Serum

FFAR4: Free Fatty Acid Receptor 4

GAPDH: Glyceraldehyde 3-Phosphate Dehydrogenase

GLP1: Glucagon-Like Peptide 1

GPCR: G-protein Coupled Receptors

G-proteins: Guanine Nucleotide-Binding Regulatory Proteins

GRP: G-protein Receptor

GRP120: G-protein Receptor 120

HG-DMEM: High Glucose Dulbecco's Minimal Essential Media

HRP: Horseradish Peroxidase

ICAM: Intracellular Adhesion Molecule

IKK: IκB Kinase

IL: Interleukin

IP_3: Inositol 1,4,5-Triphosphate

IκB: Inhibitor of NF-κB

JNK: c-Jun N-terminal kinase

LDLs: Low Density Lipoproteins

LPS: Lipopolysaccharide

MSR: Macrophage Scavenge Receptor

NFDM: Nonfat Dry Milk

NF-κB: Nuclear Factor-κB

Ox-LDLs: Oxidized Low Density Lipoproteins

PBS: Phosphate Buffered Saline

PD: Phosphate Buffered Saline – Calcium Deficient

PGE_2: Prostaglandin E2

pIKK: Phosphorylated IκB Kinase

PIP_2: Phosphatidylinositol 4,5-Bisphosphate

PLC: Phospholipase C

PVDF: Polyvinylidene Difluoride Membrane

SEM: Standard Error of the Mean

TAB1: TAK1 Binding Protein 1

TAK1: Transforming Growth Factor-β Activated Kinase 1

TBS: Tris Buffered Saline

TLR4: Toll-Like Receptor 4

TNF-α: Tumor Necrosis Factor-α

TTBS: Tween 20 in Tris Buffered Saline

TV: 0.25% Trypsin and 0.1% EDTA in PD

VCAM-1: Vascular Cell Adhesion Molecule-1

VLA4: Very Late Antigen 4

6 Introduction

6.1 Background

Atherosclerotic plaques affect people of all backgrounds. Initiation of atherosclerotic plaques is observed in areas of low or oscillatory shear stress on the endothelium such as curved arteries. Endothelial cells express mechanoreceptors which sense changes in shear stress and respond with phenotypic changes [1,2]. The low and oscillatory shear stress in these regions leads to an inflammatory response that increases the permeability of the endothelium to low density lipoproteins (LDLs) leading to the buildup and oxidation of LDLs in the subintimal layer of vessels [1,3–5]. During this phenotypic change, nuclear factor kappa B (NF-κB) in the cytoplasm is released from inhibitor of NF-κB (IκB) and translocates to the nucleus where it acts as a transcription factor [6–10]. NF-κB is activated once I kappa B Kinase (IKK) is phosphorylated, pIKK then phosphorylates I kappa B (IκB) which is subsequently degraded leaving NF-κB free to translocate to the nucleus [7,11,12]. Once NF-κB translocates to the nucleus, this transcription factor upregulates genes of inflammation like adhesion molecules [1,7,8,10,13]. This transcription factor becomes activated in the endothelial cells in regions experiencing low and oscillatory shear stress [1] and cells covering human plaques [14].

Activation of NF-κB upregulates inflammatory mediators like vascular cell adhesion molecule 1 (VCAM-1) [15,16]. VCAM-1 is a member of the immunoglobulin superfamily of cell adhesion molecules which includes intracellular adhesion molecule (ICAM)-1 and ICAM-2 [17]. VCAM-1 expression on endothelial cells recruits monocytes to the site of inflammation [15], via a three step process known as diapedesis [18–20]. First, monocytes are weakly bound by selectins which cause them to roll along endothelial cells until they contact adhesion molecules and

become tethered. Monocytes express vary late antigen 4 (VLA4), which binds to endothelial VCAM-1 molecules until the monocyte is firmly adhered. After firmly adhering, these monocytes enter the vessel wall and differentiate into macrophages.

These macrophages express scavenger receptors that bind to and uptake ox-LDLs [3,5,6,21]. As ox-LDLs increase within these macrophages, the likelihood of apoptosis increases [22]. These apoptotic cells release cytokines to attract other macrophages to begin clearing the apoptotic cells, a process known as efferocytosis [23]. Subsequently, the macrophages remove the macrophages that have undergone apoptosis, which reduces the size of the atherosclerotic plaque [23]. But as the plaque progresses, the phagocytic macrophages can become overwhelmed leaving behind foam cells that die and accumulate in a necrotic core [22,23].

The production of cytokines is not limited to cells that have undergone apoptosis; foam cells produce cellular signals which attract smooth muscle cells, by directing their migration into the subintima [22]. These previously quiescent smooth muscle cells migrate into the intima and change to a proliferative phenotype [24]. The smooth muscle cells secrete growth factors, like platelet derived growth factor, which stimulates other smooth muscle cells to proliferate and the vessel intima begins to grow inward forming a subintimal layer, enlarging the atherosclerotic plaque [3,6]. The proliferative smooth muscle cells also secrete a collagenous extracellular matrix leading to a fibrous cap surrounding the atherosclerotic plaque [24].

Limiting plaque formation and progression has become a significant focus for many people today through manipulation of their diet. A diet that includes a large amount of cholesterol, which can be a source of LDLs [25], accelerates the development of atherosclerotic plaques [26]. Cholesterol is not the only lipid in our diet. Most of the lipids we consume are triglycerides [27]. According to the American Heart Association, elevated intake of triglycerides is

associated with an increased risk of cardiovascular disease [28,29]. As atherosclerosis is a leading cause of cardiovascular disease and mortality in developed countries, reducing lipid consumption is the goal of a healthy diet [30,31]. Thankfully, not all lipids increase risk of atherosclerosis. The triglycerides in our diet are composed of three fatty acids (FAs) bound to glycerol. The FAs with long carbon chains, based on the orientation of the bonds between the carbons in the chain, determine whether FA will elevate or reduce the inflammatory response. Saturated FAs, which contain no carbon-carbon double bonds, stimulate an inflammatory response via activation of NF-κB [32]. Unsaturated FAs contain at least one carbon-carbon double bond and exist in either a *trans*- (hydrogen atoms on adjacent sides of the double bond) or *cis*- (hydrogen atoms on the same side of the double bond) form. Ingestion of *trans*-FAs increases VCAM-1, a marker of inflammation [33]. FAs in the *cis*- conformation, specifically the omega three FAs, are anti-inflammatory [6,9,34–38].

While omega three FAs, like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) can be synthesized from essential FA, α-linoleic acid, a majority of the circulating omega three FAs are ingested [39]. In human trials, combined EPA and DHA treatment decreased circulating proinflammatory cytokines [40]. Furthermore, EPA treatment decreased the expression of VCAM-1 in human umbilical vein endothelial cells [41]. These anti-inflammatory effects are proposed to involve the binding of omega three FAs to free fatty acid receptor four (FFAR4) [9,42]. FFAR4, formerly known as G-protein receptor 120 (GRP120), has an affinity for long chain omega three FAs like EPA and DHA [43]. This receptor was suggested to reduce inflammatory signals by inhibiting translocation of NF-κB to the nucleus [38]. The anti-inflammatory effects of omega three FAs were demonstrated in cells such as Caco-2 [44,45], RAW264.7 [37,46] and EA.hy926

[9], but only one of these is an endothelial cell line (EA.hy926). Even fewer studies have evaluated the inflammatory response in mouse brain endothelial (bEnd.3) cells.

These bEnd.3 cells appear to retain some normal endothelial cell functions [47]. Earlier work from Böggemeyer and colleagues [48] showed VCAM-1 expression was upregulated in bEnd.3 cells by spirochete infection. More recently, Wu and colleagues [49] showed EPA treatment rapidly increased intracellular calcium in bEnd.3 cells. As the bEnd.3 cells express FFAR4, respond to EPA and express the inflammatory mediator VCAM-1, we investigated the role of FFAR4 in mediating the anti-inflammatory effect (or the reduction of inflammatory response) of EPA on endothelial cells. In particular, we evaluated the impact of EPA on the inflammatory response by quantifying changes in signaling molecules pIKK and IκB and the expression of VCAM-1. This will provide a better understanding of how omega three FAs can reduce inflammation, and potentially stabilize atherosclerotic plaques.

6.2 Purpose

Atherosclerosis is a disease that affects many people worldwide. The hallmark of this disease is atherosclerotic plaques which develop in regions of blood vessels subjected to low or oscillatory shear stress, like branchpoints and curvatures. Mechanoreceptors in the endothelial cells lining the vessel lumen detect changes in shear stress leading to phenotypic changes [1,2]. Not only do these phenotypic changes increase permeability to LDLs, they also change connexin expression in these areas of low or oscillatory shear stress [50]. There are three gap junction proteins present in endothelial cells: connexin 37, 40, and 43 [50,51]. Furthermore, connexin 43 (Cx43) expression was elevated in the endothelium at branchpoints and areas experiencing low or oscillatory shear stress [50] where inflammatory responses are occurring. Due to this finding, previous work in this laboratory used Cx43 as a marker of inflammation in order to evaluate the

effect of EPA on bEnd.3 cells [52]. Although treatment with EPA decreased Cx43 expression at 48 hours (consistent with a decrease in inflammation), blockage of FFAR4 with AH-7614 (10 μM) decreased Cx43 expression. This was an interesting finding as FFAR4 was proposed to block the activation of NF-κB [38], which means inhibition of FFAR4 should have increased Cx43 expression [53]. In order to better understand the role of FFAR4 in the anti-inflammatory response to EPA, we evaluated the NF-κB mediated inflammatory response in endothelial cells and how it is affected by FFAR4. In order to evaluate the effects of FFAR4 on the inflammatory response in bEnd.3 cells following treatment with EPA, we assessed EPA/FFAR4-mediated changes in NF-κB signaling molecules pIKK and IκB along with inflammatory mediator VCAM-1 (a protein directly related to the recruitment of leukocytes to the site of inflammation by endothelial cells).

6.3 Scope

Atherosclerosis describes the development of plaques that affects people worldwide. Understanding the processes leading to plaque development is important in developing treatment for those afflicted, as well as strategies to slow plaque development (and stabilization). To do this, one must investigate the endothelial cells, whose damage leads to atherosclerosis. Omega three FAs have been shown to have anti-inflammatory effects and this study aims to determine if FFAR4 is involved in the omega three FAs initiated anti-inflammatory effects in bEnd.3 cells.

6.4 Assumptions

1. bEnd.3 cells are a model of endothelial cell function that allows the study of the inflammation using *in vitro* techniques in order to understand the *in vivo* response.
2. The inflammatory response of the bEnd.3 cells is similar to normal *in vivo* endothelial cells.

3. bEnd.3 cells are generating an inflammatory response during resting conditions (as they are stressed) which can be attenuated upon treatment with anti-inflammatories like EPA.

6.5 Hypothesis

Treatment of cultured endothelial cells (bEnd.3) with 30 μM EPA will decrease the inflammatory response via FFAR4. The involvement of FFAR4 will be evaluated with the FFAR4 agonist (TUG-891) which is expected to decrease inflammation as evidenced by an increase in IκB, and a decreased in pIKK and VCAM-1. This response is expected because a decrease in pIKK will mean less degradation of IκB. If less IκB is degraded, the extent of NF-κB activation should decrease which would decrease VCAM-1 expression. The involvement of FFAR4 in the EPA response will be evaluated with a FFAR4 antagonist (AH-7614). Cells were treated with EPA and AH-7614 at the same time to solidify the involvement of FFAR4. If FFAR4 is involved, the antagonist is expected to decrease IκB and increase pIKK and VCAM-1 (i.e., an increased inflammatory response should be observed when the action of EPA on FFAR4 is reduced).

6.6 Significance

Atherosclerosis is a problem affecting many families throughout the world. This disease is caused by a local inflammatory response in the vessel wall that is initiated within the endothelium. The inflammatory response is evaluated by monitoring NF-κB via signaling molecules IκB and pIKK which are decreased and increased, respectively, by inflammation. Once activated, NF-κB increases the transcription of inflammatory genes, like VCAM-1. The anti-inflammatory effects of omega three FAs on endothelial cells involved a decrease in translocation of NF-κB to the nucleus as well as a decreased expression of VCAM-1. Because

bEnd.3 cells mirror the function of *in vivo* endothelial cells in this experiment, the information gained by studying the anti-inflammatory effects of omega three FAs can be applied to endothelial cells. This could then lead to a better understanding of how to treat or even prevent the inflammatory disease atherosclerosis.

7 Review of Literature

7.1 The Formation of Atherosclerotic Plaques

Atherosclerosis is a disease that does not discriminate by race, gender, or even age. The prevalence of atherosclerosis is on the rise [54]. Atherosclerotic plaques consist of a fibrous cap composed of collagen secreted by smooth muscle cells, a necrotic core formed by the death of macrophages, and lipid laden foam cells (figure 1) [3,6,55]. As these plaques enlarge, they impinge on the vessel lumen thereby reducing blood flow to the tissue downstream of the plaque [56]. Not only do plaques lead to a decrease in blood flow to areas of the body, like the muscle of the heart, but they can rupture, leading to thrombosis [55]. Thrombosis typically occurs when the fibrous cap bursts and blood in the vessels comes into contact with the lipid-rich core of the plaque [55].

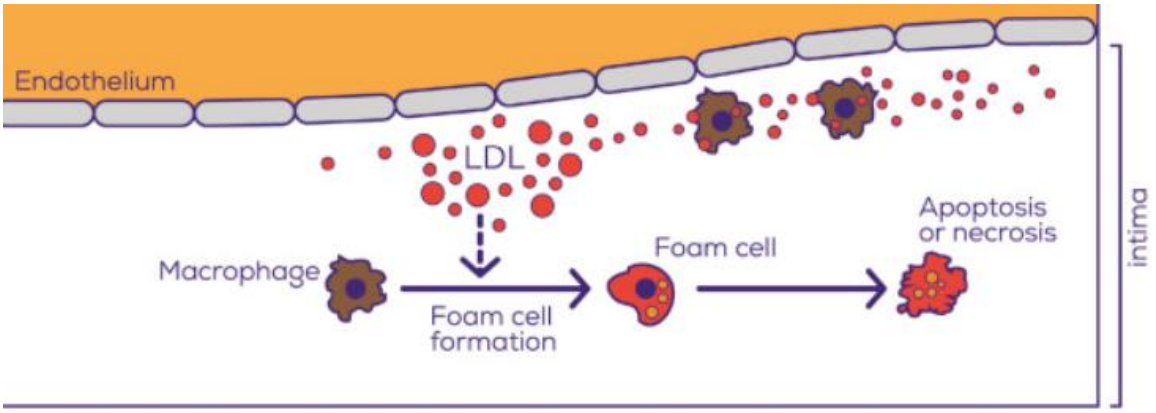

Figure 1: The Progression of Atherosclerotic Plaques

Atherosclerotic plaques begin with the collection of low-density lipoproteins (LDLs) within the intima of the vessels. This leads to the recruitment of macrophages which engulf these foreign materials. Once filled with lipids, these macrophages can form foam cells and lead to the progression of a necrotic core [57].

The risk of forming these atherosclerotic plaques is increased by a diet high in cholesterol [26]. Diets high in cholesterol can lead to atherosclerosis in people of all ages including a fetus of a hypercholesterolemic mother [4]. The accumulation of LDLs in the subendothelial region of the vessel wall results from a high cholesterol diet [4,58]. These LDLs accumulate in arteries at regions experiencing low or oscillating shear stress including downstream of branch points and curved arteries [2]. In these areas, the endothelial cells express mechanoreceptors which mediate phenotypic changes in response to low and oscillatory shear stress, leading to endothelial cell injury causing an increase in permeability of the endothelium to LDLs [1,2]. This allows LDLs to accumulate in the subintima of the vessel, where they are oxidized [1,3–5]. This accumulation of ox-LDLs is accompanied by an inflammatory response which leads to the recruitment of monocytes that differentiate into macrophages [59,60].

The uptake of ox-LDLs by macrophages that was first demonstrated by Goldstein and colleagues [61] is mediated by macrophage scavenger receptors (MSR) [21]. Early in the progression of atherosclerosis, MSRs mediate macrophage uptake of ox-LDL, as well as removal of debris from the destruction of apoptotic cells [62] which is known as efferocytosis [23]. The formation of the necrotic core depends on the efficiency of efferocytosis, the balance between removing cellular materials and accumulating foam cells which become necrotic. The process of efferocytosis effectively removes apoptotic cells early in the process. However, as the plaque progresses, the macrophages are overwhelmed and become foam cells that lead to the progression of the necrotic core of the atherosclerotic plaque [22,23].

Macrophages that have accumulated ox-LDLs secrete cytokines [62,63] and growth factors [22,64] which stimulate smooth muscle cells to migrate towards the intima and proliferate. The chemical signals released will drive the conversion of smooth muscle cells from a contractile to a synthetic phenotype [24,65]. These synthetic smooth muscle cells produce an excess of extracellular matrix proteins (collagen) leading to the fibrous cap covering the atherosclerotic plaque [22,24]. The fibrous cap is important as it stabilizes the plaque, preventing it from rupturing; a thin fibrous cap puts the plaque at risk of rupturing [22,55]. Thin fibrous caps are not the only reason a plaque ruptures as the enlargement of the necrotic core can also cause the plaque to rupture [55]. If left untreated, these atherosclerotic plaques can continue to grow (occluding blood flow) and rupture which releases the plaque to travel downstream and occlude blood flow. Even worse, the ulceration in the vessel left after the plaque ruptures is a prime location for the formation of a thrombus that can grow very quickly and occlude the vessel [55].

7.2 Atherosclerosis: An Inflammatory Disease

The recruitment of macrophages following endothelial cell injury, leading to the formation of atherosclerotic plaques, is mediated by an increased expression of adhesion molecules VCAM-1 and ICAM-1 [18,66]. VCAM-1 and ICAM-1 play a role in recruiting immune cells to the site of inflammation and have been observed in the endothelium overlying atherosclerotic plaques [18,67]. While ICAM-1 is constitutively expressed on the surface of endothelial cells, VCAM-1 is mostly inducible [67]. Induction of VCAM-1 is initiated by the presence of ox-LDLs, suggesting a transcriptional response to increased ox-LDL concentration [16,68]. These ox-LDLs then induced the activation of the non-receptor tyrosine kinase focal adhesion kinase (FAK), which in turn activates NF-κB [16]. Inhibition of FAK decreases the binding of NF-κB to its promoter sequence, which reduced VCAM-1 expression [69]. More importantly, VCAM-1 plays a larger role in the progression of atherosclerosis as partial disruption of the molecule caused a significant decrease in the progression of atherosclerotic plaques in mice fed a cholesterol rich diet [15]. Induction of VCAM-1 is mediated by a number of pathways. Inflammation induced translocation of NF-κB increases the expression of VCAM-1 [16].

Due to its role in the inflammatory process, VCAM-1 is an important molecule in the development and progression of atherosclerotic plaques. This inflammatory process is initiated by recruiting monocytes, which occurs in a three step process known as diapedesis [20]. The monocytes contact the endothelial cells and roll across them allowing for contact with the monocyte expressed receptor VLA4, which mediates the firm adhesion to VCAM-1 on the endothelial cells [70]. Then monocytes undergo transendothelial migration [20], which occurs predominately via paracellular migration [71]. Once they enter the vessel wall, monocytes differentiate into macrophages [72].

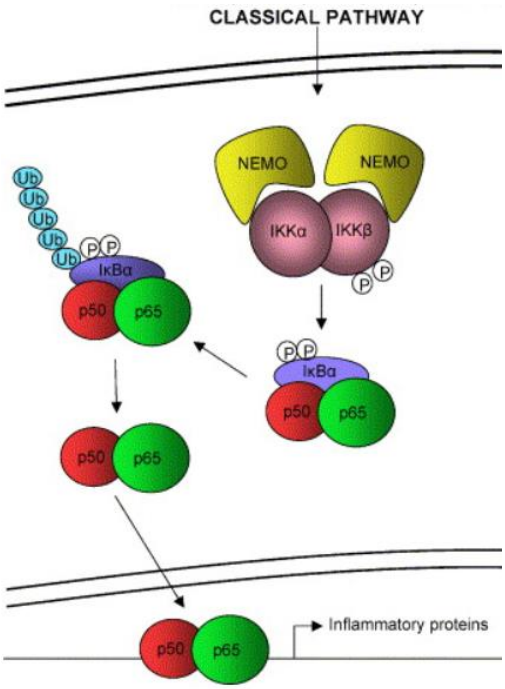

Figure 2: Classical NF-κB Pathway

Shown here is the phosphorylation of IκB by pIKK and the subsequent polyubiquitination. This leads to IκB degradation, freeing NF-κB to bind to the promotor sequence of inflammatory genes [73].

VCAM-1 expression can be induced via the transcription factor NF-κB. There are five members of the NF-κB family: RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) [12]. These family members are characterized by a conserved 300-amino acid sequence known as the Rel homology domain at the amino terminus which is involved in the dimerization process, the interaction with inhibitory proteins, and the binding to DNA [12,74]. All of these NF-κB proteins exist in the cytoplasm of unstimulated cells as homodimers and heterodimers bound to inhibitory proteins [12]. Inhibitory proteins, like IκB, stop the translocation of NF-κB into the nucleus. Inflammatory cytokines activate IKK by phosphorylation (now pIKK) which will phosphorylate IκB on two N-terminal serine residues. This phosphorylation of IκB leads to its polyubiquitylation and degradation, freeing NF-κB to translocate to the nucleus and bind to specific DNA promoter sequences of target genes (figure 2) [12]. Of the NF-κB family members mentioned earlier, the most important heterodimer in the inflammatory process is p50 and p65

[7,10]. Specifically, the p65 subunit is upregulated by c-Jun N-terminal kinase (JNK) which is activated in areas more prone to atherosclerosis [7].

Under conditions where the inflammatory response is not active, NF-κB is bound to IκB. There are seven IκB family members, IκBα, IκBβ, BCL-3, IκBε, IκBγ, and two precursor proteins p100 and p105 [12]. These proteins have five to seven ankyrin repeats that form cylinders which bind the dimerization domain of NF-κB dimers [75]. IκBα and IκBβ bind to the p50/p65 dimers masking the nuclear localization sequence, which keeps NF-κB in the cytoplasm [76,77]. IκB degradation is a tightly regulated process in order to keep the cellular activity functioning properly. Degradation is mediated by IKK, a complex of two kinase subunits, IKKα and IKKβ, and a regulatory subunit NF-κB essential modifier (NEMO) [12]. Upon phosphorylation of IKK, the IKKβ subunit phosphorylates IκBα at Ser32 and Ser36 as well as IκBβ at Ser19 and Ser23. Once phosphorylated, IκBs are ubiquitinated by the Skp1-Culin-Roc1/Rbx1/Hrt-1-F-box family of ubiquitin ligases which leads to proteasomal degradation [11]. Following degradation of IκB, NF-κB is released and binds to promoter sequences containing κB sites [12].

7.3 Fatty Acids and How They Effect Atherosclerosis

Lipids are involved in three general functions: formation of cellular membranes, energy sources, and signaling molecules. These polar lipids contain a hydrophobic tail and a hydrophilic head and make up cellular membranes. It is this amphipathic characteristic which allows cells create organelles. Cell membranes do not just provide a barrier between the environment and the inside of the cell, but they contain proteins required for processes within the cell. These energy dense molecules also function as an energy storage substrate in the form of triglycerides [27,78]. Triglycerides are broken down into three FAs and a glycerol molecule by lipolysis. The long chains

of carbon molecules contained in the FA tails can be cleaved by beta oxidation to acetyl CoA in the mitochondria and enter the citric acid cycle.

In terms of structure, FAs with no carbon-carbon double bonds are saturated whereas the unsaturated FAs contain at least one carbon-carbon double bond. The double bonds in these unsaturated FAs are in the *cis*- (hydrogens on the same side of the double bond leading to a kinking of the molecule) or *trans*- (hydrogens on opposite sides of the double bond leading to a straight chain) configuration. FAs are also described by the length of the carbon chain: short chain FAs (six and fewer carbons), medium chain FAs (six-twelve carbons), and the long chain FAs (greater than twelve carbons). The effects of FAs are dependent on the length of the carbon chain as short chain [79] and medium chain [80] FAs have proinflammatory effects, while long chain FAs have both proinflammatory [81] and anti-inflammatory effects [9,36,37,41,45,46]. Because of the anti-inflammatory effects of the long chain FAs, the term FAs in this review refers to long chain FAs.

The human body can synthesize most of the fats it requires to function, although there are some essential FAs that must be absorbed through the diet. These essential FAs are long chain FAs derived from linoleic acid and α-linoleic acid, omega six and omega three FAs respectively. These are considered essential FAs because the human body does not have a mechanism to insert a double bond into a FA nine carbons from the delta carbon end [82]. The type of unsaturated FA (omega six and omega three) is defined by the position of the first double bond [35]. Although humans cannot synthesize α-linoleic acid, we can synthesize EPA and DHA from the α-linoleic acid [43]. The efficiency of converting α-linoleic acid to EPA and DHA is very low. Thus, a majority of the EPA and DHA in the body is from intake of those FAs from wild caught oceanic fish.

Humans are told to ingest essential FAs because they have many health benefits. Although the omega six FAs are proinflammatory [81], omega three FAs get the most attention for their anti-

inflammatory effects [9,9,36,37,45,46]. Treating endothelial cells with DHA reduced leukocyte adhesion by decreasing expression of ICAM-1 [9]. Work on cultured macrophages determined that DHA inhibited the translocation of NF-κB to the nucleus [9] and suppressed inflammasome production [38]. Similarly, Tanaka and colleagues [41] showed EPA had anti-inflammatory effects in human umbilical vein endothelial cells as evidenced by a reduction in expression of VCAM-1. Recent work has suggested the anti-inflammatory effects of EPA are mediated via FFAR4.

7.4 Free Fatty Acid Receptor Four (FFAR4)

The recent discovery that the G-protein receptor (GRP)120 receptor, previously an orphan receptor, has an affinity for long chain omega three FAs has suggested another mechanism that might mediate the anti-inflammatory effects of omega three FAs [43]. G-protein coupled receptors (GPCRs) have seven transmembrane domains and relay physiological signals through guanine nucleotide-binding regulatory proteins (G-proteins) [83]. These G-proteins have three subunits, an α, β, and γ subunit and relay signals via second messengers. Upon stimulation of the GPCR, the Gα subunit dissociates leaving the Gβγ dimer, resulting in two functional subunits that can activate various signaling pathways, although, G-proteins are typically identified by the function of the α-subunit [83]. Of these α-subunits, $G\alpha_s$ stimulates adenylyl cyclase which increases cyclic adenosine monophosphate (cAMP) while $G\alpha_i$ inhibits adenylyl cyclase thereby decreasing cAMP [43]. $G\alpha_q$ activates phospholipase C (PLC) which promotes the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) and leads to an increase in intracellular calcium from the endoplasmic reticulum [43].

Although GPCRs have different effects based on the associated G-protein, the activation of GPCRs is specific to its ligand. There are four GPCRs that have been linked to FAs, GPR40, GRP41, GPR43, and GPR120 [43]. GPR43 and GPR41, known as FFAR2 and FFAR3, respectively,

are both activated by short chain FAs [84] and are observed in colonic L cells to mediate the secretion of glucagon-like peptide 1 (GLP1) [85]. GPR40, also known as FFAR1, binds to long chain FAs and is expressed in pancreatic beta cells where it stimulates the secretion of insulin [86]. The second receptor for long chain FAs is GRP120, or FFAR4, and was deorphanized in 2005 by Hirasawa and colleagues [87]. This receptor was stimulated by FAs causing an increase in $G\alpha_q$ signaling [87].

FFAR4 can mediate a multitude of responses based on the cell type. Activation of FFAR4 increases the release of GLP1 from enteroendocrine cells within the mouse colon [87]. Stimulation of FFAR4 also increases the release of insulin following glucose administration, meaning that this receptor could have pharmacological implications beyond inflammation [88]. This receptor was implicated in brown adipose tissue function due to elevated mRNA [89]. Activation of FFAR4 increases the thermogenic gene activation and browns adipose tissue suggesting this receptor stimulates thermogenesis [89]. As evidenced from the information above, FFAR4 has been proposed to be involved in a number of important physiological functions.

Although FFAR4 associates with $G\alpha_q$, there are multiple proposed pathways by which this receptor might exert its anti-inflammatory effects. Oh and colleagues [37], showed the omega three induced anti-inflammatory effects of FFAR4 resulted from inhibition of IKKβ (less degradation of IκB) and JNK [37]. These effects were prevented by knocking down FFAR4. Stimulation of FFAR4 results in β-arrestin2 mediating receptor internalization which binds to TAK1 binding protein 1 (TAB1). Interestingly, toll like receptor (TLR) activation with lipopolysaccharide (LPS) stimulates inflammation which causes phosphorylation of transforming growth factor-β activated kinase 1 (TAK1) upstream of JNK and IKKβ, leading to their activation. So, the binding of the FFAR4/β-arrestin-2 complex to TAB1 inhibits the binding of TAB1 to TAK1, thus inhibiting TAK1 activation and reducing the inflammatory response. Upon treatment with DHA, the

activation of TAK1 decreases suggesting DHA inhibits the interaction of TAK1/TAB1 with TAB2 [9]. Upon stimulation with both GW9508 and TUG-891 (agonists for FFAR4), the interaction of the TAK1/TAB1 complex with TAB2 was attenuated. The binding of TAB2 to TAB1/TAK1 activates the complex, which phosphorylates extracellular regulated kinase 1/2 (ERK1/2). ERK1/2 stimulates the translocation of NF-κB to the nucleus. Once NF-κB enters the nucleus, it binds to the promoter sequence of early growth response protein (Egr-1) causing an upregulation of Egr-1, which upregulates ICAM-1 expression. This suggests FFAR4 disrupts the binding of TAB2 to TAK1/TAB1.

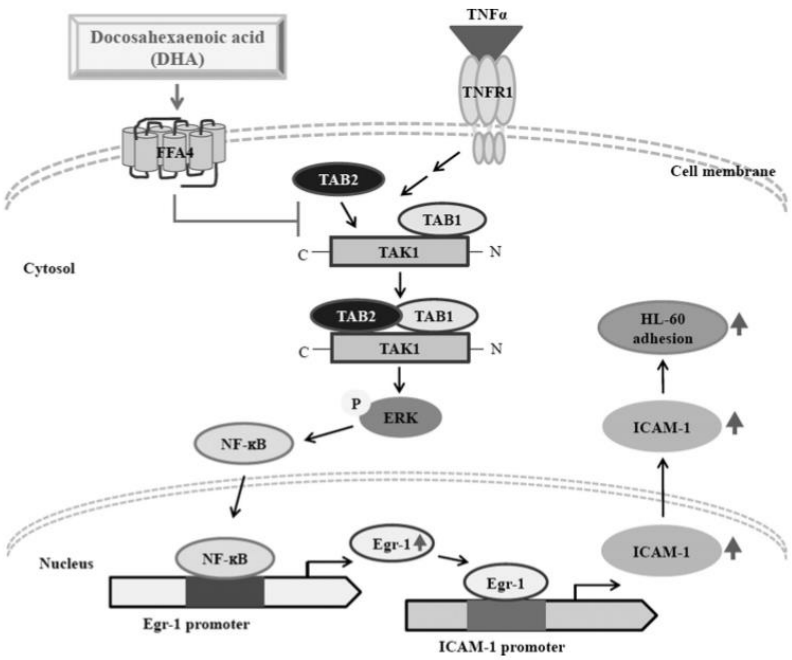

Figure 3: Proposed Mechanism of FFAR4 Anti-Inflammatory Effects

Shown here is the mechanism occurring following activation of FFAR4 with DHA attenuating the TNF-α induced inflammatory response. TAB2 interaction with the TAB1/TAK1 complex is inhibited by FFAR4. This inhibits the downstream effects which decreases the inflammatory response. ERK phosphorylation is inhibited causing a decrease in the activation of NF-κB. The expression of Egr-1 would then be decreased, leading to a decreased expression of ICAM-1. This would then decrease cell adhesion [9].

8 Methodology

8.1 Cell Culture

Mouse brain endothelial (bEnd.3; ATCC) cells were grown in 100 mm cell culture plates using high glucose Dulbecco's minimal essential media (HG-DMEM; Thermo Scientific D6429) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals) to drive proliferation. Cells were placed in a 37°C incubator at 5% carbon dioxide and 100% humidity. These cells were regularly lifted from the plate and split between plates to grow enough cells to start an experiment. In the laminar flow hood, the media was removed and replaced with 10 mL of phosphate buffered saline – calcium deficient (PD; in mM: 137 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 Na_2HPO_4). PD was then removed and replaced with 0.25% Trypsin and 0.1% EDTA in PD (TV; Thermo Scientific: 2520056). TV was removed and the plate was placed into the 37°C incubator for ~4 minutes, or until the cells lifted. Once lifted, cells were either plated at a lower density on a plate to grow more or combined and counted with a hemocytometer in order to plate 800,000 cells per plate to begin an experiment.

8.2 Endothelial Response to Eicosapentaenoic Acid (EPA)

An EPA stock solution of 30 mM was prepared by adding EPA (NU Chek Prep: U-99A-M1-D) to 100% ethanol. The stock solution was added to 10% FBS HG-DMEM media (1:1000 dilution) to make a 30 μM EPA solution. A second vehicle control media (0.1% ethanol solution) was generated by a 1:1000 dilution of 100% ethanol into 10% FBS HG-DMEM. The media was removed from the 12 confluent bEnd.3 plates and replaced in 6 of the plates with the 30 μM media. The 0.1% ethanol solution was added to another 6 bEnd.3 cell plates.

8.3 Protein Isolation

After the 12 plates were prepared, protein was isolated from the 13th plate to serve as the untreated control. The plate of bEnd.3 cells was placed on ice and washed 3 times with cold phosphate buffered saline (PBS; in nM: 137 NaCl, 2.7 KCl, 1.8 KH_2PO_4, 10 Na_2HPO_4, 6.5 NaH_2PO_4, 1 $CaCl_2$, and 0.5 $CaCl_2$). After the final rinse of PBS, cells were scraped (Fisherbrand: Cat #08-100-241) and transferred via pipet into a labeled 1.5 mL centrifuge tube. These cells were then centrifuged for 5 minutes at 7,500 RPM at 4°C (Eppendorf Centrifuge 5424 R). After centrifuging the cells, the supernatant was removed, leaving only the cell pellet. Then 35 μL 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 to the pellet, which was then sonicated to break the cell membranes (Misonix XL-2000 Series). After sonication, the cells were spun for 10 minutes at 15,000 RPM at 4°C. The sample was stored in a freezer at -20°C. The technique outlined above was repeated at 0.5, 1.5, 3, 6, 12, and 24 hours on a 0.1% ethanol control and a 30 μM EPA treated plate.

8.4 Protein Preparation

After isolating protein from all 13 plates, the protein concentration of each sample was determined using the BCA assay (Thermo Scientific: 23225) following the manufacturer's directions. To prepare each sample to run in a gel at 25 μg per well, an amount of sample protein was diluted to 4 μg/μL and β-mercaptoethanol (Bio-Rad CAT #161-0710; 10% of sample volume) was added. Samples were heated to 70°C for 10 minutes (Fisher Scientific: Dry Bath Incubator) and stored at -20°C.

8.5 Western Blotting

8.5.1 Gel Electrophoresis

Running buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS, pH 8.3) was prepared from a 20X stock solution. Na Bisulfate (antioxidant) was added to the running buffer for a 5 mM solution. An SDS-polyacrylamide gel (Sigma: 4-20% gradient PCG2016) was placed in a gel electrophoresis cassette and running buffer with the antioxidant was added to the cassette. Equal volumes of sample (4 μg/mL) were pipetted into each well [a pre-stained, low molecular weight ladder was added to an additional well (Thermo Scientific: PI 26616)]. Running buffer was then added to the tank and the gel was run at 200 volts (170 mA: E-C Apparatus Corporation: EC570-90) for 30-45 minutes to separate the protein by molecular weight.

8.5.2 Protein Transfer

Transfer buffer (25 mM Tris base, 192 mM glycine, and 20% methanol) was prepared from a 20X stock solution. Polyvinylidene difluoride membrane (PVDF; Advansta: L-08008-001) was activated in menthol (Sigma: M3641), and the filter paper (Whatman) trimmed to fit the transfer apparatus. A sandwich with a mesh, 1 piece filter paper, gel containing the protein, PVDF, another piece of filter paper, and another piece of mesh was prepared carefully, ensuring no air bubbles. The sandwich was then placed into the transfer tank, which was placed in a cold-water bath. Protein was transferred at 100 volts (170mA) for 4 hours. Once complete, the membrane was removed from the sandwich and placed in a box (protein side upward) and immersed in Tween 20 in Tris buffered saline (TTBS; 0.5% Tween 20, 500 mM Tris Base/Tris-HCl, 150 mM NaCl, pH 7.6) and stored at 4°C until probed.

8.5.3 Probing

Each membrane was probed individually with antibodies for IκB (Cell Signaling: 4812S), pIKK (Cell Signaling: 2697S), and VCAM-1 (Cell Signaling: 32653S). An antibody (Ab) was diluted

(1:1000) in 1% nonfat dry milk (NFDM: Bio-Rad 170-6404)-TTBS to generate a primary Ab solution to probe for a protein. A secondary biotinylated goat anti-rabbit Ab (Invitrogen: 656140) was diluted to 1:2000 in 1% NFDM-TTBS to generate a secondary Ab solution to probe for the primary Ab. Finally, the streptavidin horseradish peroxidase (HRP; Thermo Scientific: 21130) was diluted 1:2000 in 1% NFDM-TTBS to generate the HRP solution to detect the secondary Ab.

After removing the TTBS a 5% NFDM-TTBS blocking solution was added to the box with the membrane. The box was rocked (Boekel Scientific: Rocker II model 260350) at room temperature for 2 hours. The membrane was washed with TTBS (3 washes at 5 minutes each) before the primary Ab solution was added. After 2 hours, the membrane was washed with TTBS (3 washes at 5 minutes each) before the secondary Ab solution was added. After 2 hours, the membrane was washed with TTBS (3 washes at 5 minutes each) and the HRP solution was then added. After 2 hours, the membrane was washed with TTBS (2 washes at 5 minutes each) followed by a single 10 minute wash with tris buffered saline (TBS; 500 mM Tris Base/Tris-HCl, 150 mM NaCl, pH 7.6) and stored at 4°C until imaged.

8.6 Quantifying

Chemiluminescence was used to image the HRP by treating the membrane with equal volumes of WesternBright ECL (Advansta: R-03031-D25) and Peroxide (Advansta: R-03025-D25). The amount of light generated by the chemiluminescence was captured with the UVP EC3 Imaging System. To quantify the intensity of signal, ImageJ software was used. For each Ab (IκB, pIKK, and VCAM-1), the intensity of light was measured for the EPA treated sample and 0.1% ethanol control at each time point (background subtraction). Thereafter, the treated sample was

normalized to the control sample isolated at the same time to determine if EPA altered protein expression.

8.7 Impact of Stimulating FFAR4 (TUG-891): IκB, pIKK, and VCAM-1 Expression

The role of FFAR4 was evaluated by treating bEnd.3 cell plates with a FFAR4 agonist (TUG-891; Tocris Cat #4601). A 10 mM stock solution of TUG-891 was generated in 100% ethanol. TUG-891 (10 μM) and 0.1% ethanol solutions of media were generated as described above for EPA. Seven confluent plates were used; 3 plates were treated with 10 μM TUG-891 and 3 plates treated with 0.1% ethanol and 1 plate was used as an untreated control. Protein was isolated from 1 untreated plate after the cell treatment began. Thereafter, protein was isolated at 0.5, 1.5, and 24 hours from an ethanol control plate and a TUG-891 treated plate.

8.8 Impact of Inhibiting FFAR4 (AH-7614): IκB, pIKK, and VCAM-1 Expression

The role of FFAR4 in the response to EPA was addressed by using the FFAR4 antagonist AH-7614 (Tocris: Cat #5256). In addition to a 30 mM EPA stock solution in dimethyl sulfoxide (DMSO; Sigma: RNBF1057), a 10 mM stock solution of AH-7614 was generated in DMSO along with combined 30 mM EPA and 10 mM AH-7614 stock solution (in DMSO). Nine confluent plates were used in this experiment: 0.5 hours before the start of the experiment, 4 plates were treated with 10 μM AH-7614 in order to ensure the receptor was bound by the antagonist. Thereafter, 2 of those plates treated with 10 μM AH-7614 while the other 2 were treated with a combined 10 μM AH-7614 and 30 μM EPA solution. Two plates were treated with 30 μM EPA and another 2 plates were treated with DMSO (0.1% solution). Protein was isolated from the untreated control plate as the experiment began. At 1.5 hours protein was isolated from an EPA treated, Ah-7614 treated, combined EPA/AH-7614 treated, and DMSO treated plate. Proteins were isolated from the remaining 4 plates at 24 hours.

8.9 Statistics

Data are presented as an average of the percent increase in protein over the time control +/- standard error of the mean (SEM). Normalized treatment data were compiled across multiple experiments and a single sample, one-tailed t-test was used to determine if there was a significant change in protein expression following EPA treatment (treatment/control at each time point; p value < 0.05). The same single sample, 1 tailed t-test was used for samples treated with EPA, AH-7614, and EPA/AH-7614, as well as TUG-891 (IκB, pIKK, and VCAM-1 signals will be divided by the treatment and control at each time point).

9 Results

9.1 EPA Time Course

To determine the impact of EPA on the inflammatory process in endothelial cells, 30 μM EPA was added to the media covering bEnd.3 cells. Protein was isolated at 0.5, 1.5, 3, 6, 12, and 24 hours to determine if there was a change in mediators of inflammation, IκB and VCAM-1. Figure 4 is a representative Western blot of VCAM-1 protein expression in control and EPA treated bEnd.3 cells. There was an early increase in VCAM-1 at 1.5 hours that decreased thereafter (3-24 hours). A summary of the data collected after 6 time course experiments are presented in figure 5 and show IκB protein increased at 0.5 hours (49%: $p < 0.05$) and decreased at 24 hours (54%: $p < 0.05$). However, there were no significant changes in protein expression of VCAM-1 (a hallmark of inflammation) in bEnd.3 cells at any time across the duration of the time course experiment. Although VCAM-1 protein expression did not change, the change in IκB protein expression suggests EPA increases inflammation.

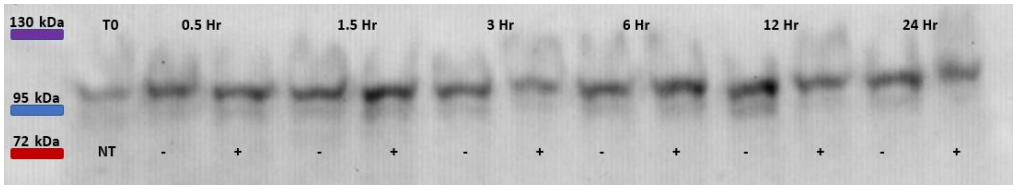

Figure 4: VCAM-1 Western Blot 20AUG20

Representative Western blot for VCAM-1 protein expression in a time course experiment. This image shows the changes in VCAM-1 expression in bEnd.3 cells treated with ethanol (0.1%: -) or EPA (30 μM: +). The samples are from left to right: time point zero (T0: no treatment - NT), the remaining time points are paired, ethanol followed by EPA treated.

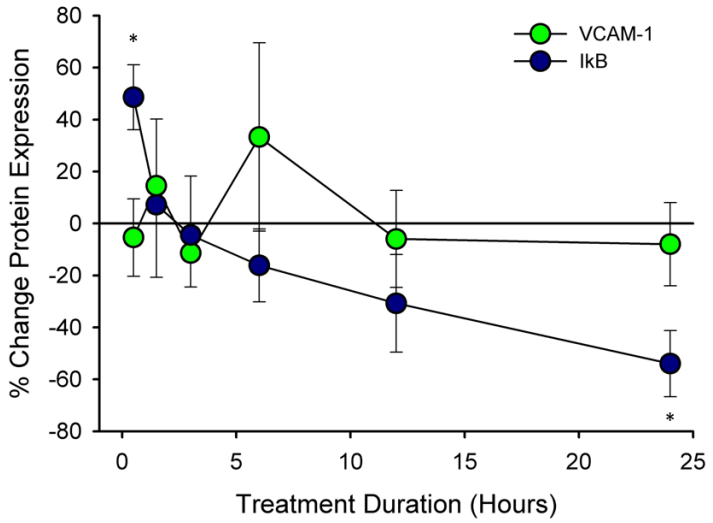

Figure 5: Changes in VCAM-1 and IκB Expression After Treatment with 30 μM EPA

Relative changes in VCAM-1 and IκB protein after treatment with 30 μM EPA. The data are presented as an average and SEM of 6 replicates for VCAM-1 and 4 replicates for IκB. The change in protein expression is shown as a percentage difference from the time control. Expression of VCAM-1 protein did not change across the 24 hours of EPA treatment. While IκB protein increased (49%: $p < 0.05$) at 0.5 hours and decreased at 24 hours (54%: $p < 0.05$). * - refers to significant change in protein over the control at the same time.

9.2 Stimulation of FFAR4 with TUG-891

In order to evaluate the role of FFAR4 on the inflammatory responses to EPA, it was necessary to determine the response of bEnd.3 cells to a specific FFAR4 agonist (TUG-891). Based on the IκB data (as there were no changes in VCAM-1 protein expression) 0.5 and 24 hours were chosen for FFAR4 agonist stimulation along with 1.5 hours due to a trend toward increased VCAM-1 expression. To better understand the inflammatory response, we evaluated another inflammatory signaling molecule (pIKK). This signaling molecule should increase as IκB decreases because pIKK phosphorylates IκB (leading to its degradation) freeing NF-κB to translocate to the nucleus to initiate the inflammatory response. Treatment of bEnd.3 cells with

10 μM TUG-891 decreased pIKK protein at 24 hours (figure 6; 18%: $p < 0.05$) at 24 hours. However, IκB and VCAM-1 protein was not altered at any of the time points.

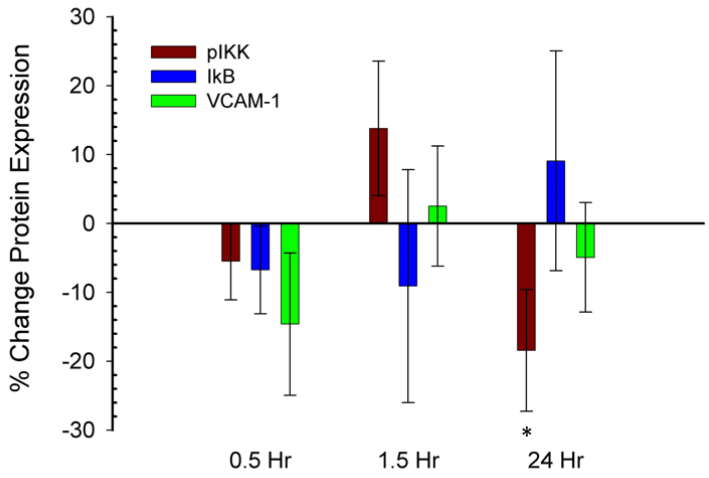

Figure 6: Changes in Protein Expression After Treatment with TUG-891

Relative changes in VCAM-1, IκB, and pIKK proteins after treatment with 10 μM TUG-891 at 0.5, 1.5, and 24 hours. Data presented are the average and SEM of 8 replicates. VCAM-1 and IκB did not change. However, after 24 hours of TUG-891 treatment, pIKK was decreased 18% ($p < 0.05$). * - refers to significant change in protein over the control at the same time.

9.3 Inhibition of FFAR4 with AH-7614

As FFAR4 stimulation did not appear to inhibit the resting inflammatory response in bEnd.3 cells, the next step was to determine the impact of FFAR4 on the EPA response. This involves the application of the FFAR4 antagonist AH-7614. In order to inhibit FFAR4, bEnd.3 cells were pretreated with 10 μM AH-7614 for 0.5 hours in order to allow time for AH-7614 to bind to FFAR4. We evaluated the change of protein at 1.5 and 24 hours after application of 30 μM EPA, 10 μM AH-7614, and a combination of EPA/AH-7614. After 1.5 hours of treatment, there was no change in pIKK expression (figure 7). However, AH-7614 treatment decreased IκB protein (33%: $p < 0.05$). More importantly, VCAM-1 protein increased ($p < 0.05$) at 1.5 hours in all treatment groups (EPA: 43%, AH-7614:53%, EPA/AH-7614: 66%). After 24 hours of treatment,

pIKK was not altered by EPA or AH-7614. However, IκB was decreased (figure 8; 51%: $p < 0.05$) by the combined EPA and AH-7614 treatment. The expression of VCAM-1 was decreased ($p < 0.05$) after 24 hours in all treatment groups (EPA: 40%, AH-7614:38%, EPA/AH-7614: 51%).

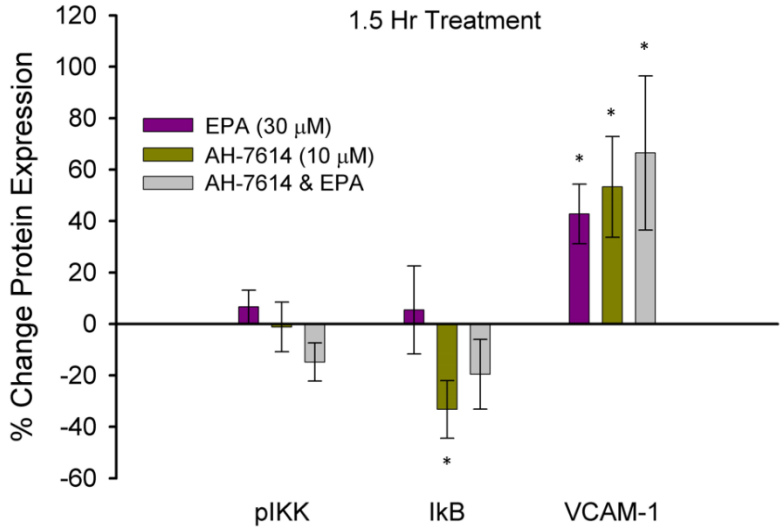

Figure 7: Changes in Protein After 1.5 Hours of Treatment with AH-7614 and/or EPA

Relative changes in VCAM-1, IκB, and pIKK proteins after 1.5 hours of treatment with 30 μM EPA, 10 μM AH-7614, or EPA and AH-7614. The data are presented an average and SEM of 5 replicates. EPA only increased VCAM-1 (43%: $p < 0.05$) whereas AH-7614 decreased IκB (33%: $p < 0.05$) and increased VCAM-1 (53%: $p < 0.05$). Combined EPA and AH-7614 treatment only increased VCAM-1 (66%: $p < 0.05$). * - refers to significant change in protein over the control at the same time.

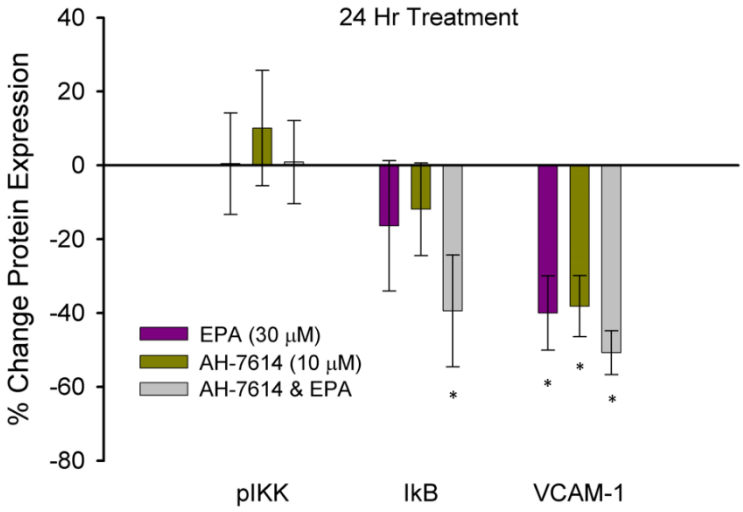

Figure 8: Changes in Protein After 24 Hours of Treatment with AH-7614 and/or EPA

Relative changes in VCAM-1, IκB, and pIKK proteins after 24 hours of treatment with 30 μM EPA, 10 μM AH-7614, or EPA and AH-7614. The data presented are an average and SEM of 5 replicates. EPA only decreased VCAM-1 (40%: $p < 0.05$) whereas AH-7614 treatment decreased VCAM-1 (38%: $p < 0.05$). Combined EPA and AH-7614 treatment decreased IκB (39%: $p < 0.05$) and VCAM-1 (51%: $p < 0.05$). * - refers to significant change in protein over the control at the same time.

10 Discussion

The data collected in this study provides conflicting evidence regarding the role of the FFAR4 in the response of bEnd.3 cultured endothelial cells to EPA. The decrease in IκB protein after 24 hours of exposure to EPA suggests the inflammatory response was enhanced at that time but that was not consistent with the lack of change in VCAM-1 expression (figure 5). In terms of the role of the FFAR4 in an inflammatory response, TUG-891 treatment only decreased pIKK (figure 6) at 24 hours which suggests a decreased inflammatory response, but it did so without changing IκB or VCAM-1. When the inhibitor of FFAR4 was evaluated with EPA, the change in expression of VCAM-1 suggested an early increase in inflammation at 1.5 hours whereas inflammation was decreased at 24 hours (figures 7 and 8). However, the response was the same for EPA and the FFAR4 antagonist: AH-7614 appeared to stimulate the same response as EPA at 1.5 and 24 hours. The inflammatory signaling molecule IκB was altered in the direction (decreased) consistent with the VCAM-1 response following 1.5 hours of inhibition of FFAR4 (figure 7). Following 24 hours of FFAR4, the alteration of the inflammatory signaling molecule IκB is no longer consistent with the VCAM-1 results (figure 8). The remainder of this paper will address reasons for the conflicting results observed as well as what this means for our understanding of EPA responses and the role of FFAR4 in the inflammatory response.

When evaluating an inhibitor of inflammatory responses in bEnd.3 cells, one assumption was that these cells are always under stress that activates the inflammatory response under control conditions. However, the extent of inflammation present under control conditions may not have been enough to allow EPA to reduce inflammation. This minimal activation of inflammation coupled with the variability in Western blots likely compromised our ability to detect substantial changes in inflammatory signaling molecules and VCAM-1. At the same time,

the protein samples isolated for the time course experiments were in the -20°C freezer for a substantial time without protease inhibitor. Due to protein degradation in these samples, a reduction in target protein likely occurred contributing to the variability in results. While this variability in Western blots is usually addressed by evaluating a constitutively expressed protein in each sample, our attempts to use the gold standard for constitutive expression (glyceraldehyde 3-phosphate dehydrogenase: GAPDH) suggested EPA may affect GAPDH expression in bEnd.3 cells (figure S1) which prevents its use as a control. Therefore, additional experiments should include a constitutively expressed protein to reduce the inherent variability in the Western blotting technique.

In order to evaluate the involvement of FFAR4 in the EPA response, bEnd.3 cells were treated with TUG-891 (FFAR4 agonist) and AH-7614 (FFAR4 antagonist). Stimulation of the FFAR4 with TUG-891 did not significantly alter VCAM-1 expression. However, AH-7614 treatment substantially altered VCAM-1 (increase after 1.5 hours followed by a decrease after 24 hours: figures 7 & 8 respectively). The limited response to the agonist (TUG-891) may reflect lack of stability in 10% FBS HG-DMEM at 37°C (as degradation rates of TUG-891 have yet to be addressed) or the rapid internalization of FFAR4 desensitizing the cells [90]. Further, differences between agonist and antagonist responses may result from issues with their affinity for the FFAR4. The affinity of TUG-891 for the FFAR4 was 5.83 (EC50 for ERK activation) [90] while AH-7614 had a higher affinity pIC_{50} = 8.1 (compound 39) [91]. Thus, 10 μM of each compound might have resulted in greater AH-7614 binding. More importantly, AH-7614 does not sterically hinder binding of the agonist. Rather, it acts as a negative allosteric modulator [92] diminishing the receptor's response to the ligand (which might explain its apparent stimulation of the FFAR4). The data collected here suggests AH-7614 was acting as an agonist for FFAR4 in

bEnd.3 cells as the responses to the FFAR4 agonist EPA were similar to AH-7614. Alternatively, AH-7614 and EPA may be stimulating a receptor other than FFAR4 to mediate these anti-inflammatory effects (although here is no evidence for this in the literature).

After evaluating the data it became apparent that the change in protein expression did not align with our original hypothesis. For the time course experiment, the decrease in IκB after 24 hours of EPA treatment suggests EPA stimulated an inflammatory response and should have increased VCAM-1. However, there was no change in VCAM-1 expression. Previous work in this laboratory evaluated the impact of EPA on Cx43 expression and only observed a decreased in Cx43 after 48 hours [52]. As Cx43 expression is increased by inflammation through the NF-κB pathway [16,53], the decrease in Cx43 suggests the reduction in inflammation to EPA might have occurred at a later time than we evaluated (i.e. 48 hours). However, AH-7614 and EPA decreased VCAM-1 after 24 hours in later experiments (figure 8). The issue with our initial data (figure 5) may be the challenges associated with time course experiments. This likely increased the variability in the data along with the degradation in samples making it difficult to find significance.

The majority of this discussion has revolved around the decreased inflammatory response after 24 hours. However, we also observed an increased inflammatory response after 1.5 hours (figure 7). This observation is not consistent with the reported anti-inflammatory effects of the FFAR4. However, previous studies evaluating FFAR4 responses used later times (6-48 hours) [6,9,42,44,46,93–95]. Only 3 studies addressed FFAR4 responses at an earlier time (4 hours) [36–38] and the earliest evaluated pERK, showing a rapid peak after 2.5 minutes [90] that would precede any changes in IκB and/or pIKK as phosphorylation of ERK activates the NF-κB pathway (figure 3) [9]. Responses to FFAR4 (a $G\alpha_q$ receptor) may initially activate the NF-κB pathway enough to

stimulate the increase in VCAM-1 (figure 7). However, the continued signal may be responsible for the anti-inflammatory response associated with FFAR4 which overcame the initial activation. Thus, more work is required to better understand the signaling pathway responsible for the anti-inflammatory effects of EPA.

11 Conclusion

While the data are not internally consistent regarding the impact of FFAR4 on the response of endothelial cells to EPA, there are several interesting outcomes from this work. In particular, we are focusing on the changes in VCAM-1 expression as a strong indicator of a change in the inflammatory response. Relative to the original hypothesis, EPA increased the inflammatory response at 1.5 hours only to decrease the response after 24 hours. The AH-7614 data are suggestive that FFAR4 plays a role in the endothelial response to EPA. However, the antagonist appears to be acting as an agonist as its response mirrored that of EPA. More work is required to strengthen the conclusion of this work.

12 Supplemental Figures

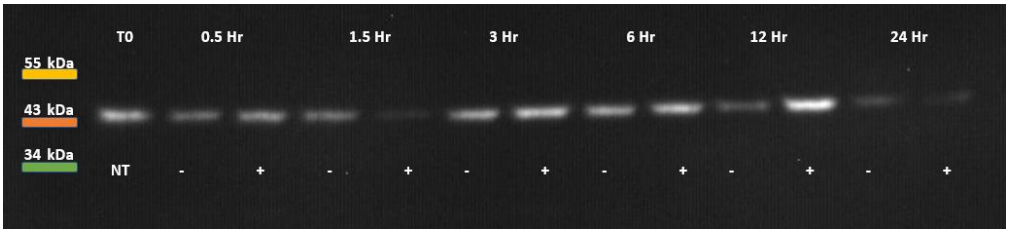

Figure S1: GAPDH Expression over Time

Expression of GAPDH at 0, 0.5, 1.5, 3, 6, 12, and 24 hours. GAPDH was evaluated in an attempt to be used as a constitutively expressed protein but was unfortunately shown to change expression over time. NT = no treatment, - = control (ethanol treatment), + = EPA treatment.

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