UNIVERSITY OF SOUTHAMPTON

Faculty of Medicine

Human Development and Health

Identifying and Understanding Anti-inflammatory Effects of 18-Carbon Fatty Acids from Sustainable Plant Sources

by

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Thesis for the degree of Doctor of Philosophy

2018

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF MEDICINE

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IDENTIFYING AND UNDERSTANDING ANTI-INFLAMMATORY EFFECTS OF 18-CARBON FATTY ACIDS FROM PLANT SOURCES

Epidemiological studies and randomised control trials demonstrate an association between high consumption of very-long chain (VLC) n-3 polyunsaturated fatty acids (PUFAs), specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and long-term health benefits. One of the key actions of EPA and DHA is to reduce inflammation. The principal source of EPA and DHA is oily fish. Current stocks of fatty fish are likely not sufficient to meet the needs of humans for VLC n-3 PUFAs. Alpha-linolenic acid (ALA), stearidonic acid (SDA), gamma-linolenic acid (GLA) and pinolenic acid (PIN) may provide sustainable land-based sources of FAs to promote human health, but their functionality has been underexplored, including in relation to inflammation. Dysfunction of vascular endothelial cells (ECs) plays a vital role in the development of atherosclerosis. Incorporation of DHA, and to a lesser extent EPA, into EC lipids has been shown to decrease cytokine-induced expression of adhesion molecules and secretion of inflammatory mediators.

The research described in this thesis explores the effects of ALA, SDA, GLA and PIN, in comparison with EPA and DHA, as well as different ratios of linoleic acid (LA) to ALA on inflammatory responses of a cultured EC line (EA.hy926 cells).

All FAs examined were incorporated into the ECs in a dose-dependent manner, with several elongation products being synthesised; of these the elongation product of PIN, eicosatrienoic acid (ETrA), was successfully identified using GC-MS. Of the two marine-derived FAs DHA had the most potent anti-inflammatory effect in EA.hy926 cells. Both EPA and DHA decreased production of all inflammatory mediators studied, cell surface expression of ICAM-1, and intracellular inflammatory proteins, and significantly modulated the expression of various inflammatory genes. DHA decreased adhesion of THP-1 monocytes to EA.hy926 cells under static conditions. Of the plant-derived FAs, GLA and PIN caused the greatest reduction in inflammatory mediator production. Gene expression was altered by both GLA and SDA; additionally, SDA reduced cell surface expression of ICAM-1. Lastly, all plant-derived FAs decreased THP-1 adhesion to EA.hy926 cells. FA effects were concentration dependent. No plant-derived FA was as potent as DHA. Silencing of the elongase 5 gene

significantly inhibited the production of DGLA and ETrA in EA.hy926 cells pre-treated with GLA and PIN. Furthermore, most of the anti-inflammatory effects of GLA and PIN were abolished by silencing elongase 5 suggesting that GLA and PIN act via their elongation products. ALA and ratios of LA to ALA containing higher concentrations of ALA were anti-inflammatory in EA.hy926 cells.

These findings confirm anti-inflammatory effects of EPA and DHA in ECs. New anti-inflammatory actions of plant-derived FAs are identified, although these FAs are less potent than DHA. Nevertheless, these findings suggest that plant-derived FAs, especially GLA and PIN have potential as sustainable anti-inflammatory alternatives to EPA and DHA. Further research on these FAs is warranted.

By Ella Baker

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DECLARATION OF AUTHORSHIP

I, Ella Baker declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Identifying and Understanding Anti-inflammatory Effects of 18-Carbon Fatty Acids from Sustainable Plant Sources

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission:

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Acknowledgements

Firstly, I am extremely grateful to my supervisors, Philip Calder and Liz Miles, for continued support, guidance and encouragement throughout my PhD. For the multitude of opportunities which they have given me, including presenting my research abroad and developing many new skills.

Special thanks to Annette West and Helena Fisk for her guidance and help with the fatty acid profile analysis. Thanks to Melissa Doherty for all her technical support across a multitude of different techniques.

I would also like to thank Kath Woods-Townsend, who gave me the opportunity to intern at Lifelab, learning a great number of new skills, and understanding the importance of outreach.

I would like to express my deepest appreciation to Carina and Helena for their unceasing support during the course of my PhD, both for their technical advice and friendship. Also to my friends Lucie, Jade, Janna and Sam who have kept me motivated throughout this process.

Finally, to my husband Jim and my parents, thank you for all the constant encouragement, kindness, and support, I would not have completed this work without you.

Definitions and Abbreviations

AA, arachidonic acid;

AcCoA, acetyl-CoA;

ALA, alpha-linolenic acid;

CAMs, cellular adhesion molecules;

CHD, coronary heart disease;

CHF, congestive heart failure;

COX, cyclooxygenase;

CRP, C-reactive protein;

CVD, cardiovascular disease;

D5D, delta-5-desaturase;

D6D, delta-6 desaturase;

DPA, docosapentaenoic acid;

DGLA, dihomo gamma linolenic acid;

DHA, docosahexaenoic acid;

DICER, endoribonuclease Dicer;

DNA, deoxyribonucleic acid;

dsRNA, double-stranded RNA;

EPA, eicosapentaenoic acid;

ELOVL5, elongase 5;

ETrA, eicosatrienoic acid;

FA, fatty acid;

FAME, fatty acid methyl ester;

FFA, free fatty acid;

FGF, fibroblast growth factor;

GLA, gamma-linolenic acid

GM, genetically modified;

HAEC, human aortic endothelial cell;

HCAEC, human coronary artery endothelial cell;

HCEC, human corneal endothelial cell;

HIMEC, human intestinal microvascular endothelial cell;

HSVEC, human saphenous vein endothelial cell;

HUVEC, human umbilical vein endothelial cell;

ICAM-1, intercellular adhesion molecule-1;

IkBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha;

IKKB, inhibitor of nuclear factor kappa-B kinase subunit beta;

IL-6, interleukin-6;

IL-8, interleukin-8;

IL-10, interleukin-10;

LA, linoleic acid;

LDL, low density lipoprotein;

LOX, lipoxygenase;

MAb, monoclonal antibody

MCP-1, monocyte chemoattractant protein 1;

MUFA, monounsaturated fatty acid;

NADH, nicotinamide adenine dinucleotide;

NADPH, nicotinamide adenine dinucleotide phosphate;

NEFA, non-esterified fatty acid;

NF-kB, nuclear factor kappa-light chain enhancer of activated B cells;

OxAc, oxaloacetate;

oxLDL; oxidised low density lipoprotein;

PCB, polychlorinated biphenyls;

PDGF, platelet-derived growth factor;

PE, phycoerythrin;

PECAM-1, platelet endothelial cell adhesion molecule;

PIM-PUFA, poly-methylene-interrupted polyunsaturated fatty acid;

PIN, pinolenic acid;

PPAR, peroxisome proliferator activated receptor;

PUFA, polyunsaturated fatty acid;

RANTES, regulated on activation, normal T cell expressed and secreted;

RBC, red blood cell;

RCT, randomised control trial;

SAA, serum amyloid A;

SDA, stearidonic acid;

SFA, saturated fatty acid;

siRNA, short interfering RNA;

TAG, triacylglycerol;

TNFα, tumour necrosis factor alpha;

Tregs, regulatory T cells;

VEGF, vascular endothelial growth factor;

VCAM-1, vascular adhesion molecule-1;

VLC, very long-chain;

VLDL, very low density lipoprotein;

n-3, omega 3;

WHO, World Health Organisation

Chapter 1: Introduction

1.1 Fatty acids

Fatty acids (FA) are widely dispersed in nature and are major components of triacylglyerols (TAG), phospholipids (PL) and other complex lipids [1]. They are structural components of living cells and have biological activities that influence cell and tissue metabolism and function.

1.1.1 Fatty acids - Chemical structure and nomenclature

FA consist of an acyl (i.e. hydrocarbon) chain, varying from 2 to 30 (or more) carbons, with a carboxyl group at one end and a methyl group at the other. The carbon of the carboxyl group is traditionally called carbon 1, and carbons 2 and 3 are usually referred to as the alpha (α) and beta (β) carbons respectively. The carbon at the methyl terminal is named the omega (ω or n) carbon. The carboxyl group readily forms ester links with alcohol groups such as propan-1,2,3-triol (i.e. glycerol) and in turn this forms acylglycerols (e.g. TAG and PL) and cholesteryl esters [2]. Saturated fatty acids (SFA) and unsaturated fatty acids (UFA) are distinguished by the absence or presence of carbon-carbon double bonds in the acyl chain. Those without double bonds are SFA and those with double bonds are UFA [3]. FA with two or more double bonds are referred to as polyunsaturated fatty acids (PUFA). Using the standard nomenclature of the International Union of Pure and Applied Chemistry (IUPAC) the number of carbons in the acyl chain determines the systematic name for a FA. There are numerous potential double bond positions and the bond may be in either a *cis* or *trans* configuration (also known as (E/Z)-isomerism)) (Figure 1.1). Classically UFA are named by identifying the carbons on which the double bonds occur, starting from carbon 1 (the carboxyl carbon). An example is octadecadienoic acid, an 18 carbon FA with *cis* double bonds between carbons 9 and 10 and 12 and 13; this is correctly identified as *cis*9, *cis*12-octadecadienoic acid or as *cis*,*cis* 9,12-octadecaadienoic acid.

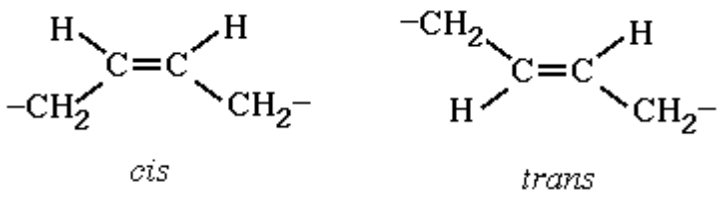

Figure 1.1 Fatty acid configuration – *cis* and *trans* double bonds (Gurr *et al*. 2002).

Short hand nomenclature for FAs is now more commonly used than systematic names. This identifies the number of carbons in the acyl chain, as well as the number and position of double bonds. In this naming system the first double bond is counted from the methyl terminus (methyl carbon as number 1) of the acyl chain and is identified as n-x, where x is the carbon on which the first double bond occurs. Therefore the short hand name for *cis*,*cis* 9,12-octadecadienoic acid is 18:2n-6. An example of a SFA is octadecanoic acid which is denoted as 18:0, indicating an acyl chain length of 18 carbons without any double bonds.

FAs are also often described by common names, which may be considered to be more concise compared to the much longer traditional naming system. This is also how they are most frequently referred to in the literature. Examples include oleic acid and linoleic acid, rather than *cis* 9-octadecenoic acid or *cis*,*cis* 9,12-octadecadienoic acid. These common names often originate from their first botanical or zoological origins. For example, oleic acid is found in the fruit of the olive tree (*Olea europaea*) [2].

Double bonds in acyl chains are generally separated by a methylene group, but can also be conjugated. Atoms that lie on the same side (*cis*) of the reference plane in the molecule are more commonly found than those found on the opposite (*trans*) side of the reference plane. *Trans* double bonds tend to occur in intermediates in the biosynthesis of FAs, and in plant lipids, some seed oils and ruminant fats (e.g. cow's milk) [2]. The rotation of *cis* double bonds means a kink is created within the acyl chain, creating a distinct molecular shape of UFA with *cis* double bonds compared to SFA or UFA with *trans* double bonds.

Table 1.1 shows the names and structures of various common FAs

Table 1.1 FA nomenclature and double bond position

Common name	Structure	IUPAC name	Short name
Palmitic acid	CH3(CH2)14COOH	Hexadecanoic acid	16:0
Oleic acid	CH3(CH2)7CH=CH(CH2)7COOH	(9Z)-9-Octadecenoic acid	18:1n-9
Linoleic acid	CH3(CH2)4CH=CHCH2CH=CH(CH2)7COOH	(9Z,12Z)-9,12-Octadecadienoic acid	18:2n-6
Alpha-Linolenic acid	$CH3CH2CH=CHCH_2CH=CHCH_2CH=CHCH_2$(CH2)6COOH	(9Z,12Z,15Z)-9,12,15-Octadecatrienoic acid	18:3n-3
Gamma-Linolenic acid	$CH3CH2CH=CHCH_2CH=CHCH_2CH=CHCH_2$(CH2)6COOH	(6Z,9Z,12Z) – 6,9,12-Octadecatrienoic acid	18:3n-3
Stearidonic acid	$CH3CH2CH=CHCH2CH=CHCH2CH=CHCH_2CH=CH$(CH2)4COOH	(6Z,9Z,12Z, 15Z) – 6,9,12,15 -Octadecatetraenoic acid	18:4n-3
Arachidonic acid	CH3(CH2)4CH=CHCH2CH=CHCH2CH=CHCH2CH=CH(CH2)3COOH	(5Z,8Z,11Z,14Z)-5,8,11,14-Eicosatetraenoic acid	20:4n-6
Eicosapentaenoic acid	CH3CH2CH=CHCH2CH=CHCH2CH=CHCH2CH=CHCH2CH=CH(CH2)3COOH	(5Z,8Z,11Z,14Z,17Z)-5,8,11,14,17-Eicosapentaenoic acid	20:5n-3
Docosahexaenoic acid	CH3CH2CH=CHCH2CH=CHCH2CH=CHCH2CH=CHCH2CH=CHCH2CH=CH(CH2)2COOH	(4Z,7Z,10Z,13Z,16Z,19Z)-4,7,10,13,16,19-Docosahexaenoic acid	22:6n-3

1.1.2 Fatty acids - metabolism and biosynthesis

FAs are naturally occurring molecules which can be provided through the diet mainly in TAG form or, in many cases, produced *de novo*. FAs are transported through the bloodstream mainly as TAG, PL or cholesteryl esters. Non-esterified fatty acids (NEFA) also circulate within the bloodstream; these are often referred to as free fatty acids (FFA). These different blood lipid pools contain a variety of FAs based on diet and on the way in which the FAs and complex lipids have been metabolised. These transport pools move FAs between tissues within the body. FAs may serve functional, storage and metabolic roles.

After ingestion, a TAG is hydrolysed by gastric and pancreatic lipases to produce a monoglyceride and two FFAs. These products are absorbed by enterocytes and are re-esterified into TAG and then assembled into lipoproteins called chylomicrons. Chylomicrons are initially released into the lymph and then into the bloodstream from where they can be utilised [4]. For example, the FAs from chylomicrons can be taken up by adipose tissue and stored.

An important metabolic role of FAs is as a source of energy which requires them to be oxidised ultimately to CO_2 and water. All FAs can be oxidised and the amount of energy yielded depends on the structure of the particular FA. FAs can be used as an energy source by most aerobic tissues, other than the brain.

Phospholipids comprise of FAs linked to a phosphoglycerol backbone and are found on the surface of circulating lipoproteins and within all cell membranes. This is the major functional role of FAs. The FA composition of the cell membrane varies depending on cell type, and is influenced by diet, metabolism, genetics, and hormone fluctuation among other factors [1]. The relative abundance of FAs within a membrane can then affect its physical nature, which in turn can influence membrane protein functions and protein movement within the membrane. Furthermore, membrane phospholipids are precursors of molecules involved in cell signalling processes. The FA composition of these signalling molecules such as ceramides, lyso-phospholipids and endocannabinoids has been shown to modify their biological activity.

1.1.2.1 Fatty acid biosynthesis *de novo*

SFAs can be synthesised from carbohydrates and proteins. The initial building block, acetyl coenzyme A (acetyl-CoA), is produced from sugar and amino acid metabolism, predominantly within mitochondria. FA biosynthesis occurs in the cytoplasm, and a transport system exists whereby citrate formed from mitochondrial acetyl-CoA and oxaloacetate in the citric acid (Krebs) cycle is moved across the mitochondrial membrane to the cytoplasm [3]. Once in the cytoplasm, citrate is cleaved to produce oxaloacetate and acetyl-CoA. Figure 1.2 depicts a simplistic cartoon of the synthesis of FAs within the cell.

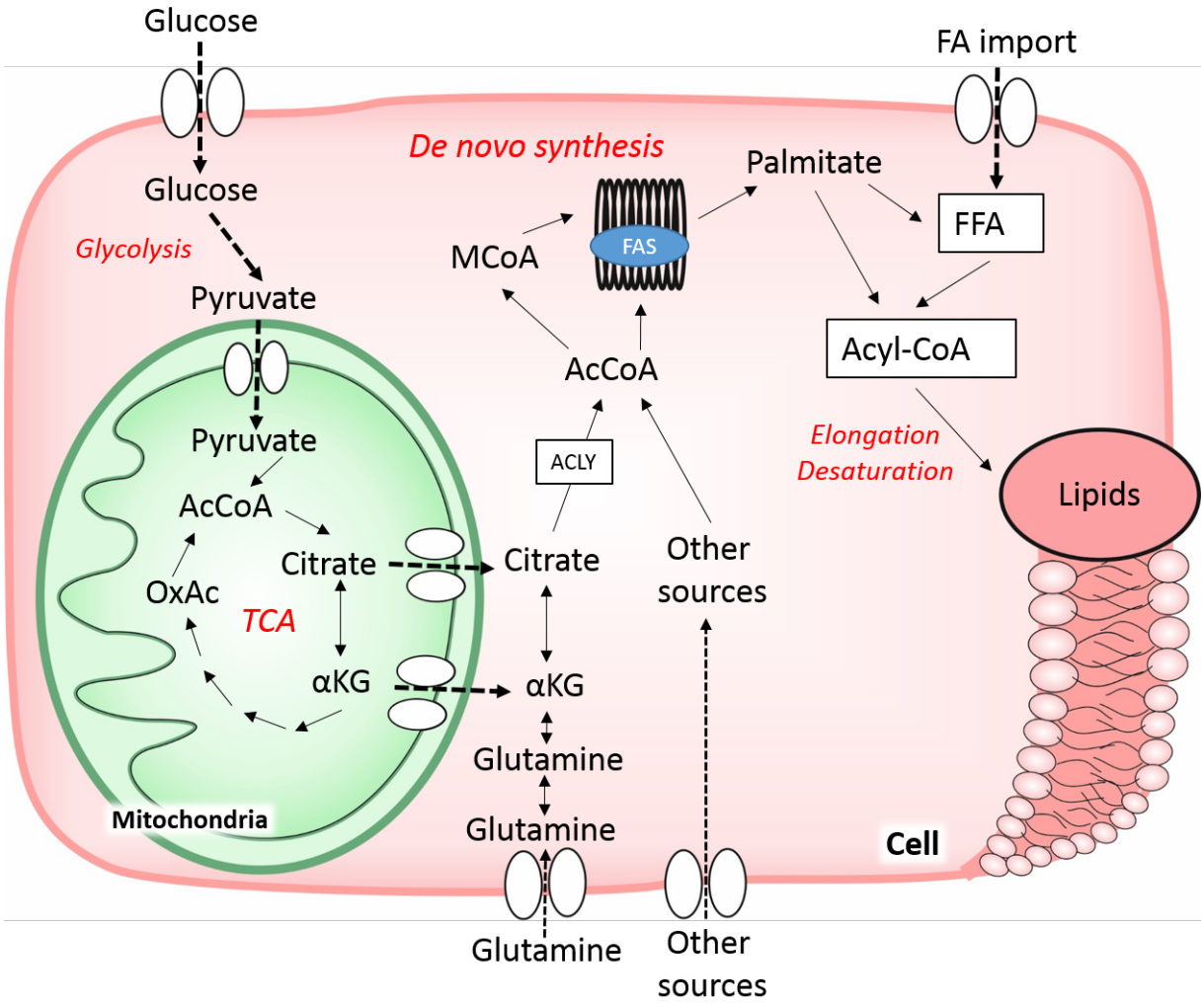

Figure 1.2 Fatty acid synthesis in cells.

AcCoA, acetyl-CoA; OxAc, oxaloacetate; αKG, α-ketoglutarate; ACLY, ATP citrate lyase; MCoA, malonyl-CoA; FAS, fatty acid synthase; FFA, free fatty acid (non esterified)

De novo synthesis of FAs is catalysed by fatty acid synthase, a multi-enzyme complex. The hydrocarbon chain is built up by the sequential addition of two carbon units, donated by malonyl-ACP. Malonate linked to acyl carrier protein (ACP) is formed from malonyl-CoA which itself is formed from acetyl-CoA. Acetyl-CoA carboxylase catalyses this conversion of acetyl-CoA to malonyl-CoA which is the rate-limiting enzyme of this pathway [3]. This rate limiting step is regulated allosterically by hormonal changes. Insulin for instance can upregulate this process meaning that insulin promotes FA biosynthesis [4]. The malonate group of malonyl-CoA then condenses with ACP to produce malonyl-ACP.

FA biosynthesis begins with malonyl-ACP being condensed with acetyl-ACP to form four-carbon acetoacetyl-ACP with the loss of one carbon as CO_2. After this follows a sequence of reduction and dehydration steps to produce four-carbon butyryl-ACP. It is this entire series of steps that is catalysed by fatty acid synthase. The cycle is repeated with butyryl-ACP replacing acetyl-ACP from the initial cycle so producing a six-carbon acyl-ACP. The cycle is repeated a further seven times to produce 16-carbon palmitoyl-ACP, which is hydrolyzed from the ACP to yield palmitic acid (16:0). The reductive steps in the FA biosynthesis pathway use nicotinamide adenine dinucleotide phosphate (NADPH) derived from the pentose phosphate pathway.

Shorter chain length SFAs can be created within some specific tissues. These tissues contain enzymes which act to release FAs at an earlier stage within the biosynthetic pathway than palmitic acid. For example, enzymes occurring in mammary glands of some species release medium-chain saturated FAs such as caprylic acid (8:0) and capric acid (10:0) [2].

In eukaryotes, enzymes termed elongases convert palmitic acid to longer-chain saturated fatty acids (LC-SFA) through elongation reactions, which occur on the endoplasmic reticulum or in mitochondrial membranes. The mitochondrial system uses acetyl-CoA as the two-carbon donor, and there is also the requirement for nicotinamide adenine dinucleotide (NADH) and/or NADPH. However, the endoplasmic reticulum is the main location for FA elongation; this uses malonyl-CoA as the two-carbon donor as well as NADPH.

Elongation converts palmitic acid to stearic acid (18:0), but a large number of FA substrates may be elongated. A total of seven elongase enzymes, which differ in their selectivity for FAs of different chain lengths and degrees of unsaturation, have been identified. Elongases 1, 3, 6, and 7 catalyze the extension of saturated

and monounsaturated fatty acids (MUFA), while elongases 2, 4, and 5 act mainly on PUFA.

UFA are synthesised by desaturase enzymes some of which catalyse the insertion of double bonds into a SFA chain. This process occurs under an oxidation reaction and involves NADH and cytochrome B5 [3]. Delta-9 desaturase, also known as stearoyl-CoA desaturase catalyses the conversion of stearic acid to oleic acid (18:1n-9) [3].

Mammals lack the enzymes that insert double bonds beyond carbon 9 in the acyl chain (counting from the carboxyl carbon). Therefore insertion of double bonds at positions 3 and 6 which involve the enzymes delta-12 (D12D) and delta-15 desaturase (D15D) only occurs in plants. Insertion of a double bond between carbons 12 and 13 of oleic acid by D12D yields linoleic acid (LA; 18:2n-6), which is further desaturated by D15D to yield alpha-linolenic acid (ALA; 18:3n-3). LA and ALA are the simplest members of the n-6 and n-3 PUFA families (Figure 1.3). These FAs are considered essential FAs, since they cannot be synthesied *de novo* by mammals but are required by mammalian cells.

Although mammalian cells are unable to synthesise LA or ALA, these FAs are the primary precursors for further desaturation and elongation. This process is believed to mainly occur within the liver [5], but there is some evidence that other tissues, including brain and testis, have high expression of the genes encoding the relevant enzymes [6, 7]. Competition exists between the conversion of n-6 and n-3 FAs as both utilise these same enzymes. The initial conversion of ALA to stearidonic acid (SDA; 18:4n-3) is catalysed by delta-6 desaturase (D6D) and is generally considered to be the rate-limiting reaction in the pathway [8, 9]. D6D has a preference for ALA over LA: the Km of rat D6D for ALA is between 29 and 33 μM while the Km for LA is between 43 and 92 μM [10]. However, greater availability of LA than ALA in most situations means that the metabolism of the former exceeds that of the latter.

The D6D reaction results in insertion of a double bond at the delta-6 position of ALA to form SDA. SDA is converted to 20:4n-3 by the addition of 2 carbons by the enzyme elongase-5. 20:4n-3 is then converted to eicosapentaenoic acid (EPA; 20:5n-3) by insertion of a double bond at the delta-5 position by delta-5-desaturase (D5D). EPA can be elongated by elongase-2 to form docosapentaenoic acid (DPA; 22:5n-3) and then to 24:5n-3 followed by desaturation that again uses D6D activity to form 24:6n-3. This desaturation seems to be catalysed by the

same D6D as in the first step of the pathway [5]. 24:6n-3 is then translocated from the endoplasmic reticulum to the peroxisomes where DHA (22:6n-3) is formed by removal of 2 carbons through β-oxidation. Recent mechanistic studies suggest conversion of EPA to DHA may be limited by the elongation enzyme product of elongase 2 which limits downstream elongation reactions and is subject to substrate competition by EPA [11].

EPA biosynthesis from SDA is considered to be more efficient than that from ALA due to it not requiring this first catalytic step, and SDA has been referred to as 'pro-EPA' because of this enhanced conversion [12].

Alternatively D6D inserts a double bond at delta-6 position of LA (see Figure 1.4). This results in the formation of gamma-linolenic acid (GLA; 18:3n-6), which is then elongated by the addition of 2 carbons by elongase 5 to form dihomo-gamma-linolenic acid (DGLA; 20:3n-6). DGLA is then desaturated by D5D, inserting a double bond at the delta-5 position yielding arachidonic acid (AA; 20:4n-6). AA can be further elongated by elongase 2 to form docosatetraenoic acid (DTA; 22:4n-6). This can under-go further desaturation by D6D to form docosapentaenoic acid n-6 (DPA; 22:5n-6).

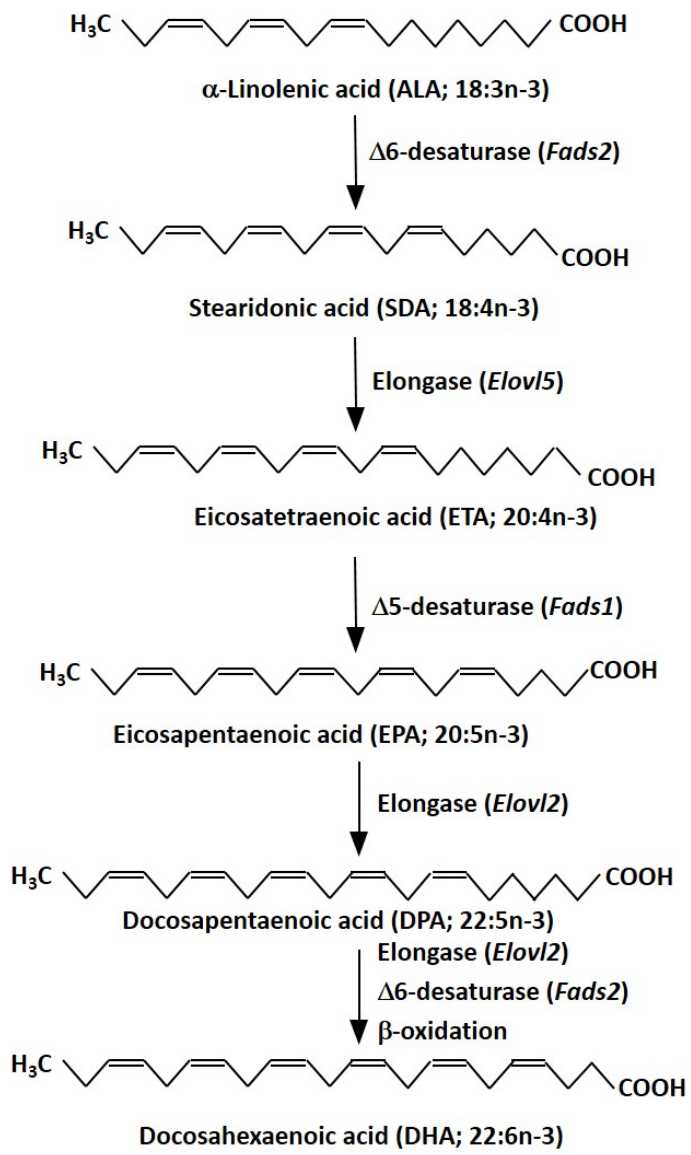

Figure 1.3 Pathway of conversion of alpha-linolenic acid to longer chain, more unsaturated n-3 fatty acids

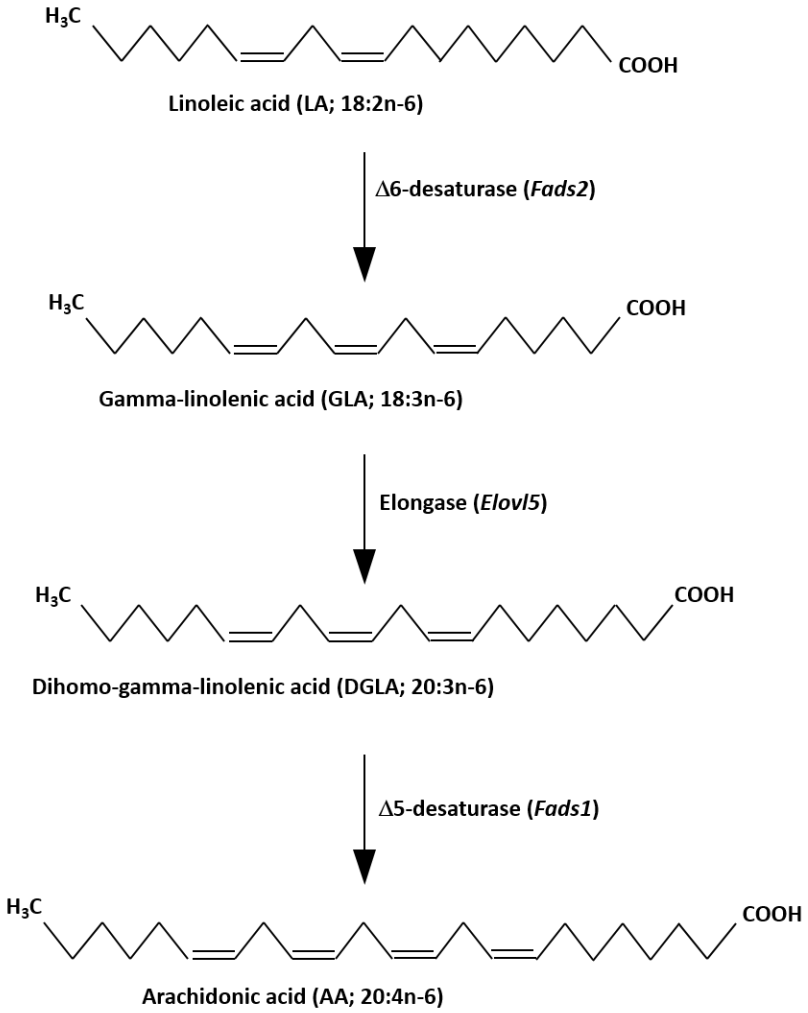

Figure 1.4 Pathway of conversion of linoleic acid to longer chain more unsaturated n-6 fatty acids

1.1.3 Fatty acids - sources and intakes

FAs in foodstuffs as well as in oils and fats are mostly esterified to a glycerol, as TAG, but some are present as esterified components of phospholipids, glycolipids and other lipids [2]. Ruminant milk contains higher proportions of short and medium chain FAs and relatively lower proportions of PUFAs. Meats from animal tissue commonly contain high levels of SFAs and MUFAs and again lower levels of PUFAs. Meat from fish is classified as either 'oily/fatty' or lean; oily fish store lipid as TAG in the flesh and are rich in the n-3 PUFAs EPA and DHA. The FA composition of lipids in plant membranes is generally consistent among different types of leaves and 90% of total FAs found in leaves is made up of ALA (ca. 56%), LA (ca.16%), palmitic acid (ca.13%), oleic acid (ca. 7%), and palmitoleic acid (ca 3%) [2].

Rich dietary sources of LA include safflower oil (~75% of total FAs) and corn and soybean oils (LA 50-60% of total FAs). Nuts and seeds with high LA content include sunflower seeds, brazil nuts and pecans.

Sources of ALA included flaxseed and flaxseed oil (ALA >50% of total FAs), soybean oil (ALA makes up 10% of total FAs), rapeseed oil, some nuts (including walnuts) and green plant tissues.

When eating a varied diet most humans consume a wide range of FAs each day, but this will vary between meals, days, seasons and will be different depending on the geographical location and cultural preferences of the person. The majority of the FAs consumed will be readily available to the bloodstream through efficient digestion and absorption.

There are large differences in fat intake between countries due to the mix of fats and oils used in food preparation and foodstuffs consumed. Average fat consumption has changed over time and continues to change; developing countries tend to have an increasing intake whereas developed countries have a decreasing intake. However, the type of fat being consumed has also changed over time, along with the proportions of different FAs in the diet. Much of this change is likely due to the shift from animal fats to vegetable oils, for example the use of margarine instead of butter. The main PUFA within the Western diet is LA followed by ALA.

In the UK the average intake of LA, the major n-6 FA in the diet, has risen to 11 g/day and LA intake is even higher in some other countries. For example average daily intake in Spain is 21.6 g. Typical intakes of ALA within the Western diet vary from 0.5-2.3 g/d [13-15]. LC n-3 PUFAs (i.e. EPA and DHA) are consumed in much lower amounts, because of the low quantities of fish and other seafood within the diet.

UK Dietary Reference Values (DRVs) recommend an intake of dietary fat of $\leq$ 35% of daily food energy intake. The UK recommendations for adults for SFA, MUFA and PUFA are < 10%, 13% and 6 to 10% of dietary energy, respectively. Various authorities including The World Health Organisation (WHO), The Committee on Medical Aspects of Food Policy (COMA) and Scientific Advisory Committee on Nutrition (SACN) recommend consumption of fish once or twice a week. SACN recommends a minimum intake of 0.45 g of LC n-3 PUFAs per day [16] .

1.1.3.1 Omega-6/omega-3 fatty acid ratio

As indicated above, in more recent years the relative intake of n-6 to n-3 FAs has drastically shifted in the Western diet, with higher intake of LA compared to ALA [17]. These FAs compete for the enzyme D6D, which is responsible for the synthesis of longer chain metabolites, which in turn lead to the production of a variety of bioactive lipid mediators.

Metabolism of LA leads to the production of AA. AA is a precursor for synthesis of eicosanoids (prostaglandins, leukotrienes and thromboxanes), which are all involved in the inflammatory response. Larger quantities of AA have been linked with pro-inflammatory status [18]. ALA is the substrate for the synthesis of EPA and DHA. EPA and DHA are also the substrate for eicosanoids and docosanoids, respectively, but those deriving from EPA and DHA have been shown to be less inflammatory than those produced from AA and this may in part explain positive health effects of EPA and DHA [19]. These pathways are described in greater detail in section 1.2.6.

Some argue the ratio of n-6 to n-3 FAs in the human diet has led to a more pro-inflammatory state and contributes to the prevalence of inflammatory diseases including atherosclerosis [18]. It is argued that greater abundance of LA in many diets today leads to the preferential metabolism of LA leading to the synthesis of AA-derived eicosanoids, and therefore more inflammation. It is possible that higher consumption of ALA or of EPA and DHA shifting the n-6 to n-3 ratio could lead to a less inflammatory or pro-resolving state, although it is important to note that some studies have reported an association of higher plasma levels of both AA and n-3 FAs with lower levels of inflammatory markers [20].

Though it is hard to estimate the amounts of ALA and LA in the diet, since these come from such varied sources, Table 1.2 shows the estimate of dietary ratios in different countries for men and women [21]. Italy, Spain and Portugal are among the countries with the highest dietary ratio of LA to ALA, whereas Finland, Sweden and Germany are consuming much lower amounts of LA. This may be an important factor when considering recommendations for n-3 FA intake.

Table 1.2 Mean daily intakes and ratios of alpha-linolenic acid (ALA) and linoleic acid (LA) in different countries for men and women (Baker *et al*. 2016 [21])

Country	Alpha-linolenic acid (ALA) g/d		Linoleic acid (LA) g/d		LA:ALA ratio	
	Men	Women	Men	Women	Men	Women
Australia/New Zealand	1.4	0.9	12.8	9.1	9.1	10.1
Austria	1.5	1.3	13.6	12.4	9.1	9.5
Belgium	1.7	1.4	16.6	12.8	9.8	9.1
Denmark	2.2	2.1	14.3	10.9	6.5	5.2
Finland	1.8	1.3	8.1	5.8	4.5	4.5
France	0.9	0.7	8.3	6.3	9.2	9
Germany	1.59	1.32	9.3	8.0	5.8	6.1
Greece	0.6	0.7	9.3	9.9	15.5	14.1
Iceland	2.5	1.4	NA	NA	NA	NA
Italy	0.8*		14.5*		18.1*	
Netherlands	1.95	1.26	17.8	12.0	9.1	9.5
Norway	1.6	1.0	12.2	7.8	7.6	7.8
Portugal	0.7*		12.1*		17.3*	
Spain	0.8*		21.6*		27*	
Sweden	1.6	1.2	9.7	7.8	6.1	6.5
UK	1.4*		11.4*		8.1*	
USA	1.7	1.3	16.8	14	9.9	10.8

* Data for men and women were combined.

1.2 Inflammation

1.2.1 Mechanisms involved in the inflammatory response

Inflammation is a part of the early response to infection and as such is an important component of host defence. Inflammation is also induced in response to tissue injury and metabolic stress. It involves enhanced movement of cells between body compartments, cellular activation and altered cellular responsiveness, altered cellular interactions, and the production of a vast number of chemical mediators. Inflammation is designed to be a destructive process. The classic signs of inflammation are redness, swelling, heat, pain and loss of function. These are each caused by or related to the influx of cells into the site of

inflammatory activity and the release of chemicals at that site. Activation of the inflammatory response triggers a biochemical cascade to up regulate vascular permeability and attract immune cells to the site of infection or stress. Although the chemical mediators produced as part of the response act to damage pathogens they can also affect the host environment. In healthy people this response is well regulated so as not to cause excessive damage. Negative feedback mechanisms such as anti-inflammatory cytokines and pro-resolving lipid mediators inhibit pro-inflammatory signalling cascades, while there is also shedding of inflammatory mediator receptors rendering cells unresponsive and activation of regulatory cells. However if the usual regulatory processes malfunction, irreparable damage to the host can be caused by the ongoing inflammation.

Inflammation can be classified as acute or chronic. The initial response known as acute inflammation is defined by the increased movement of plasma and leukocytes from the blood into a site of infection or injury [22]. This response is short term and will usually resolve within hours to days. If the inflammation becomes prolonged it is known as chronic, whereby there is a shift in the cell types present at the sight of infection or injury. Chronic inflammation is characterised by the simultaneous destruction and healing of the tissues by the on-going inflammatory process. In some cases destruction of the host environment dominates over healing, and disease occurs [22].

1.2.2 Chemotaxis and cell migration

Under normal physiological conditions, the endothelial lining of the vasculature is both anti-thrombogenic and anti-adhesive allowing blood and its components to be free flowing. However in an inflammatory state the endothelium is rendered adhesive for circulating leukocytes, initiating the process of leukocyte migration [23]. Figure 1.5 depicts the adhesion, activation and migration of leukocytes. This process is controlled by adhesion molecules and chemokines expressed on the surface of the venular endothelium in inflamed tissues. The precise pattern of leucocyte migration is determined by the exact nature of the interaction between circulating leukocytes and the endothelium. Chemokines activate the circulating cells causing them to bind to the endothelium initiating leucocyte migration across the endothelium. Once in the tissues, the leukocytes migrate towards the site of infection or injury by a process called chemotaxis which is a process of chemical attraction along a concentration gradient. Monocyte chemoattractant

protein (MCP-1) and regulated on activation, normal T cell expressed and secreted (RANTES) are examples of chemoattractants.

Regulatory T cells (Tregs), have been shown to play a role in inflammatory control and homeostasis during endothelial activation. This cell type carry out their immunosuppressive functions via several mechanisms. One suppressive mechanism of Tregs is the secretion of anti-inflammatory cytokines including IL-10, TGF-β, and IL-35 [24]. Both IL-10 and TGF-β have been shown to have atheroprotective properties [24], Ait-Oufella *et al*. demonstrated that depletion of peripheral Tregs by anti-CD25 monoclonal antibodies increased atherosclerotic lesion size and vulnerability in the atherogenic mouse model apolipoprotein E gene deficient (*ApoE*−/−) mice [25].

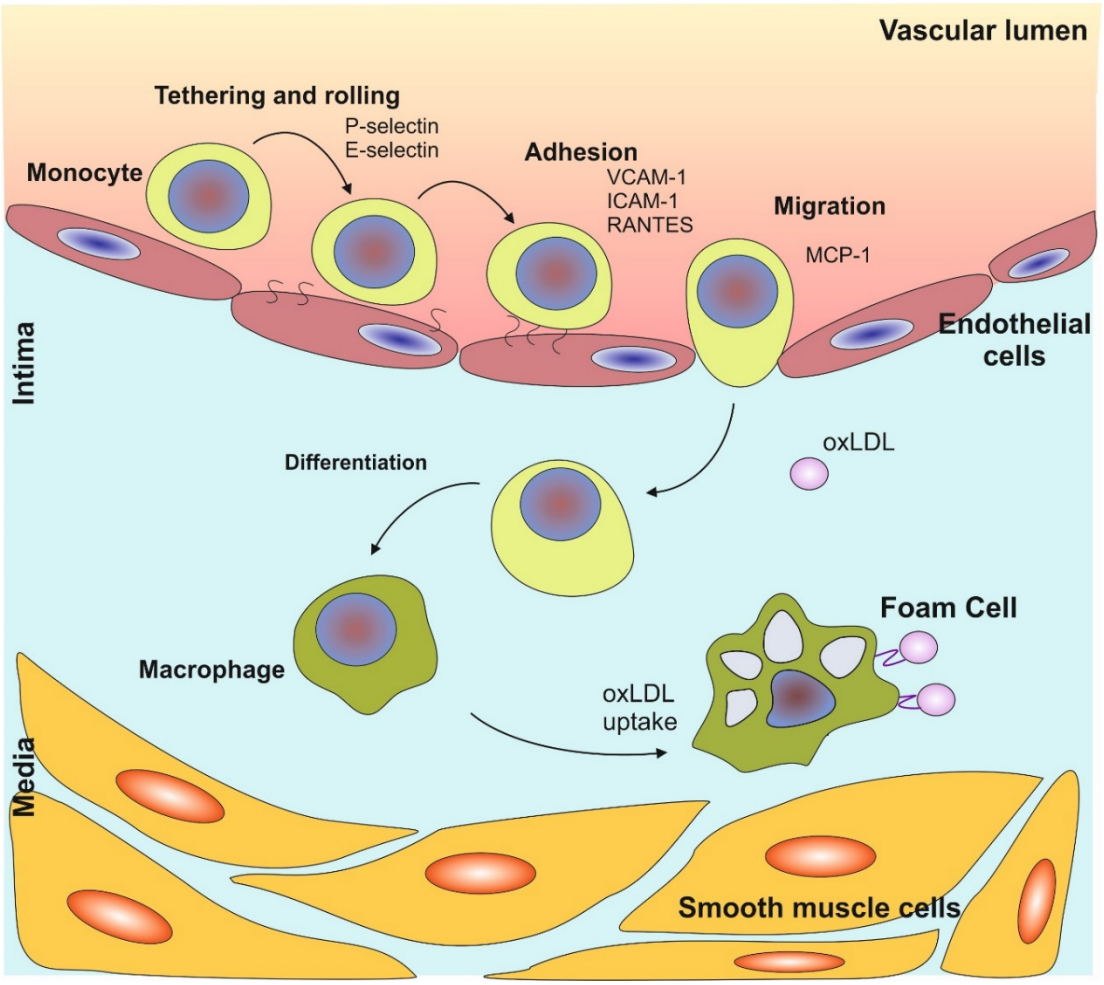

Figure 1.5 Overview of the process of adhesion of monocytes to the endothelium and migration into the sub-endothelial space

Inflamed endothelial cells express cell adhesion molecules and inflammatory cytokines, these recruit monocytes to the site of inflammation. Monocytes then begin to roll along the monolayer forming weak interactions, once firmly attached they begin to cross into the intima where they differentiate into macrophage. These then take up oxidised LDL (oxLDL) becoming lipid-laden or 'foam cells' Baker *et al.* [26].

Leukocyte recruitment involves their rolling, adhesion and migration into the intima controlled by various chemokines and adhesion molecules.

The earliest step of leukocyte transmigration involves tethering to and rolling of leukocytes along the vascular endothelium; initially these molecular interactions are weak and reversible between the endothelial and leukocyte selectins (E-selectin, P-selectin and L-selectin) and their counter ligands, such as PSGS-1 (P-selectin glycoprotein-1) and ESL-1 (E-selectin ligand-1) [27-30]. Leukocyte slowing during the rolling promotes the attachment of specific high affinity G-protein coupled receptors by endothelial cell (EC) activating factors such as chemokines. This strengthens ligand binding of integrins by increased affinity of the molecules [31]. Integrin activation is critical for leukocyte migration, by mediating the firm adhesive interaction of leukocytes to ECs as well as the flattening of leukocytes over the endothelium [23]. The β2 and β1 family of integrins are the major integrins that are involved in this stage of leukocyte/EC interaction. Integrins bind to EC counter ligands such as intercellular adhesion molecule 1(ICAM-1) and vascular cell adhesion protein 1 (VCAM-1). Table 1.3 shows examples of adhesion molecule pairs.

Table 1.3 Examples of adhesion molecule pairs.

Adhesion molecule	Tissue distribution	Ligand
Immunoglobulin superfamily		
ICAM-1	Endothelial cells, monocytes, T and B cells, dendritic cells, keratinocytes, chondrocytes, epithelial cells	LFA-1
VCAM-1	Endothelial cells, kidney epithelium, macrophages, dendritic cells, myoblasts, bone marrow fibroblasts	VLA-4
PECAM-1	Platelets, T cells, endothelial cells, monocytes, granulocytes	PECAM-1, αVβ3
Selectin Family		
E-selectin	Endothelial cells	ESL-1
L-selectin	Lymphocytes, neutrophils, monocytes	CD34
P-selectin	Megakaryocytes, platelets and endothelial cells	PSGL-1

Integrin Family		
VLA-1 to VLA-4	Endothelial cells, resting T cells, monocytes, platelets and epithelial cells	Various molecules including laminin, fibronectin, collagen, and VCAM-1
β1α7	Endothelial cells	Laminin
β1α	Platelets and megakaryocytes	Fibronectin
β2	Widely distributed	Collagen, Laminin, vitronectin
LFA-1	Leukocytes	ICAMs-1 to 3

Once adhered, leukocytes become activated, causing then to de-adhere and migrate through the vessel wall by traversing the EC layer and the basement membrane, two distinct but adjacent and interacting barriers [23]. Migration of leukocytes between ECs is described as diapedesis, and is initiated by the integrin-dependent phase of adhesion [22]. The shedding of L-selectin from the leukocyte must occur for this to happen and this is promoted by the release of certain factors including IL-8. A major leukocyte integrin is leukocyte-function associated antigen (LFA)-1 which binds to ICAM-1 and ICAM-2 and triggers the leukocyte searching of endothelial boundaries [22].

1.2.3 NF-kB

NF-kB is a transcription factor family which modulates several homeostatic cell functions including inflammation. NF-kB is formed from five family member protein monomers (RelA (p65), RelB, cRel, p50 and p52) [32]. It is a key transcription factor involved in inflammatory responses and regulates various genes encodoing pro-inflammatory proteins including adhesion molecules, cytokines and cyclooxygenase-2 (COX-2) [33, 34].

Figure 1.6 NF-kB signalling pathway [40], which is activated by a plethora of stimuli including cytokines, pathogens and oxidants. Under normal conditions NF-kB dimers are inhibited by an inhibitory subunit called inhibitor of NF-kB (IkB) in the cytoplasm. IkBα prevents NF-kB subunits from migrating to the nucleus, displaces them from promoter sites and induces their proteolysis. When the cell is stimulated, IkB kinase becomes activated which acts to liberate NF-kB dimers through phosphorylation of IkBα on Ser32 and 36. These dimers are then free to interact with kB promoter regions in the nucleus and therefore initiate gene transcription.

It is suggested n-3 PUFAs act on the NF-kB pathway to decrease expression of adhesion molecules and production of inflammatory cytokines and COX-2 metabolites [35]. EPA has been shown to decrease phosphorylation of IkB in human monocytes and to decrease endotoxin-induced activation of NF-kB [36, 37]. DHA has also been shown to reduce NF-kB activation in stimulated macrophages [38] and dendritic cells [39] again via decreased IkB phosphorylation [38].

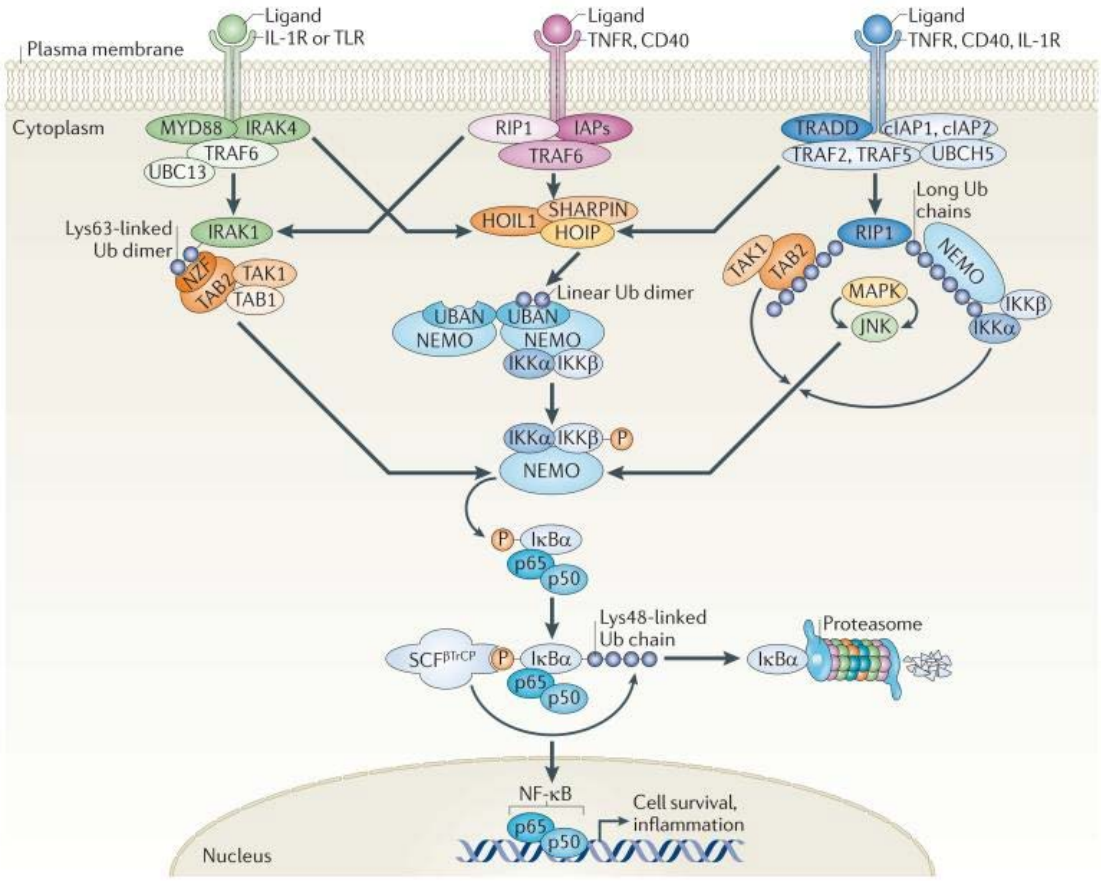

Figure 1.6 NF-kB signalling pathway [40]

1.2.4 Chronic inflammation

Despite inflammation playing a key role in the host's defence, chronic inflammation has been recognised as a contributor to the pathology of many destructive conditions, including rheumatoid arthritis, inflammatory bowel disease, asthma, and psoriasis [22]. Concentrations of inflammatory mediators have been shown to be significantly elevated at sites of disease and in the systemic circulation in these diseases [41].

Recently chronic inflammation has been identified as playing a role in other conditions such as obesity and atherosclerosis. Patients with these conditions have been shown to have moderately elevated levels of inflammatory mediators in the systemic circulation and an infiltration of inflammatory cells within disease sites such as the blood vessel wall and adipose tissue [42]. This inflammation is termed low grade chronic inflammation.

1.2.5 Atherosclerosis

Atherosclerosis, which is a “hardening” (or narrowing) of the arteries, is a major cause of cardiovascular disease (CVD). Inflammation caused by endothelial dysfunction and leukocyte infiltration into the blood vessel wall plays a central role in all stages of atherosclerosis development and progression. Initial lesion development begins with focal endothelial dysfunction [43] induced by chronic inflammatory processes within the arterial wall. Figure 1.7 shows the processes involved in atherosclerosis and the interaction between ECs and inflammatory cells.

Arteries are tubular vessels comprising of 3 layers; an inner endothelium layer, a middle intimal layer and a tunica externa. The outer layer contains connective tissue and the vasa vasorum, the middle layer comprises primarily of smooth muscle cells, and the inner layer vessel is lined by ECs which are in direct contact with free flowing blood [44].

Trapping of low-density lipoproteins (LDL) in the sub-endothelial layer of the arterial wall triggers inflammation. The LDL is retained by the intima by binding to the proteoglycan and undergoes oxidative modification to form oxLDL [45]. oxLDL causes a low-grade chronic inflammatory response in the vessel wall, leading to increased expression of cell-adhesion molecules, chemokines and pro-inflammatory cytokines, causing a cascade of events. Cell adhesion molecules promote the adhesion of blood monocytes to the endothelium and increased secretion of chemokines including MCP-1 which in turn promotes the migration of these monocytes into the intima where they undergo differentiation into macrophages [46]. Macrophages in the intima then proceed to engulf and modify oxLDL through scavenger receptors forming lipid-laden cells termed ‘foam cells’. These cells are characteristic of an atherosclerotic plaque [46]. The chronic inflammatory response is then sustained through the macrophage secretion of various cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-alpha (TNFα). These enhance endothelial permeability, allowing entry of more LDL and monocytes. As the inflammation advances, macrophages and ECs release a variety of peptide growth factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), which act as fibrogenic mediators. These promote smooth muscle

cells to proliferate and migrate to the site of early lesions. Here they form a dense extracellular matrix that acts as a cap which covers the accumulating lesions [47].

Increasing levels of IL-1 and TNFα continue to drive the inflammatory response, and stimulate the production of IL-6. This sustains the inflammation and induces distal inflammatory responses, including hepatic synthesis of acute phase cytokines such as C-reactive protein (CRP) and serum amyloid A (SAA) [44]. Thus, inflammation seems to be central to the initiation and progression of atherosclerosis [48-50]. In accordance with this, a recent meta-analysis showed that risk of mortality from vascular disease is greater in those with a higher CRP concentration [51]. Table 1.4 gives a summary of the key mediators involved the atherosclerotic process.

Advanced atherosclerotic lesions may not be stable. The underlying inflammatory processes act to simultaneously decrease collagen production, which is stabilising, and increase the activity of matrix metalloproteinases which degrade the dense extra-cellular matrix of the lesion cap. This potentially leads to lesion weakness and cap rupturing. Plaque rupture exposes the pro-thrombotic plaque interior to blood and initiates platelet aggregation and clot (thrombus) formation. IL-6 can also contribute to thrombus formation through stimulation of expression of the potent procoagulant tissue factor [47, 52]. Extensive clotting may cut off blood flow to the affected organ initiating a cardiovascular event (e.g. myocardial infarction, stroke) and may be fatal.

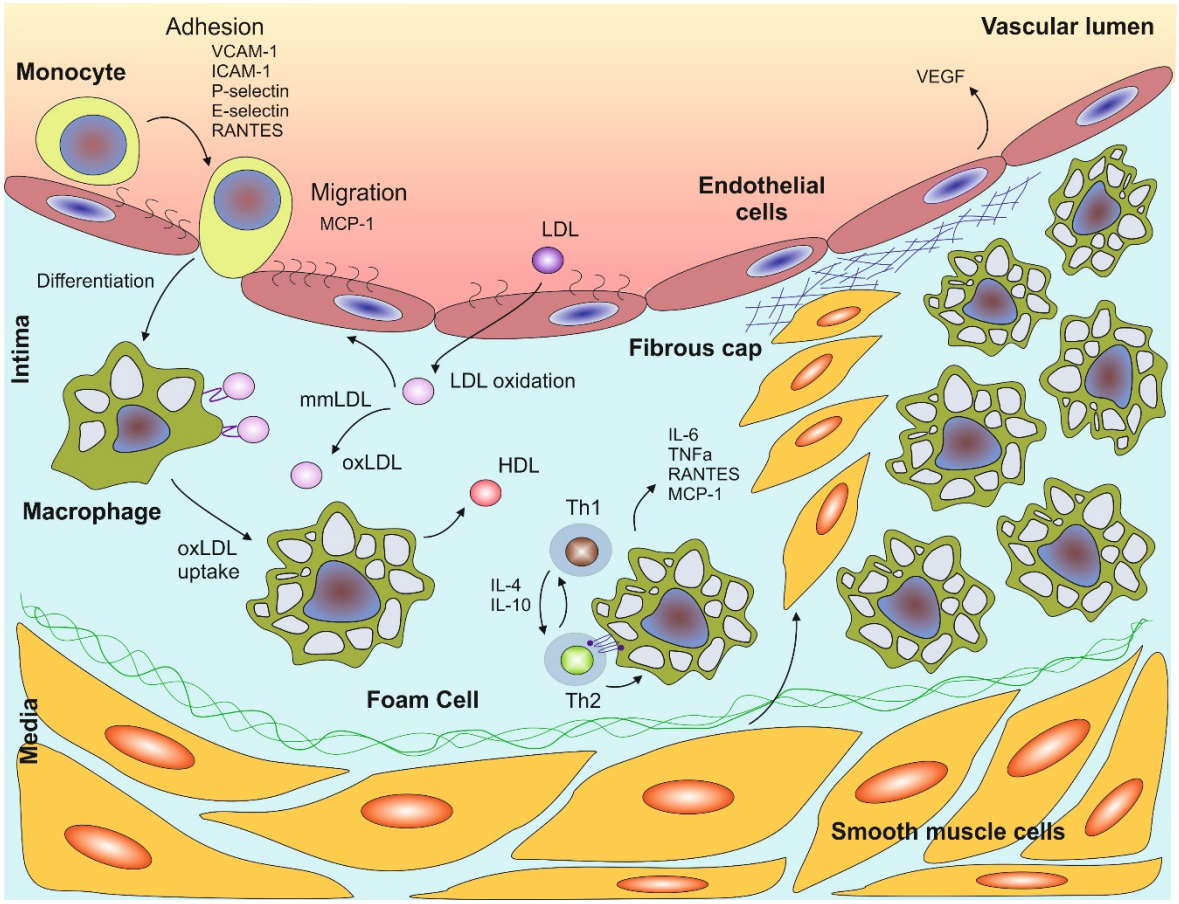

Figure 1.7 Events in the development of an atherosclerotic plaque

Monocytes adhere to endothelial cells expressing adhesion molecules and begin to migrate across the endothelial monolayer into the initima where they differentiate into macrophages. LDL is oxidised within the intima forming oxidised LDL (oxLDL). oxLDL is then taken up by the macrophages via scavenger receptors forming lipid-laden 'foam cells'. Macrophages exert both pro-atherogenic effects causing movement of smooth muscle cells from the media which begin to secrete extracellular matrix proteins. This then forms a fibrous plaque.

Table 1.4 Summary of key inflammatory mediators involved in atherosclerosis

Mediator	Abbreviation	Source	Target cells and Effects Relevant to Atherosclerosis
Interleukin 6	IL-6	T cells, B cells, Macrophages, Fibroblasts, Smooth muscle cells, Endothelial cells	Proliferation and migration of smooth muscle cells via mediating VEGF and TNFα effects
Interleukin 8	IL-8	Macrophages, Smooth muscle cells, Endothelial cells, Monocytes, T cells	Induces chemotaxis in target cells and is a promoter of angiogenesis
Interleukin 1	IL-1	Macrophages, Lymphocytes, Endothelial cells, Smooth muscle cells	Stimulates endothelial and smooth muscle cells through NF-kB and MAPK pathways [53]
Interferon γ	IFN-γ	Th1 cells, Natural killer cells, Sooth muscle cells	Promotes Th1 immune response/secretion of Th1-associated cytokines, inhibits extracellular matrix synthesis by smooth muscle cells [53]

Tumour necrosis factor α	TNFα	T cells, B cells, Monocytes, Macrophages, Smooth muscle cells, Natural Killer cells	Stimulates expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, E and P-selectin and MCP-1 through NF-kB pathway [54] and thus promotes chemotaxis and adhesion
TNF related apoptosis-inducing ligand	TRAIL		Promotes smooth muscle cell proliferation and neointima formation after arterial injury [55]
Vascular endothelial growth factor	VEGF	Endothelial cells, Macrophages, Platelets	Stimulates proliferation and survival of endothelial cells and promotes angiogenesis and vascular permeability [56]
Monocyte chemoattractant protein-1	MCP-1	Endothelial cells, Fibroblasts, Smooth muscle cells	Chemoattractant and activates monocytes and T cells. Responsible for the transmigration of monocytes into the intima at sites of lesion formation [57].

Regulated on activation, normal T expressed and secreted	RANTES/CCL5	Expressed in atherosclerotic plaque, Adipose tissue	Chemoattractant towards monocytes and memory T cells. Adhesion of monocytes to the luminal surface of the endothelium [58].
Intercellular adhesion molecule 1	ICAM-1	Endothelial cells, Leukocytes	Adhesion of monocytes to the luminal surface of the endothelium [58]
Vascular cell adhesion molecule 1	VCAM-1	Endothelial cells	Mediation of leukocyte-endothelial cell adhesion and signal transduction as well as augmenting the production of ICAM-1. It may also have a role in the monocyte transmigration into atherosclerotic plaques [58]
E-selectin	E-selectin	Endothelial cells	Rolling and recruitment of leukocytes [58]
P-selectin	P-selectin	Endothelial cells – Weibel Palade bodies, Platelets	Rolling and recruitment of leukocytes [58]

L-selectin	L-selectin	Leukocytes (neutrophils, monocytes, subsets of T cells)	Tethering, rolling of leukocytes [59]
Platelet and endothelial cell adhesion molecule	PECAM-1	Leukocytes, Endothelial cells	Stimulates diapedesis of leukocytes [59]

1.2.6 Functions of fatty acids in inflammatory processes

1.2.6.1 Saturated fatty acids

SFAs are distributed across cell membrane phospholipids which can contain high levels of palmitic and stearic acids. Gangliosides often have high levels of stearic acid and SFAs are also found in ceramides, sphingolipids and diacylglycerols which are all signalling molecules [1].

Plasma membrane microdomains known as lipid rafts are generally dense in phospholipids and sphingolipids containing SFAs. These domains have been shown to act as signalling platforms, and therefore the makeup of these will influence cell signalling and function [60].

Fatty acylation of proteins is a way in which FAs may exert their function; this mechanism helps to both traffic proteins among different organelles and to anchor proteins to the inner leaflet of the plasma membrane [61, 62]. Both palmitic and myristic acids can covalently modify certain proteins involved in signalling. Palmitic acid links to internal cysteine residues via thioester bonds [62] and myristic acid links via an amide bond to an N-terminal glycine residue of the substrate protein [61].

SFAs actions on transcription factors can influence gene expression. Sterol response element binding proteins (SREBPs) are a family of transcription factors which regulate at least 30 genes involved in synthesis and metabolism of FAs, TAG phospholipids and cholesterol [63]. As well as genes for low-density lipoprotein (LDL) receptor, fatty acid synthase and stearoyl-CoA desaturase, SREBPs also regulate genes in the liver involved in the packaging of lipids and lipoproteins for export into bile. It has been reported that certain SFAs of 10-18 carbon chain length can upregulate levels of mRNA for PGC-1α a cofactor for these SREBPs, therefore having the ability to modulate lipid metabolism [64]. SFAs are also known to bind to the transcription factor hepatocyte nuclear factor 4 (HNF4) [65] and may also act as coactivators of the heterodimeric nuclear receptor liver X receptor/retinoic acid X receptor (LXR/RXR) [66] which promote hepatic secretion of very low density lipoproteins (VLDL).

Cell culture models have demonstrated induced activation and translocation of the inflammatory transcription factor NF-kB by palmitic and lauric acids [38, 67]. This promotes upregulation of expression of several genes involved in inflammation including various pro-inflammatory cytokines and COX-2. It is

suggested that the actions of SFAs on the NF-kB pathway may involve toll-like receptor 4 (TLR4) activation, as it is demonstrated that SFAs induced lipid raft arrangement can facilitate TLR4 signalling [1, 68].

Thus, SFAs play an important role in normal cellular function. As described above they influence cell signalling in many cell types through their roles in lipid rafts, phospholipids, sphingolipids, gangliosides and in covalent modification of proteins. They also influence FA, cholesterol and TAG biosynthesis as well as inflammatory processes through effects on regulation of transcription factors involved in lipid metabolism and inflammation. Through these actions they can also affect various factors in determining risk of cardiometabolic disease. SFA intake has been linked to increased risk of cardiovascular disease; this effect may in part be due to upregulation of inflammation by SFAs.

1.2.6.2 EPA and DHA

EPA and DHA predominately exert their functional effects through incorporation into cell membrane phospholipids [69, 70].

The incorporation of PUFAs into phospholipids have been shown to disrupt membrane order which leads to greater membrane fluidity [71]. As a result they can also influence the activity of membrane bound proteins such as signalling enzymes, receptors and ion channels [72] leading to modulation of cell responses. EPA and DHA have been shown to modify raft platforms leading to modulation of intracellular signalling pathways, altered transcription factor activation, and, ultimately, different gene expression patterns [72]. Regulation of inflammation by EPA and DHA may be due to effects on transcription factors such as NF-kB [36], and peroxisome proliferator-activated receptors (PPAR) [73].

Higher quantities of EPA and DHA within cell membrane phospholipids can also influence inflammatory responses through modification of lipid mediator production. Healy *et al* demonstrate that dietary supplementation of EPA and DHA lead to increases of both these n-3 PUFAs within neutrophil phospholipids, partially at the expense of AA [74]. This can change inflammatory responses since EPA can be metabolised by the same enzymes as AA generating an alternative series of bioactive lipid mediators. AA is the dominant substrate for synthesis of eicosanoids – these include prostaglandins, leukotrienes and thromboxanes (Figure 1.8). These eicosanoids play a role in inflammatory regulation, platelet

aggregation and immunity [75]. Eicosanoids are produced via the COX and lipoxygenase (LOX) pathways. When there are higher levels of EPA and DHA within cell membrane phospholipids it is has been shown that there is a decrease in production of the AA derived eicosanoids and an increase in the EPA and DHA derived mediators [1]. These alternative mediators act in a less inflammatory or perhaps even an anti-inflammatory manner.

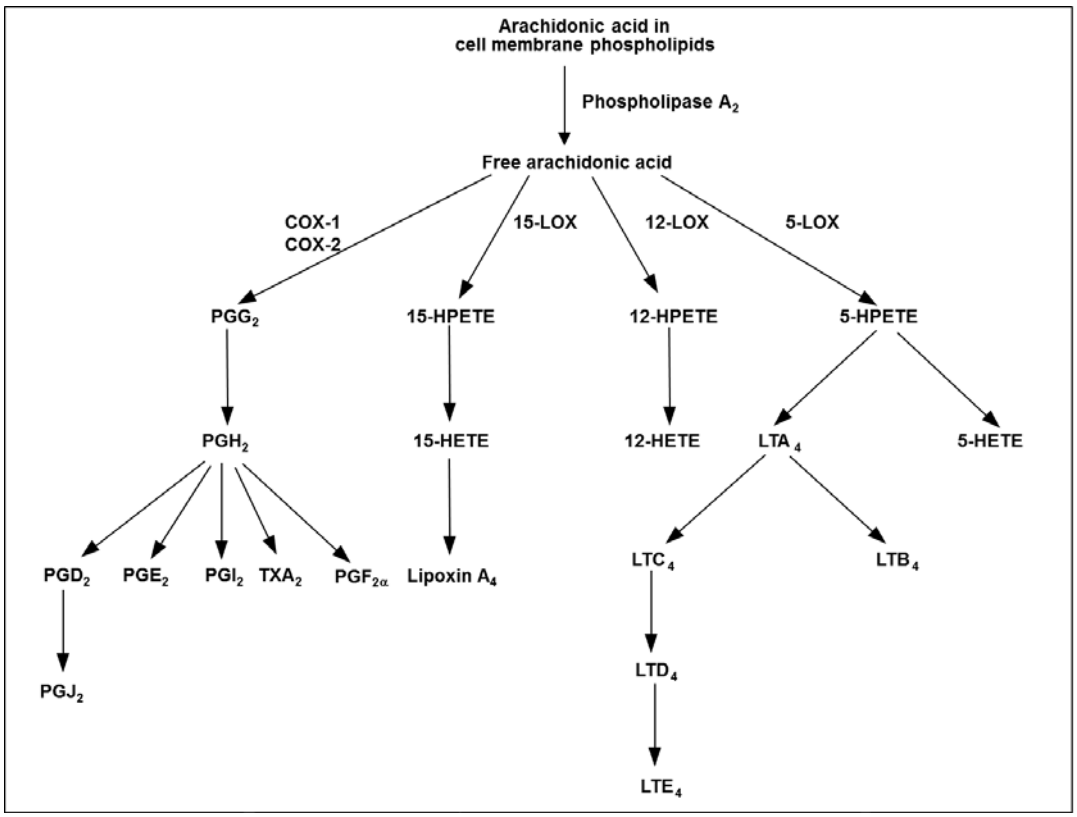

Figure 1.8 Overview of the pathway of eicosanoid synthesis from arachidonic acid [76].

EPA and DHA are also substrates for the generation of resolvins, protectins and maresins. EPA derived mediators are termed E-series resolvins and are formed via a series of reactions involving COX-2 and 5-lipoxygenase (Figure 1.9). DHA derived resolvins are termed D-series resolvins and are derived via LOX pathways. DHA is also the substrate for synthesis of protectins and maresins. These EPA and DHA derived mediators have both been shown to exert anti-inflammatory effects in cell culture and animal models [77-79]. An example of the inflammation resolving properties of these substrates are the inhibition of transendothelial migration of neutrophils by resolvin E1, resolvin D1 and protectin D1 [78, 79].

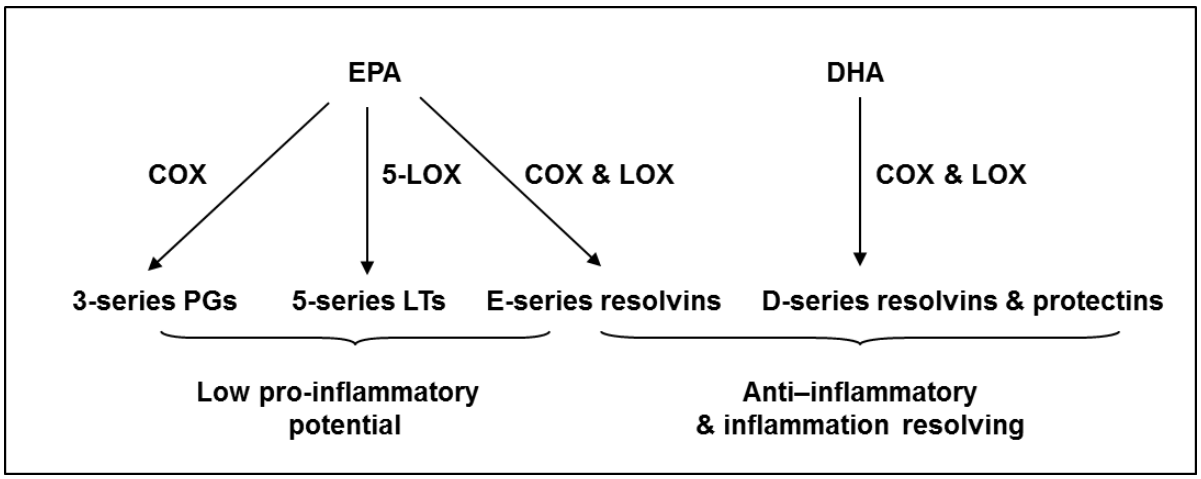

Figure 1.9 Outline of the pathway of resolvin and protectins produced by EPA and DHA via LOX and COX.

The way in which these alternative mediators act to modulate and resolve inflammatory and immune function may be a predominant activity of EPA and DHA.

Finally, free EPA and DHA can also act to modulate inflammation through binding to plasma membrane bound and cystolic receptors [80]. The G-protein-coupled receptor GPR120 has been shown to have a greater affinity for long chain n-3 FAs compared to other FAs [81]. GPR120 is expressed on the surface of macrophages and adipocytes [80].

1.3 Long chain n-3 fatty acids in CVD

The findings of many epidemiological studies and randomised control trials (RCTs) demonstrate a positive association between consumption of VLC n-3 PUFAs, specifically EPA and DHA, and long-term health benefits [82], including a reduction in cardiovascular disease (CVD) morbidity and mortality [83-85], better visual and neurological development [86] and improvements in inflammatory conditions including arthritis [87] and asthma [88]. Such effects may be mediated by modification of the biophysical properties of cell membranes [89-92], changes in specific cell signalling pathways and altered gene expression [93, 94]. The primary dietary source of EPA and DHA is seafood especially oily fish, although they are found in lower amounts in many other foods of animal origin.

The first evidence of the efficacy of EPA and DHA in CVD originated from early epidemiology studies carried out among the Greenland Inuits, native Alaskans and the Japanese, who all consumed high levels of EPA and DHA. These studies consistently showed an inverse relationship between incidence of CVD and other chronic diseases and the amounts of LC n-3 PUFAs within the diet, leading to the theory that dietary marine n-3 PUFAs may be protective against chronic diseases including CVD [95, 96]. Many of the conditions which n-3 PUFAs protect against, including CVD, have an inflammatory component, suggesting that the anti-inflammatory effects of EPA and DHA might be important in disease prevention.

1.3.1 Evidence

The ability of n-3 PUFAs to down-regulate various inflammatory mechanisms suggests they may be significant in controlling development and severity of inflammatory diseases and that they may also be able to alleviate some inflammatory conditions.

One such disease which is influenced by a (low grade) inflammatory state is CVD. Following an observed lower risk of CVD in populations where high levels of EPA and DHA are traditionally consumed such as Greenland, Alaska and Japan, studies were carried out to examine the effect of fish, fatty fish and n-3 PUFAs on those who consumed a ‘western diet’, looking at various cardiovascular risk factors, such as blood lipids, blood pressure and thrombosis, and on disease outcomes, such as coronary heart disease rate and mortality, cardiovascular mortality, myocardial infarction and stroke incidence [97, 98].

Meta-analyses investigating coronary heart disease have been carried out on these studies. He *et al.* analysed frequency of fish consumption and relative risk of coronary heart disease mortality among 13 cohorts and 11 independent studies. They reported a significantly reduced risk of developing coronary heart disease (by 38%) in those who ate more fish portions, up to 5 per week, compared to those who ate less than 1 portion of fish a month [99].

In another meta-analysis He *at al* analysed data from 9 cohorts and 8 independent studies where they examined fish consumption on stroke incidence. Again higher fish consumption was associated with a decreased risk of stroke, in those eating >5 portions of fish a week there was a 31% reduction in risk compared to those who rarely or never ate fish [100].

Chowdhury *et al*. also concluded higher consumption of EPA and DHA was associated with lower risk of coronary disease [101].

Several intervention studies have been carried out in individuals who have existing CVD examining the effect of n-3 PUFA supplementation, with varying results reported.

The Diet and Reinfarction Trial (DART) was the first controlled trial that examined the effect of dietary fish (~300 g/week) or fish oil (500-900 mg EPA + DHA/day) on the secondary prevention of myocardial infarction. A total of 1015 men who had survived a myocardial infarction had a supplemented diet of either 2 portions of fatty fish or fish oil a week. Compared to the control group there was a 29% reduction in all-cause mortality risk during the first 2 years [102].

The GISSI-Prevenzione trial examined 11,324 patients who had recently survived a myocardial infarction [103]. Subjects were randomised into 4 diet groups; n-3 PUFAs (850 mg EPA + DHA (1:2 ratio EPA/DHA)), n-3 PUFAs with vitamin E (300 mg), vitamin E or no supplementation. It was found that n-3 PUFAs significantly decreased the primary endpoint, which was non-fatal myocardial infarction, stroke and death, by 15%. There were also significant reductions in the secondary outcomes.

The GISSH-HF study examined the effect of n-3 PUFA supplementation on mortality from heart failure; 7,000 patients were randomised into 2 groups, 850 mg EPA + DHA /d or control (placebo). A reduction of 9% in all-cause mortality was observed in the n-3 PUFA supplemented group [104].

Another study which tested the hypothesis that EPA could be an effective preventative measure against major coronary events was the Japan EPA lipid intervention study (JELIS). The intervention was carried out in 18,645 hypercholesterolaemic Japanese patients taking statins. They observed a reduction of 19% in risk of mortality in groups which were supplemented with 1.8 g/d EPA plus 5 mg/d simvastatin or 10 mg/d pravastatin compared to statins alone [84].

These large RCTs indicate positive outcomes of n-3 PUFA supplementation on CVD mortality. However some recent trials describe a lack of effect of n-3 PUFAs on the same CVD outcomes.

The Alpha Omega trail examined the effect of supplementation with a fortified margarine for 40 months in 4,837 patients who had survived a myocardial infarction. Two of the margarines provided 400 mg/d EPA + DHA, a lower dose than used in the positive studies described above. EPA + DHA had no effect on rate of major CVD events [105].

The SU.FOLOM3 trial supplemented 2,501 patients who had history of myocardial infarction, ischaemic stroke or unstable angina with folate B vitamins and/or 600 md/d EPA +DHA, again a fairly low dose compared with many other studies. It was reported that supplementation with n-3 PUFAs had no significant effect on fatal and non-fatal CVD events [106].

Lastly the Omega trial followed a year long supplementation of 900 mg/day EPA + DHA in 3,851 patients who had survived myocardial infarction [107]. The supplementation was shown to have no effect on major CVD events, total mortality or sudden cardiac death.

However these newer studies show some limitations, including low sample size and using low doses of EPA and DHA. Yates *et al*. also suggest that background medication may influence the efficacy of n-3 PUFAs in secondary prevention of CVD events and mortality [108].

Though CVD encompasses many conditions the most common underlying cause is the formation of atheromatous plaques. Libby *et al*. describe these plaques as having lipid and inflammatory components [109]. As described previously, they develop when ECs become dysfunctional and promote an influx of leukocytes, especially monocytes, into the intimal layer of the artery wall. Many studies have examined the effect of EPA and DHA on endothelial and leukocyte function. These

studies have examined the effects on adhesion molecule expression and on production of cytokines involved in inflammation. Several cytokines which induce EC adhesion molecules involved in leukocyte recruitment can be measured as markers of anti-inflammatory properties of n-3 PUFAs. These studies have examined several different exposure durations and concentrations of n-3 PUFAs with different stimuli.

De Caterina *et al*. describe reduced E-selectin and VCAM-1 protein and mRNA levels in human saphenous vein endothelial cells (HSVEC) treated with 10 μM DHA for 24 hr prior to 6 hr TNFα stimulation compared to TNFα stimulation alone [110]. Another study reported that 65 μM DHA and EPA decreased IL-Iβ induced ICAM-1, VCAM-1 and E-selectin mRNA in human umbilical endothelial cells (HUVEC) [111]. Wang *et al.* also saw inhibition of TNFα induced VCAM-1 and ICAM-1 protein expression in human aortic endothelial cells (HAEC) after exposure to 20-160 μM DHA [112].

Leukocyte adhesion assays can examine the significance of the modulation of adhesion molecules expressed by ECs. In vitro adhesion assays carried out in the absence of hemodynamic factors imposed by flow demonstrate that DHA inhibits the adhesion of both monocytes [112] and neutrophils [113] to ECs. Yates *et al.* also describe DHA and EPA as modulating different stages of leukocyte recruitment. DHA inhibited E-selectin surface expression after TNFα stimulation and therefore reduced the recruitment of neutrophils from flow. EPA however did not affect neutrophil recruitment, but adherent neutrophils were unable to migrate across the EC monolayer efficiently [114]. Since EPA and DHA have been described as modulating both ECs and leukocyte function this may be their mechanism of action in CVD risk reduction.

1.3.2 Recommendations for intake of fish, fatty fish and long chain n-3 PUFA

Due to the significant health benefits provided by the n-3 PUFAs EPA and DHA, outlined above (section 1.3.1) there is potential for these FAs to be used both in a therapeutic setting but also for general health, and so various authorities have made intake recommendations. In the UK, The Committee on Medical Aspects of Food Policy (COMA) and Scientific Advisory Committee on Nutrition (SACN) recommend consumption of two portions of fish per week, one of which to be oily, providing the equivalent of approximately 450 mg/day n-3 PUFAs (SACN 2004/COMA) [16]. The current Dietary Guidelines for Americans 2015 (DGA)

recommend consumption of 230 g of cooked seafood per week, including fish that is rich in n-3 FAs, such as salmon [115]. The American Heart Association (AHA) recommends the consumption of at least two 100 g portions of fish a week, particularly oily fish (e.g. Salmon, mackerel or herring) for risk reduction of CVD [97]. For those with established CVD, the recommendation is for approximately 1 g of EPA and DHA daily, preferably from oily fish. European recommendations for n-3 PUFAs and fish, primarily for the prevention on CVD, are approximately 250–500 mg/day preferably through consumption of oily fish [116].

1.4 Sustainability

The importance of the LC n-3 PUFAs in human health means there is increasing demand for fish and fish-derived products within the human diet, and the evidence suggesting that LC n-3 PUFAs are beneficial for certain aspects of cardiovascular health has meant n-3 LC PUFAs therapies have expanded rapidly. The suggested recommendations of between 250 and 1000 mg/day of EPA and DHA, as summarised above (section1.3.2) cannot be met from the current global supplies of EPA and DHA [117] .

Phytoplankton are responsible for biosynthesising EPA, DHA and other LC FAs. These are then consumed by zooplankton and from here enter the marine food chain. The principal sources of dietary EPA and DHA in humans are fish and fish oil supplements. The total estimated global production of EPA and DHA is around 530,000 metric tons (The Global Organisation for EPA and DHA n-3s (GOED)). Only a portion of this, however, contributes to human usage with the total available EPA/DHA for human consumption being only 200,000 metric tons which is 15% of the calculated global human requirement (1.3 million metric tons) based on recommendations for intake [117] .

Approximately 75% of the global wild fish stocks are rated as fully overexploited [118]. Fish farming (aquaculture), now provides over half of all fish for human consumption, according to the United Nations Food and Agriculture Organisation (FAO), and this needs to expand in order to meet the needs of humans. However, aquaculture faces problems such as disease, and the build-up of carbon dioxide in the aquaculture systems. Dealing with these issues causes an annual losses of billions of pounds. Aquaculture systems also use fish oil, which adds to the increasing burden on fish and fish oil stocks. Decreased dependence on fish oil in

aquaculture results in lower levels of EPA and DHA within the farmed fish, meaning they are less nutritious to humans.

Furthermore, fish oil product quality is dependent on the season and location of the fish from which the oil is derived, and can be affected by ocean pollution. Many consumers are worried about the build-up of chemicals, such as mercury, in some types of fish and what harm this can cause. This can be off putting leading to people choosing not to eat fish.

Others simply do not find fish palatable, or have a vegan or vegetarian diet and therefore do not eat fish. This group of people would benefit from a more readily viable alternative plant source.

Non-fish sources of supplemental EPA and DHA are fermented algal oils and krill oil [117]. However these contribute a very small percentage of total available EPA and DHA. There are arguments that krill harvesting and krill oil production are not environmentally friendly [119].

The lack of readily available sources of preformed EPA and DHA to meet the needs for human nutrition indicates a very clear demand for alternative sources of n-3 FAs and perhaps other FAs that have similar biological properties. Plants may provide a sustainable alternative source of bioactive FAs that can support human health.

1.4.1 Alternative sources of bioactive fatty acids

There are new sources of n-3 PUFAs including marine algal or algae-like microbial oils, which could eliminate many of the taste and odour problems associated with fish and avoid the environmental issues with the fish oil-based processes. For some time *Crypthecoduinium cohnii* has been used as a source of DHA for infant formula [120]. Another algal source, S*chizochytrium,* produces both EPA and DHA and is currently used to create commercial oils for the food market an use as supplements [121]. Algal sources are both environmentally friendly and without ocean borne contaminants, though at present they represent <2% of human EPA+DHA consumption [117], possibly as refining of algal oils is still an expensive process.

Another alternative includes n-3 PUFAs derived from plants, including ALA and SDA. ALA is a metabolic precursor of EPA and DHA, whilst SDA is an intermediate on this same pathway (Figure 1.3).

Sources of ALA include green plant tissues, various nuts (e.g. walnuts), rapeseed oil (also known as canola oil), soybean oil (in which ALA contributes 10% of total FAs), and flaxseeds and flaxseed oil (in which ALA contributes > 50% of total FAs) [21]. ALA is the predominant n-3 PUFA consumed by those who do not regularly take VLC n-3 PUFA supplements or eat oily fish. Consumption of ALA in Europe, Australia and North America typically ranges from 0.6 to 2.3 g/d in adult men and 0.5 to 1.5 g/d in adult women [116, 122-126]. Despite a higher dietary intake of ALA relative to EPA and DHA (approximately 25- and 15- fold greater [122]), concentrations of ALA within plasma and cell and tissue lipids are lower than those of EPA and DHA, apart from in adipose tissue stores [21].

There are few natural sources of SDA; it is found in Echium oil, where it contributes about 9-16% of FAs [127-129]. Levels of SDA can be substantially increased in soybean oil by genetic modification [130]. SDA is potentially a better substrate than ALA for the biosynthesis of VLC n-3 PUFAs. SDA levels in human blood, cells and tissues are normally very low.

These precursor FAs represent potentially safe and sustainable sources of health benefiting n-3 FAs.

Plants can also provide n-6 PUFAs which may have beneficial properties. These include GLA.

GLA is found in some leafy green vegetables, vegetable oils and nuts, including: evening primrose (*Oenothera beinnis*) oil [131] and borage (*Borage officinalis*) oil where is comprises up to 25% of total FAs [132]. It is also found in hemp seed, oats and barley. GLA is found on the same metabolic pathway as LA and can be synthesised de novo from LA. GLA can be further elongated to produce DGLA. DGLA is a precursor of eicosanoids via COX and LOX oxidation, producing series 1 prostaglandins (prostaglandin E1(PGE_1)) and thromboxanes as well as series 3 leukotrienes [133]. PGE_1 has been found to exert anti-inflammatory properties including inhibition of smooth muscle cell proliferation associated with atherosclerotic plaque development [134, 135].

Another FA is pinolenic acid (PIN) which is found in pine nut oil (present at about 15% of FAs) [136]. PIN is an isomer of GLA and is part of a group of unusual PUFAs known as poly-methylene-interrupted polyunsaturated fatty acids (PMI-PUFA). These PMI-PUFAs contain two or more double bonds of the *cis*-configuration separated by at least one polymethylene group [137]. There has been little exploration into the functionality and potential benefits of these types

of FAs. Szu-jung Chen *et al*. describe incubation of microglial cells with PIN to reduce several LPS stimulated pro-inflammatory mediators including IL-6, prostaglandin E_2 (PGE_2) and TNFα [137].

The latter of these FAs have been less explored and so may present an alternative source of biologically beneficial PUFAs.

1.5 Aim and Objectives

Inflammation plays a key role in the progression of atherosclerosis and CVD. N-3 PUFAs from marine sources have been shown to reduce both inflammation and cardiovascular mortality. Due to these health benefits various bodies have recommended an intake of oily fish once to twice a week. Global supplies of fish are not sufficient to achieve this intake and so a sustainable source of health benefiting PUFAs need to be found. Plants provide some n-3 and n-6 PUFAs that may be of human health benefit but their effects on inflammation have been under explored.

This project aims to further explore the anti-inflammatory effects of plant-derived 18-carbon PUFAs using an inflammatory cell model. It compares the anti-inflammatory effects of marine-derived n-3 PUFAs (EPA and DHA) to those of plant-derived PUFAs (ALA, SDA, GLA and PIN) using an EC line, with an emphasis on the inflammatory responses involved in atherosclerosis development. Further exploration will be carried out to investigate the possible mechanisms involved. The overall objective of the project is to identify plant-derived PUFAs that may be sustainable sources of anti-inflammatory FAs to replace EPA and DHA in the promotion of human health.

Figure 1.10 depicts the structures of plant-derived FAs used in this research (ALA, SDA, GLA and PIN) alongside the structures of the marine-derived EPA and DHA.

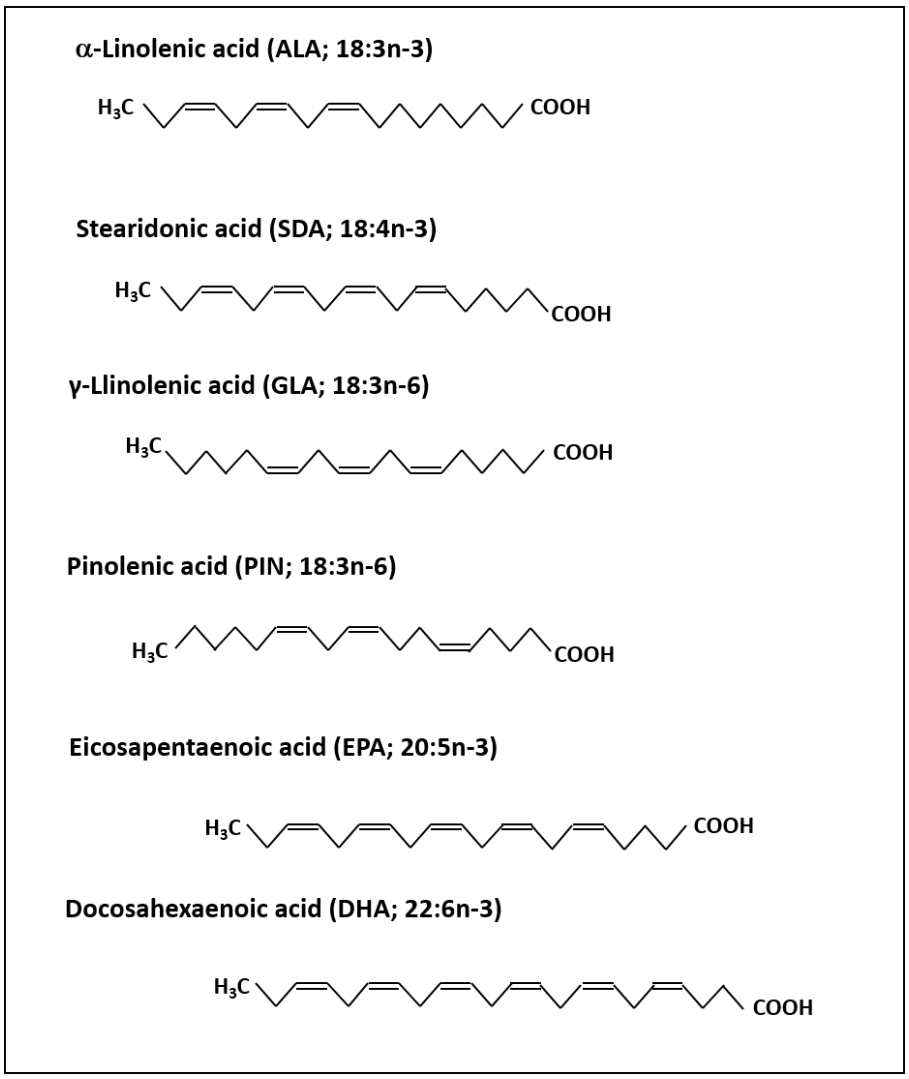

Figure 1.10 Chemical structure of FAs to be used in this project

Chapter 2: Establishing an Inflammatory Endothelial Cell Model

2.1 Introduction

ECs play a crucial role in inflammation related to the vessel wall as they are the main barrier of exchange and contact between the blood and the tissue. The adhesion of leukocytes to ECs influences atherosclerosis development and progression (section 1.2.5). Adhesion molecules such as VCAM-1, ICAM-1 and E-selectin are all critical in the tethering, activation, adhesion and transmigration of leukocytes across the endothelium monolayer. Under inflammatory conditions, monocyte recruitment to the endothelium is enhanced by upregulated expression of these adhesion molecules and increased production of other inflammatory mediators, such as MCP-1, IL-8 and RANTES [44]. VEGF plays a role in endothelial proliferation and promotes vascular permeability and angiogenesis (Table 1.4), which may be necessary for plaque formation [138, 139]. Increased VEGF induces IL-6 production which then enhances migration of smooth muscle cells and therefore atherosclerotic plaque development (Table 1.4). Others have also shown VEGF to promote and alter the rate of atherosclerotic plaque development [140].

Of these proteins, ICAM-1 and VCAM-1 are both reported to be constitutively expressed by ECs [141] and their expression can be up-regulated by cytokines and other inflammatory stimuli. It has been reported that pro-inflammatory cytokines such as IL-1β and TNFα markedly increase the expression of these adhesion molecules [141] and the enhanced expression is dependent on both mRNA and protein synthesis [142].

Development of atherosclerosis is closely linked to EC function and responses, and endothelial dysfunction is recognised as the first step in the atherosclerotic process, including the development of atherosclerotic plaques. HUVECs have played a major role as a model system for the study of regulation of EC function and are widely used. These cells originate from the umbilical vein which carries oxygenated blood and therefore is much like an artery. Also a similar pattern of adhesion and diapedesis of leukocytes to both arterial endothelial monolayers and HUVECs is seen under both static and flow conditions [110, 143, 144]. Therefore HUVECs provide an appropriate model for the investigation of arterial disease.

EA.hy926 cells are an immortalised HUVEC cell line generated in 1983 by fusion of primary HUVECs with cell line A549 (human lung epithelial cells) [145, 146]. EA.hy926 cells are frequently used and have been extensively characterised, showing them to possess many of the innate properties of primary HUVECs [147].

TNFα has been shown to be crucially involved in the pathogenesis and progression of atherosclerosis. In the vasculature, TNFα alters EC function as well as EC-leukocyte interactions [148], likely through increases in the expression of many pro-inflammatory genes and rapid increases in cell adhesion molecules [149]. TNFα is used frequently as a stimulus in inflammatory EC models, in order to explore the effects of different interventions [110, 150-152].

As mentioned above, IL-6, IL-8, VEGF, RANTES, MCP-1 and ICAM-1 are all involved in the development and progression of atherosclerosis. It was therefore chosen to examine the effect of various FAs on these mediators in EA.hy926 cells. The literature also suggests EA.hy926 produce the majority of these mediators [153-155], which have also been described to increase in after TNFα stimulation [154, 155].

In the experiments described in this chapter the effect of TNFα at various concentrations on the secretion of a variety of inflammatory mediators by EA.hy926 was explored. By inducing an inflammatory response in EA.hy926 cells using TNFα, pro- and anti-inflammatory effects of FAs could then be examined.

2.2 Aim and Objectives

The aim of the research described in this chapter was to optimise the conditions for studying inflammatory responses of cultured EA.hy926 cells.

The specific objectives were to:

- Use the pro-inflammatory cytokine TNFα to induce an inflammatory response in cultured EA.hy926 cells
- Identify the effects of different doses of TNFα on production of an array of different inflammatory markers over time, including adhesion molecules, chemokines, and cytokines
- Use an optimised model to examine the appearance of mRNA for PPARα, PPARγ, NF-kB and MCP-1 and the upregulation of NF-kB and COX-2 protein following TNFα stimulation of cultured EA.hy926 cells

2.3 Methods

2.3.1 Reagents

The reagents and materials used for these experiments are described in Appendix A.

2.3.2 Endothelial cell culture

The EC line EA.hy926 (ATCC CRL-2922) was obtained from ATCC. The cells were thawed and subcultured following the supplier's instructions. Briefly cells were washed once in Dulbeccos's Modified Eagle's Medium (DMEM) and resuspended in 5 mL culture medium, which consisted of DMEM supplemented with 10% FBS, glucose (4500 mg/L), L-glutamine (0.4 mM), penicillin, streptomycin and HAT (100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine). Cells were cultured at 5-7 x 10^5 cells/ml and maintained at 37°C in 5% CO_2. Prior to use in experiments, cells were grown in T-175 flasks until confluent.

2.3.2.1 Inflammatory mediator production

Cells from confluent cultures were scraped from culture flasks and resuspended in culture medium (section 2.3.2) at a density of 2 x 10^5 cells per mL. Cells were seeded at 1 x 10^4 cells per well in 96-well flat bottom plates and incubated for 48 hours at 37°C. Following incubation, supernatant was removed and replaced with new medium and cells were stimulated with TNFα at different concentrations (0.1, 1, 10 and 100 ng/mL) for different durations (2, 4, 6, 12, 24 and 48 hours). TNFα concentrations used were based upon those reported in the literature. At each time point supernatant was removed and stored at -20°C until analysis (see section 2.3.3).

2.3.2.2 Gene expression

Confluent EA.hy926 cultures were scraped from flasks and resuspended in culture medium at a density of 5.5 x 10^5 cells per mL. Cells were seeded at 5.5 x 10^4 cells per well in 6-well flat bottom plates, and incubated for 48 hours at 37°C with DMEM, followed by incubation with either DMEM or TNFα at 1 ng/mL for different durations (30 minutes, 1, 2, 6 and 9 hours). At each time point, cells were removed, resuspended in BL-TG buffer following ReliaPrep™ RNA Cell Miniprep System instructions and stored at -80°C until analysis (see section 2.3.4).

2.3.2.3 Protein expression

Confluent EA.hy926 cultures were scraped from flasks and resuspended in culture medium at a density of 5.5 x 10^5 cells per mL. Cells were seeded at 5.5 x 10^4 cells per well in 6-well flat bottom plates, incubated for 48 hours at 37°C with DMEM, followed by incubation with either DMEM or TNFα at 1 ng/mL for different durations (0, 15 minutes, 30 minutes, 1, 3, 4, 16, 18, 20 and 24 hours). At each time point, cells were removed by scraping and resuspended in RIPA buffer (200 µL per well) and stored at -80°C until analysis (see section 2.3.6).

2.3.3 Detection of inflammatory mediators

Concentrations of cytokines and adhesion molecules secreted by EA.hy926 cells were determined simultaneously using Human Magnetic Luminex Screening Assay kits purchased from R&D systems.

2.3.3.1 Principle of Luminex assay

Luminex assays consist of analyte-specific antibodies pre-coated onto colour-coded magnetic particles. Immobilised antibodies bind to the analytes of interest. Unbound substances are then washed away, and samples are incubated with biotinylated antibody cocktail specific to analytes of interest. Samples are washed again to remove any unbound substances, and incubated further with streptavidin-phycoerythin conjugate which binds to the biotinylated antibody. Unbound substances are again washed away and microparticles are resuspended in wash buffer and read using the Bio-Plex 200 analyzer.

Beads are read by dual-laser flow-based detection. One laser classifies the bead and identifies the analyte which is being detected. The second laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound. Figure 2.1 depicts the basic principles of the luminex assay.

The immunoassay kit was selected to detect 6 analytes: IL-6, IL-8, MCP-1, VEGF, ICAM-1 and RANTES.

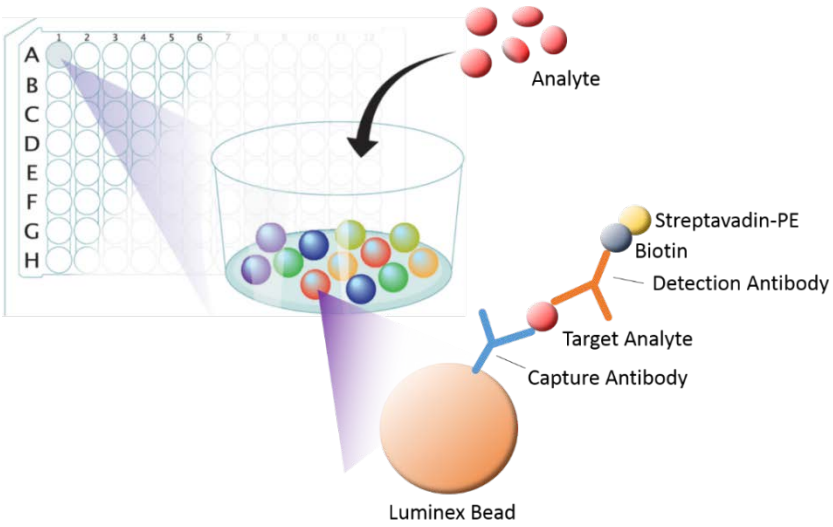

Figure 2.1 Schematic of Luminex assay principle

2.3.3.1.1 Assay procedure

Reagents and standards were prepared according to the manufacturer's instructions; undiluted samples of culture medium were analysed. Microparticle cocktail (50 µL) and 50 µl of neat sample or standard were loaded on to a 96-well clear bottomed black plate which was then covered with a foil seal. Plates were incubated for 2 hours on a horizontal orbital microplate shaker at 250 rpm. Plates were then attached to a magnetic mircoplate device and washed 3 times with 100 µL wash buffer. Biotin Antibody Cocktail (50 µL) was added to each well and plates covered with a foil seal and incubated for 1 hour on a horizontal plate shaker at 250 rpm. The washing step was repeated, 50 µL of Streptavidin-PE was added to each well and the plate was covered with a foil seal and incubated for 30 minutes on a plate shaker at 250 rpm. The washing step was repeated and each well was resuspended in 100 µL wash buffer and plates were then incubated for 2 minutes on a plate shaker at 800 rpm. Plates were then analysed on the Bi-Plex 200 (Bio-Rad) using the Bio-Plex Manager 6.1 software.

2.3.4 Analysis of gene expression using RT-qPCR

The effect of TNFα on gene expression at the different time points was analysed by RT-qPCR.

2.3.4.1 Isolation of RNA

Total cellular RNA was extracted from cells using ReliaPrep™ RNA Cell Miniprep System following the manufacturer's instructions. All components were made according to manufacturer's specifications. Lysates were thawed and

resuspended in 85 µL 100 % isopropanol and transferred to a ReliaPrep™ Minicolumn and collection tube. Lysates were then centrifuged at 11600 rpm for 30 seconds. RNA wash solution (500 µL) was added and centrifuged at 11600 rpm for 30 seconds. DNase 1 (30 µL) was added directly to Minicolumn membrane and incubated at room temperature for 15 minutes. Following incubation, 200 µL column wash solution was added and centrifuged at 11600 rpm for 15 seconds. RNA wash solution (500 µL) was added and centrifuged at 11600 rpm for 30 seconds. Minicolumns were then transferred to new collection tubes and 300 µL RNA wash solution added and centrifuged at 11600 rpm for 2 minutes. Minicolumns were then transferred to elution tubes, 15 µL of nuclease free water added and centrifuged at 11600 rpm for 1 minute. Minicolumns were then discarded. RNA quantity and quality were assessed by Nanodrop and integrity was analysed by an Agilent Bioanalyzer (see section 2.3.4.2.3). The elution tube containing purified RNA was stored at -80ºC.

2.3.4.2 RNA analysis

Isolated RNA quality and quantity was assessed by Nanodrop analysis and bioanalyzer. Measuring the concentration and purity of the RNA is crucial for determining the amount of each sample to use in RT-qPCR.

2.3.4.2.1 NanoDrop Spectrophotometer (NDS) analysis

NanoDrop analysis provides the concentration and quality of RNA. RNA (1 µL) is loaded between the NanoDrop pedestals and direct light is passed through it. Nucleic acids absorb light in the UV wavelength (with an absorbance maximum at 260 nanometers). The Beer-Lambert equation is used to correlate the calculated absorbance with concentration in ng/uL.

The ratio of absorbance at 260 and 280 nm and at 260 and 230 nm is used to assess the purity of the RNA. A 260/280 ratio of ~2.0 is generally accepted as “pure” for RNA. If the ratio is appreciably lower, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. The 260/230 values are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

2.3.4.2.2 RNA quantification

RNA was quantified using the NanoDrop, which uses the Beer-Lambert equation to correlate the calculated absorbance with concentration in ng/uL. Total yield is

obtained by multiplying the RNA concentration by the final total purified sample volume.

2.3.4.2.3 Agilent Bioanalyzer analysis

The RNA Bioanalyzer determines RNA concentration, integrity and ribosomal ratios. Using electrophoretic separation on microfabricated chips, RNA samples are separated and detected via laser induced fluorescence detection. The Bioanalyzer software generates an electropherogram and gel-like image and displays results including sample concentration and the ribosomal ratio of the 5S, 18S to 28S ribosomal subunits. These subunits are indicative of intact RNA. The electropherogram provides a detailed visual assessment of the quality of an RNA sample.

The Agilent Bioanalyzer software also assess integrity using the RNA integrity number (RIN). The RIN software algorithm allows for the classification of eukaryotic total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. Figure 2.2 is an example of good quality RNA with a RIN score of 10.

Melissa Doherty (Laboratory manager) carried out the procedure. 1 µL RNA per sample was loaded onto a chip and read on an Agilent Bioanalyser (Bioanalyzer RNA Total Eukaryote 2100 Nano).

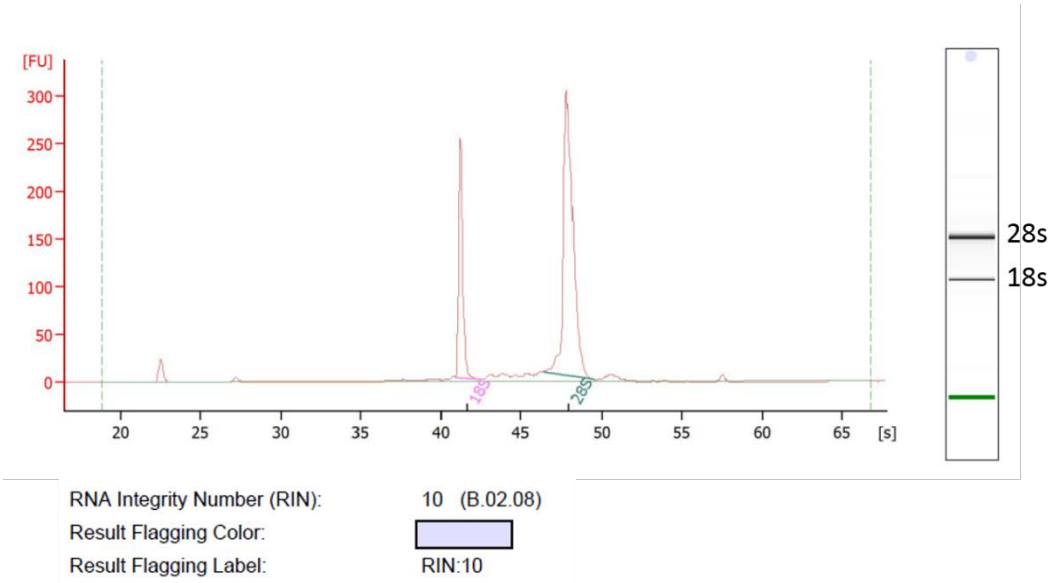

Figure 2.2 Example of RNA analysis by Bioanalyzer RNA Total Eukaryote 2100 Nano

2.3.4.3 cDNA synthesis

GoScript™ Reverse Transcriptase was used to synthesise cDNA from total RNA. All components were made according to manufacturer's specifications. Purified RNA was heated to 70ºC for 5 minutes with random primers and immediately chilled on ice for 5 minutes. Purified RNA and primer mix (5 µL) were added to 15 µL of reverse transcription reaction mix. Samples were then loaded into a Thermal Reactor and programmed to anneal at 25ºC for 5minutes, followed by extension at 42ºC for an hour and inactivation at 70ºC for 15 minutes.

2.3.4.4 Principle of RT-qPCR

PCR involves many rounds of DNA synthesis, which requires a template, primer DNA polymerase and nucleotides. Primers flank the target for amplification or 'amplicon', DNA synthesis then proceeds across the region between the primers. Each round of DNA synthesis, or cycle, involves a series of reactions: denaturation, annealing of primers and primer extension. These occur under temperature cycling. Products of each cycle are used as templates for further DNA synthesis, resulting in exponential amplification of the target sequence. See Figure 2.3, which shows the amplification process.

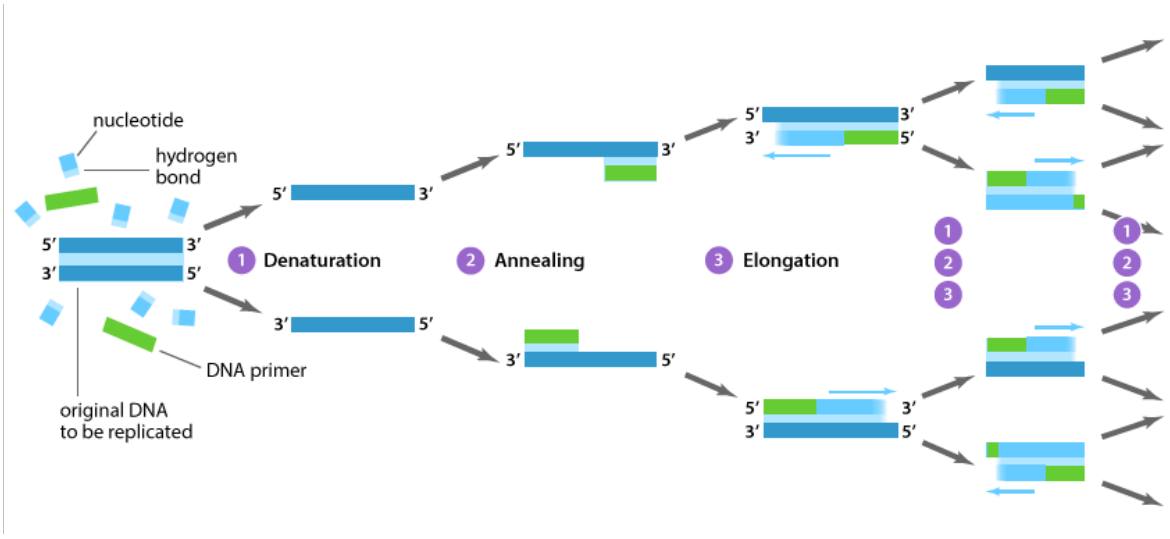

Figure 2.3 Schematic of PCR sequence amplification

Quantitative reverse transcription PCR (RT-qPCR) utilises the principles of PCR in order to measure the proportional increase in the amount of cDNA of interest with each round of PCR.

Initially RNA is extracted from the tissue of interest and levels of specific mRNA transcribed from the gene of interest measured. Reverse transcriptase then forms complimentary DNA (cDNA) by using RNA as a template. This gives cDNA for every RNA extracted, in the same proportions that are present in the tissue. Specific primers, which only anneal to the cDNA specific to the gene of interest, measure the point at which the amount of amplified DNA reaches a threshold level (known as a Ct value). The earlier the amount of DNA within a sample reaches the 'threshold level', compared to other samples, the more cDNA template was present; and therefore the mRNA was more highly expressed in the original sample. Levels of DNA can be measured by use of a dye that specifically binds to double-stranded DNA (e.g. SYBR Green), or addition of a probe, such as TaqMan.

TaqMan probes work by using a fluorescence emitting dye (e.g. FAM) that is bound to one end of a short DNA sequence with a molecule of quencher (e.g. TAMRA) at the other, Figure 2.4. While these molecules are bound in close proximity to each other, no fluorescence is emitted. Once probes bind to specific sites and Taq DNA polymerase uses its exonuclease activity to 'chew up' the oligonucleotide sequence the dye and quencher move further apart and the dye emits a fluorescent signal. One unit of fluorescence is emitted per copy of the gene to which it specifically binds; therefore, the amount of fluorescence detected is proportional to the amount of mRNA for the gene of interest present. This signal is recorded by the qPCR machine and converted into Ct values as described above.

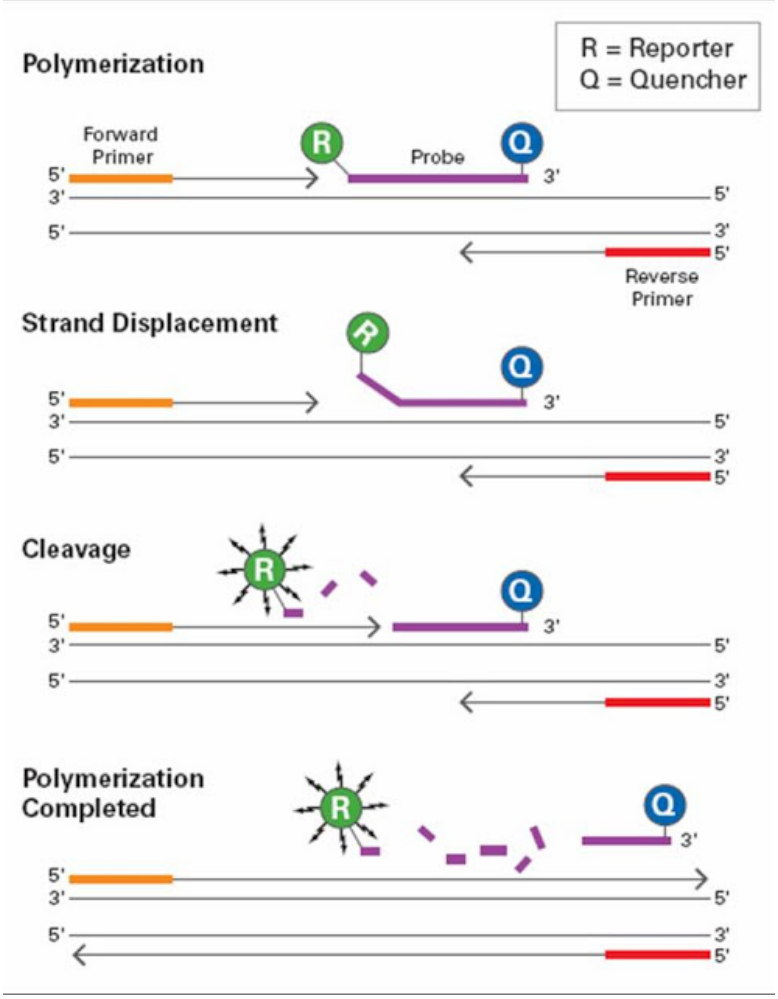

Figure 2.4 Schematic of RT-qPCR using TaqMan probes

2.3.4.4.1 Assay procdure

The expression of NF-kB, PPARα, PPARγ, MCP-1 and beta-2-microglobulin (B2M) was assessed by qRT-PCR with TaqMan® Gene Expression primers (see Appendix A for primer details). cDNA was diluted to 5 ng/µL. Using a 96-well qPCR plate, 4 µL of sample was added to 16 µL of reaction mix (prepared according to the manufacturer's specifications) per well. The plate was sealed and centrifuged at a low speed for 1 minute. The plate was then loaded into a thermal cycler with a cycle of 50ºC for 2 minutes, followed by a cycle of 95ºC for 10 minutes, then 40 cycles of 95ºC for 15 seconds and finally a cycle of 60ºC for 1 minute.

2.3.5 Relative gene expression quantification

Relative gene expression data were calculated using double delta Ct (ΔΔCt) values expressed as fold change of the target gene in test samples relative to control samples.

Average Ct values for housekeeping genes and target gene in experimental and control conditions were calculated. Delta Ct (ΔCt) values were calculated from the differences between target test gene and target reference gene as well as control test gene and control reference gene. Then the difference between these two gives the double delta Ct value (ΔΔCt). 2^ΔΔCt is then calculated to express the fold change [156].

$$\text{Relative gene expression} = 2^{-\Delta\Delta Ct}$$

$$\begin{aligned}\Delta\Delta C_t &= (C_{tTarget}-C_{tRef})_{Test} - (C_{tTarget}-C_{tRef})_{Control} \\ &= (C_{tTest}-C_{tControl})_{Target} - (C_{tTest}-C_{tControl})_{Ref} \\ &= \Delta C_{tTarget} - \Delta C_{tRef}\end{aligned}$$

Figure 2.5 Equation for relative gene expression calculation

2.3.6 Detection of protein expression

The effect of TNFα on protein expression at the different time points was analysed by Western blotting.

2.3.6.1 Protein extraction

Total cellular protein was extracted from cells using Radioimmunoprecipitation assay buffer (RIPA) buffer. Lysates were previously collected and resuspended in RIPA buffer (section 2.3.2.3). These were thawed and mixed every 10 minutes with a pipette for a total of 30 minutes. Lysates were then centrifuged at 11600 rpm for 10 minutes. Supernatant containing cellular proteins was aspirated into an Eppendorf tube for protein to be quantified by the bicinchoninic acid (BCA) assay. Samples were then stored at -80ºC for further analysis.

2.3.6.2 Protein quantification

Protein quantification was performed using the BCA assay (Thermo Fisher Scientific).

2.3.6.2.1 Principle of the BCA assay

The BCA assay is used to determine the total concentration of protein in a solution. The BCA Protein Assay combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with the selective colorimetric detection of the cuprous cation (Cu^{1+}) by BCA. The biuret reaction is the first stage where chelation of copper with protein in an alkaline environment forms a light blue complex. In this reaction peptides containing three or more amino acid residues form a coloured chelate complex with cupric ions in an alkaline environment containing sodium potassium tartrate. Secondly is the colour development reaction: BCA reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-coloured reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentration.

2.3.6.2.2 Assay procedure

Reagents and standards were prepared according to the manufacturer's instructions; undiluted samples were analysed. Sample or standard (10 µL) followed by 200 µL of working reagent were loaded on to a clear flat bottomed 96-well plate which was then covered with foil. Plates were placed on a horizontal plate shaker at 250 rpm for 2 minutes and then incubated for 30 minutes at 37°C. Plates were then read on a Multiskan EX (Thermo LabSystems) plate reader at 540 nm.

2.3.6.3 Principle of Western blot

Western blotting is a technique used to detect and analyse proteins based on their ability to bind to specific antibodies. The specificity of the antibody–antigen interaction enables the target protein to be identified in a complex protein mixture.

First, proteins are separated from each other based on their size. Second, antibodies are used to detect the protein of interest. Finally, a substrate that reacts with an enzyme is used to view the antibody/protein complex. Protein samples are subjected to electrophoresis on an SDS-polyacrylamide gel and then electro transferred onto a PVDF or nitrocellulose membrane. The transferred protein is detected using specific primary and secondary enzyme labelled antibodies.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate proteins according to their electrophoretic mobility which depends on charge, molecule size and structure. Polyacrylamide gel (PAG) is a three-dimensional mesh polymer composed of acrylamide and a cross-linker (methylene bisacrylamide). Electrophoresis separates charged particles which move toward the opposite electrode through the mesh polymer, under the influence of an electric field, with smaller proteins migrating faster and larger proteins running slower.

The PAG is a separating gel topped by a stacking gel and secured in an electrophoresis apparatus. Sample proteins are solubilised by boiling in the presence of SDS and equal amounts of the protein in solution are loaded into a gel lane, and the individual proteins separated electrophoretically. 2-mercaptoethanol or dithiothreitol are added to reduce disulfide bonds in the proteins.

After separating proteins by SDS-PAGE, the gel is placed over a sheet of nitrocellulose and the protein in the gel is electrophoretically transferred to the nitrocellulose. The nitrocellulose is then incubated in blocking buffer (5% skimmed milk solution) to "block" non-specific binding of proteins. The nitrocellulose is then incubated with a specific antibody for the protein of interest, washed and then incubated with a second antibody, which is specific for the first antibody. The second antibody will typically have a covalently attached enzyme and chemiluminescent substrate is used for detection, light being emitted can be detected with a photo imager. This allows for the amount of the protein of interest to be analysed from a complex crude mixture of proteins.

2.3.6.3.1 Assay procedure

Samples were diluted in dH_2O and Laemmli loading buffer to achieve a concentration of 30 ng of protein per sample per well. Proteins were denatured by heating at 95°C for 5 minutes. Precast 10% SDS-PAG (Optiblot, Abcam) were placed in a Bio-Rad tank with running buffer. Prepared sample (35 µL) were then loaded into individual wells, along with Prism Ultra Protein Ladder (10-245kDa). The tank was connected to an electrode and electrophoresis was run at 80 V for approximately 10 minutes (until samples passed through the stacking portion of the gels) then at 180 V for 1.5-2 hours, until samples reached the bottom of the gel.

Proteins were then transferred onto nitrocellulose membrane. Gel and membranes are ‘sandwiched’ as shown in Figure 2.6

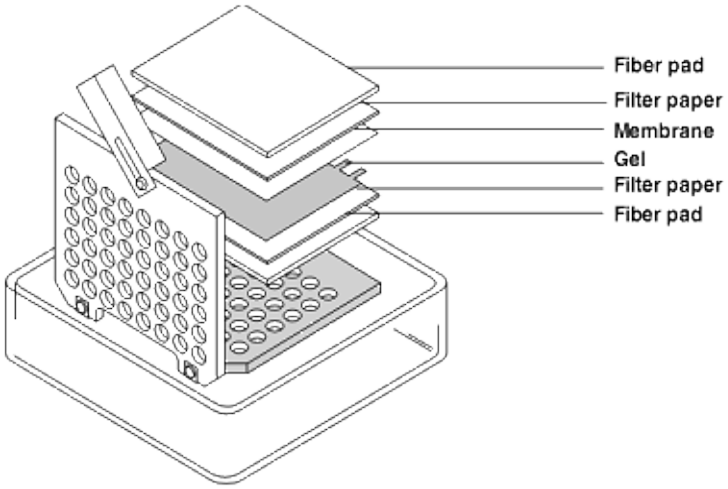

Figure 2.6 Depiction of 'transfer sandwich' for transfer of proteins from SDS-PAG to nitrocellulose membrane

The sandwich was placed into an electrode chamber and then into a tank depicted in Figure 2.7 with transfer buffer and an ice pack and run at 100 V for 1 hour at 4°C.

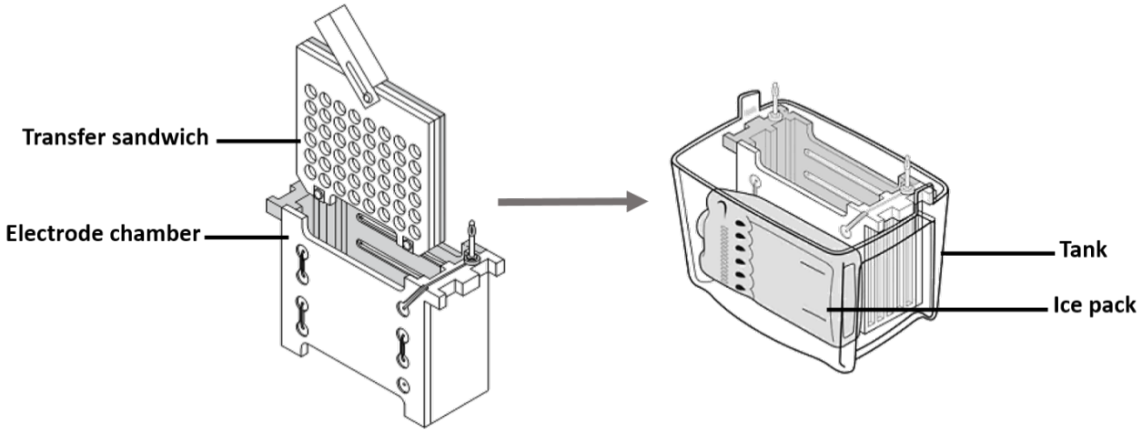

Figure 2.7 Depiction of protein transfer apparatus.

After transferring of proteins, membranes were blocked with blocking buffer (PBST 0.05% tween + 5% milk) for 1 hour at room temperature, with agitation, and then incubated with primary antibody (see appendix for primary antibodies and concentrations used) over night at 4°C again with agitation. Membranes were then washed 3 times with PBST (0.05% tween) for 5 minutes and then incubated with a secondary antibody (Goat Anti-Rabbit IgG H&L (HRP) see appendix for

concentration) at room temperature for 1 hour with agitation. Membranes were then washed as previously described and 2 mL of SuperSignal (Thermo Fisher Scientific) added per membrane to visualise using Bio-Rad Chemidoc XRS.

2.3.6.4 Quantification of western blots with ImageJ

ImageJ is used to compare the density of bands on a western blot. Protein bands were quantified as relative amounts from western blot films using the ratio of each protein band relative to the lane's loading control (reference protein). Regions of interest were defined for each protein of interest, reference and film background. Density measurements of these were then taken in imageJ. In Excel pixel densities were then inverted for all bands and their backgrounds, and net values calculated by deducting the inverted background value from the inverted protein of interest value. After calculating the net bands for protein of interest and loading controls a ratio of net band value over the net loading control of that lane is calculated. The final relative quantification values are the ratio of net band to net loading control.

2.4 Results

2.4.1 Optimization of the effect of different TNFα concentrations on inflammatory mediators in EA.hy926 cells

The effect of different concentrations and durations of TNFα exposure on the production of 6 analytes (ICAM-1, IL-6, IL-8, MCP-1, RANTES and VEGF) was examined. The aim was to identify optimal conditions for use in future experiments with FA exposure. The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30.

Figure 2.8 to Figure 2.13 show the concentrations of soluble ICAM-1, IL-6, IL-8, MCP-1, RANTES and VEGF within the supernatant following stimulation of EA.hy926 cells with 0.1 to 100 ng/mL TNFα for 2, 4, 6, 12, 24 and 48 hours. At 2, 4 and 6 hours exposure to all TNFα concentrations no differences were observed in the levels of any of analytes examined compared to the very low concentration seen in medium itself. After 12 hours exposure to 10 ng/mL TNFα MCP-1, VEGF, IL-6 and IL-8 concentrations were all increased with no change in RANTES or ICAM-1 concentrations. After 24 hours exposure to TNFα at 1, 10 and 100 ng/mL

concentrations of all 6 of the analytes increased. Based on these findings incubation of TNFα at 1ng/mL for 24 hours was selected as a condition at which to study the effect of FAs on these analytes in EA.hy926 cells.

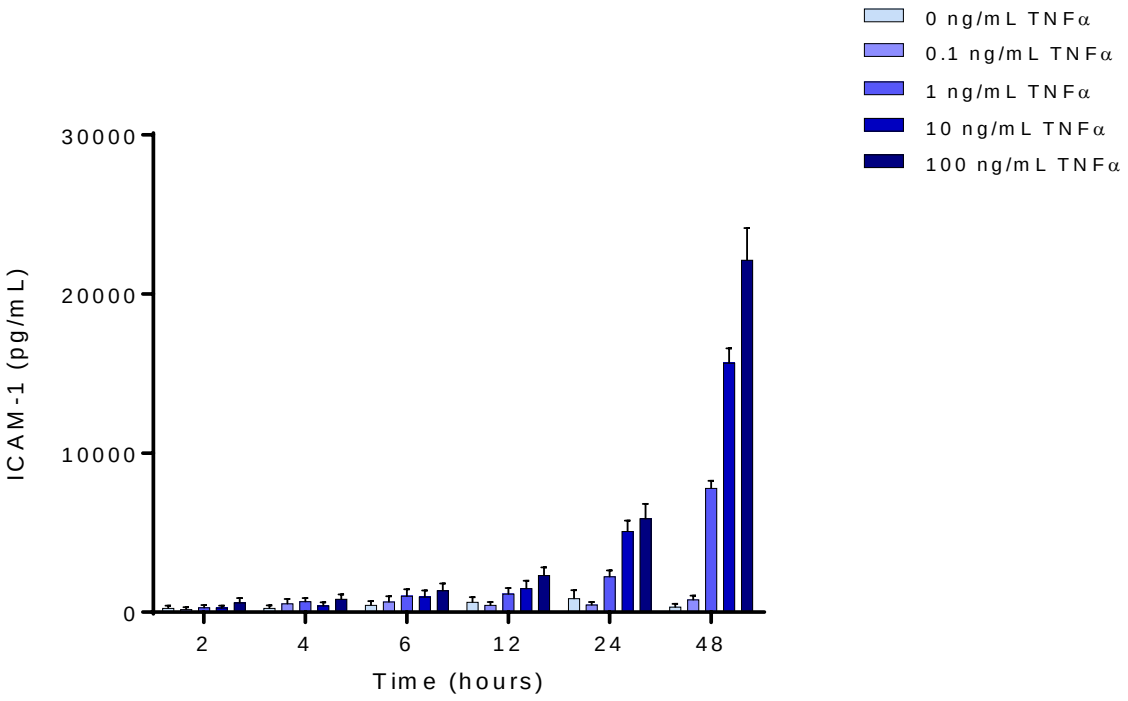

Figure 2.8 Effect of TNFα stimulation on ICAM-1 production by EA.hy926 cells

Mean (±SEM) (n = 3) production of ICAM-1by EA.hy926 cells exposed to TNFα (0-100 ng/mL) for 2 to 48 hours. ICAM-1 was measured in the supernatant of endothelial cell cultures.

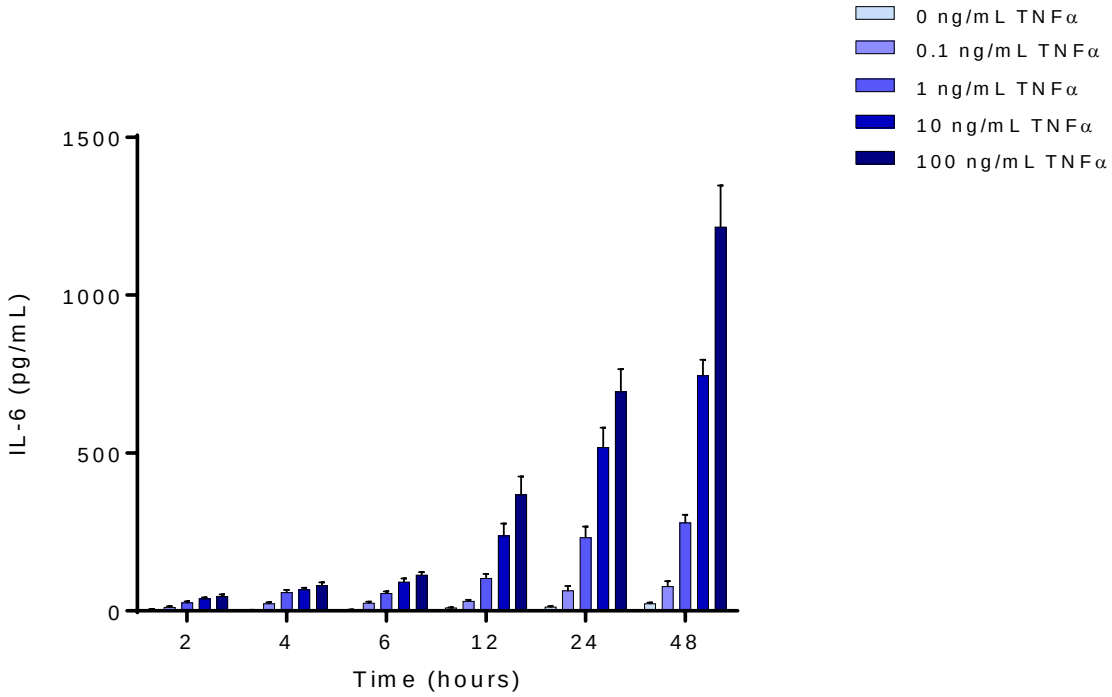

Figure 2.9 Effect of TNFα stimulation on IL-6 production by EA.hy926 cells

Mean (±SEM) (n = 3) production of IL-6 in EA.hy926 exposed to TNFα (0-100 ng/mL) for 2 – 48 hours. IL-6 was measured in the supernatant of endothelial cell cultures.

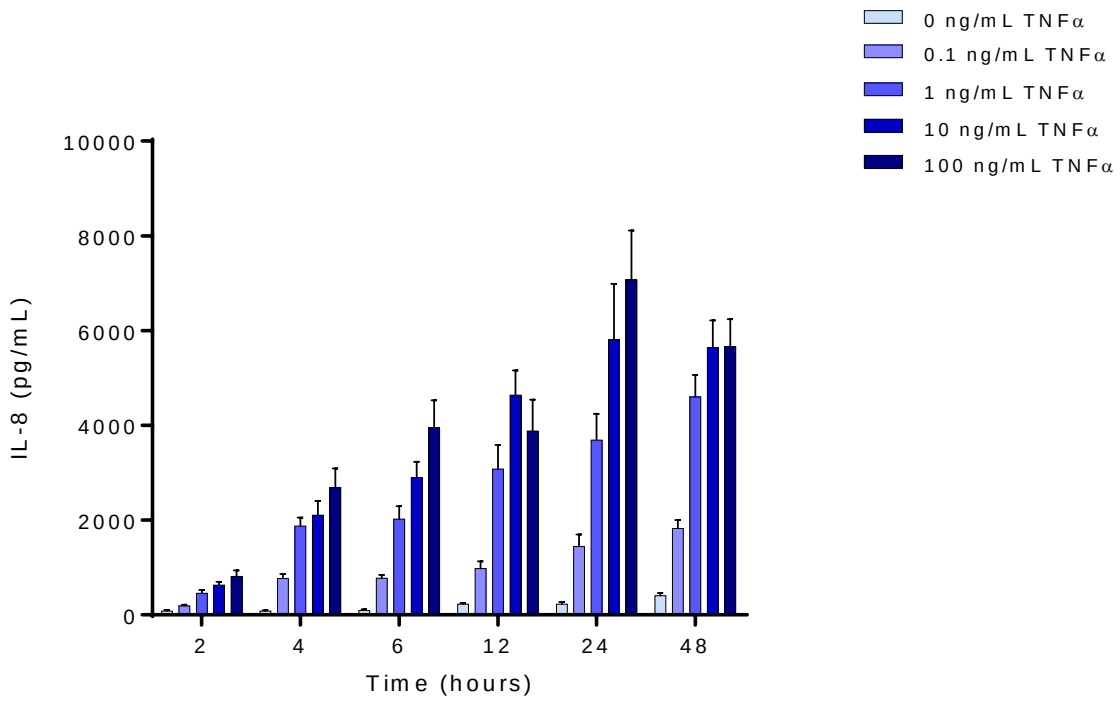

Figure 2.10 Effect of TNFα stimulation on IL-8 production by EA.hy926 cells

Mean (±SEM) (n = 3) production of IL-8 in EA.hy926 exposed to TNFα (0-100 ng/mL) for 2 – 48 hours. IL-8 was measured in the supernatant of endothelial cell cultures.

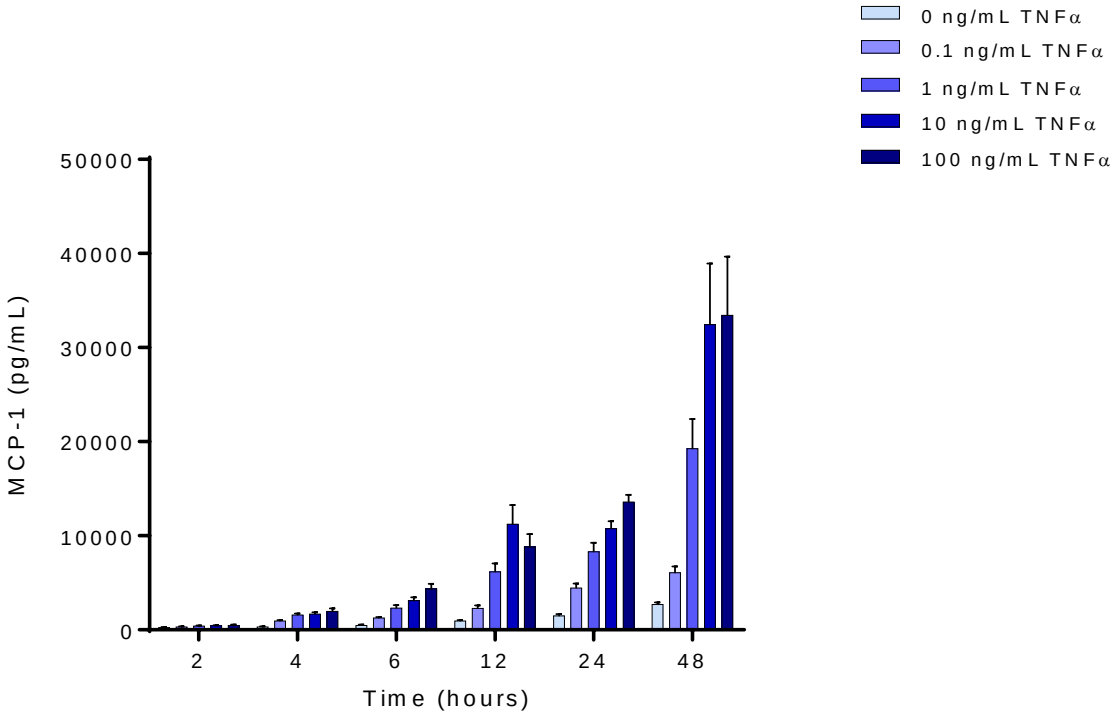

Figure 2.11 Effect of TNFα stimulation on MCP-1 production by EA.hy926 cells

Mean (±SEM) (n = 3) production of MCP-1 in EA.hy926 exposed to TNFα (0–100 ng/mL) for 2 – 48 hours. MCP-1 was measured in the supernatant of endothelial cell cultures.

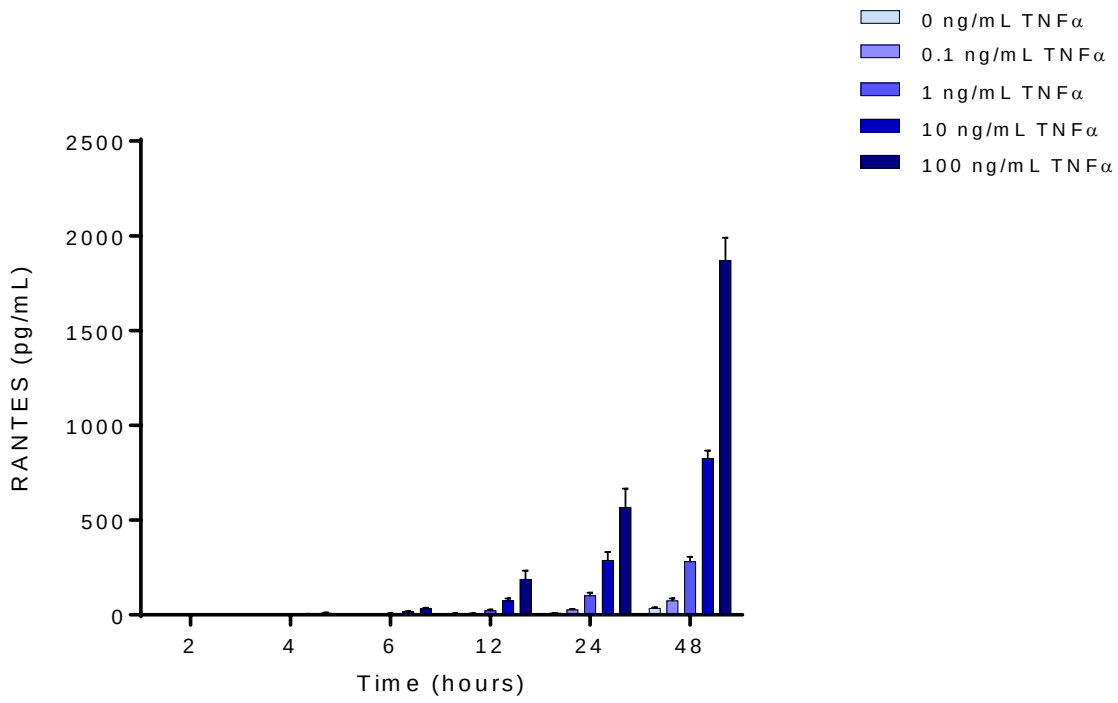

Figure 2.12 Effect of TNFα stimulation on RANTES production by EA.hy926 cells

Mean (±SEM) (n = 3) production of RANTES in EA.hy926 exposed to TNFα (0-100 ng/mL) for 2 – 48 hours. RANTES was measured in the supernatant of endothelial cell cultures.

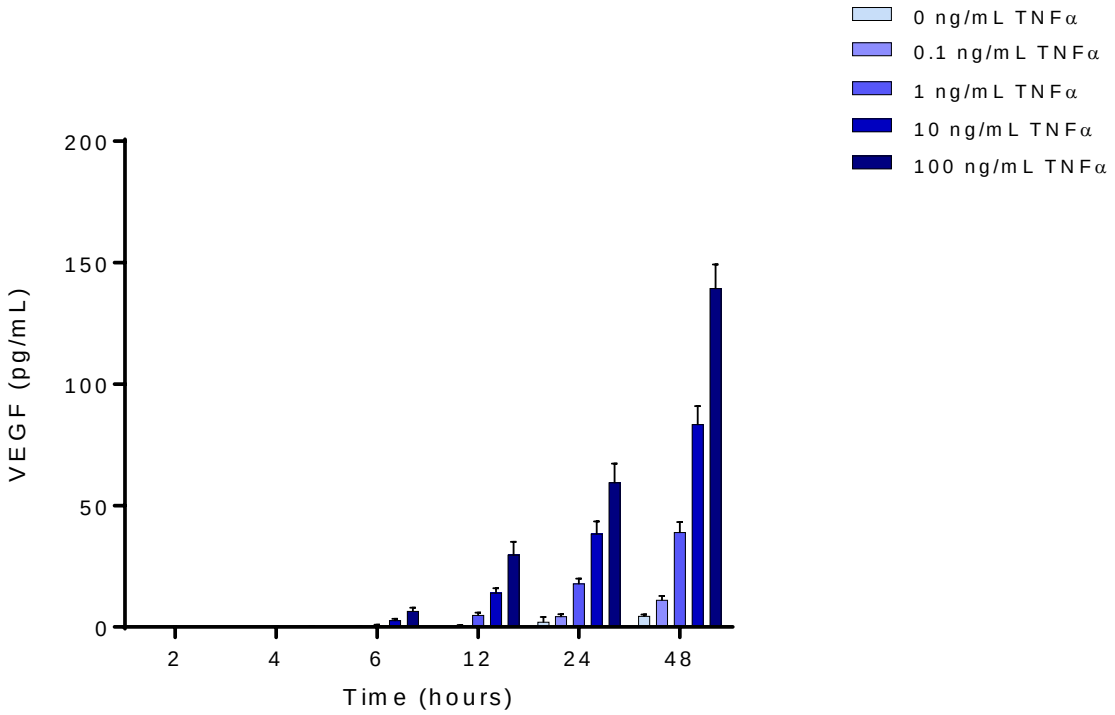

Figure 2.13 Effect of TNFα stimulation on VEGF production by EA.hy926 cells

Mean (±SEM) (n = 3) production of VEGF in EA.hy926 exposed to TNFα (0-100 ng/mL) for 2 – 48 hours. VEGF was measured in the supernatant of endothelial cell cultures.

2.4.2 RNA integrity

Total cellular RNA isolation was carried out using ReliaPrep™ RNA Cell Miniprep columns (section 2.3.4.1).

Analysis of RNA using Agilent Bioanlyzer (RNA Total Eukaryote 2100 Nano) was performed by Melissa Doherty, Laboratory manager (section 2.3.4.2.2). RNA quantity and quality were also assessed using the NanoDrop (section 2.3.4.2.1).

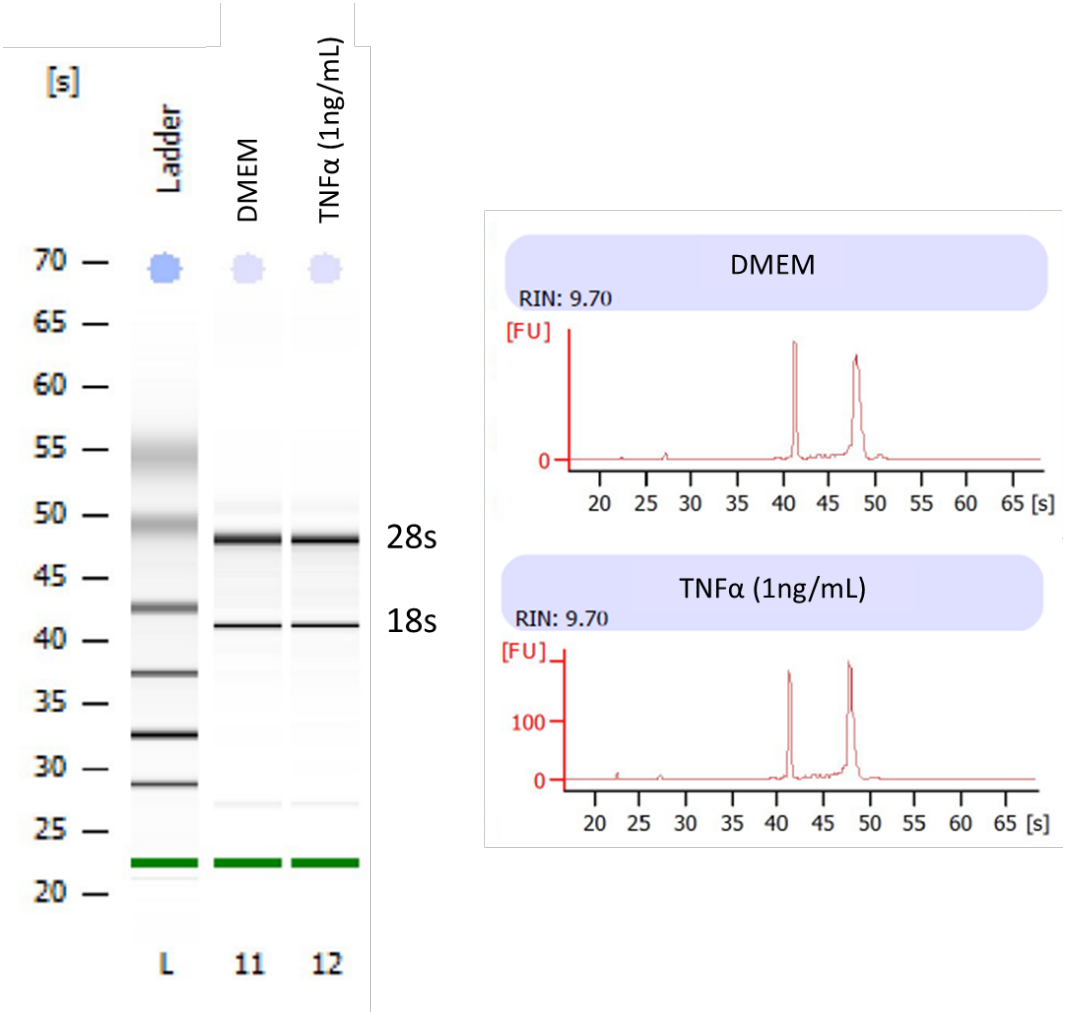

Figure 2.14 RNA analysis by Agilent Bioanalyzer

RNA samples were analysed using the Agilent Bioanalyzer (Figure 2.14). EA.hy926 cells were incubated with DMEM alone or were stimulated with TNFα at 1 ng/mL. RNA was shown to have high RIN scores indicative of good quality. Both control (DMEM alone) and stimulated EA.hy926 cells had RIN scores of 9.70 (Figure 2.14).

All RNA samples were analysed via the NanoDrop for quality and quantity (data not shown). Table 2.1 depicts representative RNA sample 260/280 and 260/230 ratios from the NanoDrop analysis. EA.hy926 cells were incubated with DMEM alone or were stimulated with TNFα at 1 ng/mL. The resukts inducate RNA of good purity.

Table 2.1 Anlaysis of RNA quality and quantity using NanoDrop

Samples	ng/µL	A260	A280	260/280	260/230
DMEM	621.32	15.533	7.288	2.13	2.2
TNFα (1ng/mL)	467.47	4.961	2.376	2.06	2.11

2.4.3 Optimization of TNFα duration on inflammatory gene expression in EA.hy926 cells

In order to understand better the effects of FAs on inflammation, it is important to explore their effects on inflammatory signalling, transcription factor activation and inflammatory gene expression. Prior to assessing the effects of FAs on inflammatory gene expression, a time course of the effect of TNFα stimulation on some genes of interest was performed. Once a time point or several time points is (are) determined, the FAs effects on inflammatory genes can be assessed. Alterations in gene expression should occur prior to the expression of analytes within the cell supernatant so therefore a time course consisting of time points at 30 minutes, 1, 3, 6 and 9 hours stimulation was chosen.

The effect of different concentrations and duration of TNFα exposure on the expression of 4 genes (NF-kB (subunit 1), MCP-1, PPARα, PPARγ) in EA.hy926 cells was examined. The aim was to identify optimal conditions for use in future experiments with FA exposure. The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30.

PPARγ mRNA was not detected under any conditions used (data not shown).

TNFα increased mRNA for NF-kB and MCP-1 at 3, 6 and 9 hours (Figure 2.15 and Figure 2.16). Optimal stimulation time with TNFα at 1ng/mL was therefore selected to be 6 hours. PPARα is an anti-inflammatory gene, and the expression of this gene was expected to decrease. There was a tendency for this at 6 hours (Figure 2.17). In order to examine the effects of various FAs on these genes, FA incubation for 48 hours followed by 6 hours stimulation with TNFα at 1 ng/mL will be used.

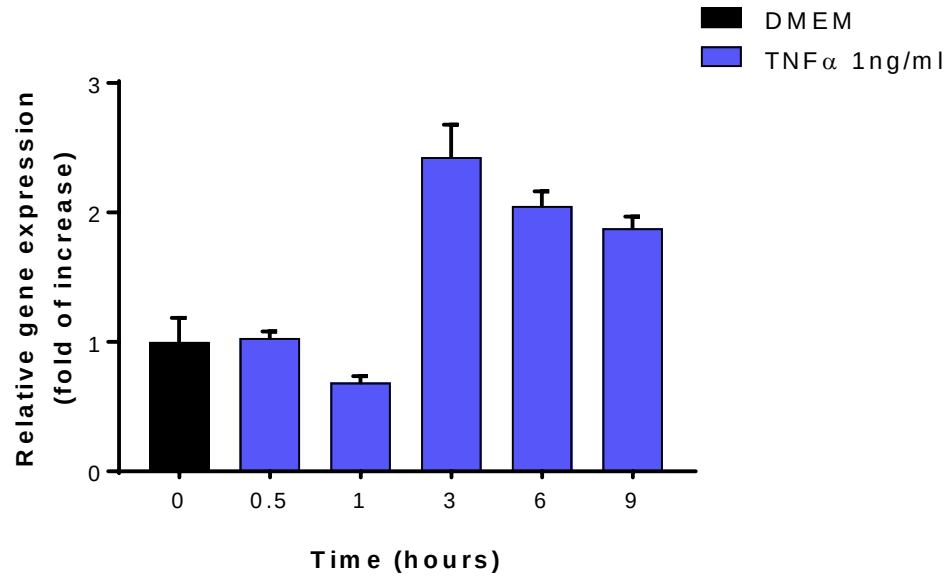

Figure 2.15 Effect of TNFα stimulation on NF-kB (subunit 1) gene expression in EA.hy926 cells

Mean (±SEM) (n = 3) gene expression of NF-kB in EA.hy926 cells after exposure to TNFα (1ng/mL). ct values normalised to B2M.

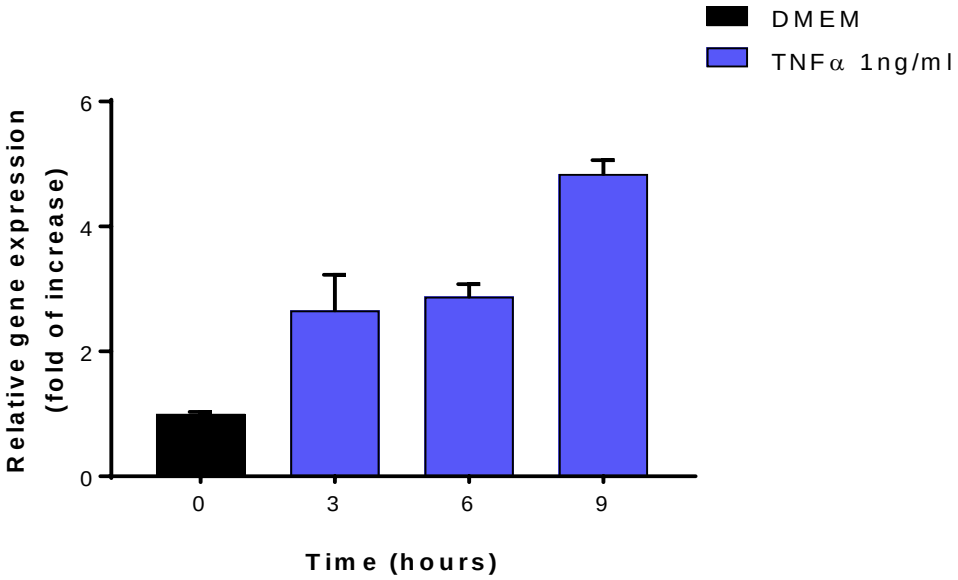

Figure 2.16 Effect of TNFα stimulation on MCP-1 gene expression in EA.hy926 cells

Mean (±SEM) (n = 3) gene expression of MCP-1 in EA.hy926 cells after exposure to TNFα (1ng/mL). Ct values normalised to B2M.

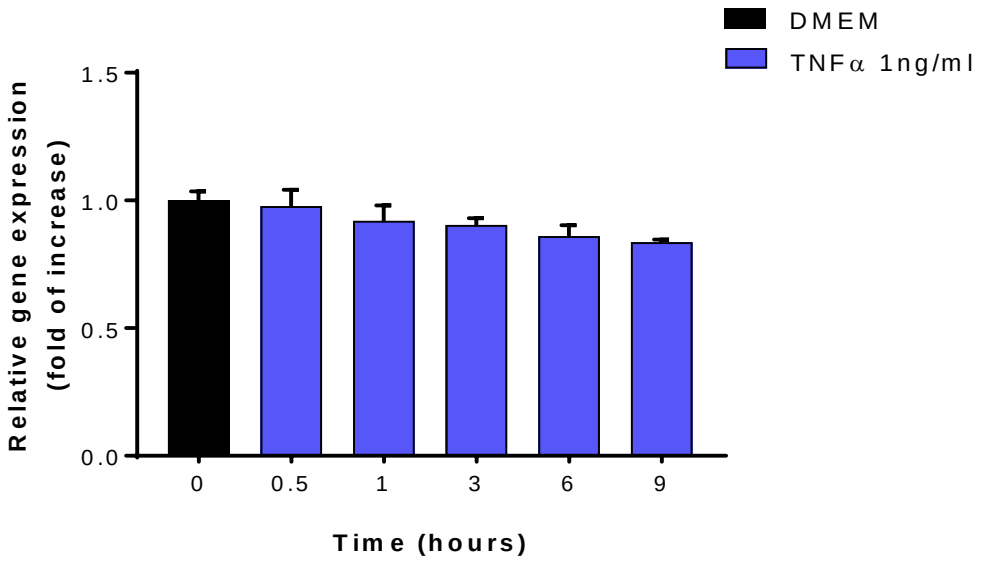

Figure 2.17 Effect of TNFα stimulation on PPARα gene expression in EA.hy926 cells

Mean (±SEM) (n = 3) gene expression of PPARα in EA.hy926 cells after exposure to TNFα (1ng/mL). Ct values normalised to B2M.

2.4.4 Optimization of TNFα duration on protein expression in EA.hy926

The effect of TNFα at 1ng/mL on cellular protein levels in EA.hy926 cells was examined. Inflammatory proteins analysed were NF-kB and COX-2. The aim was to identify optimal conditions for use in future experiments with FA exposure. The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30.

Activation of NF-kB occurs in the very early stages following stimulation. Therefore NF-kBp65 levels were measured at 15 minutes, 30 minutes, 1, 3, 4, and 24 hours after stimulation with TNFα at 1ng/mL. Activation of NF-kB leads to the phosphorylation of NF-kBp65 (RelA) a subunit of NF-kB (section 1.2.3), phosphorylated NF-kBp65 is liberated and translocates to the nucleus. NF-kB dimers then bind at kB site-containing DNA and cause a cascade of inflammatory events. Therefore NF-kBp65 (non-phosphorylated) levels indicate inactive NF-kB and so should initially decrease after stimulation.

Figure 2.18 shows the cellular concentrations of NF-kBp65 following stimulation of EA.hy926 cells with 1ng/mL TNFα for 15 minutes, 30 minutes, 1, 3, 4, and 24 hours. There was a decrease in the levels of NF-kBp65 after 15 minutes to 1 hour compared to control unstimulated cells (0 hours). Levels then start to increase from 3 to 24 hours. In order to examine the effects of FAs on the levels of NF-kBp65 cells will be stimulated for 1 hour in future experiments.

Figure 2.19 shows the cellular quantities of COX-2 following stimulation of EA.hy926 cells with 1ng/mL TNFα for 16, 18, 20 and 24 hours. At 6 hours exposure to TNFα at 1ng/mL a small increase in COX-2 was observed compared to control unstimulated cells (0 hours). After 16 hours exposure to TNFα at 1 ng/mL quantities of COX-2 peak and then slowly start to decrease over time from 18 – 24 hours. Based on these findings incubation of TNFα at 1ng/mL for 16 hours was selected as a condition at which to study the effect of FAs on COX-2 in EA.hy926 cells.

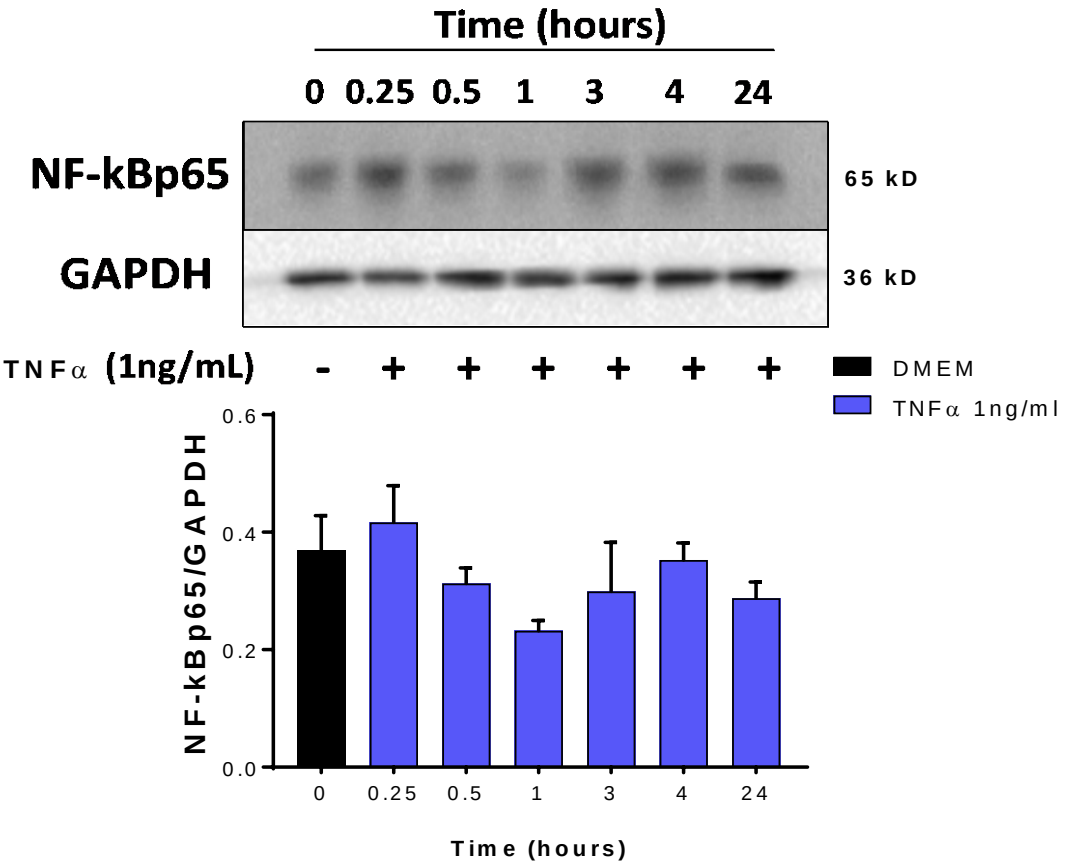

Figure 2.18 Effect of TNFα stimulation on cellular NF-kBp65 in EA.hy926 cells

Mean (±SEM) (n = 2) protein expression of NF-kBp65 in EA.hy926 cells after exposure to TNFα (1ng/mL). GAPDH (37kDa) NF-kBp65 (65kDa). Protein calculated as a ratio of NF-kBp65 to GAPDH.

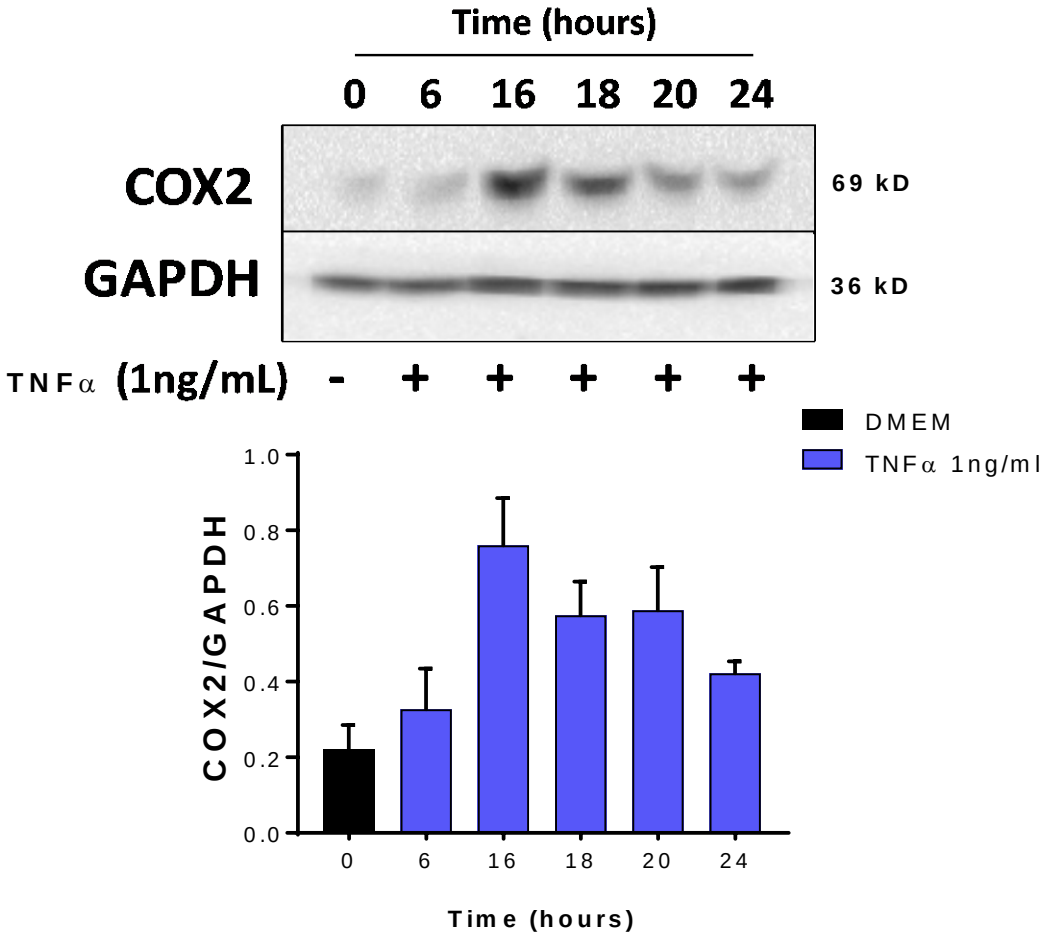

Figure 2.19 Effect of TNFα stimulation on cellular COX-2 protein levels in EA.hy926 cells

Mean (±SEM) (n = 2) protein expression of COX-2 in EA.hy926 cells after exposure to TNFα (1ng/mL). GAPDH (37kDa) COX-2 (75kDa). Protein calculated as a ratio of COX-2 to GAPDH.

2.5 Discussion

The aim of the research described in this chapter was to use the pro-inflammatory cytokine TNFα to induce an inflammatory response in cultured EA.hy926 cells and to identify the effects of different doses of TNFα on production of an array of different inflammatory markers over time, including adhesion molecules, chemokines, and cytokines, with the objective of optimising the conditions for studying inflammatory responses of cultured EA.hy926 cells. This aim and the objectives were met. The research described in this chapter produced a repeatable working model of inflammation using the EC line EA.hy926.

Luminex was used to quantify analytes secreted by EA.hy926 cells. The optimal conditions for the production of ICAM-1, IL-6, IL-8, MCP-1, RANTES and VEGF by EA.hy926 cells were determined. These conditions were TNFα stimulation at 1 ng/mL and for 24 hours. These conditions produced an increase in the concentrations of all of the analytes assessed. Using these results TNFα stimulation at 1 ng/mL was then used to analyse optimal stimulation time for gene expression and cellular protein expression.

RNA was shown to have high RIN scores (RIN 9.70) and therefore was of high quality as assessed by Bioanalyzer RNA Total Eukaryote 2100 Nano. RT-qPCR was used to quantify gene expression after TNFα stimulation (1 ng/mL) in EA.hy926 cells. A time point of 6 hours TNFα stimulation was determined as optimal for assessing increased gene expression of NF-kB and MCP-1.

Western blot was used to quantify protein expression in TNFα stimulated (1 ng/mL) EA.hy926 cells. The time course of appearance of the two proteins assessed varied. The optimal time point for lowered expression of NF-kBp65 was 1 hour of TNFα stimulation, and the optimal time point for enhanced expression of COX-2 was 16 hours of TNFα stimulation.

These conditions will be used in research described in later chapters to examine the effects of FAs on the expression and secretion of the various inflammatory analytes. EA.hy926 cells will be stimulated with TNFα at 1 ng/mL using an optimal time point for each type of analysis; 24 hours for secretion of inflammatory mediators, 6 hours for gene expression and 1 and 16 hours for intracellular protein expression.

The NF-kB activation pathway was described previously in section 1.2.3. Phosphorylated NF-kBp65 leads to the translocation from the cytosol to the nucleus and transcription of inflammatory genes including NF-kB itself. Therefore in Figure 2.15 and Figure 2.18 there is an initial decrease in NF-kB gene expression and NF-kBp65 protein concentrations after stimulation, this then increases after time with further stimulation (3 – 24 hrs). Protein expression after FA incubation will be analysed at the earlier time point (1 hour) and gene expression at the later time point of 6 hours. This will allow for the study of effect of FAs on both the quantity of NF-kB being released on activation and then the ongoing expression of NF-kB.

PPARα has been shown to increase in THP-1 monocyes with incubation with EPA [157]. This gene is anti-inflammatory, and since the gene has a tendency to decrease at 6 hours this time point will also be chosen to examine the effects of FAs.

These genes are representative of genes which will be further examined after FA incubation. NF-kB and PPARα are examples of transcription factors and MCP-1 is an example of a cytokine.

Figure 2.20 depicts the changes in gene expression, intracellular proteins and cytokine secretion in EA.hy926 over time after stimulation with TNFα at 1ng/mL. As expected stimulation of EA.hy926 lead to the increased activation of NF-kB which in turn increases gene expression leading to production of inflammatory mediators.

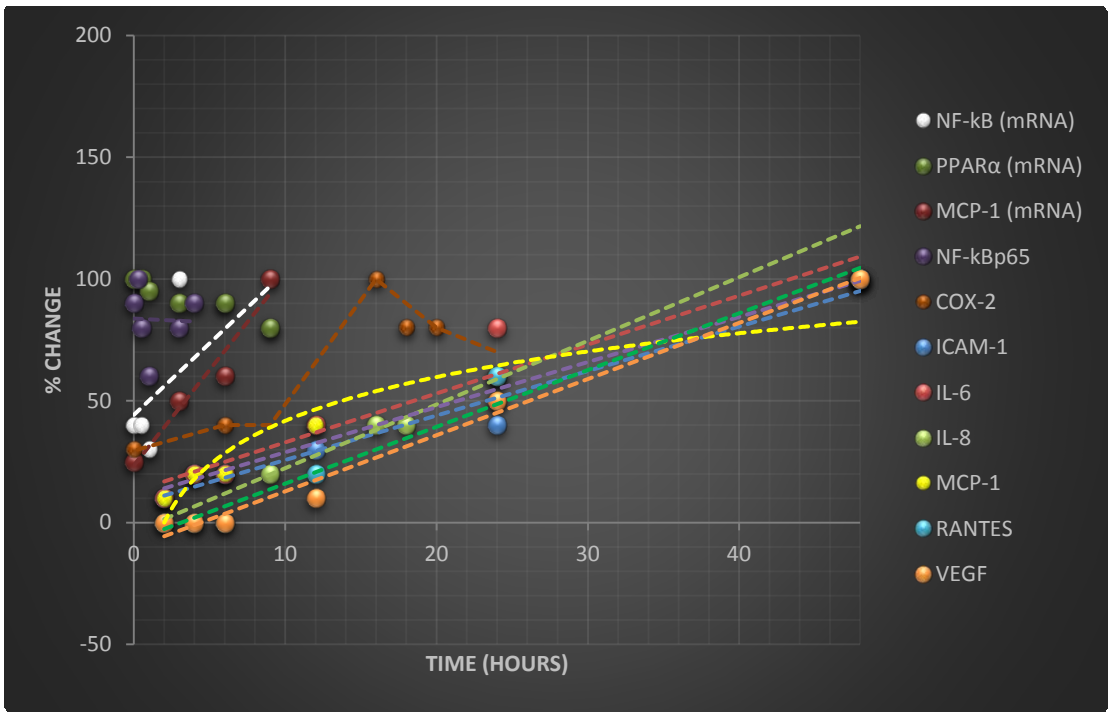

Figure 2.20 Increases (% change) in gene expression, protein concentrations and secretion of mediators over time in EA.hy926 stimulated with TNFα (1ng/mL)

In order to analyse the effect of FAs on the inflammatory response, cells will be incubated with FAs for 48 hours prior to TNFα stimulation. This incubation time was chosen based on previous work carried out within our group and has been demonstrated as an adequate time for FA incorporation into ECs by others [158]. Pre-treatment with FAs has also been shown to have modulating effects compared to adding FAs concomitantly with stimulation [111], it is more relevant when assessing protective effects of FAs. Incorporation of FAs into cells after 48 hours will be measured using gas chromatography (GC). This is vital since change in FA composition will be important in determining the effect of the different FAs on the inflammatory response.

Based on the results from the Luminex, RT-qPCR and Western blot TNFα optimisation Figure 2.21 depicts the experimental procedure for future research. The experimental design will remain consistent; FA treatment for 48 hours followed by (up to) 24 hour TNFα treatment.

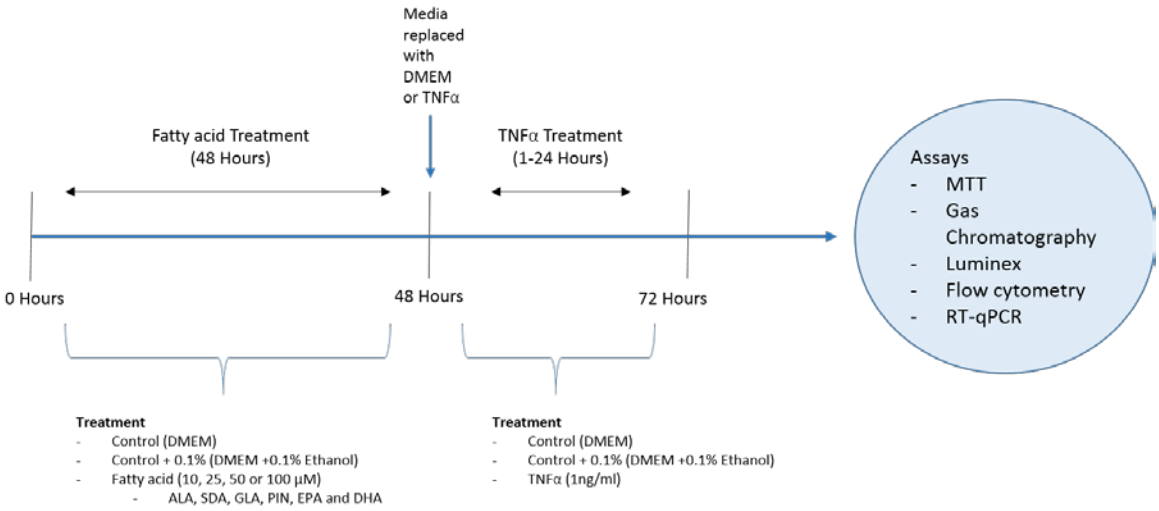

Figure 2.21 Experimental design for EA.hy926 incubation with fatty acids followed by TNFα treatment.

ALA, alpha-linolenic acid, DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLA, gamma-linolenic acid; MTT, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; PCR, polymerase chain reaction; PIN, pinolenic acid; SDA, stearidonic acid; TNFα, tumour-necrosis factor alpha

Chapter 3: Fatty Acid Incorporation into Cultured Endothelial Cells

3.1 Introduction

FAs exist within the membrane of cells, mainly in phospholipids, and from here can exert many of their biological effects (section 1.2.6.2). These include changes in lipid rafts (signalling platforms) and in lipid mediator generation and therefore FAs can modulate how the endothelium interacts with inflammatory cells and with external signals. It has been shown that when there are higher levels of EPA and DHA within cell membrane phospholipids there are alterations in cell signalling and increases in the production of EPA and DHA derived mediators [1] leading to less inflammation.

Supplementation with various FAs have been shown to increase the levels of FAs in cellular lipid pools [74, 159, 160], in a dose-dependent manner [159, 161]. Cells may also metabolised FAs including the generation of elongation products and these FAs may have their own biological functions.

Exposure of EA.hy926 cells to TNFα stimulated an inflammatory response, with increasing inflammatory mediator production, enhanced inflammatory gene expression and altered levels of selected cellular proteins seen over the period of 48 hours (Chapter 2). Different time points for TNFα stimulation were determined as optimal, with a maximum of 24 hour stimulation of TNFα at 1 ng/mL. The purpose of the experiments described in Chapter 2 was to establish conditions to investigate the effect of different FAs on EC inflammatory responses.

EPA and DHA, have been shown to reduce inflammatory responses of HUVECs and other ECs [110, 152, 162], including many aspects involved in development of atherosclerosis [110, 112, 163, 164]. However the effects of plant-derived FAs on inflammation in ECs is less well explored, and those who have predominantly use ALA treatment [165-168].

Although the observed effects of FAs on EC and other cell types most likely occur as a result of altered cell membrane compositions, few explored effects of EPA and DHA on inflammation have also described their incorporation into cells [110, 114]. Of those who have examined effects of ALA, SDA, GLA or PIN, very few report the incorporation of these FAs into the cells or how these FAs are being metabolised by the cells [166].

Therefore in order to understand effects of FAs on the inflammatory responses of ECs FA composition changes were considered important to assess. Incorporation

and metabolism of FAs of interest into the cells was determined by the membrane FA composition analysis using gas chromatography (GC).

These examinations will help to better understand any effect of plant-derived 18-carbon FAs (compared with EPA and DHA) on endothelial function and how this may help to find more sustainable health benefiting FAs.

3.2 Aim and Objectives

The aim of the research described in this chapter was to assess the incorporation of different FAs into cultured EA.hy926 cells.

The specific objectives were to:

- Determine the viability of EA.hy926 cells after culture with TNFα and various FAs;
- Determine the FA composition of EA.hy926 cells after culture with various FAs.

3.3 Methods

3.3.1 Reagents

Reagents and materials used for experiments are described in Appendix A.

3.3.2 Fatty acid dilution.

ALA, SDA, GLA, PIN, EPA and DHA were dissolved in 100% ethanol to prepare 100 mM, 50 mM, and 25 mM stock solutions. Dilutions were made from stocks in DMEM (complete medium) to create working concentrations of each FA. This gave an ethanol concentration of 0.1% in working concentrations. FAs were diluted according to requirements for each experiment. Prior to each experimental procedure GC analysis was used to verify FA stock concentrations (data not shown).

3.3.3 Viability of EA.hy926 cells treated with fatty acids

3.3.3.1 Principle of MTT assay

The MTT Assay measures the cell viability and cytotoxicity. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, by the action of dehydrogenase enzymes, to generate NADH and NADPH resulting in insoluble formazan. This intracellular purple formazan is then solubilised using isopropanol or other solvents, and the concentration quantified by spectrophotometric means.

3.3.3.1.1 Assay procedure

The MTT assay was performed on EA.hy926 cells to assess any toxicity of each individual FA and TNFα.

As described previously (section 2.3.2), confluent EA.hy926 cultures were scraped from flasks and resuspended at a density of 2 x 10^5 cells per mL. Cells were seeded at 1 x 10^4 cells per well in 96-well flat bottom plates, incubated for 48 hours at 37°C with various FAs at a concentration of 10, 25, 50 or 100 µM followed by 24 hour incubation with either DMEM or TNFα at 1 ng/mL

After treatment, MTT at 5 ng/mL was added to wells (100 µl/well), and incubated for 4 hours at 37°C. After incubation, 85 µL supernatant was removed and 75 µL DMSO added to each well and wells were then incubated at 37°C for a further 5 minutes. Plates were then read on the plate reader at 540 nm. Controls consisted of cells incubated with DMEM alone and DMEM with 0.1% ethanol. Controls in DMEM alone were considered to be 100% viable; effects of treatments were determined as a % of control.

3.3.3.2 Visualisation of EA.hy926

As described previously (2.3.2.1) confluent EA.hy926 cells were scraped from flasks and resuspended at a density of 2 x 10^5 cells per mL. Cells were seeded at 1 x 10^4 cells per well in 96-well flat bottom plates, and incubated for 48 hours at 37°C with the FAs at a concentration of 50 µM followed by 24 hour incubation with TNFα at 1 ng/mL. EA.hy926 cells were then observed and imaged using the fluorescence microscope Nikon Elipse Ti with NIS elements software (version 4.30). Images were taken at a magnification of 10x.

3.3.3.3 Principle of gas chromatography

Gas chromatography (GC) is a technique that can be used for analysis of lipids which are made volatile by modification of chemical groups. Fatty acid methyl esters (FAMEs) are separated by the different temperature at which they become volatile. Factors that influence this are carbon chain length, and number and position of double bonds. With a longer chain length there is an increase in temperature at which the FAs enter the vapour phase (i.e. boiling point). However with an increase in double bonds there is a decrease in boiling point. Differences in FAME – silica lining interactions are used to separate the FAMEs.

Usually a methyl group is added to the carboxylic end of the FA to form a FAME by reaction with a methyl donor such as methanol, in the presence of a catalyst, such as sulphuric acid. This allows for the separation of a wide range of FAs by lowering the boiling points to lower temperatures than required for the native FAs.

The gas chromatograph consists of a heated injection port, a fused silica capillary column which is located within an oven and a detector (Figure 3.1).

FAMEs are injected into the gas chromatograph port and are heated to 200-300ºC and become volatile.

FAMEs are transported into the capillary column via a stream of helium (or sometimes hydrogen). The capillary column is at a lower temperature than the injection port causing the FAMEs to condense on the column lining. The column is then heated and FAMEs dissociate at their individual boiling point.

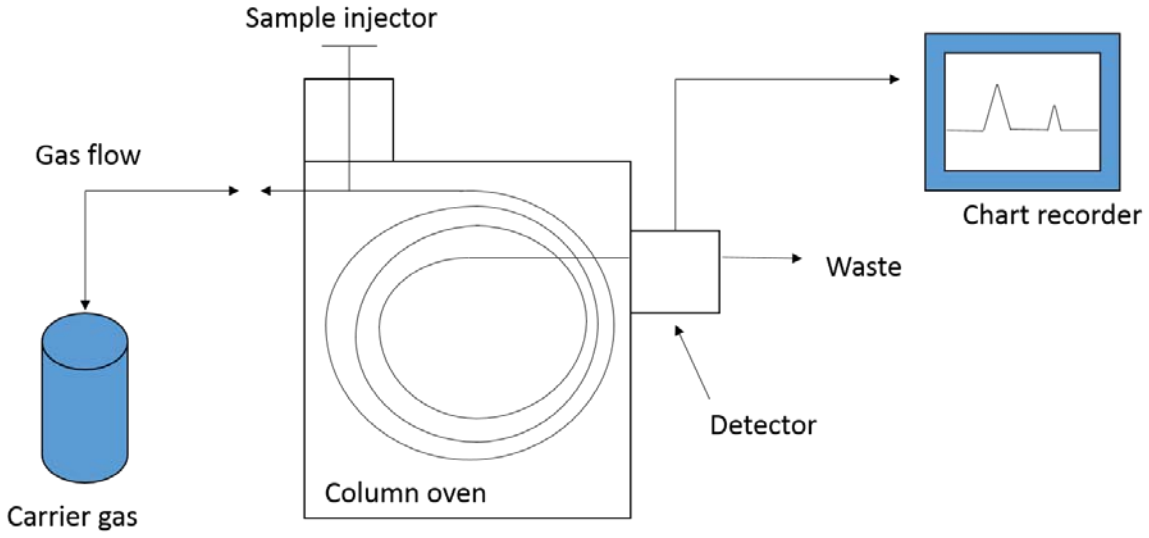

Figure 3.1 Schematic of a gas chromotograph system.

The flame ionisation detector (at 250°C) consists of a hydrogen flame that surrounds the end of the column. The flame causes combustion of the FAMEs creating an ion current proportional to the amount of FAMEs in the sample. A chromatogram is produced which contains a series of peaks, each corresponding to one FAME. The area under the peak is proportional to the mass of FAME injected onto the column.

3.3.3.3.1 GC procedure – Incorporation of FAs into EA.hy926

Figure 2.21 depicts the experimental design. Confluent cultures were scraped from flasks and cells resuspended at a density of 4×10^5 cells/mL in either culture medium with supplements (control) or culture medium with supplements containing FAs at a concentration of 10 or 50 μM. Cells were seeded at 1×10^6 cells per T25 flask and incubated for 48 hours at 37°C. Following incubation cells were scraped, centrifuged at 1200 rpm for 5 minutes, and resuspended in DMEM (washed); this was repeated twice. Cell number was determined using a Beckman Coulter cell counter.

Cells were centrifuged at 1200 rpm for 5 minutes and resuspended in 0.9% NaCl at a volume to achieve 1.25×10^6 cells/mL.

3.3.3.4 Principle of Gas Chromatography/Mass spectrometry

The Gas Chromatography/Mass Spectrometry (GC/MS) instrument combines the features of gas-liquid chromatography and mass spectrometry to identify different substances. The GC component separates chemical substances and the MS component identifies fragments of those substances at the molecular level.

The GC portion separates the mixture into individual substances when heated based on their volatility. The heated gases are then carried through a column within an inert gas (such as helium). Separated substances then flow into the MS. Spectra of compounds are collected as they exit the chromatographic column by the mass spectrometer, which identifies the chemicals according to their mass-to-charge ratio (m/z). MS can provide detailed structural information on most compounds that they can be precisely identified.

MS was performed by Magda Minnion, a research technician working for Professor Martin Feelisch.

3.3.3.4.1 GC/MS procedure – Incorporation of pinolenic acid into EA.hy926 cells

Figure 2.21 depicts the experimental design. Confluent cultures were scraped from plates and resuspended at a density of 4 x 10^5 cells per mL in either culture medium with supplements (control) or culture medium with supplements containing PIN at a concentration of 50 µM. Cells were seeded at 1 x 10^6 cells per T25 flask, and incubated for 48 hours at 37°C. Following incubation cells were scraped, centrifuged at 1200 rpm for 5 minutes, and resuspended in DMEM (washed); this was repeated twice. Cell concentration was determined using a Beckman Coulter cell counter.

Cells were centrifuged at 1200 rpm for 5 minutes and resuspended in 0.9% NaCl at a volume to achieve 1.25 x 10^6 cells/mL.

3.3.4 EA.hy926 cell fatty acid analysis by gas chromatography

3.3.4.1 Lipid extraction and formation of FAMEs

Total lipid was extracted from cell pellets. Pellets were resuspended at 1.25 x 10^6 cells/mL in 0.9% NaCl, 800 µl of which was added to 5 mL chloroform:methanol (2:1) containing BHT (50 mg/L) anti oxidant and 1 ml 1 M NaCl. Tubes were vortexed and centrifuged at 2000 rpm for 10 minutes. The lower phase was collected and dried under N_2 at 40ºC. FAMEs were produced by adding 1 mL of methanol containing 2% (v/v) H_2SO_4 to each sample and heating at 50ºC for 2 hours. Samples were allowed to cool and 1 mL of neutralising solution was added (0.25M $KHCO_3$ (25.03g/l), 0.5M K_2CO_3 (69.10g/l)). FAMEs were then dissolved in 1 mL of hexane and dried under N_2. Samples were resuspended in 150 µL of dry hexane and transferred to a GC autosampler vial.

3.3.4.2 GC analysis

GC analysis was performed in a Hewlett-Packard 6890 chromatograph fitted with a SGE BPX-70 capillary column (30 m x 0.2 mm x 0.25 µm) or WCOT fused silica CP-SIL88 capillary column (100 m x 0.25 mm) and a flame ionising detector. The split ratio was programmed to 2:1 and 1 µL of sample injected. Temperature of the injector port was set to 300ºC. Helium was used as the FAME carrier gas. A heating cycle was used to condense the FAMEs. Initially, the oven was heated to 115°C for 2 min, this was then increased by 10ºC every minute until reaching 200ºC which was held for a further 18 minutes. The column reached a maximum

temperature of 245ºC. FAME histograms produced were analysed with Agilent ChemStation software. In order to calibrate and identify FAs according to FAME retention time two standards were used; 37 FAMEs and menhaden oil. Figure 3.2 shows a typical GC trace of a sample of cultured EA.hy926 cells.

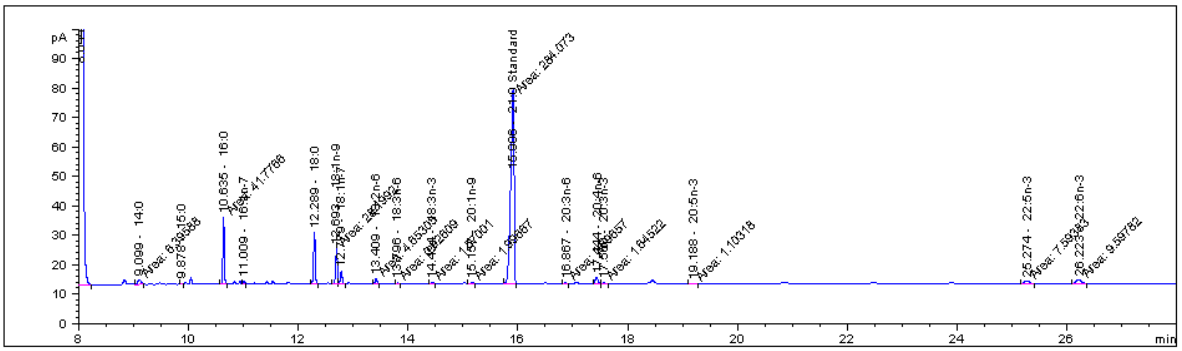

Figure 3.2 Chromatogram of EA.hy926 cells incubated in culture medium for 48 hours.

3.3.5 Statistical analysis

FAME histograms were analysed with Agilent ChemStation software and FA concentrations calculated as concentration per $1x10^6$ cells. Data are expressed as mean ± standard error; data collection analysis were performed in PRSIM and Excel. Multiple group differences were compared using one way ANOVA.

3.4 Results

3.4.1 Viability of EA.hy926 cells treated with TNFα and Fatty Acids

FAs were dissolved in ethanol and further diluted in DMEM before EA.hy926 exposure. Cells were incubated for 48 hours with FAs and then further exposed to TNFα at 1 ng/mL for 24 hours (see Figure 2.21 for experimental design). The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30.

Cell mitochondrial activity was measured using the MTT assay and viability was calculated as a % of control (DMEM). Ethanol at 0.1% did not affect cell viability. Cells exposed to 1 ng/mL TNFα alone were shown to be viable over this time

course (>90%). FAs combined with TNFα exposure had differential effects on viability according to both FA and FA concentration (Figure 3.3 to Figure 3.8).

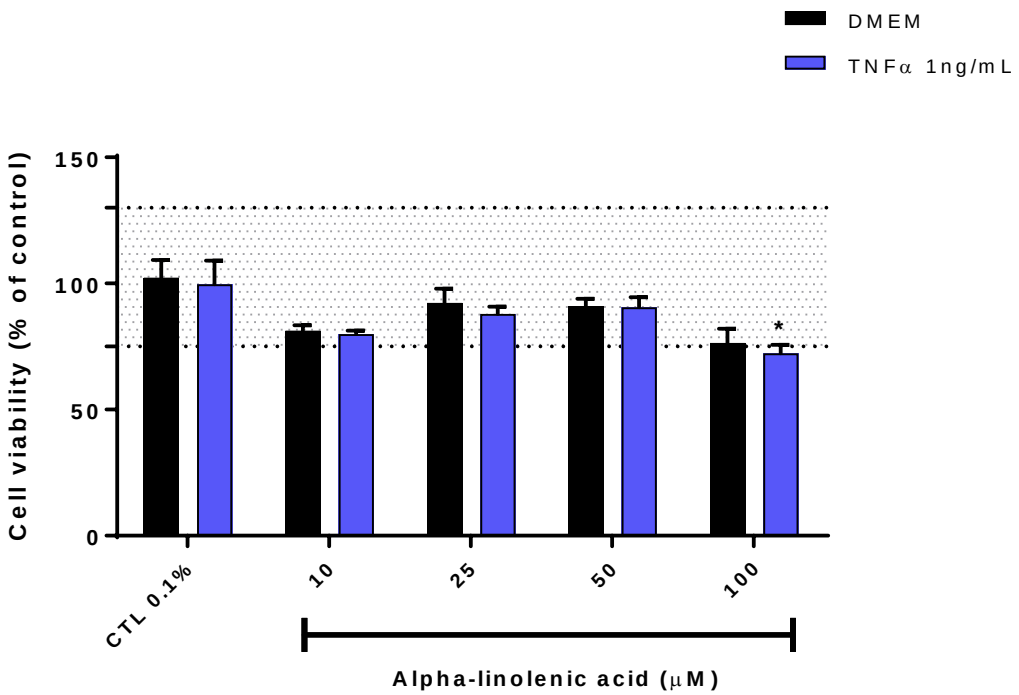

Figure 3.3 Effect of ALA treatment at 10, 25, 50 and 100 μM with or without TNFα on EA.hy926 cell viability

Mean (±SEM) (n = 3) % of control (EA.hy926 + DMEM) cell viability after 48 hour exposure to ALA (10, 25 and 50 μM) followed by 24 hour TNFα (1 ng/mL). Two way ANOVA; CTL 0.1% (TNFα) vs FA (TNFα), *p<0.05

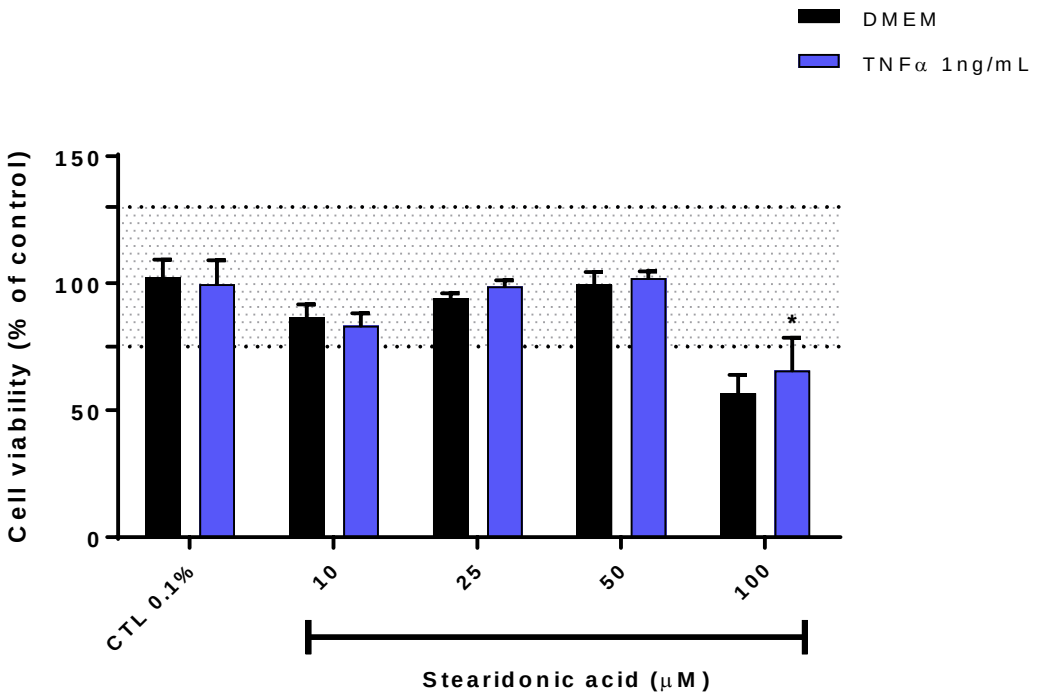

Figure 3.4 Effect of SDA treatment at 10, 25, 50 and 100μM with or without TNFα on EA.hy926 cell viability

Mean (±SEM) (n = 3) % of control (EA.hy926 +DMEM) cell viability after 48 hour exposure to SDA (10 25 and 50μM) followed by 24 hour TNFα (1 ng/mL). Two way ANOVA; CTL 0.1% (TNFα) vs FA (TNFα), *p<0.05

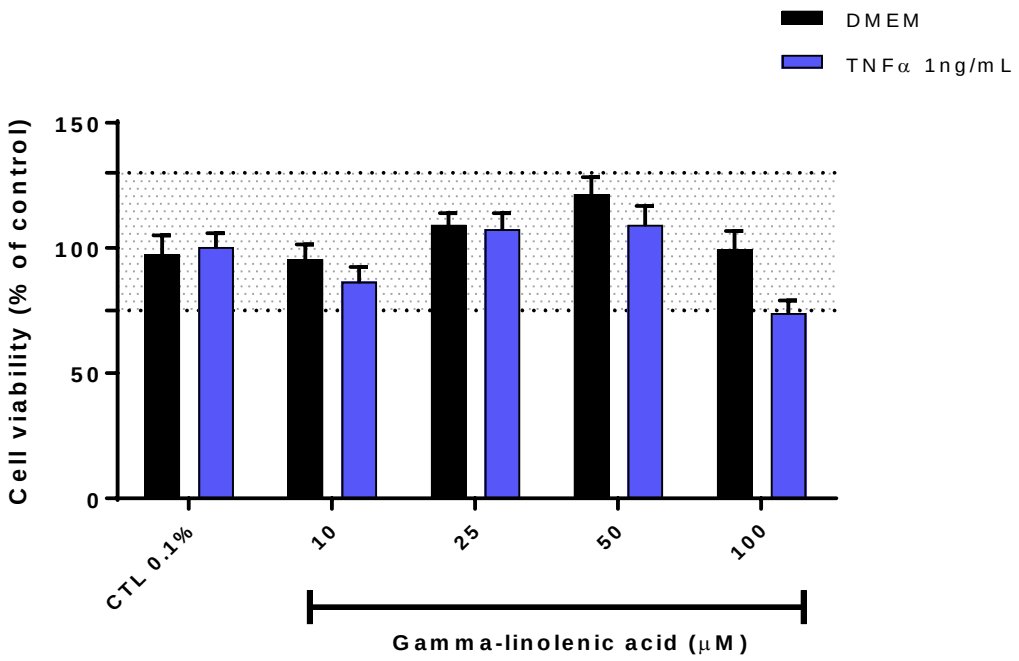

Figure 3.5 Effect of GLA treatment at 10, 25, 50 and 100μM with or without TNFα on EA.hy926 cell viability

Mean (±SEM) (n = 3) % of control (EA.hy926 +DMEM) cell viability after 48 hour exposure to GLA (10 25 and 50μM) followed by 24 hour TNFα (1 ng/mL)). Two way ANOVA; CTL 0.1% (TNFα) vs FA (TNFα)

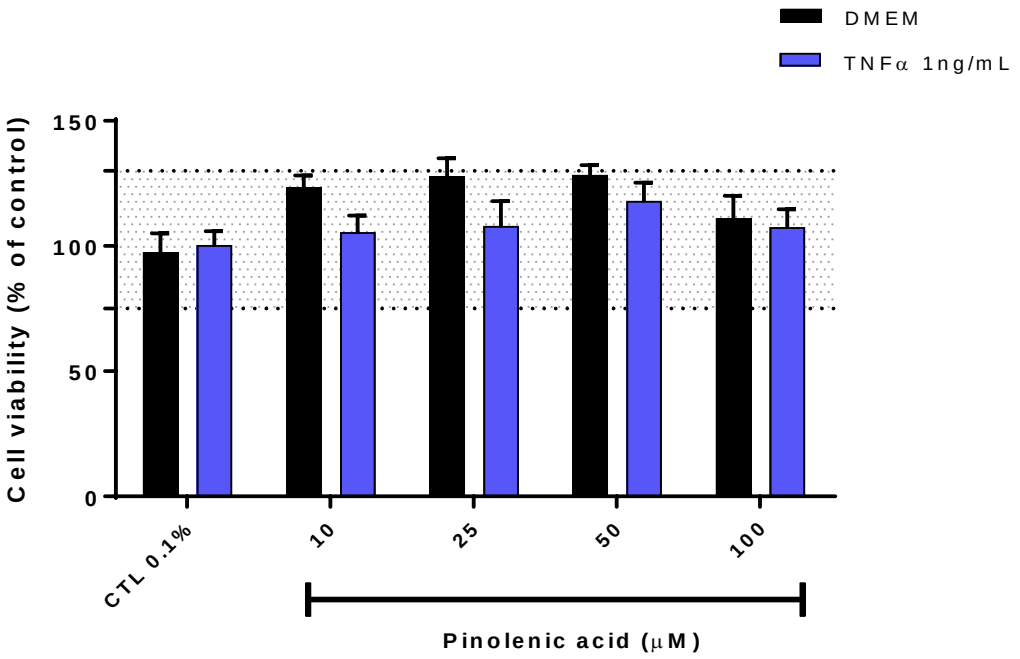

Figure 3.6 Effect of PIN treatment at 10, 25, 50 and 100μM with or without TNFα on EA.hy926 cell viability

Mean (±SEM) (n = 3) % of control (EA.hy926 +DMEM) cell viability after 48 hour exposure to PIN (10 25 and 50μM) followed by 24 hour TNFα (1 ng/mL)). Two way ANOVA; CTL 0.1% (TNFα) vs FA (TNFα)

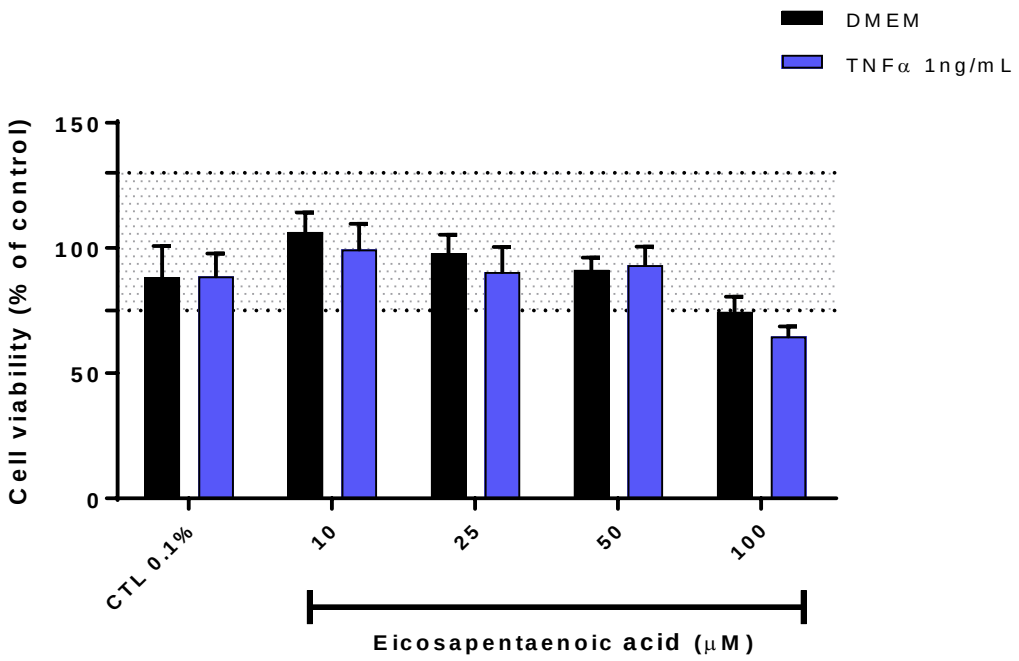

Figure 3.7 Effect of EPA treatment at 10, 25, 50 and 100μM with or without TNFα on EA.hy926 cell viability

Mean (±SEM) (n = 3) % of control (EA.hy926 +DMEM) cell viability after 48 hour exposure to EPA (10 25 and 50μM) followed by 24 hour TNFα (1 ng/mL)). Two way ANOVA; CTL 0.1% (TNFα) vs FA (TNFα)

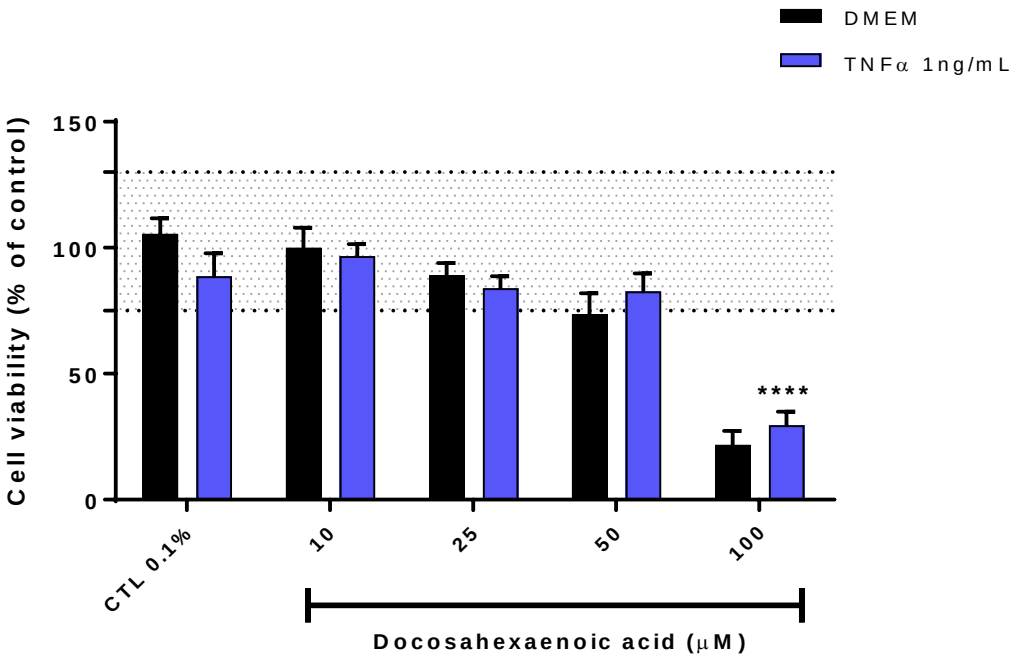

Figure 3.8 Effect of DHA treatment at 10, 25, 50 and 100μM with or without TNFα on EA.hy926 cell viability

Mean (±SEM) (n = 3) % of control (EA.hy926 +DMEM) cell viability after 48 hour exposure to DHA (10 25 and 50μM) followed by 24 hour TNFα (1 ng/mL)). Two way ANOVA; CTL 0.1% (TNFα) vs FA (TNFα), ****p<0.0001

Results showed that after 48 hour FA treatment followed by 24 hour 1ng/mL TNFα exposure the various FAs had a dose-dependent effect on EA.hy926 viability. Figure 3.3, Figure 3.4 and Figure 3.8 show a significant reduction in viability of cells cultured with ALA ($p<0.05$), SDA ($p<0.05$), and DHA ($p<0.0001$) at 100 μM compared to stimulated control cells (<60%, <70%, <25% viability, respectively). EPA too showed a reduction in cell viability at 100 μM (<60%); however this was not a significant effect (Figure 3.7). Viability for these FAs was acceptable (>75%) at concentrations of 50 μM and lower. GLA and PIN did not affect EA.hy926 cell viability even at 100 μM. Based on these results a maximum concentration of 50 μM for each FA was chosen for future experiments.

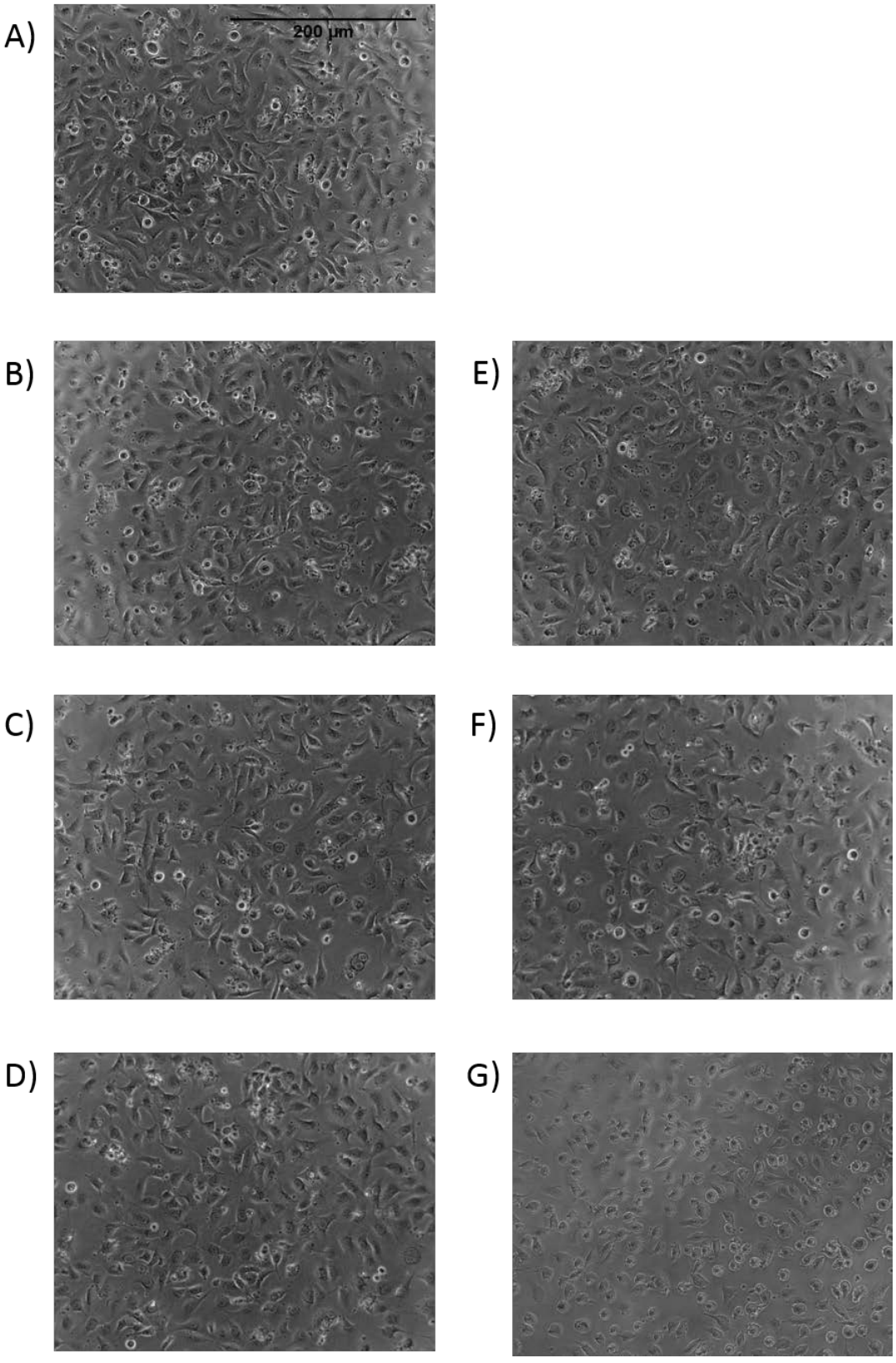

Figure 3.9 Visualisation of TNFα stimulated EA.hy926 after FA exposure at 50 µM

EA.hy926 cells without prior incubation with FA (control; CTL (A)) or with 48 hours prior exposure to ALA (B), SDA (C), GLA (D), PIN (E), EPA (F) and DHA (G) (50 µM) followed by 24 hour TNFα (1 ng/mL). EA.hy926 visualised by fluorescence microscope Nikon Elipse Ti at a magnitude of 10x under transmitted light

Photographs of EA.hy926 cells were taken at the highest concentration of FA (50 µM) and longest exposure of TNFα (24 hours at 1ng/mL) (Figure 3.9). These photographs depict healthy confluent cell monolayers after FA and TNFα exposure, EA.hy926 cells exposed to ALA, SDA, GLA, PIN, EPA and DHA are comparable to control stimulated cells (DMEM). Interestingly DHA treated cells appear to be slightly smaller in diameter.

3.4.2 FA incorporation into EA.hy929 cells

FAs were dissolved in ethanol and further diluted in DMEM, EA.hy926 cells were incubated with the various FAs to allow incorporation into the cell membranes. Appendix B shows the entire FA composition of EA.hy926 cells incubated in the control medium without added FAs. The data shown are from 3 separate experiments each conducted in duplicate, EA.hy926 were used at a passage of >30. Figure 3.10 to Figure 3.15 show the change in FA composition after 48 hour treatment with each FA at a concentration of 10 µM and 50 µM.

After 48 hour FA treatment, FAs were successfully taken up by EA.hy926 cells in a dose-dependent manner. Incubation with the different FAs lead to changes in membrane composition compared to control cells (DMEM alone) and to the appearance of specific metabolic elongation products.

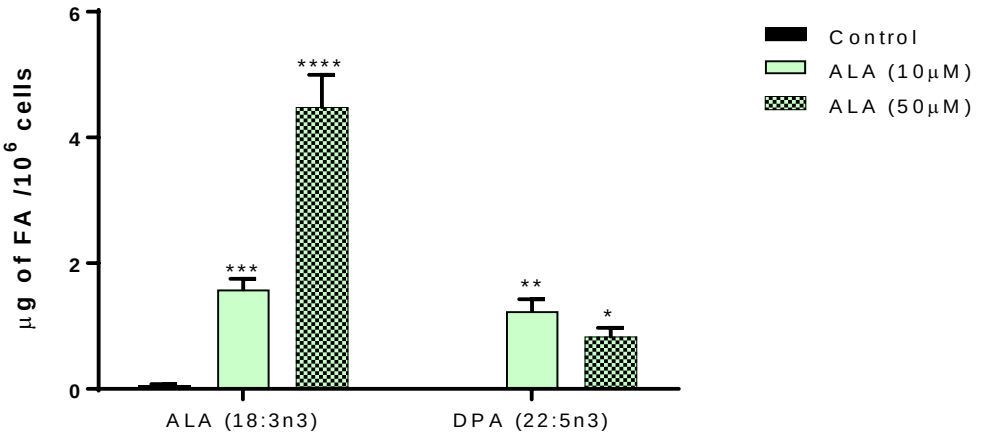

Figure 3.10 Incorporation of ALA and appearance of an elongation product (DPA) after incubation of EA.hy926 cells with ALA for 48 hours

Mean (±SEM) (n = 3) µg of FA/10^6 cells compared to control (EA.hy926 +DMEM) after 48 hour exposure to ALA (10 and 50 µM). Two way ANOVA; control vs FA, *p<0.05, **p<0.01, ****p<0.0001

There was a significant dose-dependent increase in ALA in EA.hy929 cells after incubation with both 10 and 50 μM ALA ($p<0.01$ and $p<0.0001$, respectively) (Figure 3.10). There were also a significant increase in DPA after 10 ($p<0.001$) and 50 μM ($p<0.05$) ALA treatment.

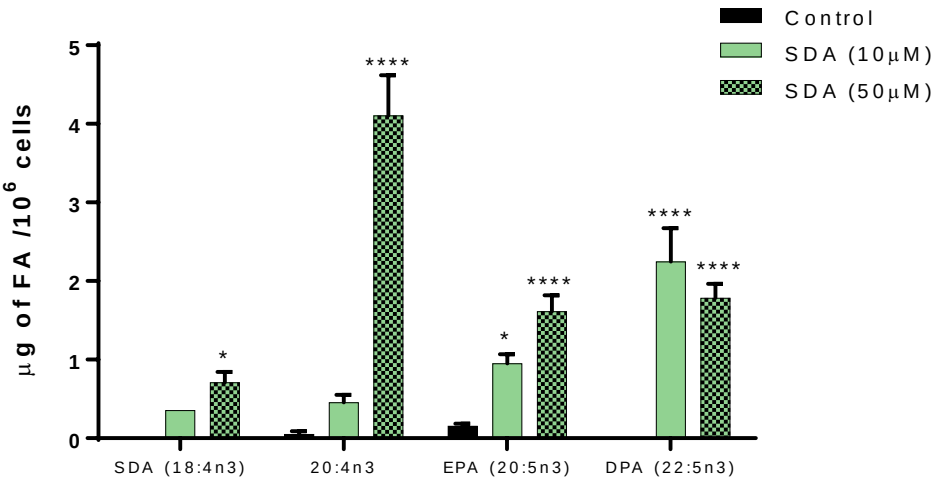

Figure 3.11 Incorporation of SDA and appearance of elongation products after incubation of EA.hy926 cells with SDA for 48 hours

Mean (±SEM) (n = 3) μg of FA/10^6 cells compared to control (EA.hy926 +DMEM) after 48 hour exposure to SDA (10 and 50 μM). Two way ANOVA; control vs FA, *$p<0.05$, ****$p<0.0001$

Incubation with SDA at 50 μM increased SDA levels significantly ($p<0.05$), and also increased levels of several metabolic elongation products including EPA and DPA (both $p<0.0001$) (Figure 3.11).

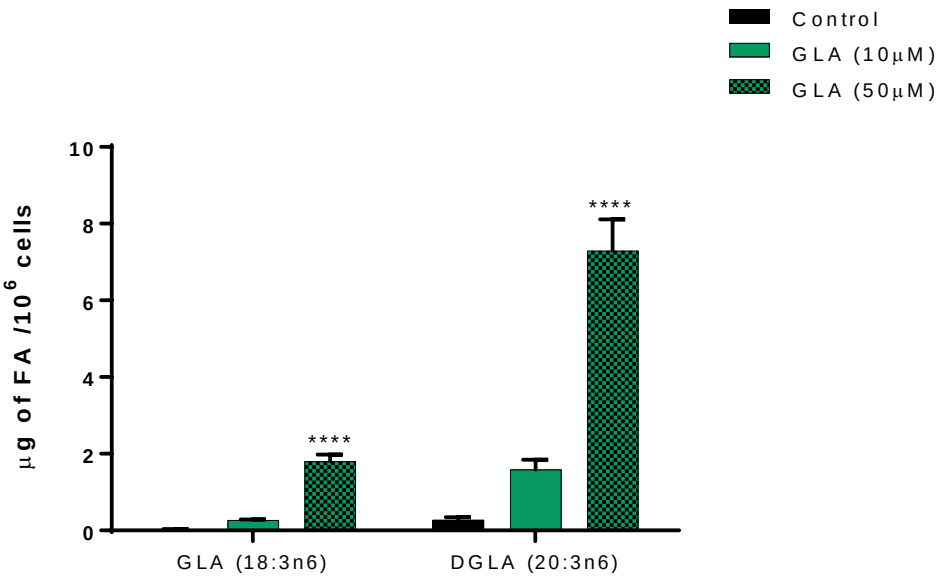

Figure 3.12 Incorporation of GLA and appearance of an elongation product (DGLA) after incubation of EA.hy926 cells with GLA for 48 hours

Mean (±SEM) (n = 3) μg of FA/10^6 cells compared to control (EA.hy926 +DMEM) after 48 hour exposure to GLA (10 and 50 μM). Two way ANOVA; control vs FA, *p<0.05, **p<0.01, ****p<0.0001

A significant increase in GLA was seen after EA.hy926 cells were exposed to 50 μM GLA (p<0.0001) (Figure 3.12). Once again significant increases in other FAs were seen when compared to control cells: the elongation product DGLA, increased significantly after exposure to 10 and 50 μM GLA (p<0.001 and p<0.0001, respectively).

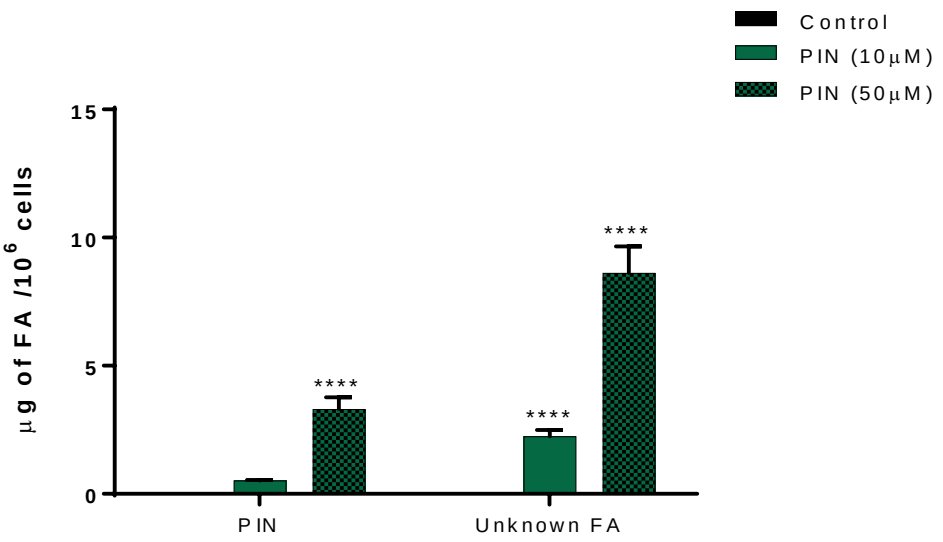

Figure 3.13 Incorporation of PIN and appearance of an unknown product after incubation of EA.hy926 cells with PIN for 48 hours

Mean (±SEM) (n = 3) µg of FA/10^6 cells compared to control (EA.hy926 +DMEM) after 48 hour exposure to PIN (10 and 50 µM). Two way ANOVA; control vs FA, **p<0.01, ****p<0.0001

PIN was significantly incorporated into EA.hy926 cells after exposure at 50 µM ($p<0.0001$) (Figure 3.13). There was also a dose-dependent increase in an unknown FA. This product was not able to be identified using GC since the current standard mixtures used did not contain this FA. Literature suggested the product to be 7,11,14. eicosatrienoic acid (ETrA), an elongation product of PIN and an isomer of DGLA [137]. GC/MS was used to identify the product (see section 3.4.3).

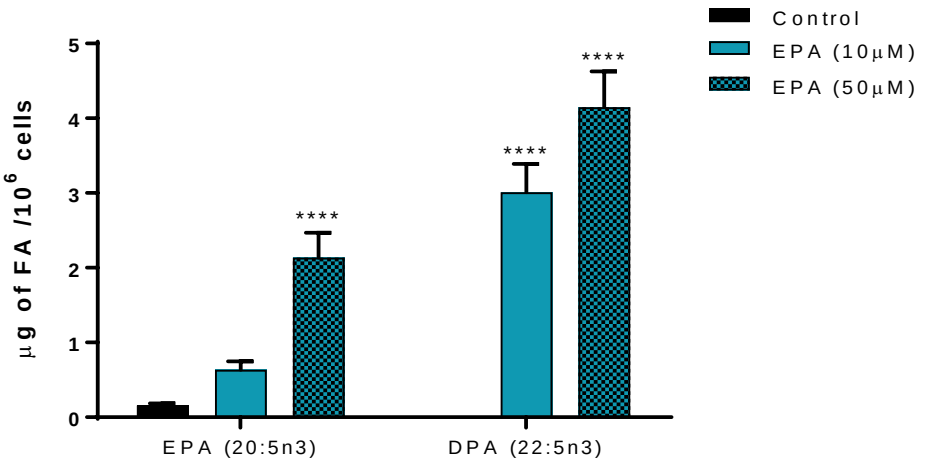

Figure 3.14 Incorporation of EPA and appearance of an elongation product (DPA) after incubation of EA.hy926 cells with EPA for 48 hours

Mean (±SEM) (n = 3) μg of FA/10^6 cells compared to control (EA.hy926 +DMEM) after 48 hour exposure to EPA (10 and 50 μM). Two way ANOVA; control vs FA, ****p<0.0001

Incubation of EA.hy926 cells with EPA at 50 μM significantly increased EPA content of the cells (p<0.0001) (Figure 3.14). There was also a significant dose-dependent increase in DPA after incubation with EPA at 10 and 50 μM (p<0.0001). Incubation with EPA did not enrich the cells with DHA.

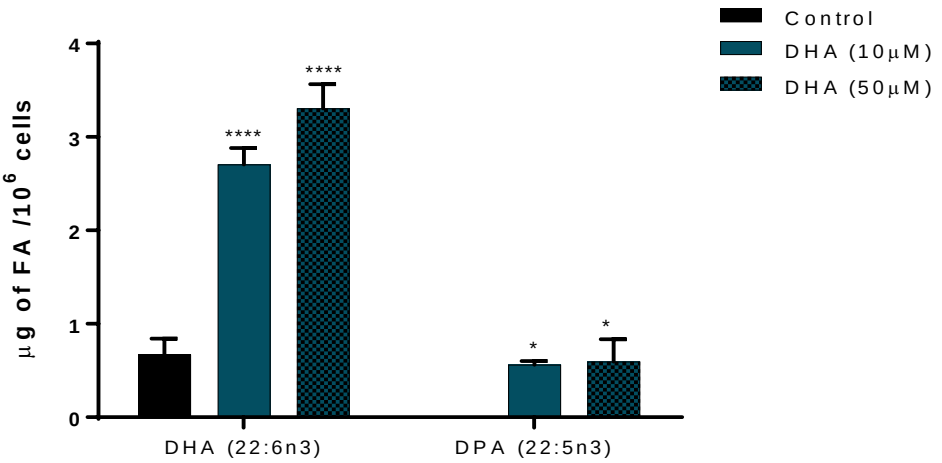

Figure 3.15 Incorporation of DHA and appearance of a metabolic product (DPA) after incubation of EA.hy926 cells with DHA for 48 hours

Mean (±SEM) (n = 3) μg of FA/10^6 cells compared to control (EA.hy926 +DMEM) after 48 hour exposure to DHA (10 and 50 μM). Two way ANOVA; control vs FA, *p<0.05, ***p<0.001, ****p<0.0001

Incubation of EA.hy926 cells with DHA at 10 and 50 μM significantly increased DHA content of the cells (p<0.0001) (Figure 3.15). There was also a significant increase in DPA after incubation with DHA at both 10 and 50 μM (p<0.05). The presence of DPA suggests retroconversion of DHA since DPA is found on the pathway prior to DHA; DPA can be synthesised from DHA by limited peroxisomal β-oxidation [169]. Incubation with DHA did not enrich the cells with EPA.

3.4.3 GC/MS results

3.4.3.1 Identification of the unknown FA produced in EA.hy926 cells after incubation with PIN

GC/MS was used to identify the unknown FA produced by EA.hy926 cells after incubation with PIN.

GC/MS confirmed the presence of the unknown FA after cells were exposed to PIN (Figure 3.16).

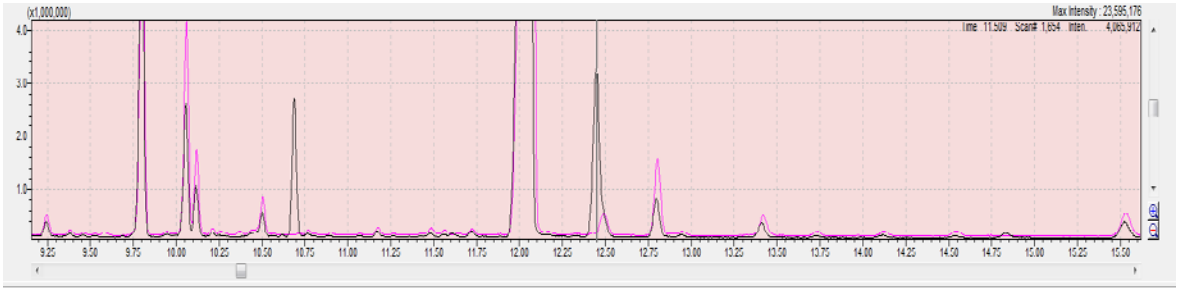

Figure 3.16 GC chromatogram depicting overlay of control EA.hy926 cells (pink line) and EA.hy926 cells incubated with PIN (50 µM) (black line)

Figure 3.16 shows the MS total ion chromatogram depicting (A) the ion fragments of the FA produced in EA.hy926 cells after 48 hours treatment with PIN at 50 µM and (B) the total ion chromatogram of eicosatrienoic acid (ETrA) taken from the Lipidhome data base [170]. Comparison of these fragments showed distinct similarities: fragments of the unknown FA and the known ETrA match suggesting the unknown FA to be an elongation product identified as eicosatrienoic acid (ETrA). This is in line with the literature whereby Szu-Jung *et al*. suggested PIN to be converted to ETrA in murine microglial BV-2 cells [137] .

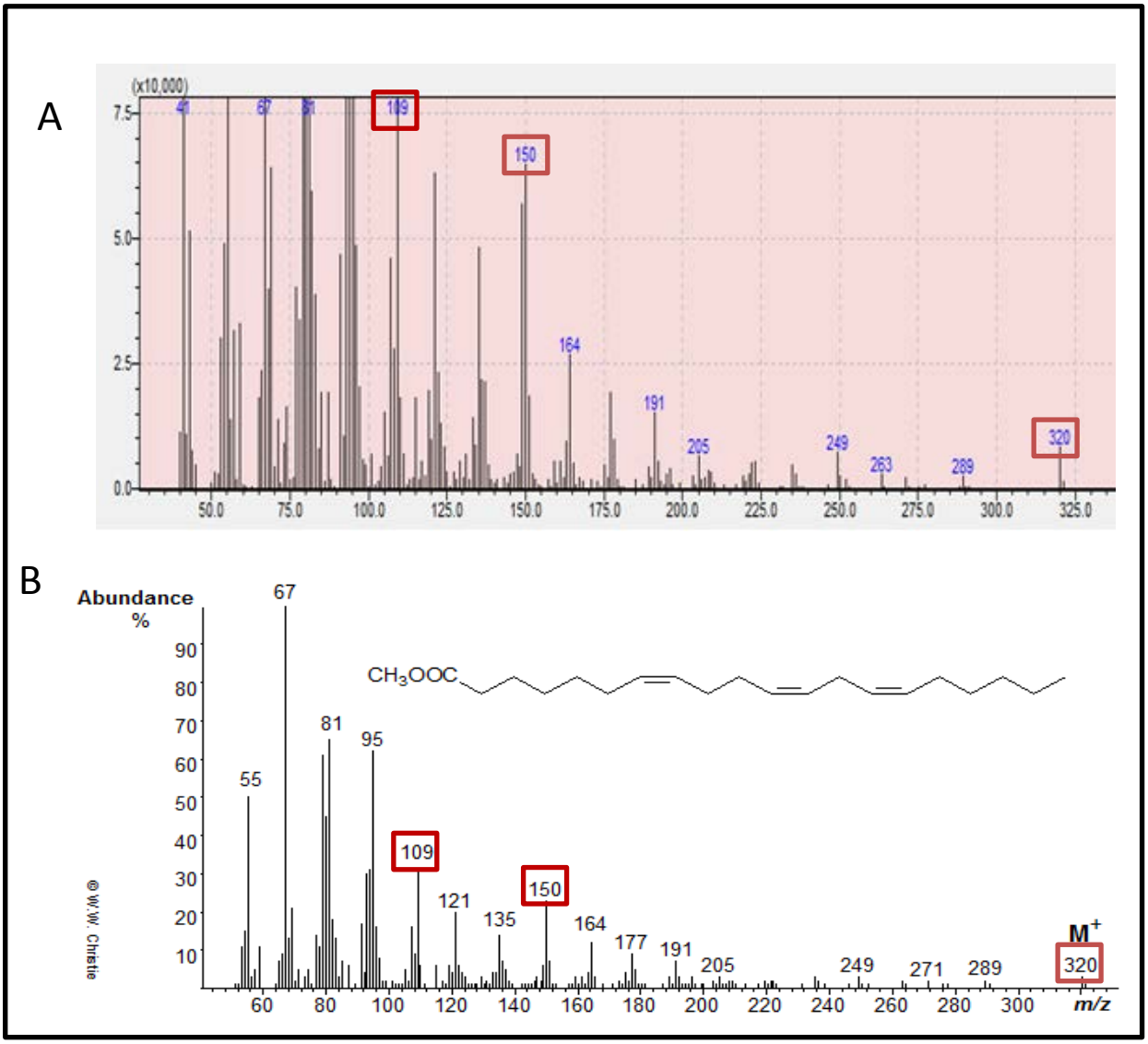

Figure 3.17 GCMS depicting unique identifying fragments of ETrA

A: MS total ion chromatogram of unknown FA present in EA.hy926 cells after exposure to PIN at 50 μM; B: Total ion chromatogram of ETrA taken from lipidhome data base [170]. Unique fragments highlighted in red.

3.5 Discussion

The aim of the research described in this chapter was to determine the incorporation of different FAs into cultured EA.hy926 cells, with the objectives of assessing the toxicity, if any, of TNFα and FAs and the composition of EA.hy926 cells after culture with the different FA. These aims and objectives were met.

TNFα exposure did not alter EA.hy926 cell viability. Furthermore, exposure to GLA or PIN for 48 hours prior to TNFα treatment did not affect cell viability even when the FAs were used at a concentration of 100 μM. In contrast, 48 hour incubation with ALA, SDA, EPA or DHA at 100 μM followed by 24 hour 1 ng/mL TNFα exposure reduced EA.hy926 cell viability to < 75% and much more than this when DHA was used. Therefore the maximal concentration of FA to be used for future experiments was determined to be 50 μM. EA.hy926 cell viability after exposure to any FA at ≤50 μM was ≥75% and this was deemed acceptable. Others also report SDA, EPA and DHA treatment at higher doses can damage and decrease viability of ECs [165, 171], therefore many others use similar concentrations of FAs in endothelial culture models [111, 112, 167, 172, 173]. However some studies have used higher concentrations (≥100 μM) [112, 162, 164, 174]. It is notable that two of the FAs that did not affect viability have 18 carbons and three double bonds. In contrast SDA has four double bonds while EPA and DHA are longer chained (20 and 22 carbons) and more unsaturated (5 and 6 double bonds). It is possible that the more unsaturated FAs (SDA, EPA and DHA) are susceptible to peroxidation in the culture medium and that the peroxidised products are damaging to the cells. This could be explored by adding an antioxidant like vitamin E to the culture medium. The three trienoic FAs (ALA, GLA, PIN) may be more resistant to such peroxidation and so not damaging to the cells.

Photographs of cells exposed to the highest concentration of FAs (50 μM) and 24 hour exposure to TNFα at a concentration of 1 ng/mL indicated that the cells looked normal and had good monolayer formation (Figure 3.9). DHA however appeared to have a slight effect on the morphology of the EA.hy926 cells. These observations, together with the data from the MTT assay, suggest that the cells were in a healthy state.

It is important to note that each of the FA stock concentrations was analysed prior to each experimental procedure by GC analysis (see Appendix C for example), as well as the concentrations of FA within complete culture medium

(2.3.2) (see Appendix C). Most publications do not indicate that the researchers verify the concentrations of the FA stock they use. Doing this ensures that the FA being used is at the correct concentration allowing for an accurate examination of the effect of the FA under study. This is one strength of the current work.

3.5.1 FA incorporation and metabolism

Exposure of EA.hy926 cell to any of the six FAs studied resulted in incorporation of the specific FA into the cells in a dose-dependent manner. EA.hy926 cells were also shown to produce several different elongation and desaturation products when exposed to the various different FAs. ALA gave rise to DPA and SDA gave rise to 20:4n-3, EPA and DPA. SDA produced more EPA and DPA than ALA did, consistent with other studies suggesting that SDA is a better precursor for EPA and DPA than ALA [12]. Since ALA gave rise to DPA alone, this may indicate quick metabolism of EPA into DPA, as a similar metabolism was seen after SDA treatment at 10 μM, where although EPA was shown to increase this was to a lower extent than DPA. Incubation with EPA produced DPA. None of ALA, SDA or EPA produced detectable DHA. These observations show that ECs, or at least the EA.hy926 cell line, have a fully intact PUFA desaturation and elongation pathway that produces DPA as its end product within the n-3 FA family. The findings also suggest that ECs cannot produce their own DHA.

Very few studies have reported ALA incorporation and FA composition of ECs after ALA treatment. Livingston *et al.* observed no significant changes in ALA composition after treatment with ALA (20 μM) and reported no changes in EPA or DHA [166]. To the best of my knowledge the incorporation of SDA has not been studied after treatment of ECs in vitro. However researchers have reported incubation with SDA in 3T3-L1 adipocytes and saw enriched levels of EPA, as well as of DHA [175]. Furthermore they reported greater enrichment of EPA after SDA treatment compared to ALA. In the current study incubation with GLA produced DGLA, but no detectable AA. These findings with cultured ECs are similar to effects seen by Chapkin *et al.* in macrophages incubated with GLA; they described no change in AA content but an increase in DGLA [176]. These observations suggest that ECs can metabolise GLA but not all the way to AA; why the n-3 FA pathway to EPA works well, while that same n-6 FA pathway to AA does not is not clear, but this may be a metabolic attempt to restrict endogenous biosynthesis of pro-inflammatory and prothrombotic AA within ECs.

Incubation with DHA produced DPA but not EPA, indicating partial retroconversion and possibly an attempt to control the extent of DHA incorporation. Others have also reported retroconversion of DHA in ECs but show increases in EPA after DHA treatment [151]. It is possible that DHA is retroconverted to EPA which is rapidly elongated to DPA, so that EPA does not accumulate but DPA does.

There are few studies of the metabolic fate of PIN, a trienoic FA with an unusual double bond configuration [177, 178]. PIN itself was well incorporated into EA.hy926 cells and produced a FA that could not be identified by standard GC. It was presumed that this was the elongation product ETrA [137]. Further analysis using GC/MS successfully identified the unknown FA as ETrA. Similarly Chuang *et al*. saw increased PIN and ETrA after treatment with PIN (50 μM) in RAW264.7 (murine macrophage) cells [179].

Of those that have described effects of EPA and DHA treatment in ECs, few have also reported the FA incorporation [110, 114]. Yates *et al*. described significant increases in both EPA and DHA after supplementation in ECs. They linked this incorporation to functional effects of these FAs including a decrease in neutrophil adhesion in DHA treated cells [114]. De Caterina *et al.* also showed that treatment with DHA enriched EC cellular phospholipids, which they directly correlated to the inhibition of cytokine induced cell surface VCAM-1 expression [110].

However, since few studies report incorporation details after FA treatment of ECs and most studies make assumptions on the extent to which the FA being examined has been incorporated, it is possible that these FAs are also elongated as seen here in the EA.hy926 cells after treatment. This is of importance since the function of FAs cannot be fully examined and understood without knowing the extent of FA incorporation and metabolism. This is another strength of the current work.

The FA elongation products being produced by EA.hy926 may also influence how the cells respond to inflammatory stimulation. ALA has been shown to have little effect on cardiovascular outcomes in human studies, whereas SDA has some effects. It is likely that this difference is due to the extent to which these FAs are elongated to their longer chain more bioactive metabolites [21]. As shown here ALA is not metabolised by ECs to the same extent as SDA, and this may influence how well these two FAs affect inflammation in EA.hy926 cells. Other studies comparing effects of SDA or ALA supplementation on blood cell VLC n-3 FA

composition report higher levels of EPA after SDA supplementation than ALA supplementation [180, 181].

However, the fact the FAs provided to the cells are metabolised to other bioactive FAs will make it difficult to unequivocally ascribe any effects seen to the specific FA provided. In order to differentiate between the effects of the parent FA and its FA metabolites, enzymatic pathways of desaturation and elongation could be blocked. This may help to define the roles of the specific FAs in inflammation.

3.5.2 Conclusions

Overall the observations made here confirm significant incorporation and metabolism of FAs provided to cultured EA.hy926 cells. These cells are able to convert precursor FAs into longer chain, more unsaturated derivatives, although they appear unable to synthesise DHA. They can retroconvert DHA however. Despite the significant changes in FA composition, the cells remain viable when FAs are used at concentrations up to 50 μM. This was further confirmed by photographs taken of the cells in the most extreme conditions (50 μM FA and 24 hour TNFα at 1 ng/mL), which showed FAs and TNFα did not affect cell monolayer formation. Together these data indicate cells to be in proper functioning state. It is assumed that much of the FA incorporation will be into the membrane of the cells. This provides a link between FA exposure and functional effects. Influence of the FAs on inflammatory responses of the ECs will be described in the following chapters.

Chapter 4: Effect of fatty acids on production of inflammatory mediators by cultured endothelial cells

4.1 Introduction

ECs are the main barrier of exchange and contact between blood and tissues and therefore immune mediators and molecules secreted by these cells play a major role in inflammatory processes. It has been described that the marine-derived n-3 PUFAs EPA and DHA show anti-inflammatory effects on ECs. Effects of PUFAs on endothelial functionality were firstly examined by de Caterina *et al.* who showed that ECs treated with DHA had both decreased cytokine-induced expression of endothelial leukocyte adhesion molecules (VCAM-1 and ICAM-1) and secretion of inflammatory mediators (IL-6 and IL-8) [110]. Another study by Ibrahim *et al.* described reduced VCAM-1 and VEGF expression as well as IL-6 and IL-8 production in IL-1β stimulated HIMEC (human intestinal microvascular endothelial cells) after DHA treatment [182]. Indeed the literature suggests that the effects of EPA and DHA on the endothelium could in part be responsible for their health benefiting actions [26].

Plant-derived FAs may also help to lower endothelium inflammation and by doing so could have the potential to ameliorate atherosclerosis. However effects of bioactive plant-derived FAs on ECs are poorly explored. Of the plant FAs, ALA and SDA, have been more frequently studied, and there are indications that these FAs have some anti-inflammatory effects [165, 167, 168, 183, 184]. However meta-analyses have indicated ALA may not have the same potency as marine-derived EPA and DHA [21, 185]. Few studies have investigated the effects of GLA [183, 186] and PIN [177, 179] on cell inflammation and those that have yield mixed outcomes.

In chapter 2 an EC based model for examining effects of FAs on inflammation was established while in chapter 3 both plant and marine-derived FAs were shown to be incorporated into and metabolised by ECs in culture without a loss in cell viability. In the context of establishing real alternatives to EPA and DHA for promotion of human health, candidate FAs need to be examined first in model systems and where they appear to be of potential benefit they can be carried forward into animal and human studies. The research described in this chapter compares the effects of four plant-derived 18 carbon FAs (ALA, SDA, GLA, and PIN) on inflammatory responses of ECs in vitro with those of EPA and DHA. This is an important first step in the search for more sustainable sources of bioactive FAs for promoting human health.

4.2 Aim and Objectives

The aim of the research described in this chapter was to compare the effects of four plant-derived and two marine-derived FAs on inflammatory responses of cultured ECs.

The specific objectives were to:

- compare the effects of four plant-derived FAs (ALA, SDA, GLA and PIN) to two marine-derived FAs (EPA and DHA) on production of various inflammatory mediators (ICAM-1, MCP-1, VEGF, IL-6, IL-8, RANTES) by cultured ECs;
- compare the effect of the plant and marine-derived FAs on the expression of ICAM-1 on the surface of cultured ECs.

4.3 Methods

4.3.1 Reagents

Reagents and materials used for experiments are described in Appendix A.

4.3.2 Production of inflammatory mediators by cultured EA.hy926 cells

As described previously (2.3.2.1) confluent EA.hy926 cells were scraped from flasks and resuspended at a density of 2 x 10^5 cells per mL. Cells were seeded at 1 x 10^4 cells per well in 96-well flat bottom plates, and incubated for 48 hours at 37°C with the FAs at a concentration of 10, 25 or 50 μM followed by 24 hour incubation with TNFα at 1 ng/mL. At each time point, supernatant was removed and stored at -20°C until analysis. Concentrations of cytokines and adhesion molecules in these supernatants were determined simultaneously using Human Magnetic Luminex Screening Assay kits by R&D using the assay described in section 2.3.3.

4.3.2.1 Cell surface expression of ICAM-1 determined by flow cytometry

Confluent EA.hy926 cultures were scraped from flasks and resuspended in culture medium at a density of 5.5 x 10^5 cells per mL. Cells were seeded at 5.5 x 10^4 cells per well in 6-well flat bottom plates, incubated for 48 hours at 37°C with

various FAs at a concentration of 25 or 50 μM followed by 6 hour incubation with either DMEM or TNFα at 1 ng/mL.

Following incubation, supernatant was removed and 2 mL cold PBS added to each well. Cells were detached by scraping and transferred to Eppendorf tubes. Cells were centrifuged at 1200 rpm for 15 mins. Pellets were then resuspended in warm PBS containing 2% FBS and transferred to a 96-well plate. The plate was centrifuged at 1200 rpm for 5 min. Supernatant was discarded and cells stained with (PE)-conjugated anti-human CD54 (ICAM-1) at 10 μg/mL for 30 min at room temperature in darkness. Unstained negative controls were also prepared; these were incubated with PBS alone.

After incubation, samples were washed with PBS containing 2% FBS, centrifuged at 1200 rpm for 5 min. Supernatant was discarded and pellets resuspended in 200 μL BD CellFIX. Plates were then stored at 4˚C overnight and were analysed the following morning. Cells were analysed in a FACS Calibur flow cytometer (Becton Dickinson) and data collected using CellQuest Pro software.

For each sample 10,000 events were collected. Forward and side scatter were used to determine the cell size and granularity and a gate was drawn around the cell population. Cells within this gate were analysed to determine PE fluorescent staining.

Figure 4.1 shows gating profiles for unstimulated and stimulated stained and unstained EA.hy926 cells.

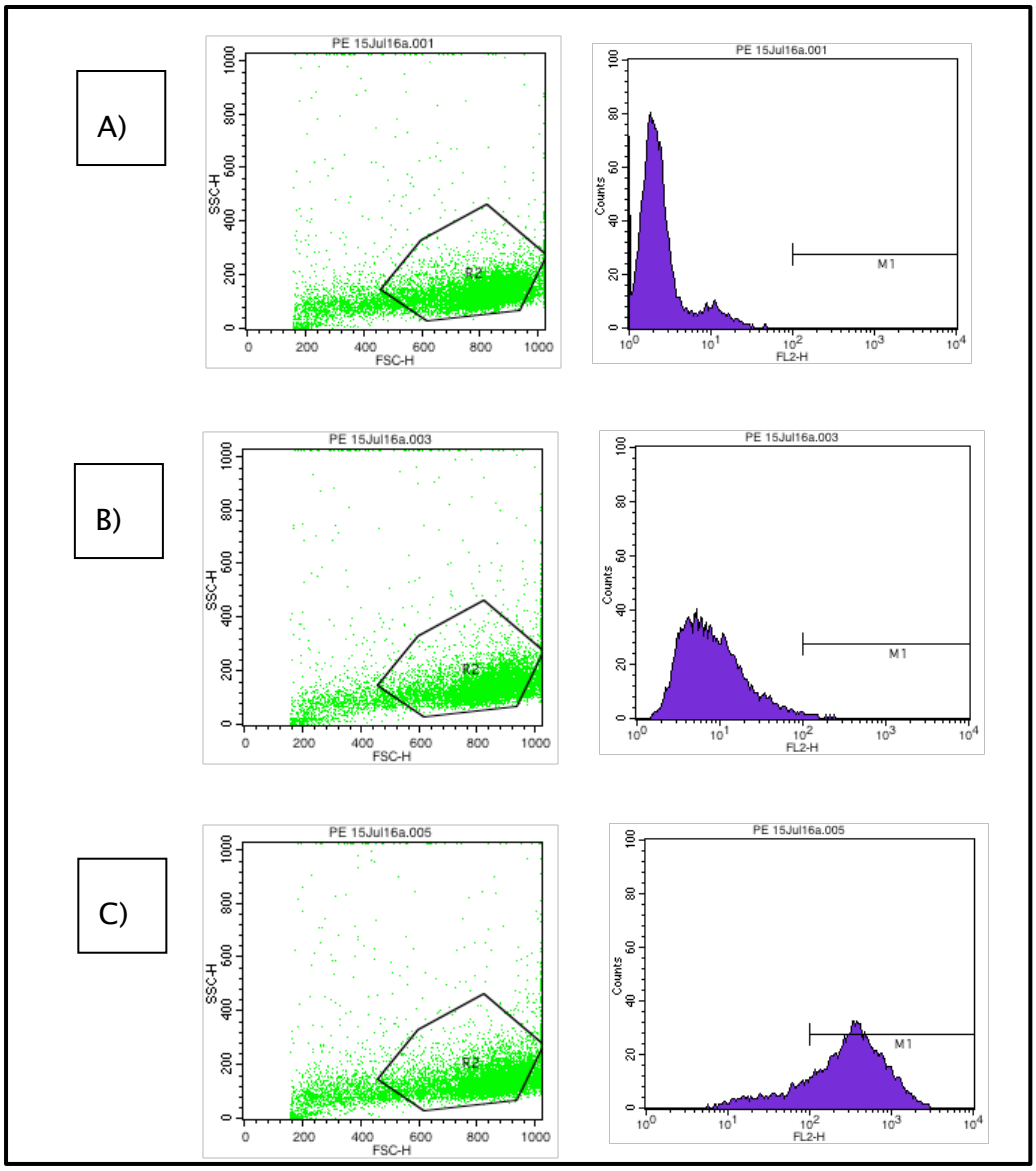

Figure 4.1 Flow cytometry plots for ICAM-1 cell surface analysis. Gating of EA.hy926 for A) unstimulated unstained cells; B) unstimulated, PE-conjugated ICAM-1 antibody stained cells; C) stimulated with TNFα (1ng/mL), PE-conjugated ICAM-1 antibody stained cells.

4.3.2.2 Principle of flow cytometry

Flow cytometry measures and analyses cells as they flow in a stream through the beam of a laser.

The flow cytometer is composed of

- a flow chamber which is designed to deliver the cells in single file at the point of measurement;

- an optic system whereby lasers create light signals (through which cells/particles are passed) which are then collected/directed by optical filters to specific detectors
- an electronic system which converts detected light signals into electrical signals. This electronic signal is processed and analysed using computer software.

The sample is injected into the centre of a stream of liquid (water or buffer). Figure 4.2 is a depiction of the main principle of flow cytometry.

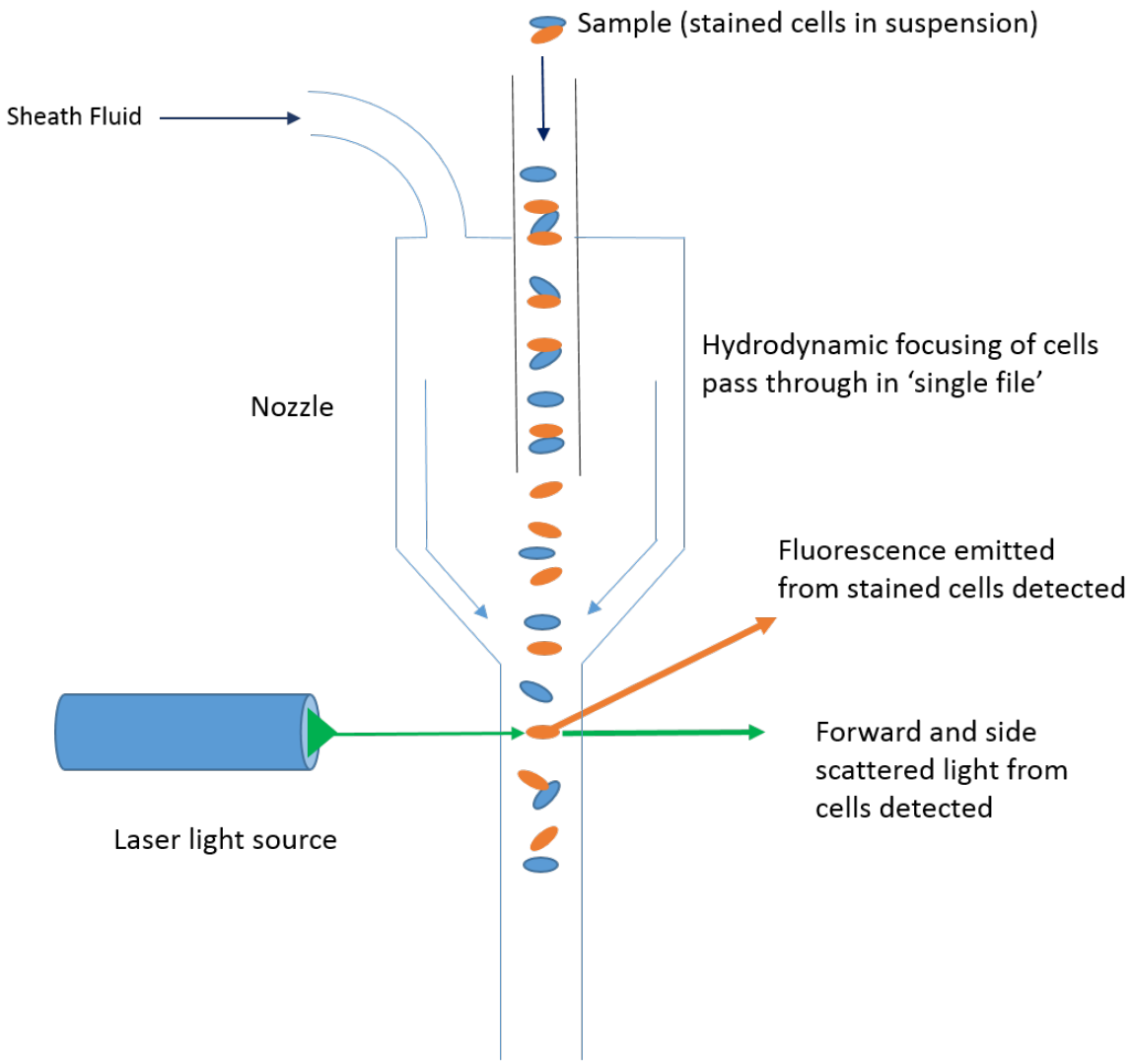

Figure 4.2 Schematic of flow cytometry principle

4.3.3 Statistical analysis

Supernatant concentrations of cytokines and inflammatory mediators were calculated using the Bio-Plex manager 6.0 software (Bio-Rad). Data are expressed as mean ± standard error; data collection analysis was performed in PRSIM and Excel. Multiple group differences were compared using one way ANOVA.

Flow cytometry data are expressed as a % of gated $CD54^{+}$ positive cells (control). Data are expressed as mean ± standard error; data collection analysis was performed in PRSIM and Excel.

4.4 Results

4.4.1 Effect of fatty acid treatment on production of inflammatory mediators by EA.hy926 cells

In order to investigate the effect of different FAs on the production of inflammatory mediators, EA.hy926 cells were exposed to FAs at concentrations of 10, 25 and 50 μM for 48 hours followed by 24 hour TNFα stimulation at 1 ng/mL. Concentrations of mediators in the culture medium after 24 hours were evaluated by Luminex assay. The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30.

TNFα treatment was previously shown to increase production of each of the analytes examined (Figure 2.8 - Figure 2.13). FA exposure was shown to have differential effects depending on the individual FA and on FA concentration (Figure 4.3 Figure 4.8). Marine-derived EPA and DHA showed the most potent anti-inflammatory effects for all analytes measured. Plant-derived GLA and PIN showed some significant anti-inflammatory effects with a tendency to decrease most of the analytes measured, whereas ALA and SDA had the least potent effects on inflammation.

4.4.1.1 ICAM-1

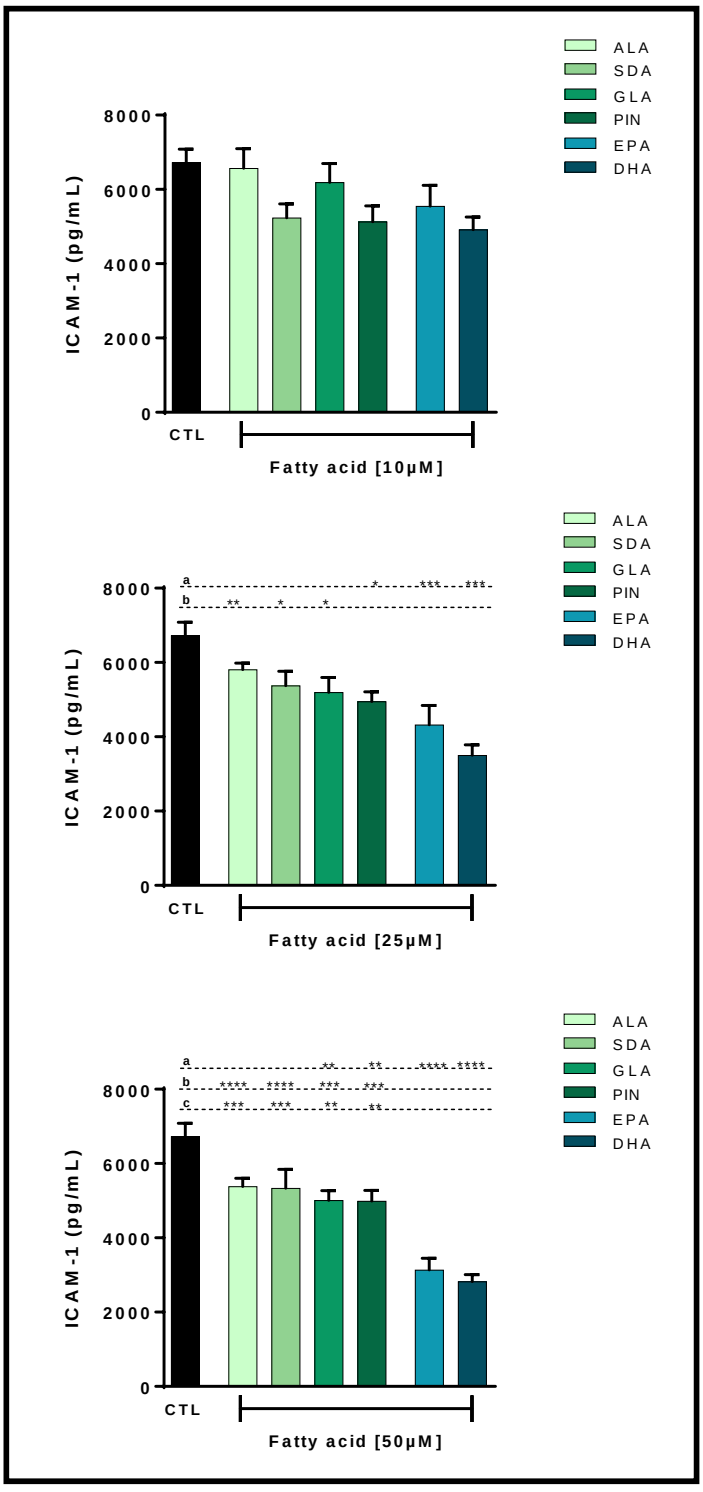

Figure 4.3 Effect of FA exposure on soluble ICAM-1 production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) concentration of soluble ICAM-1 in the medium of TNFα activated EA.hy926 cells without prior incubation with FA (control; CTL) or with 48 hours prior exposure to ALA, SDA, GLA, PIN, EPA and DHA (10, 25 and 50 µM) followed by 24 hour TNFα (1 ng/mL). One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

4.4.1.2 IL-6

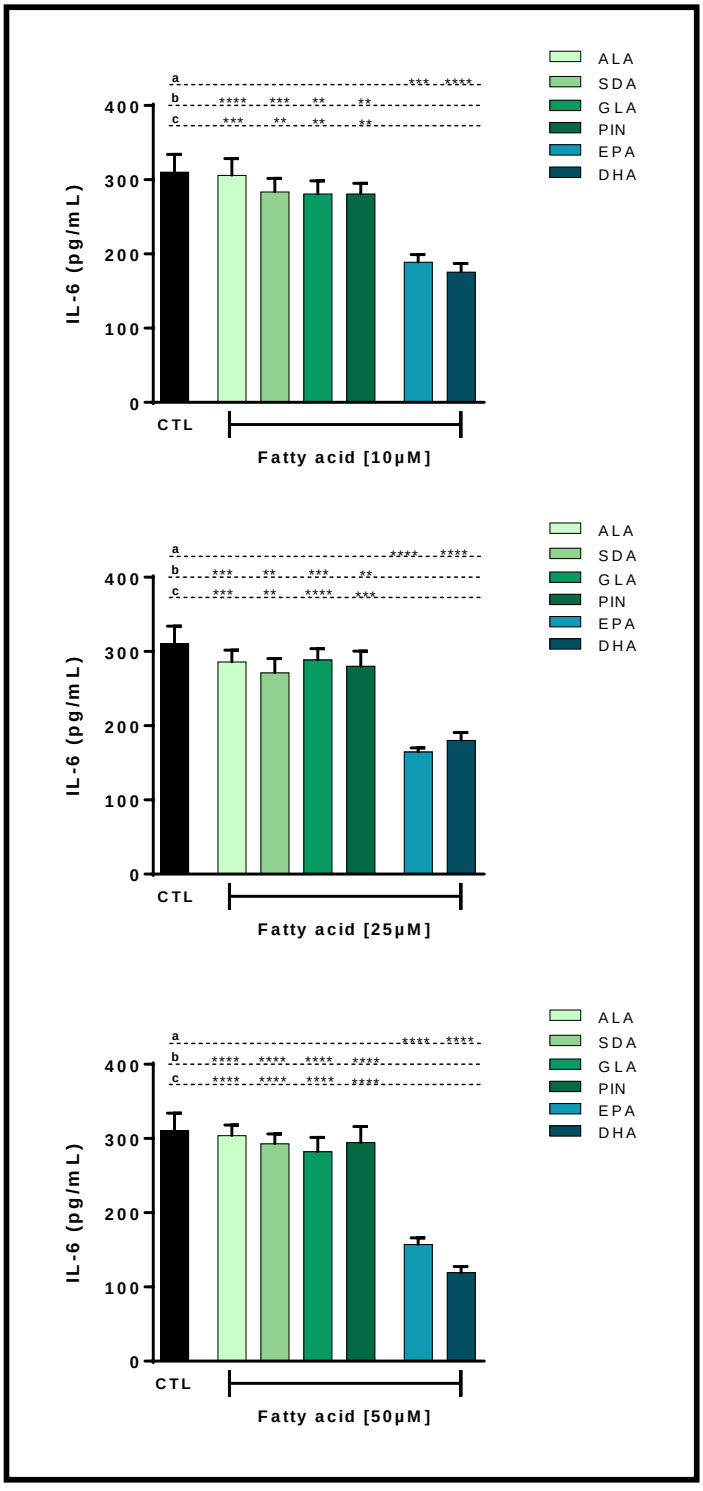

Figure 4.4 Effect of FA exposure on IL-6 production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) production of IL-6 in activated EA.hy926 (control) or 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (10, 25 and 50µM) followed by 24 hour TNFα (1ng/mL). IL-6 was measured in the supernate of endothelial cell cultures. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

4.4.1.3 IL-8

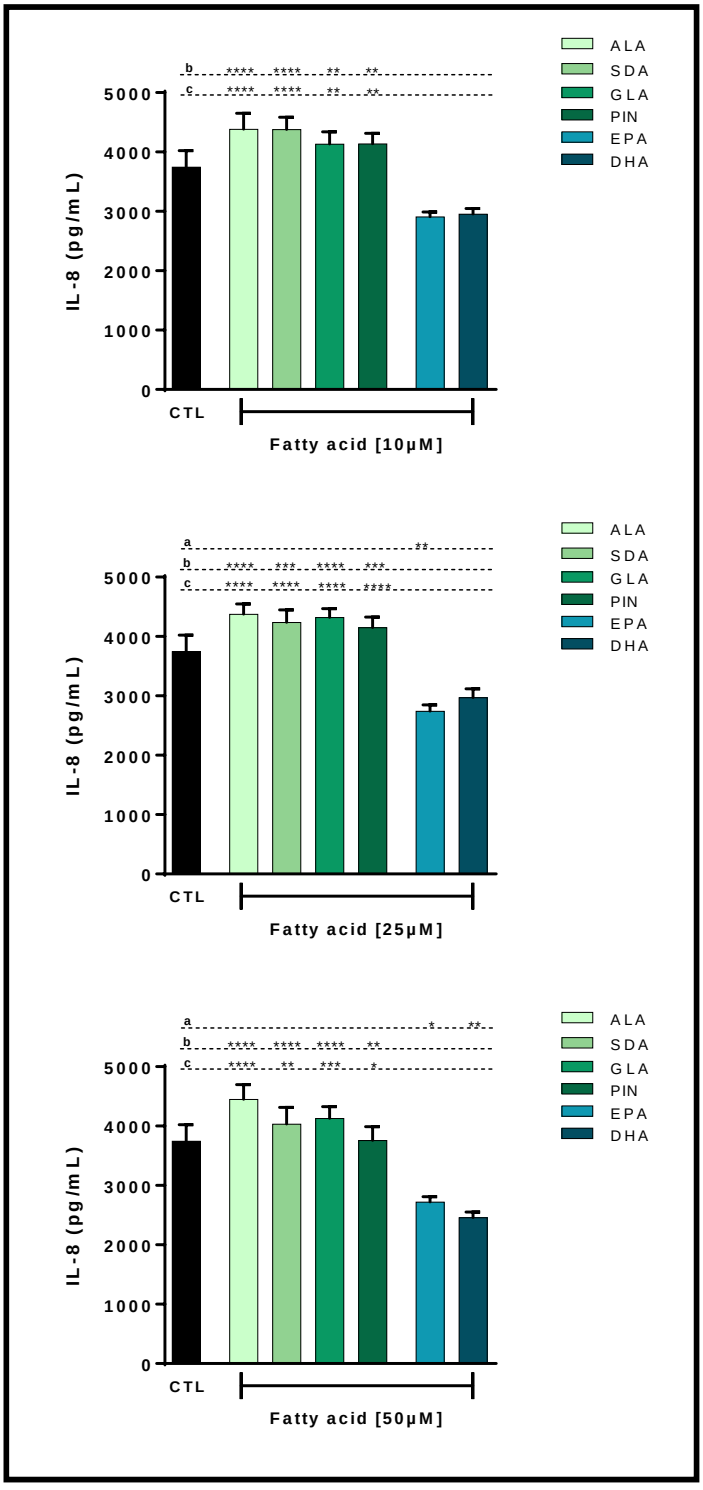

Figure 4.5 Effect of FA exposure on IL-8 production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) production of IL-8 in activated EA.hy926 (control) or 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (10, 25 and 50µM) followed by 24 hour TNFα (1ng/mL). IL-6 was measured in the supernate of endothelial cell cultures. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

4.4.1.4 MCP-1

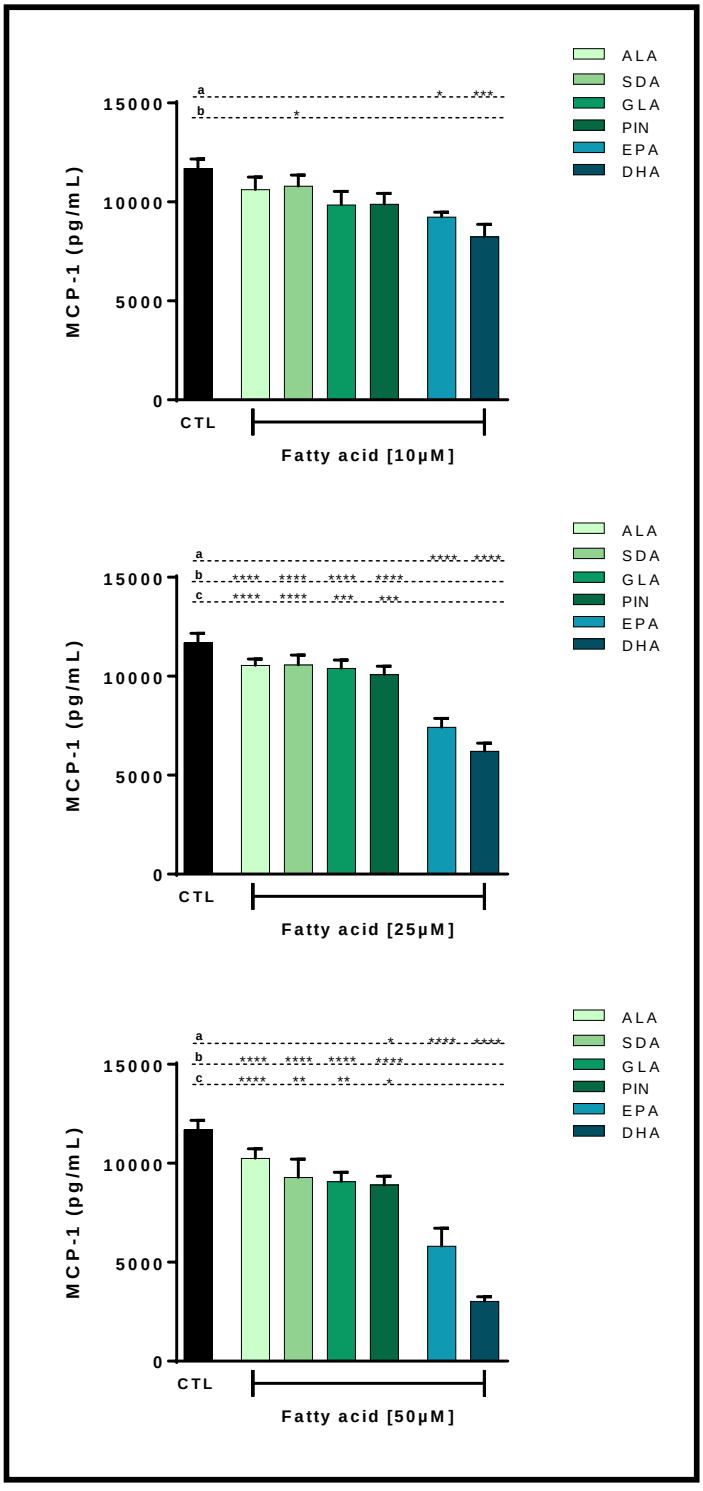

Figure 4.6 Effect of FA exposure on MCP1 production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) production of MCP-1 in activated EA.hy926 (control) or 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (10, 25 and 50µM) followed by 24 hour TNFα (1ng/mL). IL-6 was measured in the supernate of endothelial cell cultures. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

4.4.1.5 RANTES

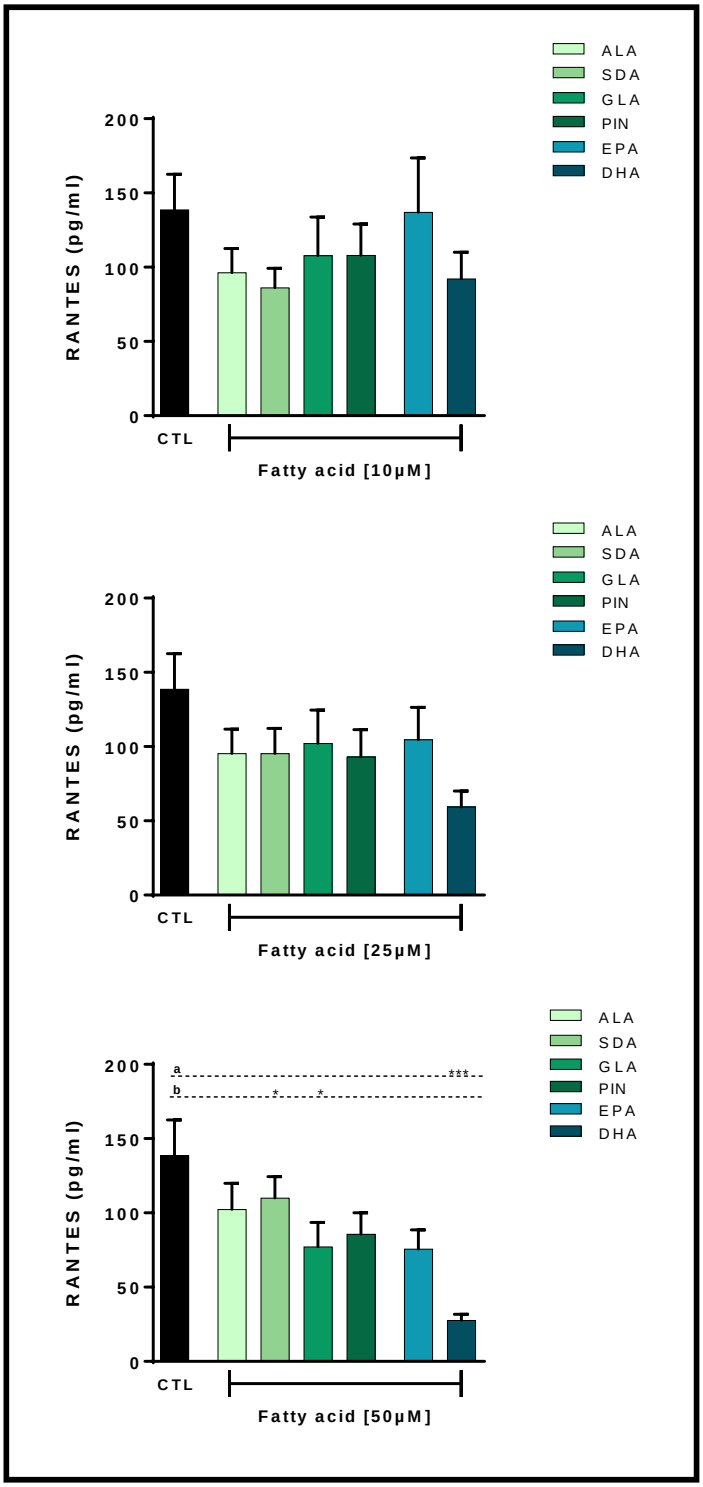

Figure 4.7 Effect of FA exposure on RANTES production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 9) production of RANTES in activated EA.hy926 (control) or 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (10, 25 and 50µM) followed by 24 hour TNFα (1ng/mL). IL-6 was measured in the supernate of endothelial cell cultures. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

4.4.1.6 VEGF

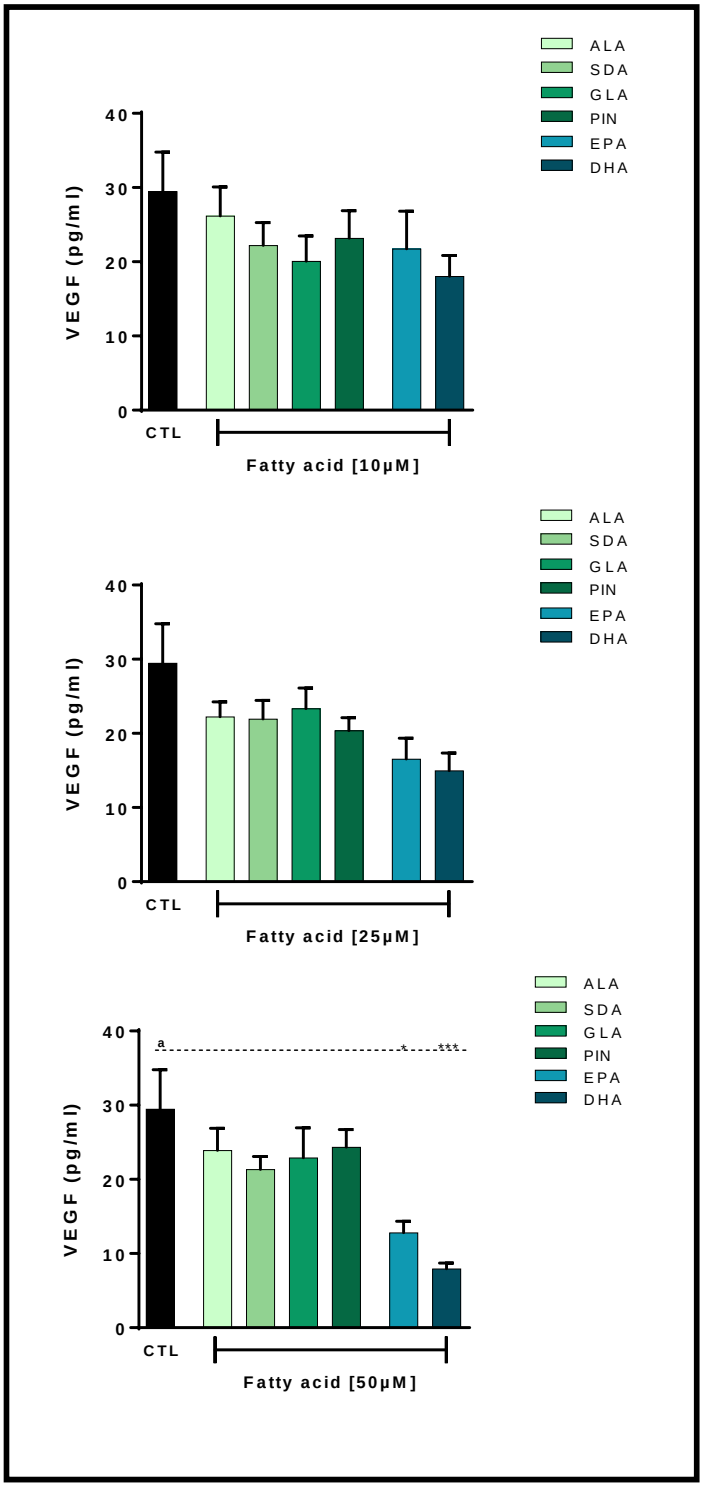

Figure 4.8 Effect of FA exposure on VEGF production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) production of VEGF in activated EA.hy926 (control) or 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (10, 25 and 50µM) followed by 24 hour TNFα (1ng/mL). IL-6 was measured in the supernate of endothelial cell cultures. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

The concentrations of all analytes examined were significantly increased by TNFα (data not shown). Differential effects were seen depending on analyte, the specific FA and the FA concentration (Figure 4.3 - Figure 4.9).

Overall DHA was shown to have the greatest inhibitory effect on production of all analytes. EPA too significantly decreased the production of many of the mediators. Plant-derived FAs, specifically GLA and PIN had a tendency to decrease some of the mediators, but not to the same extent as marine-derived FAs.

ALA did not significantly affect the production of any of mediators studied. At 50 μM ALA showed some tendencies to decrease production of MCP-1 and of soluble ICAM-1.

SDA performed similarly to ALA, with no significant effects on production of any of the mediators but there was a tendency for SDA to decrease soluble ICAM-1 and MCP-1 production when used at 50 μM.

When comparing ALA or SDA to EPA and DHA, the marine-derived FAs had a significantly greater inhibitory effect on the mediators secreted. At all concentrations, both ALA and SDA treated cells had significantly higher secretion of IL-8 when compared to the cells treated with marine-derived FAs EPA and DHA. At 10, 25 and 50 μM EPA and DHA were significantly different from ALA (10 μM ($p<0.0001$; 25 μM ($p<0.0001$, $P<0.001$) and 50 μM ($p<0.01$, $p<0.0001$) respectively).

GLA treatment of EA.hy926 cells at 10 μM or 25 μM did not significantly decrease any of the analytes examined. At a concentration of 50 μM, GLA significantly decreased soluble ICAM-1 secretion by EA.hy926 cells ($p<0.01$). Treatment with GLA at 50 μM also showed a tendency for inhibition of MCP-1 (p0.051).

GLA showed a greater anti-inflammatory effect than either ALA or SDA. Comparison of the effects of GLA to those of EPA and DHA indicated a less anti-inflammatory potency of this plant-derived FAs. MCP-1 production was significantly decreased by both EPA and DHA at 50 μM ($p<0.0001$), this was significantly different in GLA treated cells when compared to EPA ($p<0.01$) and DHA ($p<0.0001$).

Of the plant-derived FAs, PIN had the most significant effects on the mediators studied, although no change was observed after treatment with 10 μM PIN, compared to control cells. However 25 μM PIN significantly decreased soluble ICAM-1 secretion ($p<0.05$), and with greater effect seen at 50 μM ($p<0.01$). PIN

also significantly decreased MCP-1 production when used at a concentration of 50 μM ($p<0.05$).

When comparing the effects of PIN with those of marine-derived n-3 FAs, EPA and DHA had greater effects. However, PIN did have a similar effect to EPA and DHA for some of the analytes examined. The decrease in soluble ICAM-1 secretion after treatment with 10 μM EPA or DHA was not significantly different from the effect of PIN. However, when comparing effects of 50 μM FAs on RANTES production, EPA and DHA had greater effects than PIN ($p<0.05$, $p<0.01$, respectively).

4.4.2 Effect of fatty acid treatment on ICAM-1 cell surface expression in response to TNFα

TNFα was shown to up-regulate ICAM-1 expression on EA.hy926 cells (Figure 4.1). Figure 4.9 - Figure 4.10 show the change in surface ICAM-1 expression in response to TNFα stimulation in EA.hy926 cells pre-treated with ALA, SDA, GLA, PIN, EPA and DHA. The FA effect is shown as a % of control (6 hour TNF 1 ng/mL stimulated cells). The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30.

FA exposure was shown to have differential effects depending on type and concentration of FA. The marine-derived n-3 FAs EPA and DHA caused a significant reduction in ICAM-1 cell surface expression (Figure 4.9 -Figure 4.10).

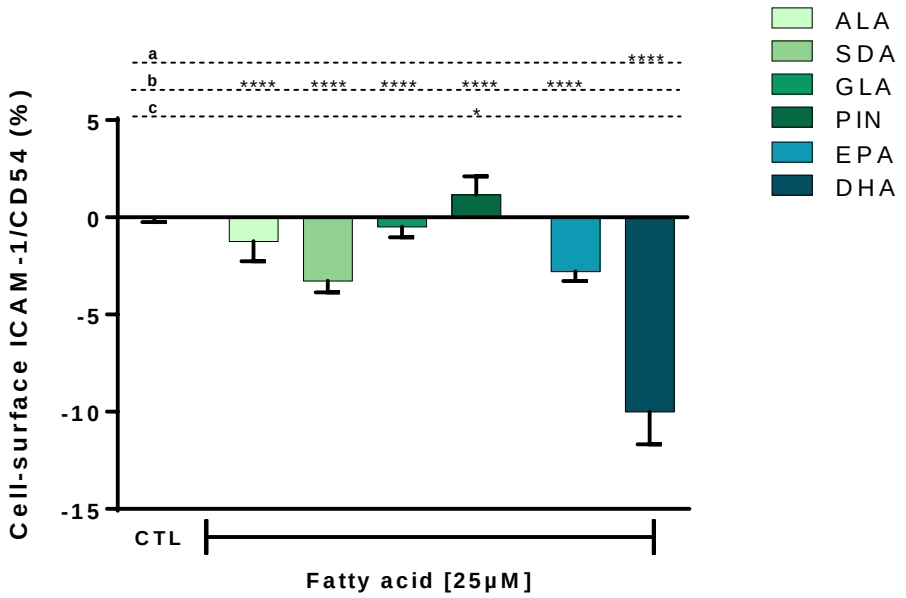

Figure 4.9 Effect of FA exposure on cell surface expression of ICAM-1 in EA.hy926 cells

Data are mean (±SEM) (n = 3) % of control (activated EA.hy926 cells) cell surface expression of ICAM-1 (CD54) after 48 hour exposure to ALA, EPA and DHA (25µM) followed by 6 hour TNFα (1 ng/mL). One way ANOVA, control vs FA; ***p<0.001, ****p<0.0001

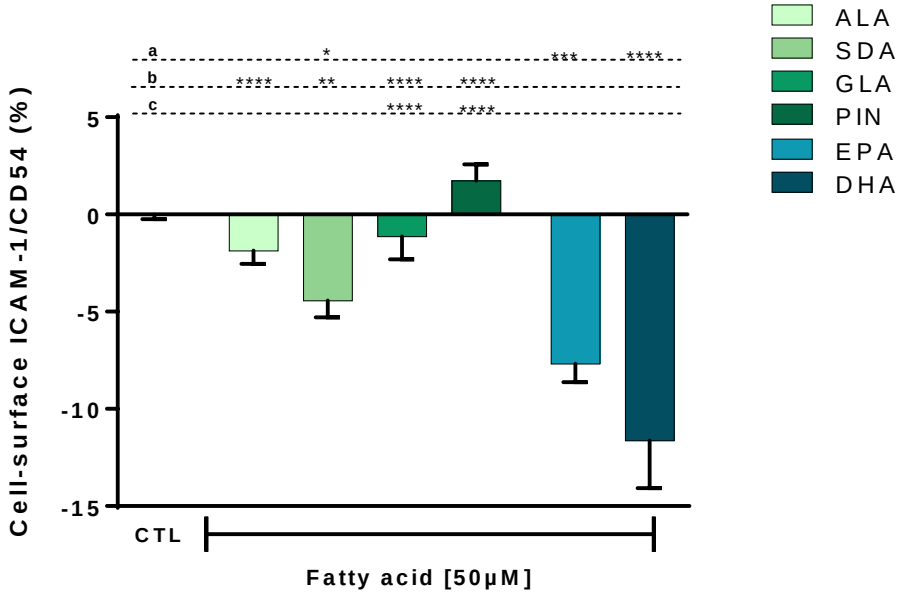

Figure 4.10 Effect of FA exposure on cell surface expression of ICAM-1 in EA.hy926

Data are mean (±SEM) (n = 3) % of control (activated EA.hy926 cells) cell surface expression of ICAM-1 (CD54) after 48 hour exposure to SDA, EPA and DHA (25 and 50 µM) followed by 6 hour TNFα (1 ng/mL). One way ANOVA, control vs FA; *P<0.05, **p<0.01, ***p<0.001, ****p<0.0001

ALA treatment did not have a significant effect on ICAM-1 cell surface expression at either concentration. ALA treated cells had significantly higher ICAM-1 expression when compared to DHA treated cells ($p<0.0001$).

SDA had a greater inhibitory effect on the cell surface expression of ICAM-1 ($p<0.05$) compared to the other plant-derived FAs, though it was less potent than both EPA ($p<0.001$) and DHA ($p<0.0001$).

GLA and PIN had no effect on ICAM-1 cell surface expression and cells treated with these two FAs had higher ICAM-1 expression than those treated with EPA or DHA at 50 μM ($p<0.0001$).

4.5 Discussion

The aims of the research described in this chapter were to compare the effects of four plant-derived FAs (ALA, SDA, GLA and PIN) to two marine-derived FAs (EPA and DHA) on production of various inflammatory mediators (ICAM-1, MCP-1, VEGF, IL-6, IL-8, RANTES) by cultured ECs and on the expression of ICAM-1 on the surface of cultured ECs. These aims and the overarching objective were met.

Marine-derived n-3 FAs showed the most potent inhibitory effect on both inflammatory mediator production by stimulated EA.hy926 cells and cell surface expression of ICAM-1 on those cells.

4.5.1 Effects of FA on inflammatory mediators

Comparison of FAs at a concentration of 50 μM revealed DHA as having the greatest inhibitory effect on mediator production compared to control (TNFα stimulated EA.hy926 cells). DHA significantly reduced production of all 6 analytes measured and the effects seen were highly statistically significant.

EPA had the second greatest inhibitory effect (at 50 μM), reducing production of five of the six analytes examined (soluble ICAM-1, IL-6, MCP-1, IL-8 and VEGF). EPA was less potent than DHA. Effects of EPA and DHA on production of some of these mediators by ECs have already been reported by others [110, 182, 187].

EPA and DHA also reduced cell surface ICAM-1 expression; this effect was also reported by De Caterina *et al.* [158]. Others have described reduction in surface ICAM-1 expression after EPA and DHA treatment [110, 150, 187].

Many others have observed anti-inflammatory effects of EPA and DHA in a variety of cell types similar to the effects seen within this inflammatory EC model. De Caterina and Libby demonstrated a decrease in in ICAM-1, IL-6 and IL-8 after n-3 PUFA treatment of ECs [188]. Grenon *et al*. too showed an effect of EPA on EA.hy926 cell adhesion molecule expression although they described little or no effect compared to control cells (TNFα activated) but did argue inhibitory effects compared to n-6 PUFAs [152]. The anti-inflammatory effects of EPA and DHA are consistent with data in the literature showing that they slow or prevent atherosclerosis in experimental animal models and lower the risk of mortality from CVD in humans [83].

Of the plant-derived FAs, GLA and PIN (at 50 μM) had the most significant anti-inflammatory effects, whereas ALA and SDA did not affect secretion of any of the inflammatory mediators examined. However, SDA at 50 μM did lower ICAM-1 cell surface expression (by 5%) when compared to the other plant-derived FAs, but this effect was less than those of EPA (-8%) and DHA (-12%). ICAM expression on the EC surface is important in the development and progression of atherosclerosis as it is involved in the attraction and adhesion of monocytes to the endothelium. SDA may therefore have a role in atherosclerosis prevention, so long as significant concentrations of it can be achieved in vivo.

Some others have explored effects of ALA on secretion of inflammatory mediators in ECs and a variety of cell types. Similar to observations made here Shen *et al.* reported no changes in IL-6 secretion after treatment with ALA (50 and 200 μM) in LPS-stimulated HUVECs [167]. In contrast, others report significant decreases in IL-6 and IL-8 after ALA treatment (200 μM) in LPS-stimulated human corneal epithelial cells (HCECs) [183]. Also in contrast to the findings made with the current model, those who have reported effects of ALA on ICAM-1 describe decreases. Shen *et al*. described decreases in ICAM-1 secretion in LPS-stimulated HUVECs (50 and 200 μM) [167]. Zhang *et al.* also describe attenuation of ICAM-1 expression after treatment with ALA (50 μM) in HUVECs exposed to high glucose [168].

Very few studies report effects of SDA exposure on inflammatory mediator production in vitro. SDA treatment (100 μM) of LPS-stimulated 3T3-L1 adipocytes had no effect on the secretion of MCP-1; furthermore the authors reported increased IL-6 secretion in LPS-stimulated 3T3-L1 adipocytes [184].

PIN decreased production of soluble ICAM-1 and MCP-1 and had a tendency to lower production of RANTES. Interestingly PIN did not decrease the abundance of cell surface ICAM-1 compared to control cells. GLA significantly reduced production of one mediator (ICAM-1), and showed a tendency to reduce production of MCP-1 and RANTES.

Studies describing effects of GLA or PIN use different cell models. Similar to observations made in this EC model, Erdinest *et al*. described no changes in either IL-6 or IL-8 levels after treatment with GLA (200 μM) in LPS-stimulated HCECs [183]. Kim *et al*. observed reduced MCP-1 production in both rat kidney epithelial cells (NRK-52E) and mesangial cells after treatment with GLA at 10 and 100 μM [189]. Treatment of EA.hy926 cells with GLA at 50 μM had a tendency to decrease MCP-1 production. EA.hy926 cell production of IL-6 was not altered by pre-treatment with PIN in this inflammatory cell model; however some reports describe decreased IL-6 secretion in LPS-stimulated murine microglial BV-2 cells after treatment with 50 μM PIN [137].

Differences in effects reported after treatment with these various FAs may be due to different cell type, concentration of FAs used and the cell stimulus used. The lack of reports of effects of plant-derived FAs on inflammatory mediator production in ECs, or in any other cell type, highlights the need for these investigations. Exploring these mediators of inflammation is important as part of the process of understanding the functional effects of these bioactive lipids, especially within the endothelium and the context of atherosclerosis.

4.5.2 Conclusions

Overall, the results obtained from the experiments described in this chapter support those in the current literature with regard to EPA and DHA, but extend these to include other inflammatory mediators and other FAs. The findings are summarised in Table 4.1. The current work adds much new information on the likely anti-inflammatory and anti-atherosclerotic potential of 18-carbon plant-derived FAs. Of these, GLA and PIN look the most promising.

Table 4.1 Summary of effects of FA treatment on inflammatory mediator production and ICAM-1 surface expression in EA.hy926 cells

	Fatty acid						Comments
Mediator production	ALA	SDA	GLA	PIN	EPA	DHA	
sICAM-1	↔	↔	↓	↓	↓↓	↓↓	Effect was dose-dependent
IL-6	↔	↔	↔	↔	↓↓	↓↓	Effect was dose-dependent
IL-8	↔	↔	↔	↔	↓	↓↓	Effect was dose-dependent
MCP-1	↔	↔	↔	↓	↓↓	↓↓↓	Effect was dose-dependent
RANTES	↔	↔	↔	↔	↔	↓	Effect was dose-dependent
VEGF	↔	↔	↔	↔	↓	↓↓	Effect was dose-dependent
Cell-surface expression	ALA	SDA	GLA	PIN	EPA	DHA	
ICAM-1	↔	↓	↔	↔	↓	↓↓	Effect was dose-dependent

Endothelial dysfunction is one of the very early stages in the development of atherosclerosis which begins with increased abundance of inflammatory signals including cytokines, chemokines and adhesion markers. Several of the FAs examined were shown to decrease production of a variety of these key mediators in stimulated EA.hy926 cells. These effects may be through modulation of gene expression including transcription factors such as NF-kB and PPARs. Therefore effects of FAs on expression of inflammatory genes within EA.hy926 cells will be further explored.

Chapter 5: Effects of fatty acids on inflammatory gene expression in cultured endothelial cells

5.1 Introduction

FAs have been shown to modulate inflammation through the alteration and activation of different inflammatory genes [65]. FAs can regulate the activity of several transcription factors including PPARs and NF-kB. This may occur through direct actions of the FAs themselves or through their oxidised metabolites and may involve direct binding of the FA or its derivative to the transcription factor altering its activity. Furthermore, FAs can act indirectly on gene expression through either their effects on pathways that involve changes in membrane lipid composition such as G-protein receptor signalling, or their effects on enzymes (mediated pathways) such as COX [65]. The effects of FAs on gene expression are cell-specific and can be influenced by FA structure [65].

In the previous chapter it was shown that the various FAs being examined had differential effects on the production of inflammatory mediators by stimulated EA.hy926 cells in culture (Chapter 4). In order to better understand these anti-inflammatory effects, gene expression and the effects on inflammatory signalling through transcription factors can be examined. This chapter describes effects of FAs on gene expression.

Prior to assessing the effects of FAs on inflammatory gene expression, a time course of the effect of TNFα stimulation on some representative genes of interest was performed. TNFα increased the level of inflammatory gene expression by EA.hy926 cells in a time dependent manner (section 2.4.3). A time point of 6 hours stimulation was identified to assess the effects of FA on inflammatory genes.

Several genes of interest were chosen to be examined; these are part of the inflammatory pathways related to the production of inflammatory mediators examined in chapter 4. Increasing or decreasing relative expression of the genes may indicate which pathway the specific FA may act upon in order to exert their anti-inflammatory effects.

Several studies have examined the effects of EPA and DHA on inflammatory gene expression pathways in HUVECs and other EC [152, 164, 172, 190]. Effects of plant-derived FAs (ALA, SDA, GLA and PIN) on inflammatory gene expression in vitro in EC have not been explored. Thus, the research described in this chapter compares the effects of four plant-derived 18 carbon FAs (ALA, SDA, GLA, PIN) on various genes involved in inflammation in ECs with those of EPA and DHA. This

will allow a better understanding of how these FAs exert their anti-inflammatory actions. The relative expression of genes encoding inflammatory mediators and transcription factors was assessed by RT-qPCR.

5.2 Aim and objectives

The aim of the research described in this chapter was to compare the effects of four plant-derived and two marine-derived FAs on inflammatory gene expression in cultured ECs.

The specific objective was to:

- compare the effects of four plant-derived FAs (ALA, SDA, GLA and PIN) to two marine-derived FAs (EPA and DHA) on the expression of genes for various inflammatory factors (NF-kB, IKKB, COX-2, PPARα, MCP-1 and IL-6) in cultured ECs

5.3 Methods

5.3.1 Reagents

Reagents and materials used for these experiments are described in Appendix A.

5.3.2 Endothelial cell culture

Confluent EA.hy926 cell cultures were scraped from flasks and resuspended in culture medium at a density of 5.5 x 10^5 cells per mL. Cells were seeded at 5.5 x 10^4 cells per well in 6-well flat bottom plates, incubated for 48 hours at 37°C with various FAs at a concentration of 25 or 50 μM, followed by 6 hour incubation with TNFα at 1 ng/mL. After 6 hours, cells were removed, resuspended in BL-TG buffer following ReliaPrep™ RNA Cell Miniprep System instructions and stored at -80°C until analysis.

5.3.3 Analysis of gene expression using RT-qPCR

Gene expression in TNFα stimulated EA.hy926 cells was analysed by RT-qPCR.

5.3.3.1 Isolation of RNA

Total cellular RNA was extracted from cells using the ReliaPrep™ RNA Cell Miniprep System as described previously in section 2.3.4.1.

5.3.3.2 RNA analysis.

Isolated RNA quality and quantity were assessed by NanoDrop analysis (section 2.3.4.2.1 and section 2.3.4.2.2) and the Aglient bioanalyzer (section 2.3.4.2.3).

5.3.4 cDNA synthesis

GoScript™ Reverse Transcriptase was used to synthesise cDNA from total RNA as described in section 2.3.4.3.

5.3.5 RT-qPCR

The expression of NF-kB (subunit 1), IKIKB (IKKB), PPARα, MCP-1, PTGS2 (COX-2), IL-6, GAPDH, RPL13A and B2M genes was assessed by qRT-PCR with TaqMan® gene expression primers; see Appendix A for primer details. The procedure was as described in section 2.3.4.4. Briefly cDNA was diluted to 5 ng/µL and 4 µL added to 16 µL of reaction mix (prepared according to the manufacturer's specifications) per well in a 96-well plate. Plates were then sealed and loaded into a thermocycler for the specific number of cycles as per the manufacturer's instructions.

5.3.6 geNorm analysis

geNorm kit (primer design) was used to identify the most stable and optimal number of reference (housekeeping) genes suitable for analysis of gene expression in the inflammatory EC model. The selection of suitable control genes is crucial for proper interpretation of RT-qPCR data. A gene expression normalisation factor can be calculated for each sample based on the geometric mean of a user-defined number of reference genes.

Duplicate samples from each condition were used; EA.hy926 cells were exposed to different FAs (ALA, SDA, GLA, PIN, EPA or DHA) at 25 and 50 µM for 48 hours followed by TNFα stimulation for 6 hours. Data were analysed using geNorm software qbase^{+}.

5.3.7 Statistical analysis

Relative gene expression data were calculated using double delta Ct (ΔΔCt) values expressed as fold change of the target gene in test samples relative to control samples. Initially the geometric mean of the ΔCt for each housekeeping is calculated for each sample, and this mean is used to calculate the relative expression (see section 2.3.5).

Data are expressed as mean ± standard error; data analysis was performed in PRSIM and Excel. Multiple group differences were compared using one way ANOVA.

5.4 Results

In order to investigate the effect of different FAs on the relative expression of various genes involved in the inflammatory pathway, EA.hy926 cells were exposed to FAs at concentrations of 25 and 50 μM for 48 hours followed by 6 hour TNFα stimulation at 1 ng/mL. RNA quantification and integrity were analysed using NanoDrop and Bioanalyzer. The expression of various genes of interest was assessed by qRT-PCR (2.3.4.4). The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30.

RNA was shown to have high RIN scores and therefore to be of good quality (Figure 5.1). TNFα treatment was previously shown to increase relative expression of the genes examined

Figure 2.8 - Figure 2.13). Quantification and identification of suitable reference genes was performed using geNorm, allowing for robust evaluation of relative gene expression. The analysis identified 3 stable reference genes for use within in this experimental design; these were B2M, GAPDH and RPL13A (Figure 5.2).

FA exposure was shown to have differential effects depending on the individual FA and on FA concentration (Figure 5.3 - Figure 5.8). Marine-derived EPA and DHA showed the most potent effects on relative expression of the genes analysed. Plant-derived SDA and GLA showed some significant effects on relative gene expression, whereas ALA and PIN had the least effect on gene expression.

5.4.1 RNA integrity

Total cellular RNA isolation was carried out using ReliaPrep™ RNA Cell Miniprep columns (section 2.3.4.1).

Analysis of RNA using Agilent Bioanlyzer (RNA Total Eukaryote 2100 Nano) was performed by Melissa Doherty, Laboratory manager (section 2.3.4.2.2). RNA quantity and quality were also assessed using the NanoDrop (section 2.3.4.2.1).

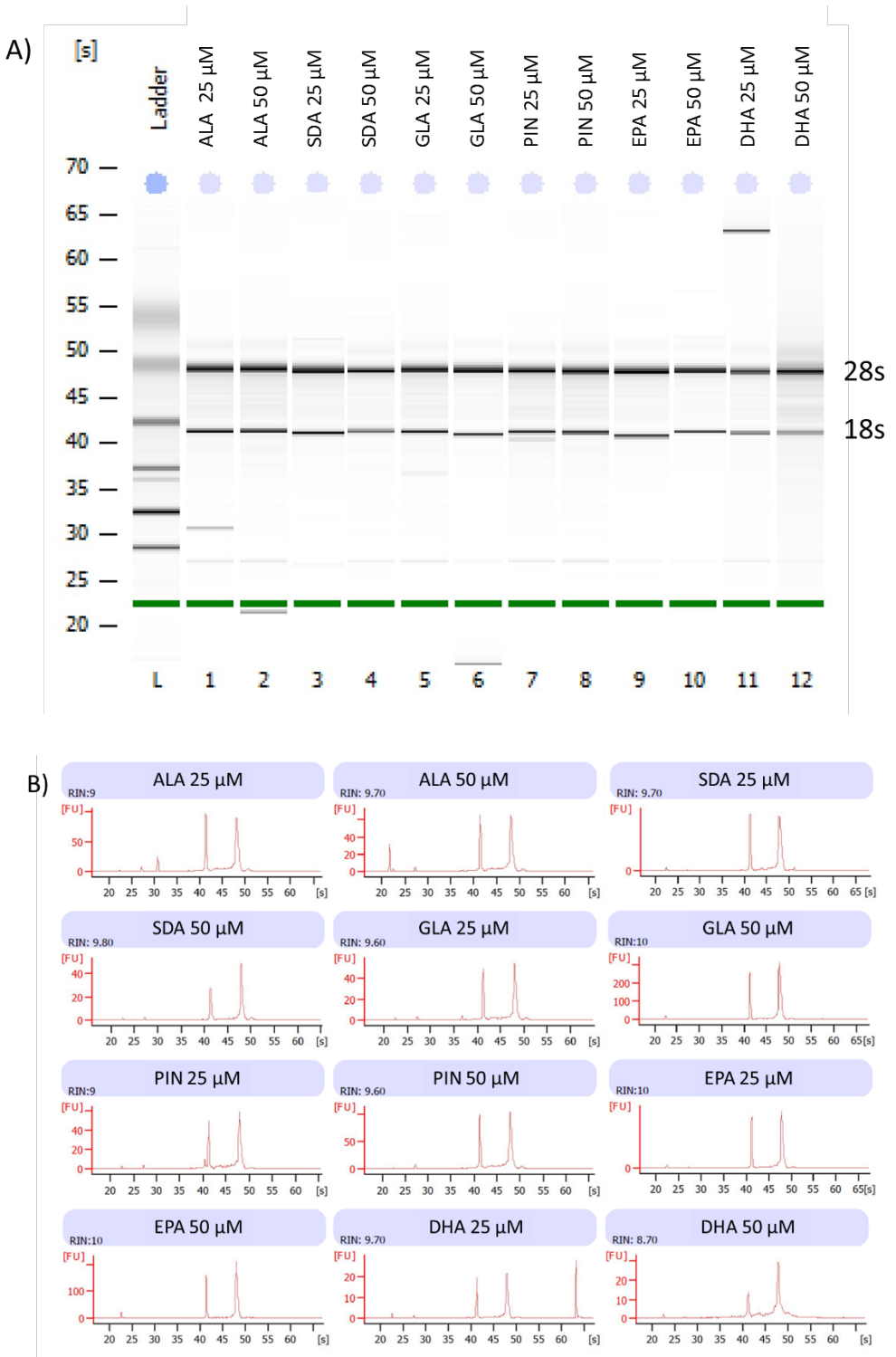

Figure 5.1 Aglient Bioanalyzer RNA integrity results.

Bioanalyzer profiles of RNA extracted from activated EA.hy926 cells (control) or 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (50µM) followed by 6 hour TNFα (1 ng/mL). A) Bands represent 18S and 28S, peaks in B) represent 18S and 28S. RIN scores were measured by Aglient bioanalyzer

All RNA samples were analysed via the NanoDrop for quantity and quality. Table 5.1 depicts representative RNA sample 260/280 and 260/230 ratios from the NanoDrop analysis. Data indicate that RNA was of good quality.

Table 5.1 Anlaysis of RNA quality and quantity using NanoDrop

Samples	ng/µL	A260	A280	260/280	260/230
ALA 25 µM	427.18	10.68	5.08	2.1	2.14
ALA 50 µM	391.28	9.782	4.719	2.07	2.09
SDA 25 µM	448.94	11.223	5.408	2.08	2.13
SDA 50 µM	325.66	8.141	3.883	2.1	2.11
GLA 25 µM	469.86	11.746	5.658	2.08	2.15
GLA 50 µM	432.34	10.809	5.142	2.1	2.13
PIN 25 µM	328.79	8.22	3.903	2.11	2.11
PIN 50 µM	255.3	6.382	3.021	2.11	2.04
EPA 25 µM	399.56	9.989	4.744	2.11	2.13
EPA 50 µM	398.26	9.956	4.7	2.12	2.1
DHA 25 µM	216.95	5.424	2.573	2.11	1.97
DHA 50 µM	270.48	6.762	3.201	2.11	2.02

5.4.2 Reference gene identification

Samples of each cells cultured in each condition were analysed using the geNorm kit (primer design) and data were analysed using the geNorm software qbase+. The optimal number of reference targets in this experimental situation was 3 (geNorm V < 0.15 when comparing a normalization factor based on the 3 or 4 most stable targets) which is shown in Figure 5.2. As such, the optimal normalization factor can be calculated as the geometric mean of reference targets RPL13A, B2M and GAPDH.

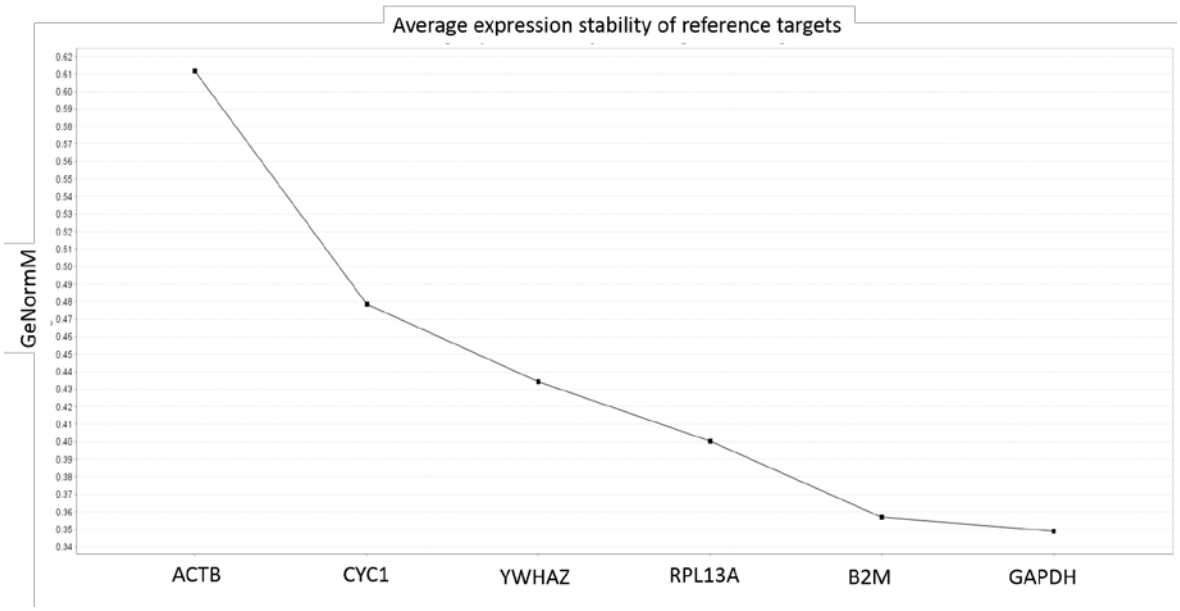

Figure 5.2 geNrom qbase+ analysis for reference genes suitable for the conditions for the inflammatory model.

5.4.3 Effect of fatty acid treatment on gene expression

The relative expression of all genes examined was significantly increased by TNFα (data not shown). Differential effects were seen depending on gene, the specific FA and the FA concentration.

Overall DHA was shown to have the greatest effect on relative expression of all genes assessed. EPA had less of an effect than DHA and only significantly modulated the expression of the IL-6 gene compared to control cells, although EPA showed trends to modulate other genes. Of the plant-derived FAs, SDA and GLA had some effects on the expression of genes examined, but not to the same extent as DHA.

5.4.3.1 NF-kB

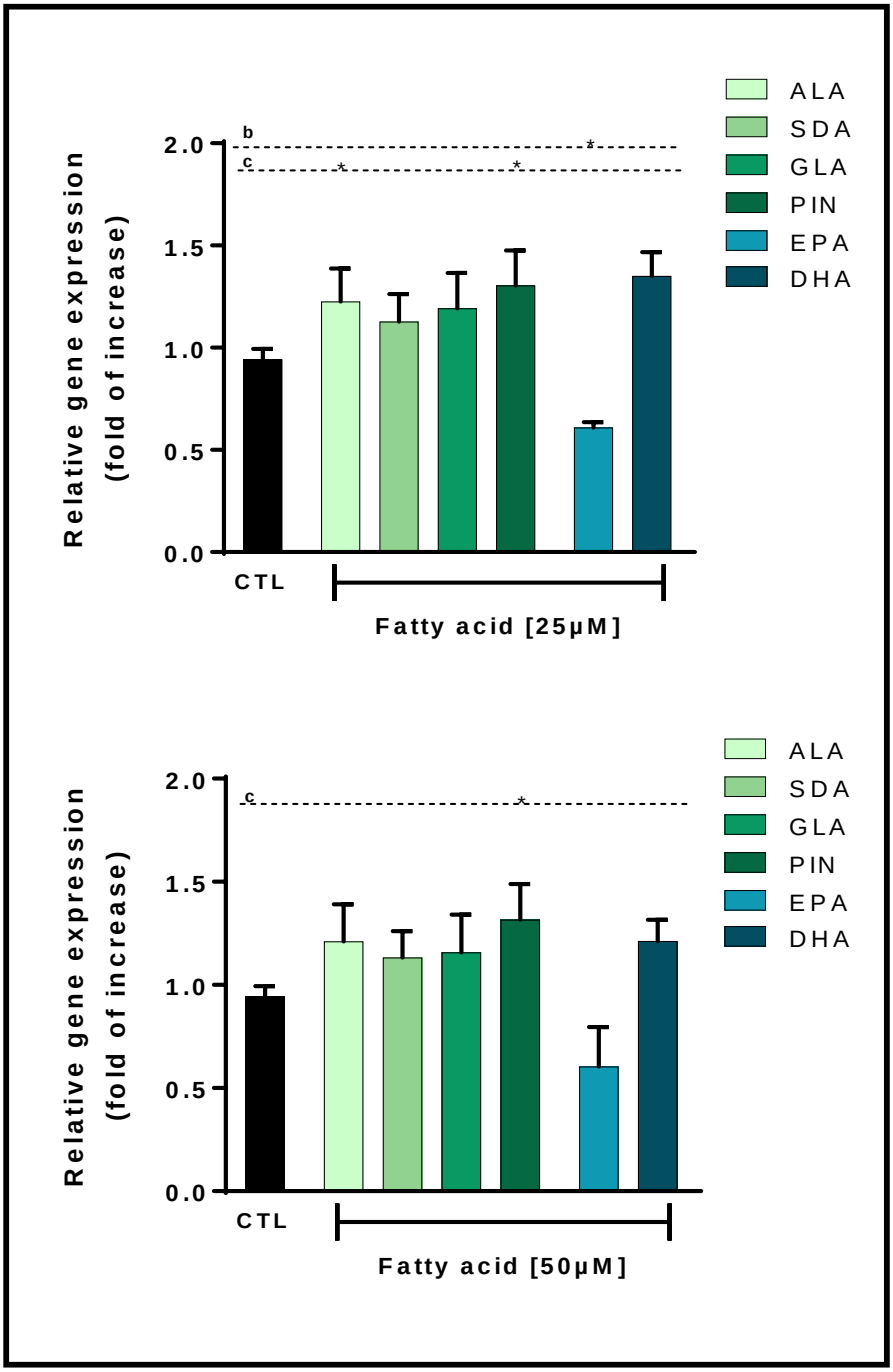

Figure 5.3 Effect of FA exposure on NF-kB (subunit 1, p50) gene expression in TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) gene expression of NF-kB (subunit 1) in activated EA.hy926 cells (control) or following 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (25 and 50 µM) followed by 6 hour TNFα (1 ng/mL). Ct values for NF-kB gene expression were normalised to RPL13A, B2M and GAPDH. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

5.4.3.2 IKKB

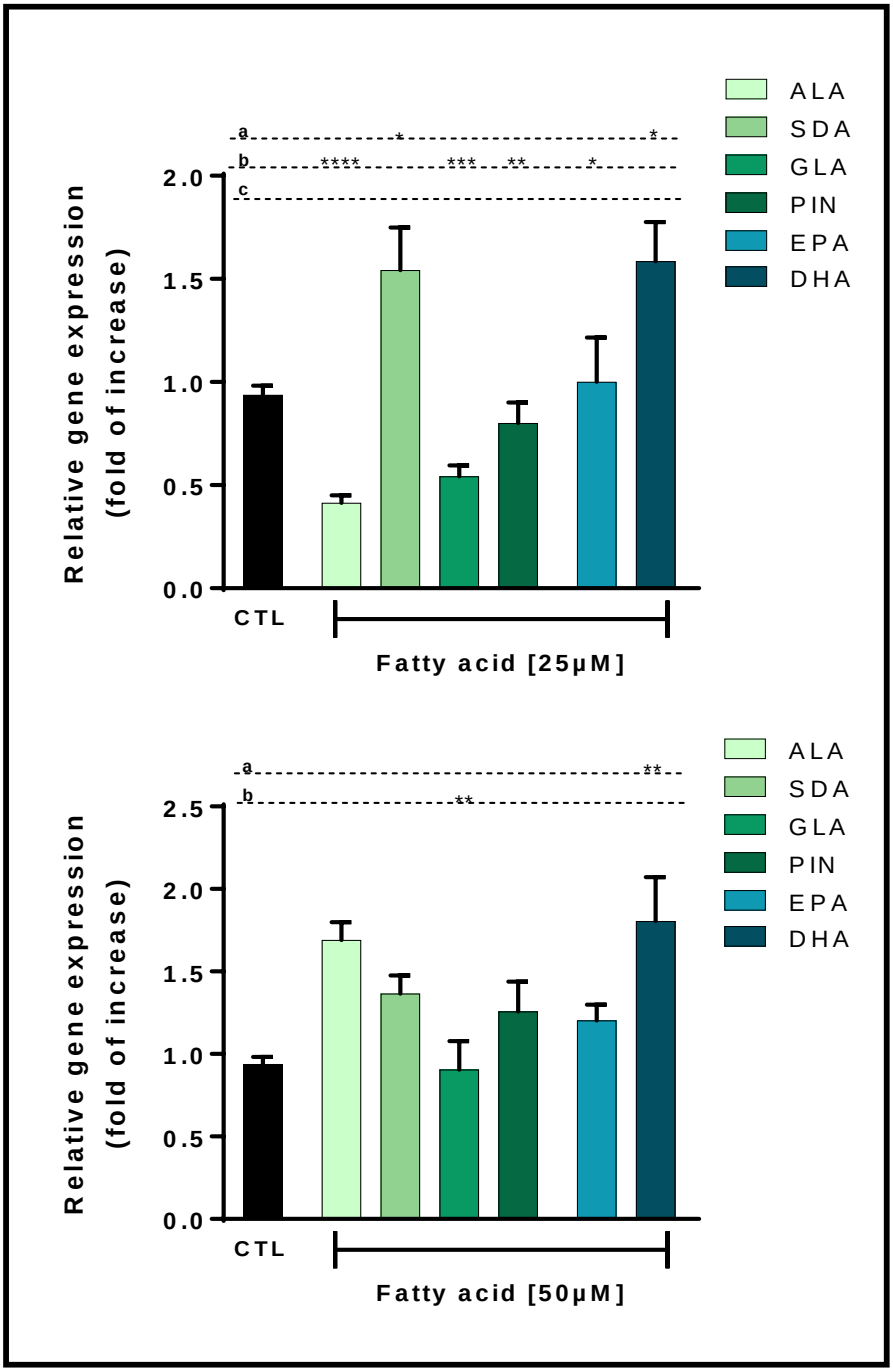

Figure 5.4 Effect of FA exposure on IKKB (IKIKB) gene expression in TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) gene expression of IKKB (IKIKB) in activated EA.hy926 cells (control) or following 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (25 and 50 µM) followed by 6 hour TNFα (1 ng/mL). Ct values for NF-kB gene expression were normalised to RPL13A, B2M and GAPDH. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

5.4.3.3 PPARα

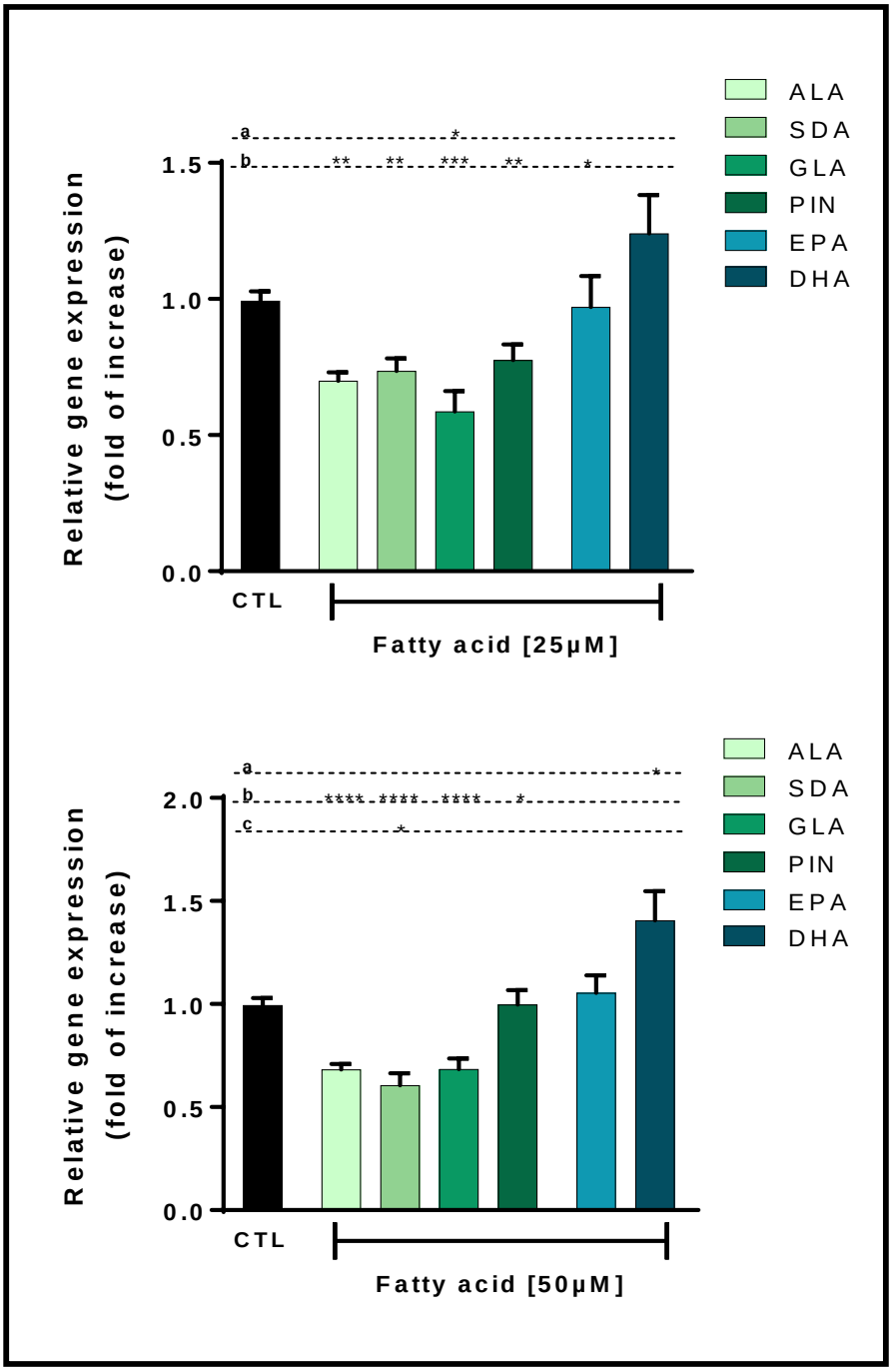

Figure 5.5 Effect of FA exposure on PPARα gene expression in TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) gene expression of PPARα in activated EA.hy926 cells (control) or following 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (25 and 50 µM) followed by 6 hour TNFα (1 ng/mL). Ct values for NF-kB gene expression were normalised to RPL13A, B2M and GAPDH. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

5.4.3.4 COX-2

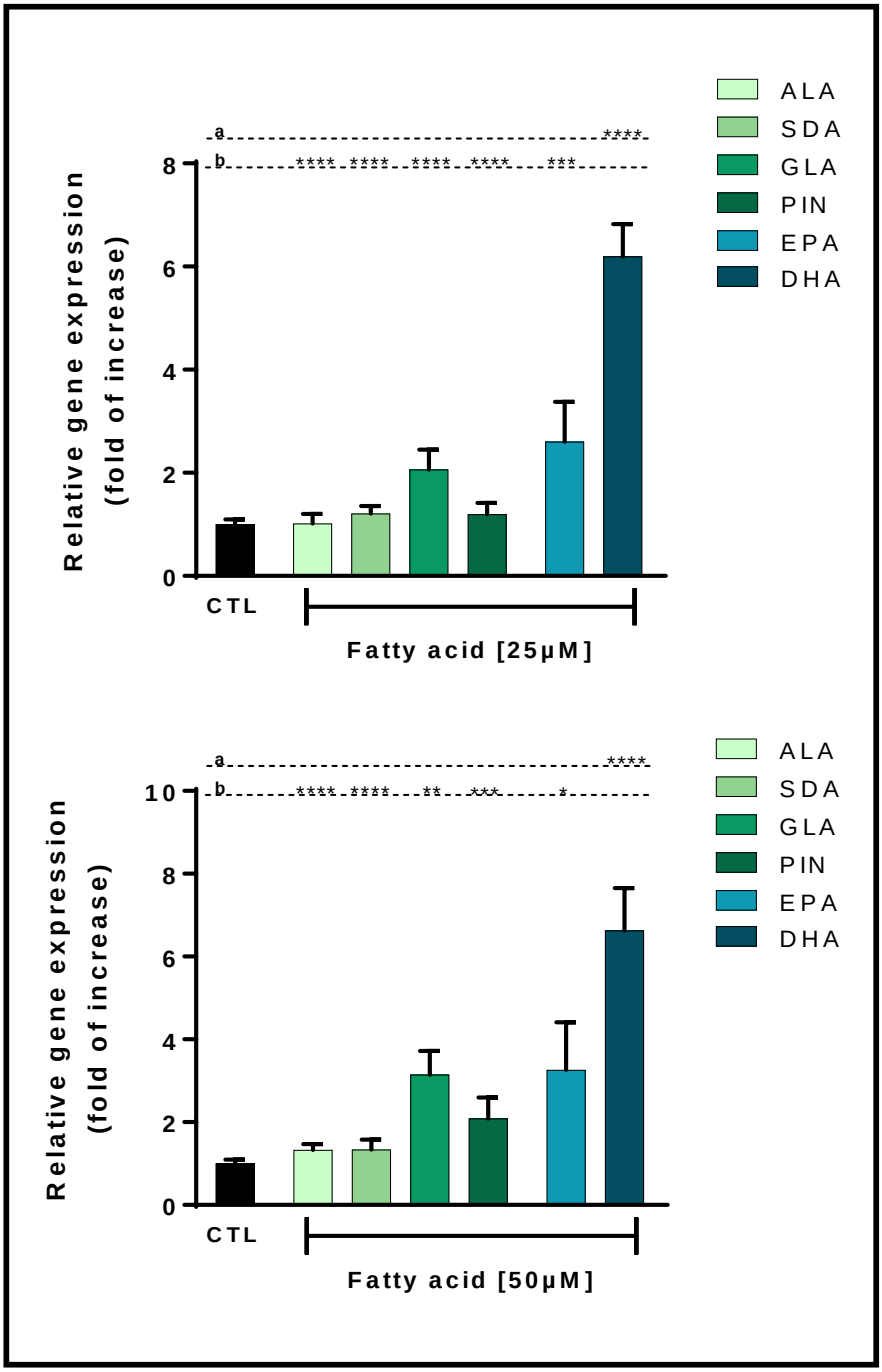

Figure 5.6 Effect of FA exposure on COX-2 (PTGS2) gene expression in TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) gene expression of COX-2 (PTGS2) in activated EA.hy926 cells (control) or following 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (25 and 50 µM) followed by 6 hour TNFα (1 ng/mL). Ct values for NF-kB gene expression were normalised to RPL13A, B2M and GAPDH. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

5.4.3.5 MCP-1

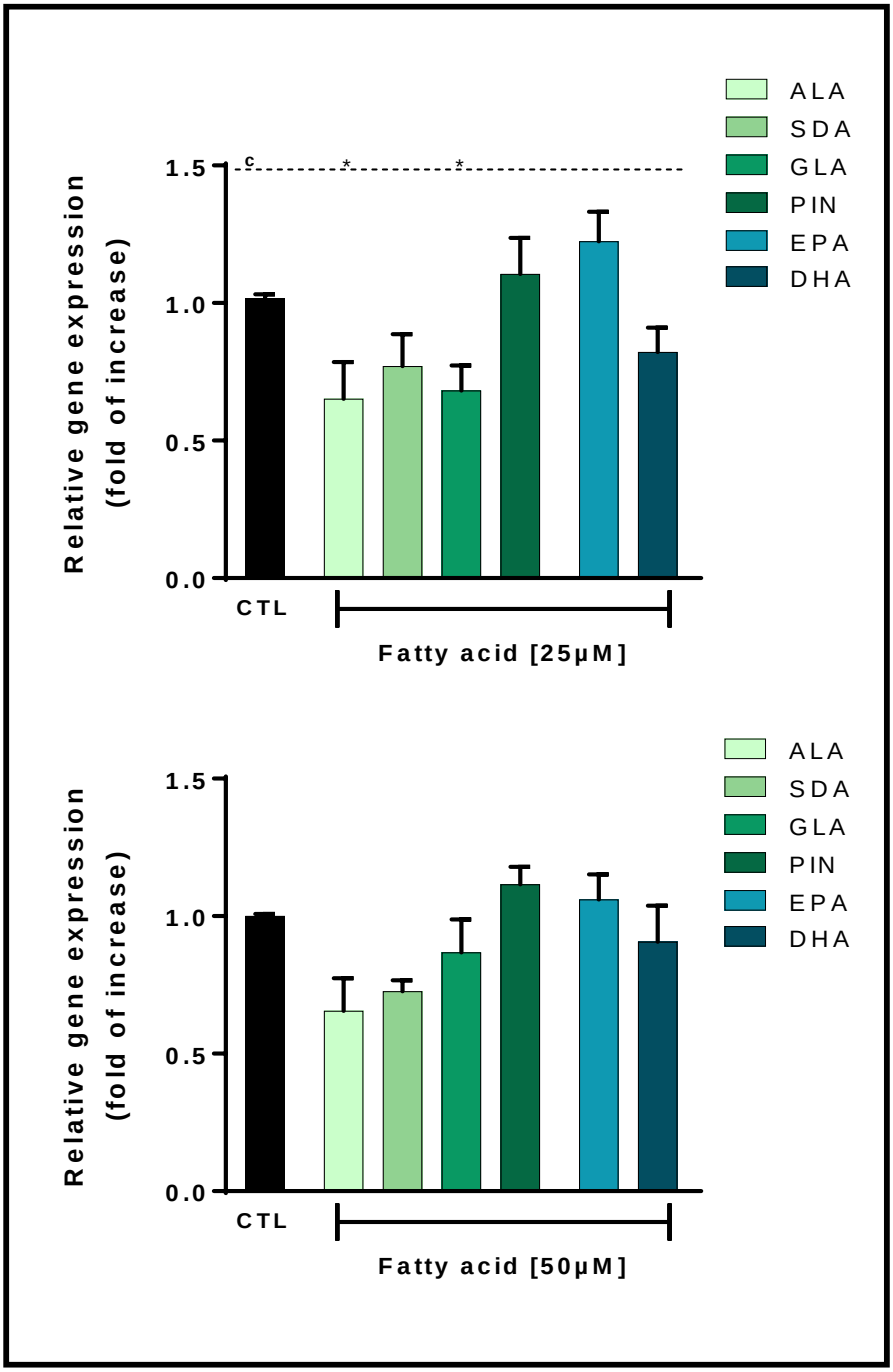

Figure 5.7 Effect of FA exposure on MCP-1 (CCL2) gene expression in TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) gene expression of MCP-1 (CCL2) in activated EA.hy926 cells (control) or following 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (25 and 50 µM) followed by 6 hour TNFα (1 ng/mL). Ct values for NF-kB gene expression were normalised to RPL13A, B2M and GAPDH. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

5.4.3.6 IL-6

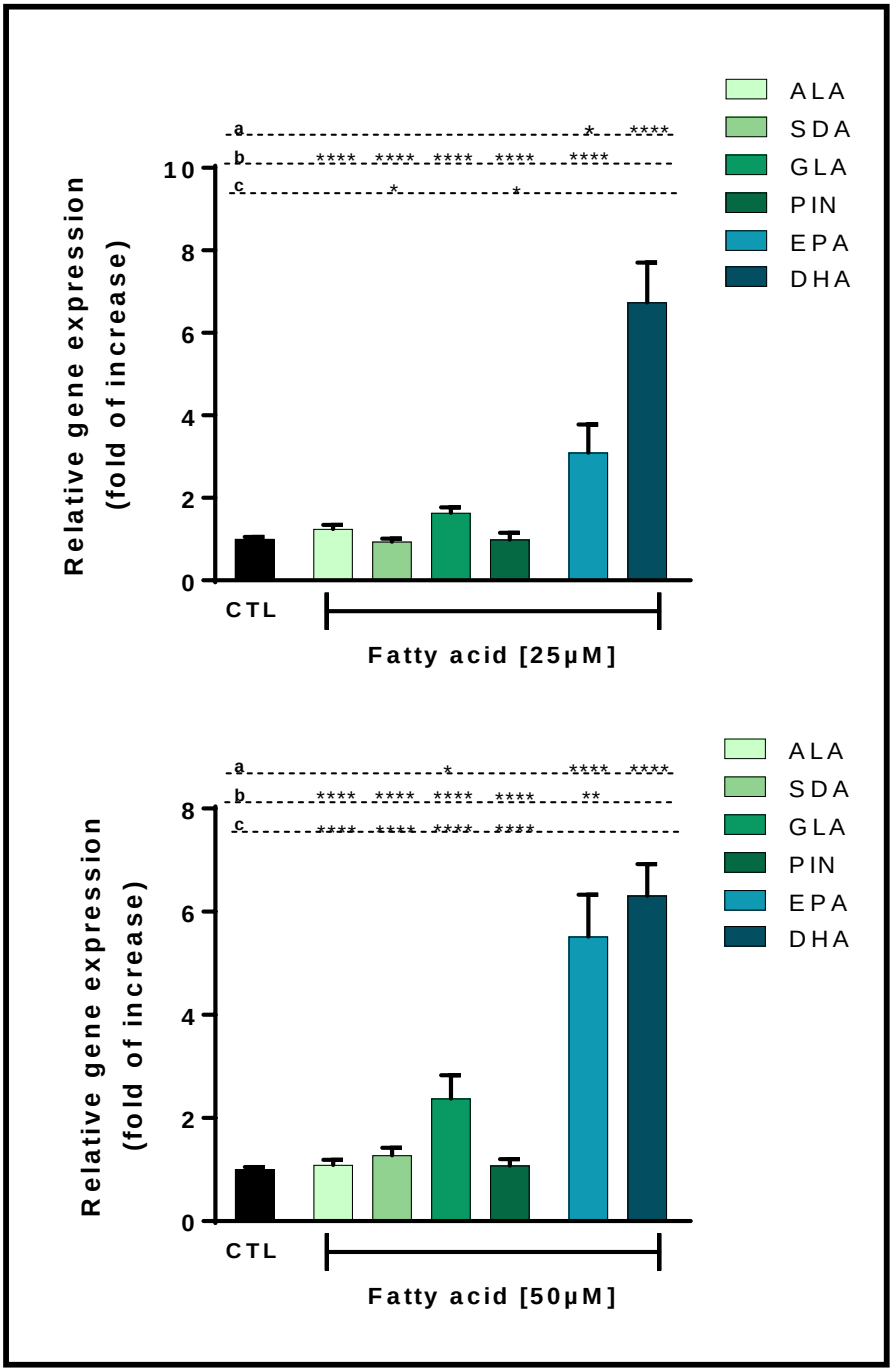

Figure 5.8 Effect of FA exposure on IL-6 gene expression in TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) gene expression of IL-6 in activated EA.hy926 cells (control) or following 48 hour exposure to ALA, SDA, GLA, PIN, EPA and DHA (25 and 50 µM) followed by 6 hour TNFα (1 ng/mL). Ct values for NF-kB gene expression were normalised to RPL13A, B2M and GAPDH. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Surprisingly in this model modulation of relative gene expression by DHA and EPA was in some instances “inflammatory” rather than “anti-inflammatory”. Relative expression of the IL-6 gene was significantly increased by both EPA and DHA at 25 μM ($p<0.05$, $p<0.0001$ respectively) and 50 μM ($p<0.0001$, $p<0.0001$ respectively) (Figure 5.8). COX-2 gene expression was also significantly increased by DHA at 25 and 50 μM ($p<0.0001$), and EPA too had a tendency to increase the expression of COX-2 gene, though not to the same extent (Figure 5.6). COX-2 gene expression may be upregulated by EPA and DHA, as these are substrates for COX-2 but yield ‘less’ inflammatory eicosanoids than AA, although increases in gene expression do not necessarily lead to increased translation and appearance of protein within the cell.

DHA at both 25 μM and 50 μM increased relative expression of the gene for IKKB, which encodes an enzyme that activates and phosphorylates IkBs; increasing IKKB may cause activation of NF-kB and therefore this effect also seems to be inflammatory.

ALA did not significantly affect the expression of any of the genes examined. At 25 and 50 μM ALA showed some tendencies to decrease relative gene expression of MCP-1 (Figure 5.7). ALA also showed some tendencies to increase the relative gene expression of both NF-kB and IKKB (Figure 5.3 and Figure 5.4).

SDA also had little effect on the expression of the genes examined, although it did significantly increase IKKB expression at 25 μM compared to control cells ($p<0.05$) (Figure 5.4). SDA also showed a tendency to increase NF-kB expression at both 25 and 50 μM and showed some slight increases in PPARα expression again at both 25 and 50 μM (Figure 5.5), although these changes were not significant.

When comparing ALA or SDA to EPA and DHA, the marine-derived FAs had significantly greater effects on relative expression of the genes examined.

At 25 μM ALA resulted in significantly higher relative gene expression of NF-kB compared to EPA ($p<0.05$) (Figure 5.3) and significantly lower gene expression of MCP-1compared to EPA ($p<0.05$) (Figure 5.7). Gene expression of IKKB after ALA treatment at 25 μM was also significantly lower than DHA at that concentration ($p<0.0001$) (Figure 5.4). PPARα gene expression after treatment with ALA at both 25 and 50 μM was significantly lower compared to DHA treatment at 25 and 50 μM ($p<0.01$, $p<0.0001$, respectively) (Figure 5.5). ALA resulted in significantly lower relative gene expression of IL-6 and COX-2 compared to DHA at both 25

and 50 μM ($p<0.0001$, $p<0.0001$, respectively) and significantly lower relative expression of IL-6 compared to EPA at 50 μM ($p<0.0001$) (Figure 5.8 and Figure 5.6).

At 25 and 50 μM SDA resulted in significantly lower relative gene expression of PPARα compared to DHA at 25 μM ($p<0.01$) and compared to both EPA and DHA at 50 μM ($p<0.05$, $p<0.0001$, respectively) (Figure 5.5). IL-6 gene expression was also significantly lower after SDA treatment compared to both EPA and DHA at both 25 ($p<0.05$, $p<0.0001$, respectively and 50 μM ($p<0.0001$) (Figure 5.8). Compared to DHA treatment, SDA treatment lead to significantly lower relative gene expression of COX-2 ($p<0.0001$) (Figure 6.1).

GLA treatment had the greatest modulating effects on gene expression among the plant-derived FAs. GLA treatment of EA.hy926 cells at 25 μM significantly decreased relative gene expression of PPARα, with a trend to increase relative gene expression at 50 μM (Figure 5.5). Treatment of EA.hy926 cells with GLA at 50 μM significantly increased relative expression of IL-6 ($p<0.05$) compared to control cells, although this what not seen at the lower concentration (25 μM) (Figure 5.8). GLA treatment at both 25 and 50 μM also had a tendency to increase the relative gene expression of NF-kB and COX-2 compared to control, although these effects were not significant (Figure 5.3 and Figure 5.6).

PIN did not significantly affect the expression of any of the genes examined. At 25 and 50 μM PIN showed some tendencies to increase relative gene expression of NF-kB and MCP-1 (Figure 5.3 and Figure 5.7). PIN treatment at 50 μM also appeared to increase relative gene expression of IKKB compared to control (Figure 5.4), although again this was not significant.

Comparison of GLA and PIN to EPA and DHA, showed EPA and DHA to have greater effects on the expression of genes examined.

GLA treatment lead to significantly lower relative gene expression of IKKB compared to DHA at both 25 ($p<0.01$) and 50 μM ($p<0.01$) (Figure 5.4).This was also seen with PIN treatment at 25 μM ($p<0.01$). PIN treatment was also significantly different compared to EPA at 25 and 50 μM for NF-kB relative gene expression ($p<0.05$) (Figure 5.3). Both GLA and PIN treatment lead to lower PPARα relative gene expression compared to DHA at both 25 ($p<0.001$, $p<0.01$, respectively) and 50 μM ($p<0.0001$, $p<0.05$, respectively) (Figure 5.5). Significantly lower relative gene expression of COX-2 and IL-6 was also seen after treatment with GLA compared to DHA at both 25 ($p<0.0001$) and 50 μM ($p<0.01$,

$p<0.0001$) as well as PIN compared to DHA at both 25 ($p<0.0001$) and 50 μM ($p<0.0001$) (Figure 5.6 and Figure 5.8). Relative IL-6 gene expression was also significantly different after PIN treatment compared to EPA treatment at 25 μM ($p<0.05$) and compared to both GLA and PIN at 50 μM ($p<0.0001$) (Figure 5.8). Lastly relative expression of MCP-1 was significantly lower after treatment with GLA compared to EPA at 25 μM ($p<0.05$) (Figure 5.7).

These results indicate the complex nature of the activity of these FAs. Changes in gene expression do not necessarily result in changes in cellular protein abundances. This is clearly seen in this inflammatory model where by EPA and DHA decrease IL-6 secretion by stimulated EA.hy926 cells yet increase IL-6 relative gene expression.

5.5 Discussion

The aims of the research described in this chapter were to compare the effects of four plant-derived FAs (ALA, SDA, GLA and PIN) to two marine-derived FAs (EPA and DHA) on the expression of various inflammatory genes (NFκB, IKIKB (IKKB), PPARα, MCP-1, PTGS2 (COX-2), IL-6) by cultured ECs. The aims and the objective were met.

Isolated RNA was shown to have high RIN scores indicative of good quality and integrity (Figure 5.1). geNorm (Primer Design) was used to identify the number of and the specific reference genes for the analysis of the RT-qPCR. Based on the geNorm analysis 3 reference genes were optimal for the conditions being examined; these were B2M, GAPDH and RPL13A (Figure 5.2). This allowed for a robust analysis of relative gene expression within this inflammatory cell model. The geometric mean of these reference genes was then used to quantify the relative expression of the genes of interest (see section 2.3.5).

Increased expression of the NF-KB gene would be considered to be pro-inflammatory in nature, and could lead to increased NF-kB protein which is a key transcription factor involved in up-regulation of expression of genes encoding many proteins involved in inflammation (Chapter 1). Increased expression of MCP-1 and IL-6 genes may lead to higher quantitates of these inflammatory mediators and therefore could also be considered to be pro-inflammatory. IKKB is a protein subunit of IkB kinase (IKK) and acts to phosphorylate the NF-kB inhibitory subunit (IkBα). This then leads to the activation of NF-kB. Increased IKKB may therefore lead to increased NF-kB activation. Increased expression of

COX-2 gene could lead to increased COX-2 protein which as described previously leads to the synthesis variety of inflammatory or resolving FA-derived mediators (section 1.2.6.2) depending on the FA which this enzyme is acting upon. In contrast PPARα is considered to have anti-inflammatory actions, mainly because it interferes with nuclear translocation of NFkB.

5.5.1 Effects of fatty acids on gene expression

Based upon the findings of chapter 4 and the literature, it was anticipated that EPA and especially DHA would lower inflammatory and enhance anti-inflammatory gene expression. However this was not seen. In fact, marine-derived n-3 FAs induced the greatest fold change increase in expression of the various different genes examined in stimulated EA.hy926 cells.

Comparison of FAs at a concentration of 50 μM revealed DHA as having the most potent effect on gene expression compared to control (TNFα stimulated EA.hy926 cells). DHA at 50 μM significantly increased relative gene expression of IKKB, PPARα, COX-2 and IL-6, compared to control cells. The effects on PPARα can be regarded as anti-inflammatory, but those on IKKB, COX-2 and IL-6 could be considered to be pro-inflammatory. EPA at 50 μM also increased IL-6 relative gene expression compared to stimulated control cells. There was a tendency for EPA to also increase COX-2 gene expression, although this was not significant. EPA at 25 μM and at 50 μM had a tendency to reduce relative gene expression of NF-kB compared to control cells.

These results indicate a complex role for EPA and DHA in modulating inflammation, with expression of both pro and anti-inflammatory genes being altered in EA.hy926 cells after treatment with these FAs. The observations seen here are also contradictory to the inflammatory proteins being secreted by the cells which indicate a very potent anti-inflammatory effect of these two derived FAs. Others too have seen different effects in the relative gene expression compared to transcription of the proteins within the cell [190, 191].

Both marine-derived n-3 FAs increased the gene expression of COX-2. Similarly Gdula-Argasinska *et al*. observed increased COX-2 gene expression after treatment of HUVECs with DHA at 80 μM for 24 hours [190]. Chene *et al.* also described increased COX-2 gene expression after EPA treatment (20 μM) in human keratinocyte HaCat cells [191].

Although an induction of COX-2 gene expression by marine n-3 PUFAs seems counterintuitive it is important to remember that COX-2 can lead to both the production of inflammatory mediators from AA and to pro resolving mediators from EPA and DHA. Therefore, increase expression of this gene may indicate a pathway by which these FAs are having an anti-inflammatory effect in this model. It is also possible that although the COX-2 gene is being overly expressed the protein is not being translated. In order to test for this COX-2 protein will be measured in experiments described in Chapter 6.

Similar to observations made here, Grenon *et al*. also describe significant increases in IL-6 gene expression after treatment with EPA at ~16 μM in non-activated EA.hy926 cells, though they did not see this in TNFα stimulated cells, which may be because a lower concentration of EPA was used [152].

IL-6 concentration was shown to be lower in the supernatant of EA.hy926 cells treated with DHA compared to control cells (Chapter 4) yet here there was an increase in IL-6 gene expression in DHA treated cells. Again it is possible the IL-6 protein is not translated, or that changes in membrane composition after DHA treatment may lead to the blocking of the secretion of the protein. One way of assessing this would be to look at the cellular content of IL-6 after DHA treatment.

Although EPA and DHA did modulate gene expression of the transcription factors in this model, others describe no effect of EPA and DHA (10, 25 and 100 μM) on the expression of PPARs or NF-kB mRNA in TNFα stimulated HUVECs [174].

Of the plant-derived FAs, at a concentration of 50 μM, only pre-treatment with GLA had significant effects on relative gene expression. GLA significantly increased IL-6 gene expression compared to control stimulated cells, with a tendency to increase COX-2 at both 25 and 50 μM. No other plant-derived FA at 50 μM had any effect on relative expression in any of the genes analysed. GLA was also seen to significantly reduce PPARα mRNA at 25 μM compared to control cells. SDA at 25 μM significantly increased relative IKKB gene expression compared to control cells. ALA and PIN were shown to have no effect on relative expression of any of the genes examined.

There is no previous research on the effects of these plant-derived FAs on gene expression in ECs. Some researchers have reported differences after treatment with ALA and GLA in other cell types. Similar to the effects of GLA in EA.hy926 cells, others observed increases in COX-2 gene expression with GLA in human

keratinocyte HaCat cells [191]. Erdinest *et al.* described ALA to significantly decrease IkBα and IL-6 gene expression in LPS stimulated human corneal epithelial cells [183]; however this was not observed in the EA.hy926 model. Wang *et al.* examined effects of ALA (20 μM) on relative gene expression of COX-2 in oxidative stress induced porcine ECs, they described no changes compared to control cells [192].

5.5.2 Conclusions

The effects of the FAs seen here indicate EPA and more especially DHA have modulatory effects on gene expression in TNFα activated ECs. Plant-derived SDA and GLA also have some effects on relative gene expression within this model. The findings are summarised in Table 5.2. The effects on gene expression are different from those described for secreted protein in Chapter 4.

Table 5.2 Summary of effects of FA treatment on inflammatory gene expression in EA.hy926 cells

	Fatty acid						**Comments**
Gene	ALA	SDA	GLA	PIN	EPA	DHA	
NF-kB	↔	↔	↔	↔	↔	↔	No changes observed
IKKB	↔	↑	↔	↔	↔	↑↑	Effect was dose-dependent
PPARα	↔	↔	↓	↔	↔	↑	Effect was dose-dependent
COX-2	↔	↔	↑	↔	↑	↑↑	Effect was dose-dependent
MCP-1	↔	↔	↔	↔	↔	↔	Effect was dose-dependent
IL-6	↔	↔	↔	↔	↑	↑↑	Effect was dose-dependent

In order to identify whether the altered gene expression after exposure to FAs is related to changes in inflammatory pathways, some proteins of interest will be examined using Western blotting in Chapter 6. Various FA treatments modulated expression of the IKKB gene. IKK activity causes activation of NF-kB; however IKK-related kinase phosphorylation may prevent the over production of inflammatory mediators since they exert negative regulation on IKKs. In order to examine this further, phosphorylated and non-phosphorylated NF-kB will be quantified using Western blotting after treatment with all FAs. COX-2 and IL-6 mRNA are shown to have large fold increases in gene expression after EPA and DHA treatment and therefore cellular content of these proteins will also be examined using Western blotting.

Chapter 6: Effect of fatty acids on inflammatory proteins in cultured endothelial cells

6.1 Introduction

Inflammatory processes of ECs play a key role in the development of atherosclerosis (section 1.2.5) and proper EC function is critical for regulation of inflammation. FAs have been shown to modulate different inflammatory proteins in ECs [158, 163, 167, 174], with different FAs having different effects (Chapter4). Chapter 4 focussed upon extracellular appearance of cytokines and related inflammatory mediators. Intracellular proteins (signalling proteins, transcription factors, enzymes) also change in response to inflammatory stimuli as part of the cascade of signalling events which eventually lead to the secretion of inflammatory cytokines, chemokines, lipid mediators and other types of mediator as well as the appearance of cell surface adhesion markers. It is possible that FAs may modulate the levels and activities these intracellular proteins as part of their role in controlling inflammatory responses.

Previous chapters have shown the effects of different FAs being explored within the context of an inflammatory model using human ECs. The model was developed in the experiments described in Chapter 2Chapter 2: and subsequent experiments showed that FAs have differential effects on inflammatory mediator production (Chapter 4) and relative expression of genes involved in inflammation (Chapter 6). The results obtained confirm potent effects of EPA and especially DHA on inflammatory processes, and describe more limited effects with treatment with several plant-derived FAs (ALA, SDA, GLA and PIN), which may offer future sustainable alternatives. However it is important to note that the effects on gene expression and on secreted inflammatory mediators did not always match. Reasons for this are not clear but may relate to effects on protein synthesis, protein modification and protein secretion.

In order to better understand the pathway of action of FAs on inflammation in ECs, several proteins involved in inflammation were examined. These include NF-kB and COX-2. These two proteins play a vital role in the pathogenesis of inflammation.

NF-kB is a transcription factor which is responsible for the modulation of inflammation as it regulates the expression of numerous genes encoding pro-inflammatory proteins including adhesion molecules, chemokines and cytokines [193]. The NF-kB complex is formed from five family member protein monomers (RelA (p65), RelB, cRel, p50 and p52) [32], and resides in the cytoplasm in an inactive from associated with a class of inhibitory proteins termed IkBs. The IkB

family members have common Ankyrin repeat domains, which regulate subcellular localisation and therefore the DNA binding and transcriptional activity of NF-kB proteins. The basis for the cytoplasmic localisation of the inactive NF-kB:IkB complex is thought to be due to masking of the NLS (nuclear localisation signals) on the NF-kB subunits by the IkB proteins [194]. Therefore IkB degradation leads to the unmasking of NLS on the NF-kB subunits allowing free NF-kB to undergo translocation to the nucleus.

NF-kB is activated through a variety of different inflammatory signals including cytokines like TNF. This activation involves several mechanisms including degradation of the IkB proteins, processing of NF-kB precursor proteins, and expression of NF-kB monomer proteins [32]. Multi-subunit protein kinase, the IkB kinases (IKK), are activated through phosphorylation, and several inflammatory cytokines, including TNFα, result in IKK phosphorylation-dependent activation of NF-kB. IKK phosphorylates NF-kB bound IkBs targeting them for ubiquitination and proteasomal degradation [195]. As IkBs degrade, free NF-kB is then able to translocate to the nucleus where it binds kB sites on DNA and activates gene expression [32]. The IkBs display a preference for specific NF-kB/Rel complexes, which may provide a means to regulate the activation of distinct Rel/NF-kB complexes. In most cells NF-kB dimers p65(relA):p50 are associated with IkBα .

In this chapter the phosphorylation of NF-kBp65 will be examined: greater phosphorylation suggests greater activity of NF-kB since, once phosphorylated, NF-kBp65 translocates to the nucleus and begins to transcribe the genes encoding various mediators involved in the inflammatory process (section 1.2.3).

COX-2 is an enzyme which acts upon FAs to produce a variety of different lipid mediators (section 1.2.6.2). AA is the dominant substrate for synthesis of eicosanoids including prostaglandins, leukotrienes and thromboxanes, which play a role in inflammatory regulation. However EPA and DHA are also COX-2 substrates generating alternative lipid mediators including resolvins, protectins and maresins. These EPA- and DHA-derived mediators have been shown to exert anti-inflammatory and inflammation resolving effects in cell culture and animal models [77-79]. Dietary supplementation of EPA and DHA leads to increases of both these n-3 PUFAs within the cellular phospholipids, partially at the expense of AA [74], and can therefore alter the inflammatory response through this mechanism.

In chapter 5, GLA, EPA and DHA were shown to increase COX-2 gene expression, this may lead to greater translation of COX-2, which in turn could change inflammatory lipid mediators and inflammatory potency depending on the FA which is being used as substrate (section 1.2.6.2). In this chapter FA treatment on COX-2 protein expression will be explored.

In Chapter 2 an EC based model for examining effects of FAs on inflammation was established. Prior to assessing the effects of FAs on inflammatory cellular proteins a time course of the effect of TNFα stimulation on COX-2 and NF-kB was performed. TNFα decreased the cellular quantity of non-phosphorylated NF-kBp65 in EA.hy926 cells in a time dependent manner; this subunit of NF-kB becomes phosphorylated in response to cellular activation, and therefore the amount of non-phosphorylated NF-kBp65 is decreased as NF-kB is activated. A time point of 1 hour stimulation was chosen to explore effects of FAs on NF-kB protein.

COX-2 is inducible under inflammatory conditions. TNFα increased the level of COX-2 in EA.hy926 cells in a time dependent manner (section 2.4.3). A time point of 16 hours stimulation was used to assess the effect of FAs on COX-2 cellular protein levels.

Previously it has been shown that several of the plant-derived FAs possess some anti-inflammatory actions and therefore may have the potential as alternatives to EPA and DHA. The pathway of action of these FAs needs to be further examined in order to be carried forward to animal and human studies. The research described in this chapter compares the effects of four plant-derived 18 carbon FAs (ALA, SDA, GLA, PIN) on expression of intracellular inflammatory proteins in EA.hy926 cells in vitro with those of EPA and DHA. This is an important step in the search more sustainable sources of bioactive FAs for promoting human health.

6.2 Aim and objectives

The aim of the research described in this chapter was to compare the effects of four plant-derived and two marine-derived FAs on inflammatory responses of cultured ECs.

The specific objective was to:

- Compare the effects of four plant-derived FAs (ALA, SDA, GLA and PIN) to two marine-derived FAs (EPA and DHA) on cellular levels of NF-kBp65, phosphorylated NF-kBp65 (pNF-kBp65) and COX-2 in cultured ECs.

6.3 Methods

6.3.1 Reagents

Reagents and materials used for the experiments are described in Appendix A.

6.3.2 Endothelial cell culture

As described previously in section 2.3.2.3 confluent EA.hy926 cell cultures were scraped from flasks and resuspended in culture medium at a density of 5.5 x 10^5 cells per mL. Cells were seeded at 5.5 x 10^4 cells per well in 6-well flat bottom plates, incubated for 48 hours at 37°C with various FAs at a concentration of 50 μM, followed by 1 or 16 hours incubation with TNFα at 1 ng/mL. At each time point, cells were removed, resuspended in RIPA buffer and stored at -80°C until analysis (see section 2.3.6).

6.3.2.1 Analysis of inflammatory intracellular proteins by EA.hy926

Concentrations of intracellular proteins from EC lysates were determined using Western blotting, as described in section 2.3.6.3.1.

6.3.2.2 Protein quantification BCA assay

The BCA assay was carried out to analyse protein concentrations in cell lysates as described in section 2.3.6.2.1.

6.3.2.3 Western blotting

The expression of NF-kBp65, phosphorylated NF-kBp65 (pNF-kBp65), COX-2 and GAPDH was assessed by Western blotting; see Appendix A for antibody details. The procedure was as described in section 2.3.6.3.1. Briefly, samples were diluted and a total of 30 ng loaded into precast 10% SDS-PAG (Optiblot, Abcam) alongside Prism Ultra Protein Ladder (10-245kDa). Gels were run for 1.5- 2 hours and then proteins transferred onto nitrocellulose membrane which were probed with antibodies for proteins of interest.

6.3.3 Statistical analysis

Western blots were quantified using ImageJ software and relative quantification values are presented as the ratio of each protein band relative to the lane's loading control (reference protein) (section 2.3.6.4).

Data are expressed as mean ± standard error; data analysis was performed in PRSIM and Excel. Multiple group differences were compared using one way ANOVA.

6.4 Results

In order to investigate the effect of different FAs on various cellular proteins involved in inflammation, EA.hy926 cells were exposed to FAs at a concentration of 50 μM for 48 hours followed by 1 or 16 hours TNFα stimulation at 1 ng/mL, depending on protein being examined. TNFα treatment was previously shown to modulate proteins of interest (Figure 2.18 and Figure 2.19). Total protein was quantified using the BCA assay and the expression of proteins of interest was assessed by Western blotting and quantified using imageJ. Data are presented as a ratio of COX-2 to GAPDH and pNF-kBp65 to NF-kBp65. The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30. 1 sample per treatment from each experiment was analysed by western blot generating 3 blots per experiment.

FA exposure was shown to have differential effects depending on the protein being examined and the individual FA (Figure 6.1and Figure 6.2). Marine-derived DHA showed the most potent effects on levels of the intracellular proteins being analysed. EPA and PIN also had some significant effects while ALA, SDA and GLA had the least effects.

6.4.1 COX-2

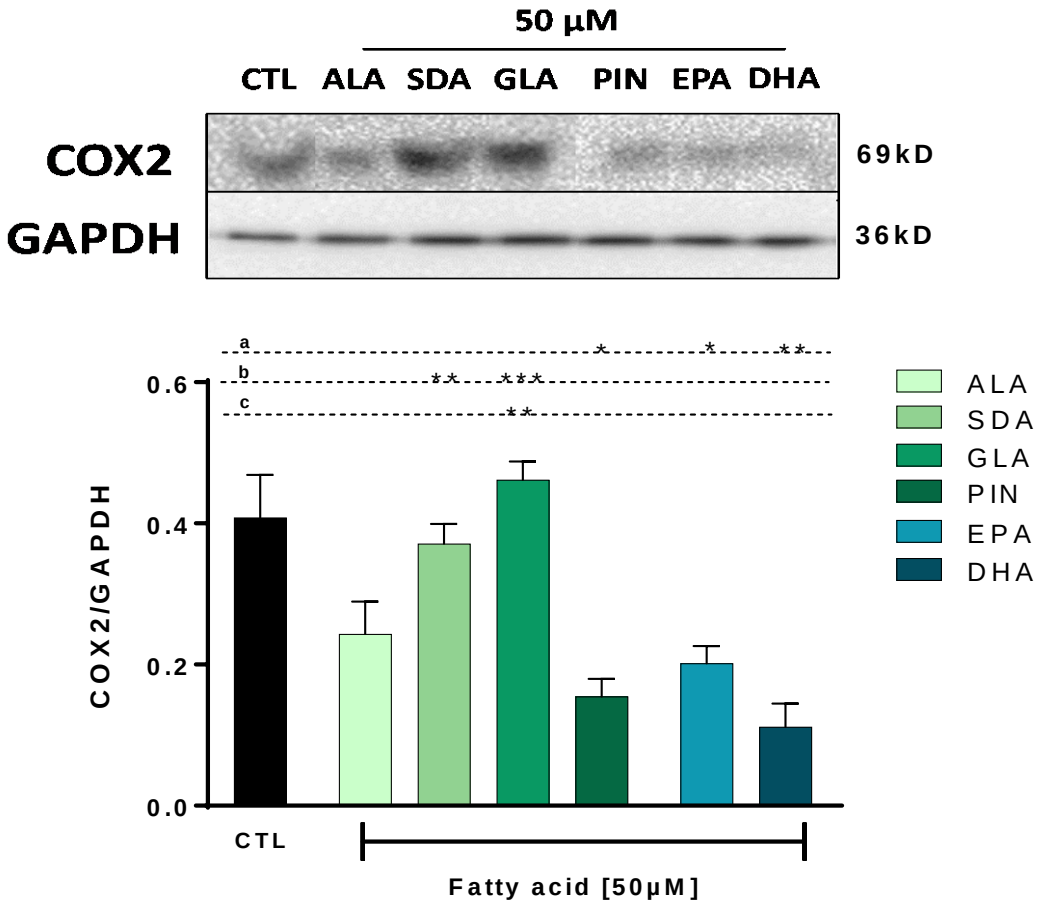

Figure 6.1 Effect of FA exposure on intracellular COX-2 in TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) cellular levels of COX-2 in activated EA.hy926 cells without prior incubation with FA (control; CTL) or with 48 hours prior exposure to ALA, SDA, GLA, PIN, EPA and DHA (50 µM) followed by 16 hours exposure to TNFα (1 ng/mL). COX-2 was measured in the whole cell lysate by Western blotting. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001

6.4.2 NF-kB

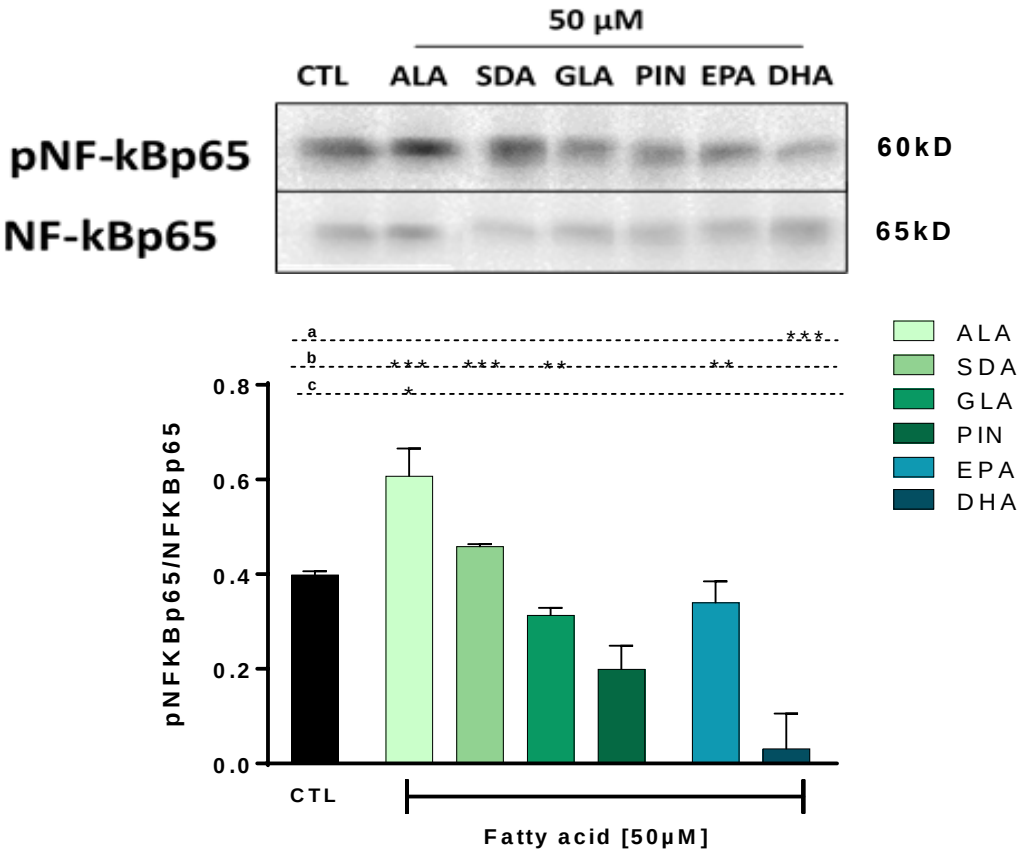

Figure 6.2 Effect of FA exposure on intracellular phosphoNF-kBp65 and NF-KBp65 quantities by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) production of pNF-KBp65 and NF-KBp65 activated EA.hy926 cells without prior incubation with FA (control; CTL) or with 48 hours prior exposure to ALA, SDA, GLA, PIN, EPA and DHA (50 µM) followed by 1 hour TNFα (1 ng/mL). pNF-KBp65 and NF-KBp65 were measured in the whole cell lysate of endothelial cell cultures. One way ANOVA a: control vs FA; b: FA vs DHA; c: FA vs EPA; *p<0.05, **p<0.01, ***p<0.001

DHA exposure at 50 µM significantly decreased COX-2 protein levels compared to control ($p<0.01$) (Figure 6.1). This contrasts with the previous, unexpected, finding that DHA treatment lead to an increase in gene expression of COX-2 in this model (Chapter 5). DHA also significantly decreased the ratio of pNF-kBp65/NF-kBp65 ($p<0.001$) and the overall quantities of both pNF-kBp65 and NF-kBp65 (Figure 6.2). EPA treatment also lead to significantly lower levels of COX-2 protein than in control cells ($p<0.05$) (Figure 6.1); again this is in contrast to previous results which showed EPA increasing COX-2 gene expression in

EA.hy926 cells (Chapter 5). However, in contrast to DHA, EPA had no effect on the pNF-Kbp65/NF-kBp65 ratio (Figure 6.2). This may indicate different anti-inflammatory mechanisms of action of EPA and DHA.

ALA, SDA or GLA had no significant effects on either of the proteins examined in this model (Figure 6.1 and Figure 6.2). However ALA treatment had a tendency to increase the ratio of pNF-kBp65/NF-kBp65 while GLA treatment had a tendency to increase COX-2 expression compared to control. This mirrors a tendency to increase COX-2 relative gene expression after treatment with GLA (Chapter 5). Treatment with SDA had no effect on either COX-2 or pNF-kBp65/NF-kBp65 ratio. EA.hy926 cells pre-treated with PIN however showed significantly decreased COX-2 expression compared to control ($p<0.05$) and a tendency to a decreased pNF-kBp65/NF-kBp65 ratio, although this was not significant (Figure 6.1 and Figure 6.2).

Comparing protein levels of COX-2 after exposure to plant-derived FAs to EPA and DHA showed significant differences between SDA and GLA treatment compared to treatment with DHA ($p<0.01$, $p<0.001$, respectively) and GLA treatment also lead to significantly higher proteins levels of COX-2 than EPA ($p<0.01$).

ALA, SDA and GLA treatments all lead to significantly higher ratios of pNF-kBp65/NF-kBp65 protein levels in stimulated EA.hy926 cells compared to DHA ($p<0.001$, $p<0.001$, $p<0.01$ respectively). Pre-treatment with ALA also produced a significantly higher ratio of pNF-kBp65/NF-kBp65 compared to EPA treatment ($p<0.05$). Furthermore EPA treatment lead to significantly higher protein ratios of pNF-kBp65/NF-kBp65 than DHA treatment ($p<0.01$).

6.5 Discussion

In order to better understand the effects of plant and marine-derived FAs on inflammatory pathways, two proteins involved in inflammation were examined after treatment of ECs with the FAs.

In this model DHA treatment (50 μM) had the strongest effect on the inflammatory proteins assessed, decreasing total COX-2 protein and the pNF-kBp65/NF-kBp65 ratio. These findings indicate an anti-inflammatory role for DHA through a decrease in proteins involved in pro-inflammatory signalling. The effect on pNF-kBp65 would be expected to lower expression of genes involved in inflammation like cytokines, chemokines, adhesion molecules and COX-2;

however this was not seen (Chapter 5) although secreted levels of cytokines and chemokines, ICAM-1 surface expression and COX-2 protein were all decreased by DHA. The decrease in COX-2 protein would be expected to result in lower production of inflammatory lipid mediators from the substrate AA. This was not examined here but is a common finding especially with cells cultured in the presence of DHA [182, 190].

Treatment with EPA was also shown to decrease COX-2 protein compared to control cells, although EPA did not reduce NF-kB activity as assessed by pNF-kBp65. The finding that EPA treatment resulted in a significantly higher ratio of pNF-kBp65/NF-kBp65 than DHA indicates differences in how these marine-derived FAs affect inflammation.

Others have described reduced quantities of COX-2 protein after treatment with EPA (20, 50 and 100 μM) and to a greater extent DHA (10, 20, 50 and 100 μM) [162] in HUVECs. Massaro *et al.* described a 50% reduction in cellular COX-2 protein levels after DHA treatment (25 μM) in IL-1α stimulated HSVECs compared to control cells [196]. Similarly Chen *et al.* described reduced COX-2 protein levels after treatment with DHA (10 and 40 μM) in rat brain microvascular ECs [197]. However some studies report no changes in COX-2 levels after treatment with EPA or DHA in VEGF-stimulated HUVECs [165].

Others have also described reduced NF-kB activity after DHA treatment of various cell types. Some reported reduced nuclear translocation of the p65 NF-kB subunit after DHA treatment in IL-1α stimulated HSVECs [196]. Huang *et al.* describe inhibition of phosphorylation of IkBα, and therefore inhibition of NF-kB activation, after EPA and DHA treatment (100 μM) in LPS-stimulated HAECs [164]. Similarly EPA treatment (50 μM) resulted in significant reductions in palmitic acid-stimulated IkB degradation and NF-kB translocation in HUVECs [198]. Like findings described here where DHA, but not EPA, reduced NF-kB via decreased phosphorylation of NF-kBp65, Wang *et al.* observed a reduction in phosphorylation of IkBα after DHA (80 μM) and not EPA (80 μM) treatment in TNFα stimulated HAECs [112]. In contrast to these findings, Goua *et al.* saw no changes in IkBα phosphorylation after EPA or DHA treatment (50 μM) in TNFα stimulated HUVECs [150].

Effects of plant-derived FAs on intracellular proteins involved in inflammation including COX-2 and NF-kB are less well described. Furthermore very few studies

have examined effects of these FAs in human endothelial cell models, highlighting the novelty of the current work.

Similar to the findings seen here, Szymczak *et al.* describe no changes in COX-2 levels after treatment with either ALA or SDA (10 μM) in VEGF-stimulated HUVECs [165]. Wang *et al.* also reported that ALA treatment (20 μM) of porcine ECs had no effect on COX-2 quantities compared to stimulated control cells, as well as describing no effects of ALA on nuclear NF-kB levels [192].

There are no studies describing effects of GLA or PIN on COX-2 or NF-kB activity in human ECs. Cao *et al.* examined the effects of GLA on NF-kB activity in LPS stimulated primary goat mammary gland epithelial cells [186]: GLA at 100 μM significantly reduced NF-kB activity, which was reported to occur via the CD36 receptor. In this model PIN had a tendency to decrease NF-kB activity, although this was not significant. Others reported increased COX-2 quantities in LPS stimulated RAW264.7 (murine macrophage) cells after PIN treatment (50 μM) [179]. However in the current EC model PIN significantly reduced COX-2 protein levels compared to stimulated control cells. The previous observations were seen using animal, not human, cell models. Therefore differences seen between the current study and previous reports may be due to a variety of factors including different cell type, FA concentration and FA incubation time as well as stimulus used.

6.5.1 Conclusions

The findings of this chapter are summarised in Table 6.1.

Table 6.1 Summary of effects of FA treatment on protein expression in EA.hy926 cells

	Fatty acid						Comments
Protein	ALA	SDA	GLA	PIN	EPA	DHA	
COX-2	↔	↔	↔	↓	↓	↓↓	
NF-kB/pNF-kB	↔	↔	↔	↓	↔	↓	Tendency after PIN treatment

DHA decreases COX-2 protein levels and the level of activated NF-kBp65 in TNFα-stimulated human ECs. Treatment with EPA and PIN decreased COX-2 protein expression, and PIN had a tendency to decrease NF-kB activity. ALA, SDA and GLA do not affect these proteins in this model and at the concentrations used. These

findings support those in the current literature with regard to DHA and to some extent EPA but suggest different mechanisms of action of EPA and DHA. These findings also indicate a role for PIN as an anti-inflammatory FA. These findings add to the understanding of anti-inflammatory actions of 18-carbon plant-derived FAs.

Chapter 7: Binding of monocytes to fatty acid treated endothelial cells

7.1 Introduction

Previously it has been shown marine FAs, especially DHA, have anti-inflammatory actions on ECs stimulated with TNFα (Chapter 4 and Chapter 6), and that some of the plant-derived FAs, particularly GLA and PIN, also act in an anti-inflammatory manner although at lower potency. Effects were identified by measuring inflammatory cytokines and chemokines being secreted from the ECs (Chapter 4) and selected inflammatory proteins within the cells (Chapter 6). There were also indications of effects of FAs on inflammatory signalling where there were differences in transcription factor activation (Chapter 6) and inflammatory gene expression (Chapter 5), although these were not always in the same direction. An effect of SDA, EPA and DHA on surface expression of the adhesion molecule ICAM-1 was also seen (Chapter 4). This might affect binding of leukocytes, especially monocytes, to the ECs. This would be important in preventing or slowing the early stages of atherosclerosis since monocyte attachment to and movement through the endothelium is a key early step in the development of atherosclerosis (section 1.2.5).

Adhesion molecules expressed on ECs play an important role in leukocyte-endothelium interactions (see section 1.2.2). The adhesion molecules expressed on ECs include selectins, members of the immunoglobulin superfamily (e.g. ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) [26].

Various reports describe reduction of surface adhesion molecule expression after treatment with EPA and DHA in a number of different EC models (see section 1.3.1), these effects could lead to a decrease in leukocyte infiltration into the blood vessel wall, reduced inflammation, and greater plaque stability [26]. It has been demonstrated that individuals consuming increased amounts of EPA and DHA have a lower risk of cardiovascular events and mortality [101, 199]. This could be explained in some part by a reduction in atherosclerosis. LC n-3 PUFAs have been shown to help to stabilise plaques [200].

Table 7.1 Studies investigating the effect of increased EPA and or DHA on adhesion markers in various endothelial cells.

The magnitude of statistically significant effects is indicated; where there was no statistically significant effect "None" is entered. (Baker *et al*. [26]).

Cell type	**EPA or DHA concentration**	**Stimulus used**	**Outcomes measured**	**Effect of EPA (approx. % change where significant)**	**Effect of DHA (approx. % change where significant)**	**Reference**
HUVECs	65 μM EPA or DHA	IL-1β	mRNA VCAM-1 ICAM-1 E-selectin	 None None None	 None None None	Collie-Duguid and Wahle (1996) [111]
HSVECs	10 μM DHA	TNFα or IL-1α	Cell surface expression VCAM-1 Protein VCAM-1 ICAM-1 E-selectin mRNA	 None NA NA NA	 -30% -23% -53% -52%	De Caterina *et al.* (1994) [110]

			VCAM-1	NA	-50%	
HUVECs	10 μM EPA or DHA	TNFα 20 hr	Cell surface expression VCAM-1 ICAM-1 E-selectin	 None None None	 None None None	Mayer *et al.* (2002) [173]
HCAECs	10 or 50 μM EPA or DHA	Ox-LDL	Protein ICAM-1 P-selectin mRNA ICAM-1 P-selectin	 -22%, -83% (10, 50 μM) -29%, -71% (10, 50 μM) -27%, -67% (10, 50 μM) -31%, -67% (10, 50 μM)	 -33%, -83% (10, 50 μM) -41%, -71% (10, 50 μM) -27%, -73% (10, 50 μM) -44%, -63% (10, 50 μM)	Chen *et al.* (2003) [172]
HUVECs	25 μM EPA or DHA	TNFα 6 hr or 24 hr	Cell surface expression VCAM-1 ICAM-1	 -8% -18%	 -15% -10%	Goua *et al.* (2008) [150]
HIMECs	5 or 25 μM DHA	IL-1β 8 or 24 hr	Protein VCAM-1 ICAM-1	 NA NA	 -37.5%, -50% (5, 25 μM) None	Ibrahim *et al.* (2011) [182]

HAECs	20-160 μM EPA or DHA	TNFα	Cell surface expression VCAM-1 ICAM-1 Protein VCAM-1 ICAM-1	 -20% (80-160 μM) None None None	 -20, -55,-70% (40-160 μM) -20% (160 μM) -70% (80 μM) None	Wang *et al.* (2011) [112]
HUVECs	5 μM DHA	TNFα	Cell surface expression E-selectin Protein E-selectin mRNA E-selectin ICAM-1	 NA NA NA NA	 -55% None None None	Yates *et al.* (2011) [114]
HAECs	100 μM EPA or DHA	LPS	Cell surface expression VCAM-1 ICAM-1 Protein	 -60% -40%	 -70% -50%	Huang *et al.* (2015) [164]

			VCAM-1	-70%	-80%	
			ICAM-1	-50%	-70%	
			mRNA			
			VCAM-1	-70%	-90%	
			ICAM-1	-40%	-70%	

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HAECs, human aortic endothelial cells; HCAECs, human coronary artety endothelial cells; HIMECs, human intestinal microvascular endothelial cells; HUVECs, human umbilical vein endothelial cells; HSVECs, human saphenous vein endothelial cells; ICAM-1, intercellular adhesion molecule 1; IL-1α, interleukin 1 alpha; IL-1β, interleukin 1 beta; LPS, lipopolysaccharide; NA, not assessed; Ox-LDL, oxidised low-density lipoprotein; TNFα, tumour necrosis factor alpha; VCAM-1, vascular cell adhesion protein 1.

Both static and flow adhesion assays have been used *in vitro* to examine the functional effect of reduced adhesion molecule expression by EPA and DHA. The studies demonstrate consistent effects. Static adhesion assays carried out by De Caterina *et al*. demonstrated DHA treatment of ECs leads to reduced adhesion of monocytes [110]. Others report treatment with both EPA and DHA reduced THP-1 or monocyte adhesion to ECs under static conditions [164, 172]. Similar observations are seen under flow conditions. Mayer *et al*. describe that treatment of HUVECs with EPA and DHA decreased both monocyte rolling and adhesion under flow conditions [173]. Furthermore Yates *et al.* suggest EPA and DHA may modulate different stages of leukocyte recruitment, where by DHA was seen to reduce recruitment while EPA was shown to inhibit migration under flow conditions [114]. Together these studies report reduction of adhesion and rolling of leukocytes after incubation of ECs with EPA and DHA whether under static or flow conditions, indicating a role for both of these methods in assessment of FA treatment.

Effects of plant-derived FAs on the adhesion of monocytes to ECs has not been explored.

Since the different FAs used in this research have different effects on ICAM-1 expression, but also on other factors involved in adhesive interactions like MCP-1 production (section 4.4.1.4), it is important to assess whether the FAs have an effect on the adhesion process itself.

7.1.1 Aim and objectives

The aim of the research described in this chapter was to optimise the conditions for studying adhesion of monocytes to cultured EA.hy926 cells and to compare the effects of four plant-derived FAs and two marine-derived FAs on the adhesion of monocytes to cultured ECs.

The specific objectives were to:

- Establish a static cell adhesion model with EA.hy926 endothelial cells and THP-1 monocytes using the Vybrant™ Cell Adhesion Assay Kit;

- Compare the effects of four plant-derived FAs (ALA, SDA, GLA and PIN) to two marine-derived FAs (EPA and DHA) on adhesion of THP-1 monocytes to cultured EC monolayers.

7.2 Methods

7.2.1 Reagents

The reagents and materials used for these experiments are described in Appendix A.

7.2.2 Endothelial cell culture

As described previously (section 2.3.2), confluent EA.hy926 cell cultures were scraped from flasks and resuspended at a density of 2 x 10^5 cells per mL. Cells were seeded at 1 x 10^4 cells per well in 96-well flat bottom plates, incubated for 48 hours at 37°C with various FAs at a concentration of 25 or 50 µM followed by 6 hour incubation with TNFα at 1 ng/mL.

7.2.3 THP-1 cell culture

The monocyte cell line THP-1 (ECACC - 88081201) was obtained from ECACC. The cells were thawed and subcultured following the supplier's instructions. Cells were cultured at 5-7 x 10^5 cells/mL in culture medium which consisted of Roswell Park Memorial Institute (RPMI) medium supplemented with 10% FBS, L-glutamine (0.4 mM), penicillin, streptomycin and HAT (100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine). Cells were maintained at 37°C in 5% CO_2. Prior to use in experiments cells were grown in T-175 flasks until confluent.

7.2.4 Static adhesion

Adhesion of THP-1 monocytes to EA.hy926 cells was determined using the Vybrant™ Cell Adhesion Assay Kit (Thermo Fisher).

7.2.4.1 Principle of the static adhesion assay

The Vybrant™ Cell Adhesion Assay Kit (Thermo Fisher) utilizes the fluorogenic dye calcein acetoxymethyl ester (calcein AM) to measure cell adhesion. Calcein AM is non-fluorescent but, once loaded into cells, is cleaved by endogenous esterases to produce highly fluorescent calcein. The cells whose adhesion is to be assessed (in this case THP-1 monocytes) are labelled with calcein AM by incubating them for 30 minutes. These cells are then incubated with the adhesion ligand bearing

cells (in this case EA.hy926 cells) for a period of time to allow the cell-to-cell adhesion to occur. After removal of non-adherent cells, calcein fluorescence is used to calculate the number of adherent cells.

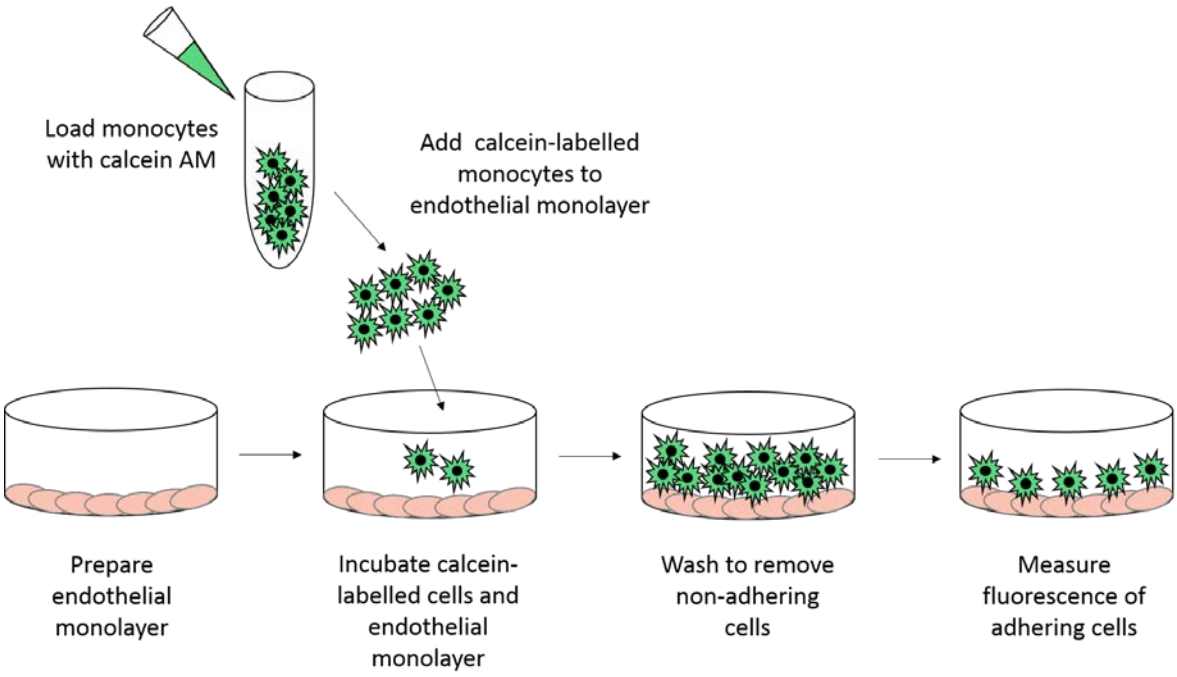

Figure 7.1 Schematic of static adhesion assay prinpicle

7.2.4.2 Protocol of the assay

Calcien labelled THP-1 cells were incubated with EA.hy926 monolayers.

EA.hy926 cells were incubated as described in section 7.2.2 . Following Vybrant cell adhesion kit assay protocol instructions, THP-1 cells were washed twice with RPMI (without supplements) and incubated with calcein (5 µM) at a density of 5 x 10^4/mL for 30 minutes.

After TNFα stimulation supernatant was removed from the EA.hy946 cell cultures and the cells were washed twice with RPMI (non-supplemented). Calcein labelled THP-1 cell suspension (5 x 10^4 cells in 100 µL) was added to each well containing 1 x 10^4 ECs. Cells were co-incubated for 1 hour at 37°C. Non adherent THP-1 cells were removed by gentle washing using a pipette, and adherent cells were washed a further 3 times with RPMI (non-supplemented). Finally 100 µL of PBS was added to each well and fluorescence was read on the Glomax Discover System (Promega) at 490 nm.

7.2.5 Visualisation of THP-1 binding

Following the static adhesion protocol as described in section (7.2.4.2) images of fluorescence-labelled THP-1 monocytes bound to EA.hy926 cells were taken with fluorescence microscope Nikon Elipse Ti using NIS elements software (version 4.30). Images were taken at a magnification of 10x under transmitted light.

7.2.6 Statistical analysis

THP-1 monocyte adhesion to FA treated TNFα stimulated EA.hy926 cells is expressed as a % of control, control being TNFα stimulated EA.hy926 cells non-FA treated. Data are expressed as mean ± standard error; data analysis was performed in PRISM and Excel. Multiple group differences were compared using one way ANOVA.

7.3 Results

In order to investigate the effect of different FAs on adhesion of monocytes to the endothelial monolayer, EA.hy926 cells were exposed to FAs at concentrations of 25 and 50 μM for 48 hours followed by 6 hours TNFα stimulation at 1 ng/mL. Monolayers were then co incubated with calcein labelled THP-1 cells for 1 hour. The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 and THP-1 were used at a passage of >30. Figure 7.2 depicts a dose-dependent increase in THP-1 monocyte adhesion with increasing TNFα stimulation of EA.hy926, these experiments were completed by Frans van Gemerden [201]. Stimulation with TNFα for 6 hours was chosen as the cell surface expression of ICAM-1 (as seen in Chapter 4) was increased at this time, and therefore this should indicate an increase in the ability of the ECs to capture monocytes at this time point. Adhesion of calcein labelled THP-1 cells was measured using Glomax Discover System at a wavelength of 490nm. Adhesion of THP-1 cells was calculated as a percentage of TNFα stimulated control cells (DMEM alone). The data shown are from 3 separate experiments each conducted in triplicate. Visualisation of THP-1 adhesion was carried out using a fluorescence microscope.

FA exposure was shown to have differential effects depending on individual FA and FA concentration. Adhesion was not significantly affected by any FA when used at 25 μM (Figure 7.3). Treatment of EA.hy926 cells with FAs at 50 μM, showed all but EPA to decrease adhesion (Figure 7.4).

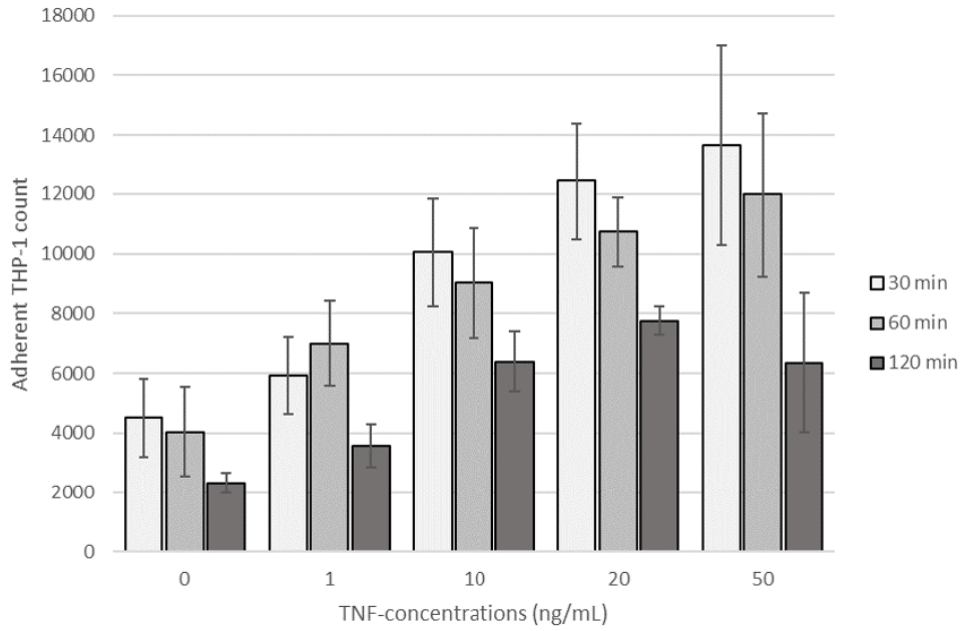

Figure 7.2 Effects of time and TNFα exposure on adhesion of THP-1 monocytes to EA.hy926.

Mean (±SEM) (n = 1) adhesion of THP-1 cells to EA.hy926 exposed to TNFα (0-50ng/mL) for 6 hours and 30, 60 and 120 minute co incubation with THP-1 cells [201].

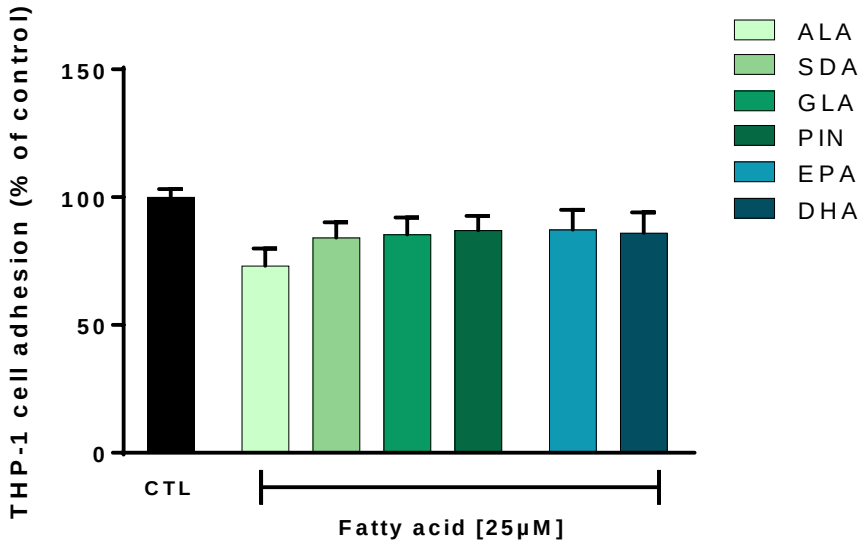

Figure 7.3 Effect of FA exposure (25 µM) on adhesion of THP-1 cells to TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) adhesion of THP-1 cells to activated EA.hy926 cells without prior incubation with FA (control; CTL) or with 48 hours prior exposure to ALA, SDA, GLA, PIN, EPA and DHA (25 µM) followed by 6 hour TNFα (1 ng/mL) and 1 hour co incubation with THP-1 cells. % adhesion of control. One way ANOVA: no significant effects.

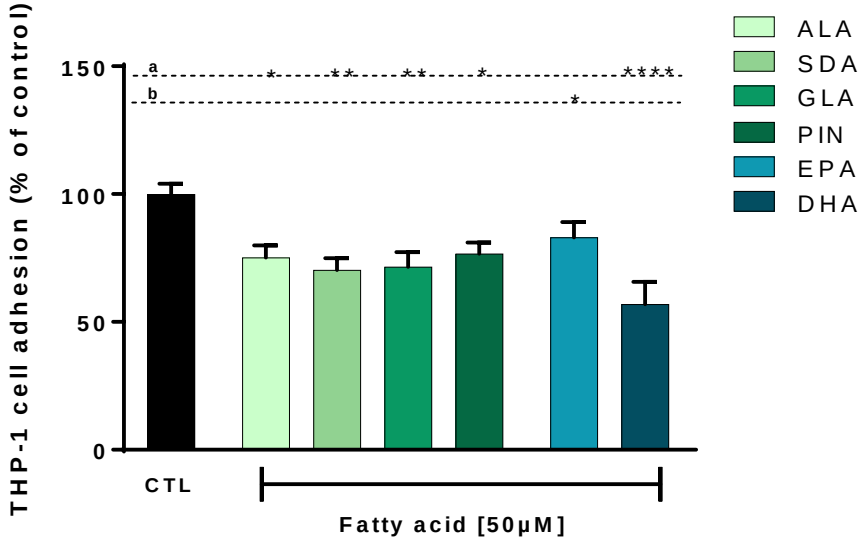

Figure 7.4 Effect of FA exposure (50 µM) on adhesion of THP-1 cells to TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) adhesion of THP-1 cells to activated EA.hy926 cells without prior incubation with FA (control; CTL) or with 48 hours prior exposure to ALA, SDA, GLA, PIN, EPA and DHA (50 µM) followed by 6 hour TNFα (1 ng/mL) and 1 hour co incubation with THP-1 cells. % adhesion of control. One way ANOVA a: control vs FA; b: FA vs DHA; *p<0.05, **p<0.01, ****p<0.0001

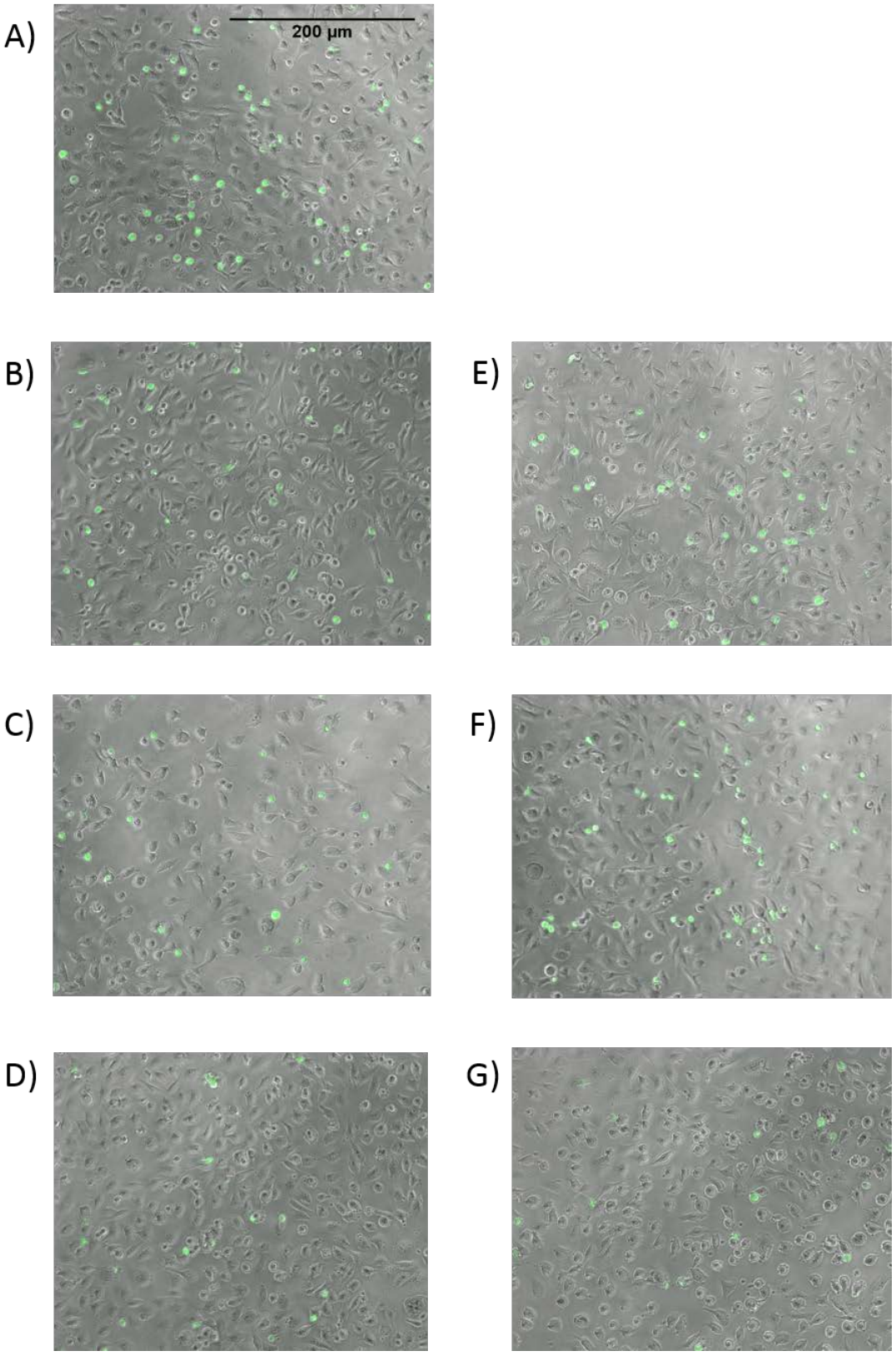

Figure 7.5 Visualisation of adhesion of calcein labelled THP-1 cells to TNFα stimulated EA.hy926 after FA exposure at 50 µM

Adhesion of THP-1 cells to activated EA.hy926 cells without prior incubation with FA (control; CTL (A)) or with 48 hours prior exposure to ALA (B), SDA (C), GLA (D), PIN (E), EPA (F) and DHA (G) (50 µM) followed by 6 hour TNFα (1 ng/mL) and 1 hour co incubation with calcein labelled THP-1 cells. Attached THP-1 cells were visualised by fluorescence microscope Nikon Elipse Ti at a magnitude of 10x under transmitted light.

Adhesion of calcein labelled THP-1 cells to EA.hy926 was shown to significantly increase with TNFα stimulation of the ECs (Figure 7.2).

Treatment of EA.hy926 cells with any FA at 25 μM did not significantly affect adhesion of THP-1 cells to the ECs (Figure 7.3).

When used at 50 μM, DHA had the most potent effect on THP-1 cell adhesion to EC monolayers, inhibiting this by ~40% ($p < 0.0001$; Figure 7.4). In contrast, EPA treatment at this concentration had no effect on adhesion (Figure 7.4). All plant-derived FAs reduced adhesion of THP-1 cells when used at 50 μM (Figure 7.4). ALA and PIN reduced adhesion of THP-1 cells by ~24% and ~23% respectively (both $p<0.05$). SDA and GLA reduced adhesion by ~30% and ~29% respectively (both $p<0.01$) compared to stimulated control cells.

Comparison of the effect of the different FAs on THP-1 cell adhesion when used at 50 μM showed ECs treated with EPA to have significantly higher levels of adhesion of THP-1 monocytes than ECs treated with DHA (Figure 7.4). No other significant differences among the different FAs used were observed. These observations again indicate that DHA and EPA may regulate the inflammatory process through different pathways. These findings also highlight the possible anti-inflammatory effects of the plant-derived FAs suggesting them as potential sustainable alternatives to marine n-3 FAs.

7.4 Discussion

Expression of adhesion markers by ECs is vital for EC-blood cell interaction. The expression of these adhesion markers allows for the attachment and movement of leukocytes across the endothelial monolayer into sites of inflammation. Dysfunction of these processes may lead to a chronic inflammatory state and eventual development of atherosclerotic lesions. ICAM-1 plays a critical role in the adhesion process. It has been shown to facilitate the transmigration of leukocytes across the endothelium [202]. Equally it has been described that EPA and DHA can help to dampen EC inflammation through the reduction of ICAM-1 surface expression on EC [110, 112, 150, 164, 172].

Several of the FAs examined within this model modulated ICAM-1 secretion and cell surface expression (Chapter 4). In order to examine how these changes affected the functionality of the cells a static adhesion model involving THP-1 monocytes was developed.

The objective to establish a static cell adhesion model with EA.hy926 ECs and THP-1 monocytes was successfully met. This model was then used to examine the effects of EC treatment with marine and plant-derived FAs on THP-1 binding.

None of the FAs at a concentration of 25 μM significantly affected the adhesion of THP-1 cells to the EA.hy926 monolayer. However after treatment with FAs at concentration of 50 μM all FAs, except EPA, significantly decreased the percentage of adhesion compared to control.

Individual effects of DHA and EPA on monocyte adhesion to ECs have been reported previously. De Caterina *et al*. reported that pre-treatment of HSVECs with DHA (10 μM) significantly reduced the adhesion of monocytes [110]. Yamada *et al*. reported significant reduction of rolling and adhesion of monocytes to LPS stimulated HUVEC after EPA exposure (50 μM) [203].

Some studies have compared effects of EC treatment with EPA and DHA. Similar to the observations made here, Wang *et al*. demonstrated reduced adhesion of THP-1 cells to TNFα stimulated HAECs after DHA exposure (80 μM), with no change in monocyte adhesion after EPA exposure [112]. These effects were also observed by Yates *et al.* under flow conditions. They describe significant reduction of adhesion of neutrophils to TNFα stimulated HUVECs after DHA treatment (5 μM) with no effects of EPA treatment (5 μM) [114]. They suggest DHA and EPA modulate different stages of leukocyte recruitment.

However, some others report decreased adhesion after treatment of ECs with both EPA and DHA. Huang *et al*. reported significant inhibition of THP-1 monocyte adhesion to LPS-activated HAECs after treatment with both EPA and DHA at 100 μM [164]. This was also reported for monocyte adhesion to oxLDL stimulated human coronary artery endothelial cells (HCAEC) where pre-treatment with both EPA and DHA (10 or 50 μM) lead to significant reduction in adhesion [172]. Mayer *et al*. too described significant reduction of rolling and adhesion of monocytes to HUVECs under laminar flow conditions after EPA and DHA treatment (10 μM) [173]. Thus an inhibitory effect of DHA treatment of ECs on adhesion of monocytes is consistently reported in the literature and is confirmed here. However the effects of EPA are not consistently observed. It is unclear why this is. Concentration does not appear to be a factor to explain the inconsistent results with EPA.

Treatment with ALA at 50 μM was shown to decrease the adhesion of THP-1 monocytes to stimulated EA.hy926 cells. Similar observations were made by Zhang *et al*. who reported ALA exposure at 50 μM inhibited neutrophil adhesion in HUVECs exposed to high glucose [168]. However others who have looked at the effects of ALA exposure at various concentrations (25, 50, 100 and 200 μM) report no change in THP-1 monocyte adhesion in LPS-stimulated HUVECs [167]. Although ALA has not been shown to modulate many of the inflammatory markers examined within this model it is possible these actions of decreased adhesion are the result of effects of ALA elsewhere in the inflammatory signalling pathway. No previous research has investigated the effects of treatment with SDA, GLA or PIN on the adhesion of monocytes to human ECs.

Reduction of adhesion after FA treatment (50 μM) may be due to interference with ICAM-1 expression in EA.hy926 cells. In Chapter 4, SDA, EPA and DHA treatment (50 μM) was shown to decrease ICAM-1 cell surface expression in EA.hy926 cells (Figure 4.10) and both pre-treatment with GLA and PIN (50 μM) also reduced ICAM-1 supernatant levels (Figure 4.3). Since ICAM-1 plays an essential role in the adhesion of monocytes to the endothelial monolayer [202], a reduction of ICAM-1 expression in EA.hy926 cells by plant FAs should inhibit THP-1 adhesion.

Figure 7.6 depicts the correlation between % adhesion of THP-1 cells and % cell surface expression of ICAM-1.

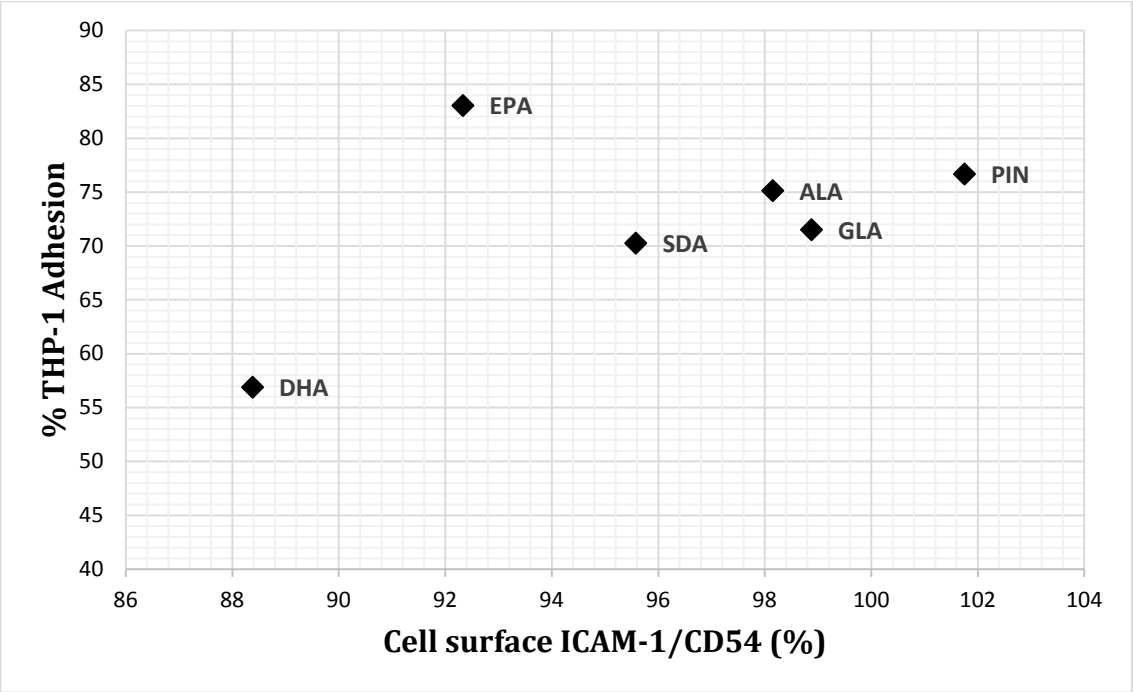

Figure 7.6 % THP-1 adhesion *vs* cell surface expression of ICAM-1 (CD34) %.

Adhesion of THP-1 cells to activated EA.hy926 cells with 48 hours prior exposure to ALA, SDA, GLA, PIN, EPA and DHA (50 μM) followed by 6 hour TNFα (1 ng/mL) and 1 hour co incubation with THP-1 cells. % adhesion of control *vs* % of control (activated EA.hy926) cell surface expression of ICAM-1 (CD54) after 48 hour exposure to SDA, EPA and DHA (25 and 50μM) followed by 6 hour TNFα (1ng/mL).

This suggests a link between effects of FAs at 50 μM on adhesion of THP-1 cells to ECs and on ICAM-1 surface expression on ECs with the exception of EPA (Figure 7.6). EPA significantly reduced ICAM-1 surface expression in EA.hy926 cells compared to stimulated control (Figure 4.10), but adhesion of THP-1 cells was unaffected by EPA (Figure 7.4). This is consistent with different modes of action of DHA and EPA in modulation of leukocyte recruitment, as suggested by Yates *et al*. [114]. It is also possible that adhesion molecules other than ICAM-1 become more important in this adhesion model. Blocking ICAM-1 using anti-ICAM-1 monoclonal antibodies (mAbs) only reduced adhesion of monocytes to stimulated ECs by 30% [204] suggesting the involvement of other contributing factors and adhesion molecules. However, the evidence obtained with the current model suggests ICAM-1 is very important in adhesion of monocytes to EA.hy926 cells.

The mechanism of how DHA reduces adhesion may involve PPARα. In Chapter 5 it was shown that treatment with DHA increased PPARα gene expression. PPARα has been shown to interfere with early stages of atherosclerosis through reduction of

leukocyte adhesion to activated ECs [205]. These effects may be mediated in part by reduction of ICAM-1: Naidenow *et al*. describe PPARα agonists to significantly induce ICAM-1 surface, intracellular protein and mRNA expression in non-stimulated ECs [206]. DHA itself is a PPARα agonist [207].

7.4.1 Conclusions

The findings are summarised in Table 7.2.

Table 7.2 Summary of effects of FA treatment on adhesion of THP-1 monocytes to stimulated EA.hy926 cells

	Fatty acid						**Comments**
	ALA	SDA	GLA	PIN	EPA	DHA	
THP-1 adhesion	↓	↓↓	↓↓	↓	↔	↓↓↓	Effect was dose dependant

Overall these results support the current literature with regard to the marine-derived FAs: DHA has a strong inhibitory effect on adhesion in this model which confirms the observations of others [110, 112, 114, 164, 172, 173] while EPA is without effect when used at 25 or 50 µM. The effect of higher concentrations of EPA are not known in this model. The new data generated here with plant FAs indicates the potential for plant-derived FAs as sustainable alternatives to marine n-3 FAs to influence inflammation and atherosclerosis, since all of these FAs at 50 µM lead to reduced monocyte adhesion.

Chapter 8: Using silencing of elongase 5 to examine whether gamma-linolenic and pinolenic acids retain their anti-inflammatory effects in cultured endothelial cells

8.1 Introduction

There is an increasing need for suitable alternative FAs for the benefits of human health, since stocks of oily fish, the richest source of EPA and DHA, are rapidly declining. GLA is derived from a variety of different plant sources including; evening primrose oil and borage oil. Older studies have reported some beneficial effects of GLA supplementation on progression of atherosclerosis in animal models [134, 208]; however there are fewer modern studies on GLA and inflammation and cardiovascular outcomes. PIN which is found in pine nuts is more unusual, and no studies report its effect on atherosclerosis; however some describe anti-inflammatory effects in rats [209, 210]. Both of these FAs have been underexplored, yet they may hold the potential to help to fill the void of decreasing abundance of EPA and DHA.

GLA and PIN have been shown to have anti-inflammatory effects in cultured human ECs (EA.hy926 cells) decreasing production of several inflammatory mediators by ECs (Chapter 4) and decreasing monocyte adhesion to ECs (Chapter 7). EA.hy926 cells metabolised GLA and PIN to their elongation products DGLA and ETrA, respectively (Chapter 3), leading to significant increases in cellular DGLA and ETrA after incubation with their precursor FA (Figure 3.12 and Figure 3.13). Thus it is possible that the identified effects of GLA and PIN are due to their elongation products. The work described in this chapter aims to better understand how GLA and PIN exert their anti-inflammatory effects in this model.

As described previously, FAs are metabolised through a series of elongation and desaturation steps (section 1.1.2). Elongase 5, encoded by the ELOVL5 gene, participates in the elongation of monounsaturated and polyunsaturated FAs of 18-20 carbons in length. Elongase 5 catalyses the first and rate-limiting reaction of the long-chain FA elongation cycle, and acts specifically toward polyunsaturated acyl-CoA with higher activity toward 18:3n-6 acyl-CoA compared to other FAs of 18-20 carbons in length [211]. GLA is elongated to DGLA by elongase5 [212]. PIN has been observed to be elongated to ETrA [177, 178], as also observed here in EA.hy926 cells (Chapter 3); however, the enzyme responsible for elongation of PIN has not been described. Similarities in the structure of GLA and PIN suggest that they will be elongated by the same enzyme.

Increases in the amount of DGLA and ETrA in cells leads to an increase in alternative substrate for the synthesis of eicosanoids which may have anti-inflammatory effects. For example, DGLA is a substrate for COX-2 [213], and is

metabolised to PGE_1, which has been shown to suppress inflammation [134, 214, 215]. DGLA is also a substrate for 5-LOX. ETrA may also act via COX and LOX metabolism [212]. It is also possible that both the precursor FAs (i.e. GLA and PIN) and the elongation products (i.e. DGLA and ETrA) act simultaneously to decrease or resolve inflammation.

To attempt to separate the effects of the precursor FAs from their elongation products, silencing of ELOVL5 using siRNA was used.

8.2 Aim and objectives

The aim of the research described in this chapter was to establish the elongation pathway of PIN and to compare the effects of GLA and PIN on inflammatory responses in EA.hy926 cells with or without elongase 5 silencing.

The specific objectives were to:

- Silence elongase 5 in EA.hy926 cells using siRNA.
- Quantify relative ELOVL5 gene expression after ELOVL5 silencing using RT-qPCR.
- Compare quantities of elongation products in cells treated with GLA or PIN with or without ELOVL5 silencing.
- Compare the effects of plant-derived FAs (GLA and PIN) on production of various inflammatory mediators (ICAM-1, MCP-1, VEGF, IL-6, IL-8, RANTES) by cultured ECs with or without ELOVL5 silencing.

8.3 Methods

8.3.1 Reagents

Reagents and materials used for experiments are described in Appendix A.

8.3.2 siRNA mediated inhibition of ELOVL5

Silencing of ELOVL5 in EA.hy926 was achieved by siRNA mediated inhibition of ELOVL5 using Santa Cruz reagents and protocol.

8.3.2.1 Principle of siRNA

RNA interference (RNAi) exercises two forms of post-transcriptional control. First RNAi can inhibit the translation of target mRNA and second RNAi can direct target mRNA destruction though the RISC complex (RNA-induced silencing complex). Figure 8.1 depicts the siRNA principle.

DICER (endoribonuclease Dicer), an enzyme which cleaves double-stranded RNA (dsRNA), first processes dsRNA leaving a two nucleotide long 3" overhang. This primes the dsRNA for binding to the RISC complex and leads to activation of the enzyme activity of argonaute, the RNAse component of the RISC complex that destroys one of the RNA strands; the remaining guide strand, through complementary binding, then leads the RISC complex to associate with and cleave the target RNA molecules.

Transfer of genetic material into the cell takes place via liposomes, which merge with the phospholipid membrane releasing the siRNA into the cell. siRNA enters the cell via lipid-based transfection, siRNA binds RISC, siRNA strands are separated, siRNA/RISC complex associates with the target mRNA and cleaves it.

Transfection of siRNA gene silencers into cultured cells provides an efficient short-term decrease in target gene expression.

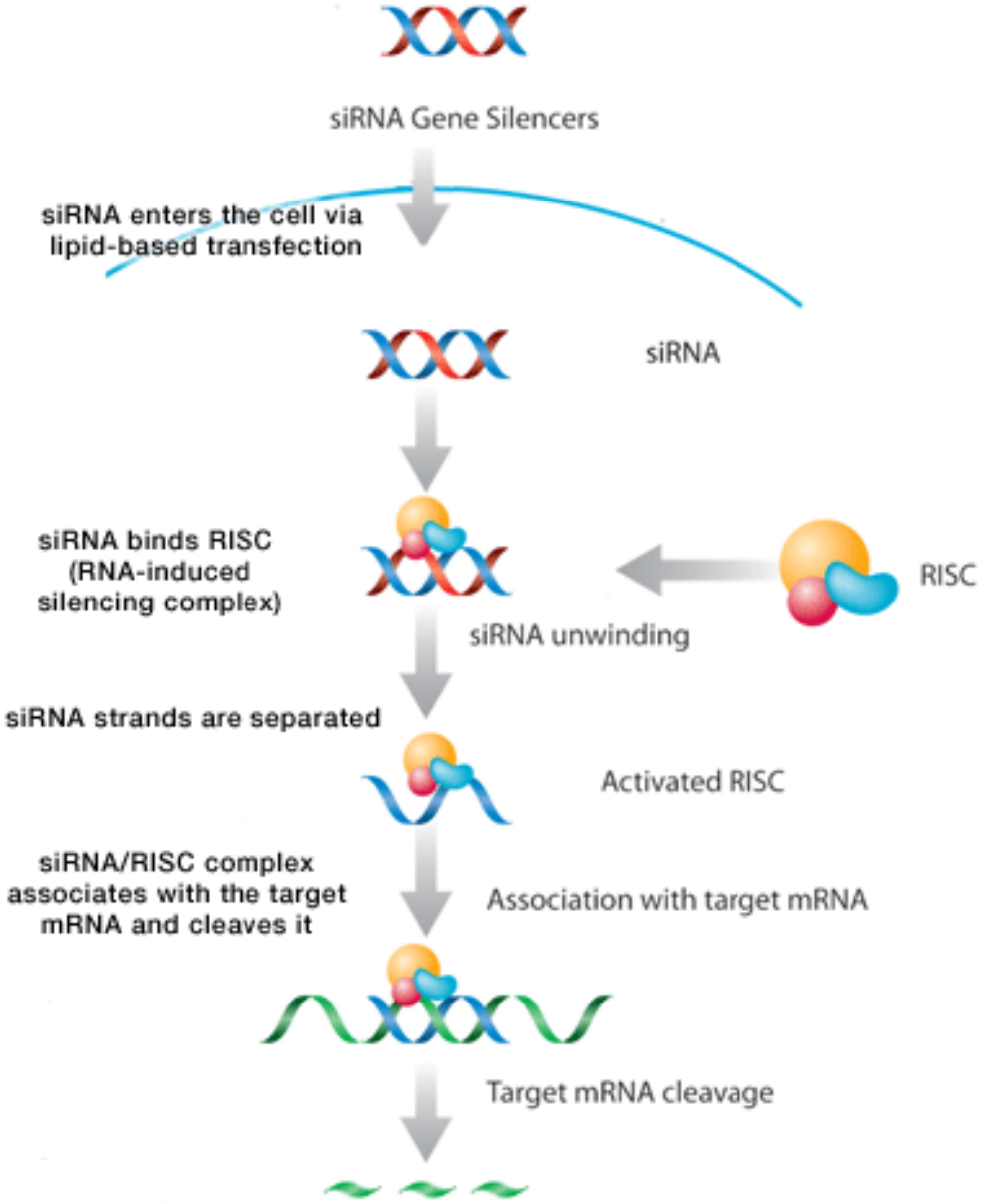

Figure 8.1 Schematic of siRNA silencing protocol (Santa Cruz).

8.3.2.2 Protocol for elongase 5 silencing

The Santa Cruz Biotechnology Inc. protocol was followed. Confluent EA.hy926 cell cultures were scraped from flasks and resuspended in antibiotic-free culture medium at a density of 6.5 x 10^5 cells per mL. Cells were seeded at 6.5 x 10^4 cells per well in 6-well flat bottom plates and incubated for 24 hours at 37°C.

Following incubation, supernatant was removed and cells were washed with 2 mL DMEM (unsupplemented). siRNA transfection reagent mixture (1 mL), containing ELOVL5 siRNA or control siRNA and siRNA transfection reagent (all Santa Cruz

Biotechnology Inc.) or supplemented DMEM was added to each well. Plates were then incubated for 6 hours at 37°C. Finally medium was removed and the cells used for different purposes.

8.3.3 Analysis of gene expression using RT-qPCR

The effect of ELVOL5 silencing on ELOVL5 gene expression in EA.hy926 cells was analysed by RT-qPCR. Following siRNA-mediated inhibition of ELOVL5, confluent cultures were incubated with either culture medium with supplements (control) or culture medium with supplements containing FAs (GLA or PIN) at a concentration of 50 μM. Cells were incubated for 48 hours at 37°C.

8.3.3.1 Isolation of RNA

Total cellular RNA was extracted from cells using ReliaPrep™ RNA Cell Miniprep System as described previously in section 2.3.4.1.

8.3.3.2 RNA analysis.

Isolated RNA quality and quantity was assessed by NanoDrop analysis (section 2.3.4.2.1 and section 2.3.4.2.2) and the Agilent bioanalyzer (section 2.3.4.2.3).

8.3.3.3 cDNA synthesis

GoScript™ Reverse Transcriptase was used to synthesise cDNA from total RNA as described in section 2.3.4.3.

8.3.3.4 RT-qPCR

The expression of ELOVL5, and the control genes GAPDH, RPL13A and B2M was assessed by qRT-PCR with TaqMan® gene expression primers (see Appendix A for primer details). The procedure was as described in section 2.3.4.4. Briefly cDNA was diluted to 5 ng/μL and 4 μL added to 16 μL of reaction mix (prepared according to the manufacturer's specifications) per well in a 96-well plate. Plates were then sealed and loaded into the thermocycler for the specific cycles according to the manufacturer's instructions

8.3.4 Analysis of cellular fatty acid composition

Following siRNA mediated inhibition of ELOVL5, confluent cultures were incubated with either culture medium with supplements (control) or culture

medium with supplements containing FAs (GLA or PIN) at a concentration of 50 μM. Cells were incubated for 48 hours at 37°C. FA composition of the cells was determined by GC using the protocol described previously in section 3.3.3.3.1. Briefly, after FA incubation the cells were scraped from the plate, collected by centrifugation at 1200 rpm for 5 minutes, and resuspended in 0.9% NaCl at a volume to achieve 1.25 x 10 cells/mL.

8.3.4.1 Gas chromatography analysis

Total lipid was extracted from cell pellets as described in section 3.3.4.1. FAMEs were prepared (section 3.3.4.1) and analysed in using Hewlett-Packard 6890 gas chromatograph (section 3.3.4.2).

8.3.5 Production of inflammatory mediators

Following siRNA mediated inhibition of ELOVL5, cells were incubated for 48 hours at 37°C with GLA or PIN at a concentration of 50 μM followed by 24 hour incubation with either DMEM or TNFα at 1 ng/mL. Supernatant was removed and stored at -20°C until analysis. Concentrations of cytokines and adhesion molecules in EC culture supernatants were determined simultaneously using Human Magnetic Luminex Screening Assay kits by R&D using the assay described in section 2.4.4.1.

8.3.6 Statistical analysis

Relative gene expression data were calculated using double delta Ct (ΔΔCt) values expressed as fold change of the target gene in test samples relative to control samples. Initially the geometric mean of the ΔCt for each housekeeping is calculated for each sample, and this mean is used to calculate the relative expression (see section 2.3.5).

Supernatant concentrations of cytokines and inflammatory mediators were calculated using the Luminex Bio Plex manager 6 (2.3.3). FAME histograms were analysed with Agilent ChemStation software (section 3.3.4) and FA concentrations calculated as μg of FA/5x10^5 cells.

Data are expressed as mean ± standard error; data analysis was performed in PRISM and Excel. Multiple group differences were compared using one way ANOVA.

8.4 Results

Previously it was shown that incubation of EA.hy926 cells with GLA or PIN lead to significant increases in the elongation metabolites DGLA and ETrA, respectively (Figure 3.12 and Figure 3.13). Treatment with these FAs also lead to decreases in production of inflammatory mediators by stimulated EA.hy926 cells (Figure 4.3 - Figure 4.8). These effects may be due to the increase in the elongation metabolites rather than to GLA and PIN themselves. In order to examine how these FAs affect the production of inflammatory mediators, EA.hy926 cells were exposed to ELOVL5 siRNA prior to further assays; including RT-qPCR analysis, GC analysis, and stimulation and inflammatory mediator production analysis. The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30.

ELOVL5 siRNA significantly decreased relative gene activity of ELOVL5 in EA.hy926 cells (Figure 8.3). Furthermore this decrease in gene activity lead to a significant decrease the production of elongation products by EA.hy926 cells incubated with GLA and PIN, with both production of DGLA and ETrA being significantly decreased (Figure 8.4 and Figure 8.5). These observations provide good evidence for the role of elongase 5 in the metabolism of both GLA and PIN in ECs.

GLA and PIN significantly decreased production of the majority of the inflammatory mediators within this model. Silencing of ELOVL5 in EA.hy926 cells incubated with GLA and PIN lead to the loss of most of these anti-inflammatory effects (Figure 8.6 - Figure 8.11).

8.4.1 RNA integrity

Total cellular RNA isolation was carried out using ReliaPrep™ RNA Cell Miniprep columns (section 2.3.4.1).

Analysis of RNA using Agilent Bioanlyzer (RNA Total Eukaryote 2100 Nano) was performed by Melissa Doherty, Laboratory manager (section 2.3.4.2.2). RNA quantity and quality were also assessed using the NanoDrop (section 2.3.4.2.1).

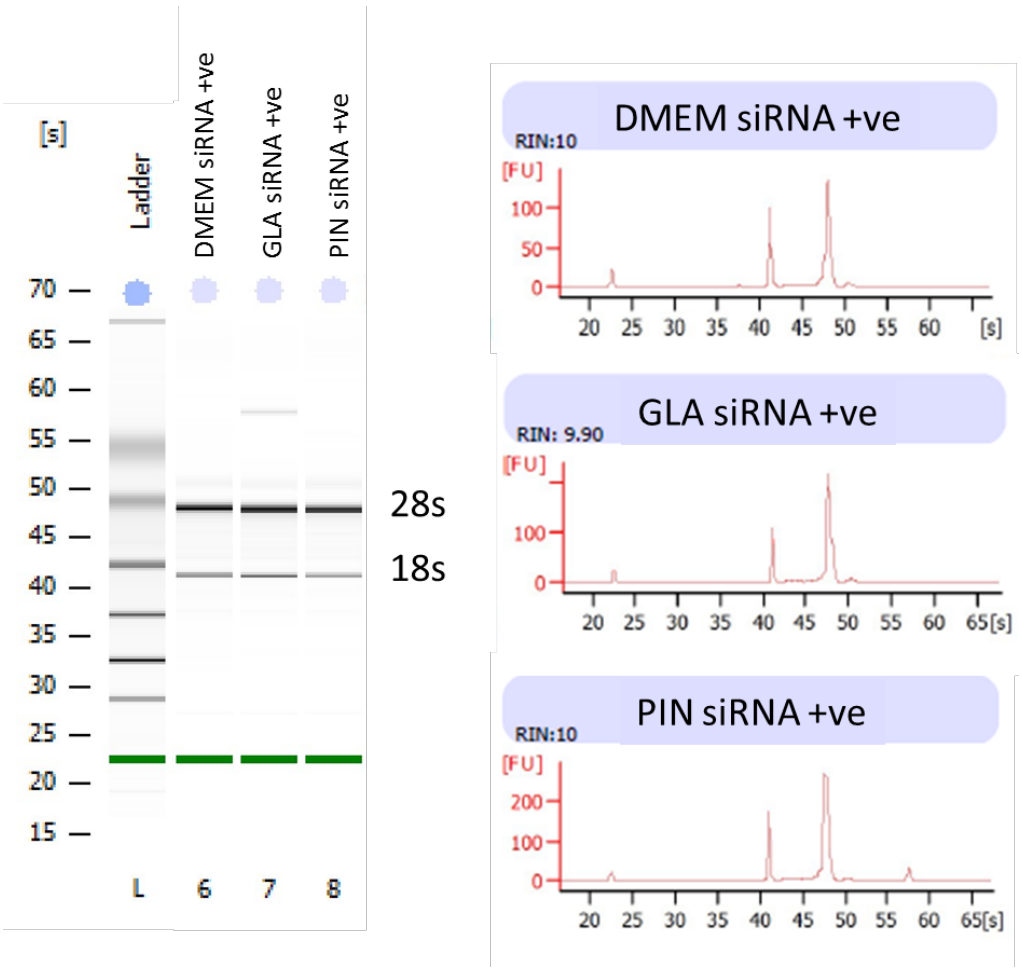

Figure 8.2 Aglient Bioanalyzer RNA integrity results.

Bioanalyzer profiles of RNA extracted from activated EA.hy926 (control) or 48 hour exposure to GLA and PIN (50μM) with ELOVL5 siRNA. RIN scores were measured by Aglient bioanalyzer

Table 8.1 NanoDrop RNA analysis results

Samples	ng/μL	A260	A280	260/280	260/230
DMEM siRNA +ve	225.9	5.648	2.65	2.13	2.09
GLA siRNA +ve	193.41	4.835	2.3	2.1	2.03
pin siRNA +ve	222.4	5.56	2.639	2.11	2.1

8.4.2 siRNA silencing of ELOVL5

Incubation of EA.hy926 cells with ELOVL5 siRNA was shown to significantly decrease relative gene expression of ELOVL5 compared to non-siRNA treated cells ($p<0.001$). ELOVL5 relative gene expression was decreased by ~90% after ELOVL5 siRNA exposure across all conditions; control (DMEM alone) (-93%), GLA (50 μM) (-87%) and PIN (50 μM) (-88%) (Figure 8.3). Control siRNA resulted in unaltered

relative gene expression in EA.hy926 cells under all conditions (DMEM, GLA and PIN) indicating the specificity of the ELOVL5 siRNA treatment.

8.4.2.1 RT-qPCR

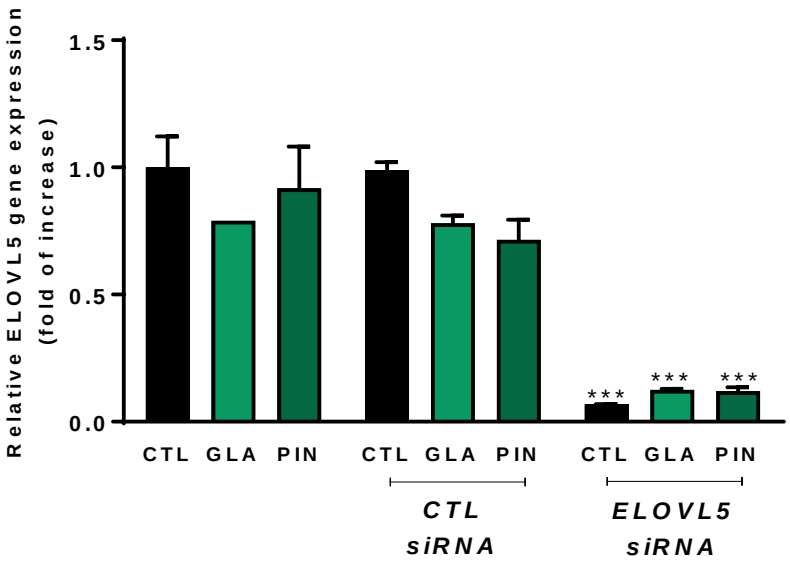

Figure 8.3 Effect of ELOVL5 siRNA on ELOVL5 gene expression in EA.hy926 cells

Mean (±SEM) (n = 3) gene expression of ELOVL5 in EA.hy926 (control (CTL)) or 48 hour exposure to GLA or PIN (50μM), with or without exposure to control siRNA (CTL siRNA) or ELOVL5 siRNA. Ct values for ELOVL5 gene expression were normalised to RPL13A, B2M and GAPDH. One way ANOVA: control treatments vs CTL siRNA and ELOVL5 siRNA; ***p<0.001

8.4.3 Fatty acid composition of EA.hy926 cells incubated with FA and with ELOVL5 silencing

The effects of GLA and PIN on FA composition of Hy.926 cells were near identical to the previous results in Chapter 3. After 48 hour FA treatment, FAs were successfully incorporated by EA.hy926 cells, with the appearance of specific metabolic elongation products depending on FA. GLA incubation of non siRNA treated cells (50 μM) lead to a significant increase in DGLA ($p<0.001$) (Figure 8.4); similarly after PIN treatment (50 μM) there was a significant increase in ETrA ($p<0.001$) (Figure 8.5).

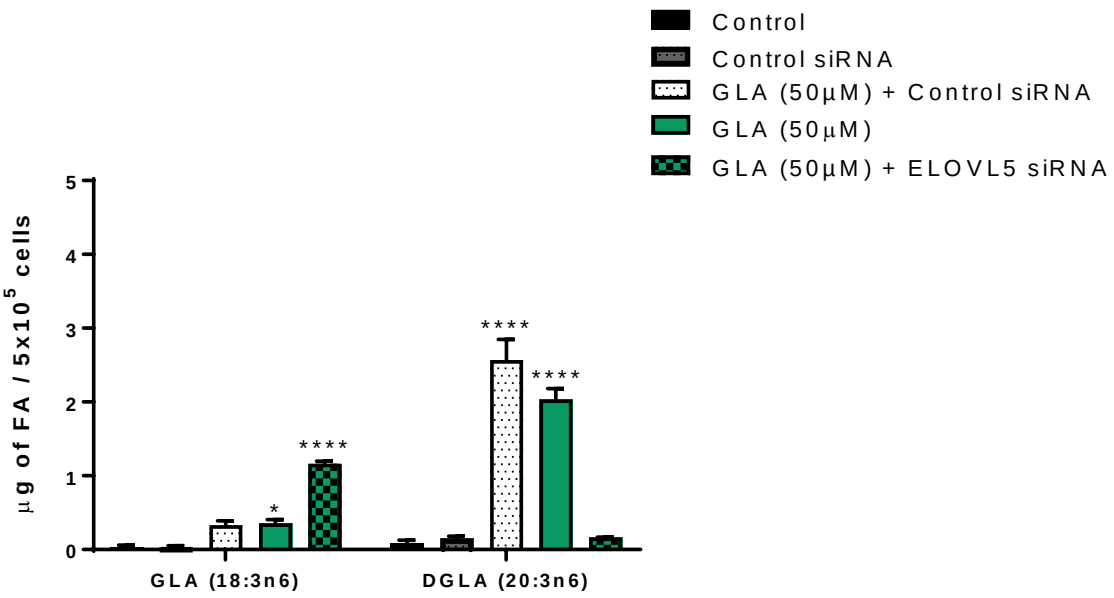

Figure 8.4 Incorporation of GLA and appearance of an elongation product (DGLA) after incubation of EA.hy926 cells with GLA with or without ELOVL5 siRNA for 48 hours

Mean (±SEM) (n = 3) µg of FA/10^6 cells compared to control (EA.hy926 +DMEM) after 48 hour exposure to GLA (50µM) with or without ELOVL siRNA pre-treatment. Two way ANOVA; control vs FA, *p<0.05, ****p<0.0001

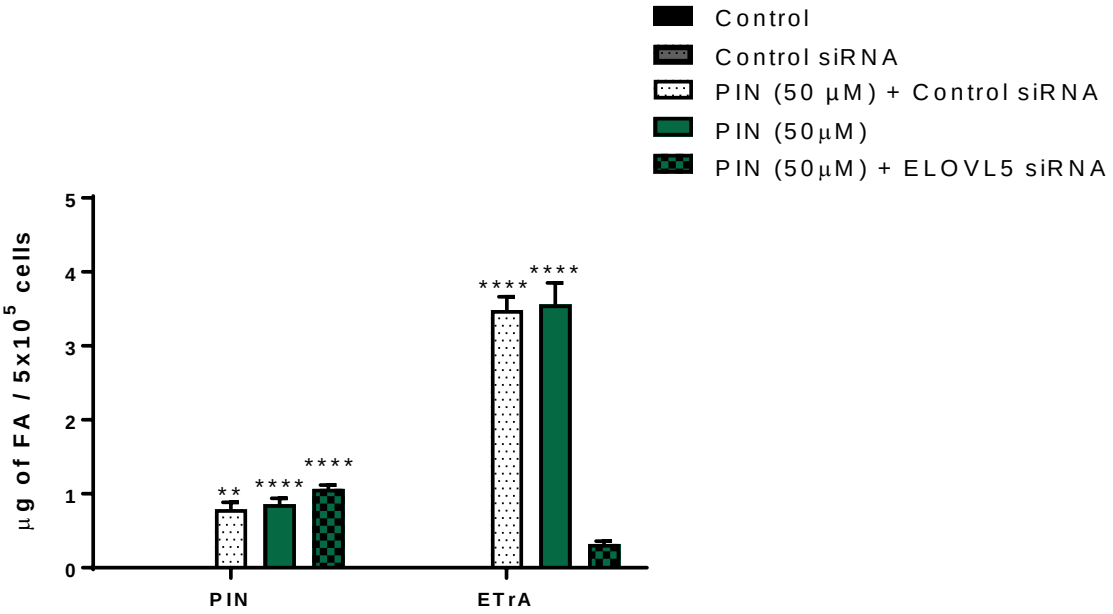

Figure 8.5 Incorporation of PIN and appearance of an elongation product (ETrA) after incubation of EA.hy926 cells with PIN with or without ELOVL5 siRNA for 48 hours

Mean (±SEM) (n = 3) µg of FA/10^6 cells compared to control (EA.hy926 +DMEM) after 48 hour exposure to PIN (50µM) with or without ELOVL siRNA pre-treatment. Two way ANOVA; control vs FA, *p<0.05, ****p<0.0001

Following ELOVL5 siRNA treatment of EA.hy926 cells, incubation with GLA or PIN no longer lead to the appearance of DGLA and ETrA (Figure 8.4 and Figure 8.5). Indicating a decrease in FA elongation. Thus the reduced ELOVL5 gene activity, as seen in the RT-qPCR results (Figure 8.3), likely lead to a decrease in elongase 5 protein, and ultimately the inability of EA.hy926 to elongate GLA or PIN. Control siRNA did not alter the ability of EA.hy926 cells to metabolise precursor FAs to their elongation products.

ELOVL5 silencing in GLA treated cells lead to a build-up of GLA in the cells (Figure 8.4), however this was not seen in PIN treated silenced cells (Figure 8.5).

The changes in composition after treatment with GLA and PIN in siRNA ELOVL5 silenced cells will enable the further examination of whether these FAs are acting through their elongation products.

8.4.4 Inflammatory mediators

In order to investigate the possible effect of DGLA and ETrA on the production of inflammatory mediators, ELOVL5 siRNA silenced EA.hy926 cells were exposed to GLA or PIN at concentrations of 50 μM for 48 hours followed by 24 hour TNFα stimulation at 1 ng/mL. Concentrations of mediators in the culture medium after 24 hours were evaluated by Luminex assay. The data shown are from 3 separate experiments each conducted in triplicate.

TNFα treatment was previously shown to increase production of each of the analytes examined (

Figure 2.8 - Figure 2.13).

GLA and PIN had differential effects depending on metabolite examined. ELOVL5 silencing inhibited the majority of these effects, but this was not seen for production of MCP-1 after GLA treatment in stimulated EA.hy926 cells.

GLA treatment lead to the significant decrease in 5 of the 6 analytes examined in this model, with PIN treatment decreasing the production of 4 of the 6.

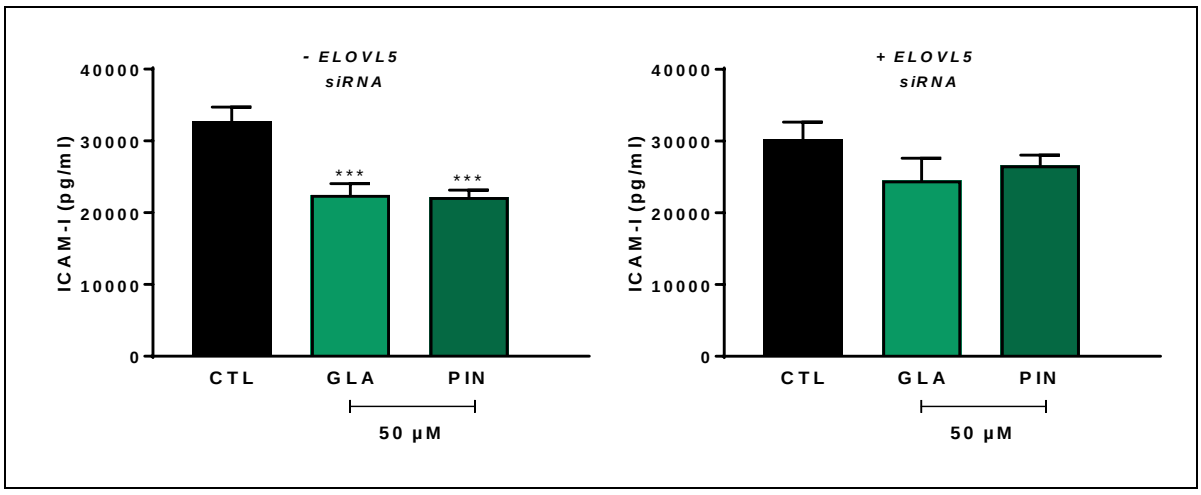

Figure 8.6 Effect of FA exposure on ICAM-1 production by TNFα stimulated EA.hy926 cells, with or without ELOVL5 siRNA

Mean (±SEM) (n = 3) production of ICAM-1by TNFα activated EA.hy926 cells without incubation with FA (control; CTL) or with 48 hours prior exposure to GLA and PIN (50μM) followed by 24 hour TNFα (1ng/mL). EA.hy926 were prior incubated without (-) or with (+) ELOVL5 siRNA. ICAM-1 was measured in the supernatant of endothelial cell cultures. One way ANOVA control vs FA, ***p<0.001.

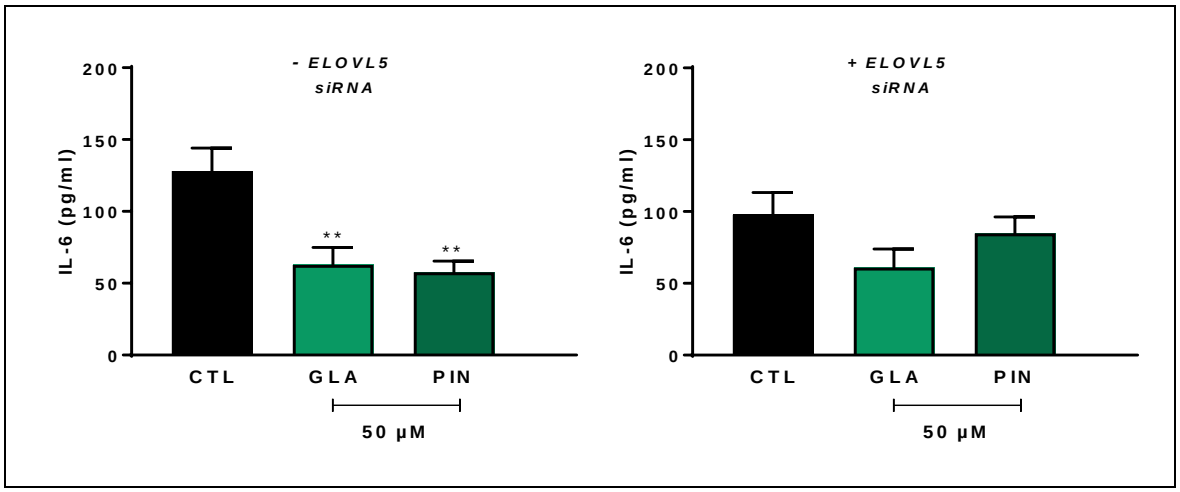

Figure 8.7 Effect of FA exposure on IL-6 production by TNFα stimulated EA.hy926 cells, with or without ELOVL5 siRNA

Mean (±SEM) (n = 3) production of IL-6 by TNFα activated EA.hy926 cells without incubation with FA (control; CTL) or with 48 hours prior exposure to GLA and PIN (50µM) followed by 24 hour TNFα (1ng/mL). EA.hy926 were prior incubation without (-) or with (+) ELOVL5 siRNA. IL-6 was measured in the supernatant of endothelial cell cultures. One way ANOVA control vs FA, **p<0.01

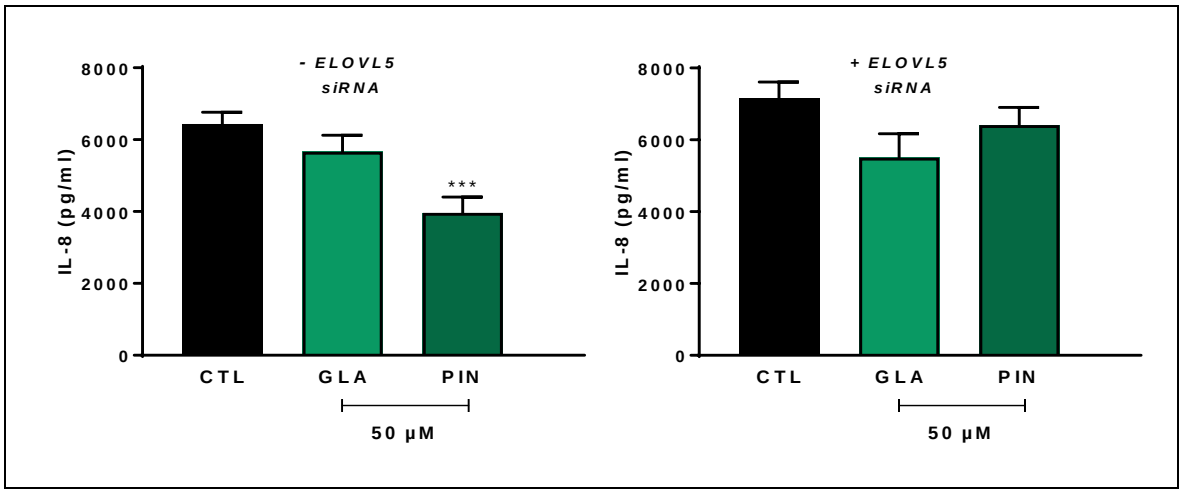

Figure 8.8 Effect of FA exposure on IL-8 production by TNFα stimulated EA.hy926 cells, with or without ELOVL5 siRNA

Mean (±SEM) (n = 3) production of IL-8 by TNFα activated EA.hy926 cells without incubation with FA (control; CTL) or with 48 hours prior exposure to GLA and PIN (50µM) followed by 24 hour TNFα (1ng/mL).

EA.hy926 were prior incubation without (-) or with (+) ELOVL5 siRNA. IL-8 was measured in the supernatant of endothelial cell cultures. One way ANOVA control vs FA, ***p<0.001.

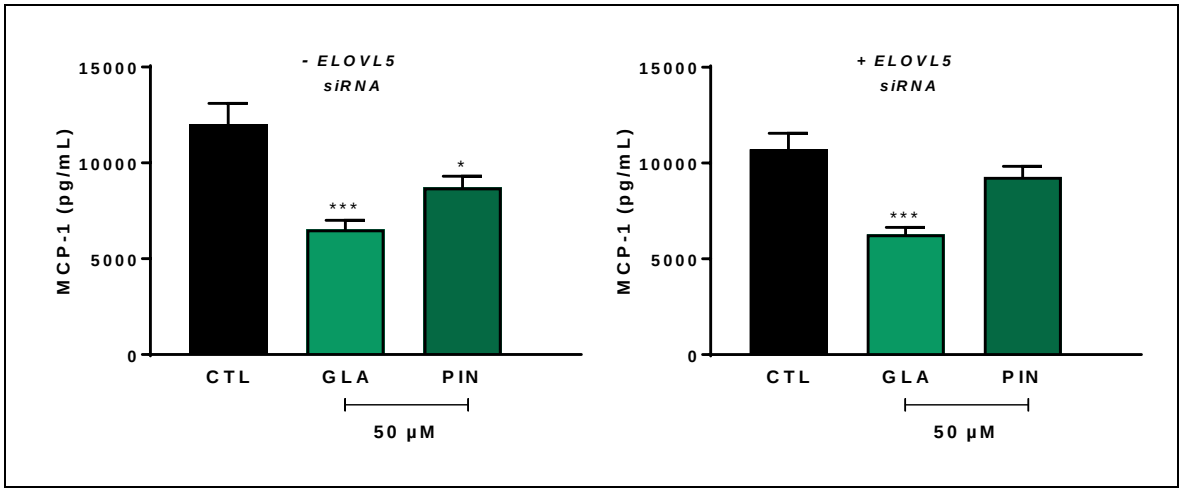

Figure 8.9 Effect of FA exposure on MCP-1 production by TNFα stimulated EA.hy926 cells, with or without ELOVL5 siRNA

Mean (±SEM) (n = 3) production of MCP-1 by TNFα activated EA.hy926 cells without incubation with FA (control; CTL) or with 48 hours prior exposure to GLA and PIN (50µM) followed by 24 hour TNFα (1ng/mL). EA.hy926 were prior incubation without (-) or with (+) ELOVL5 siRNA. MCP-1 was measured in the supernatant of endothelial cell cultures. One way ANOVA control vs FA, *<p.0.05, ***p<0.001.

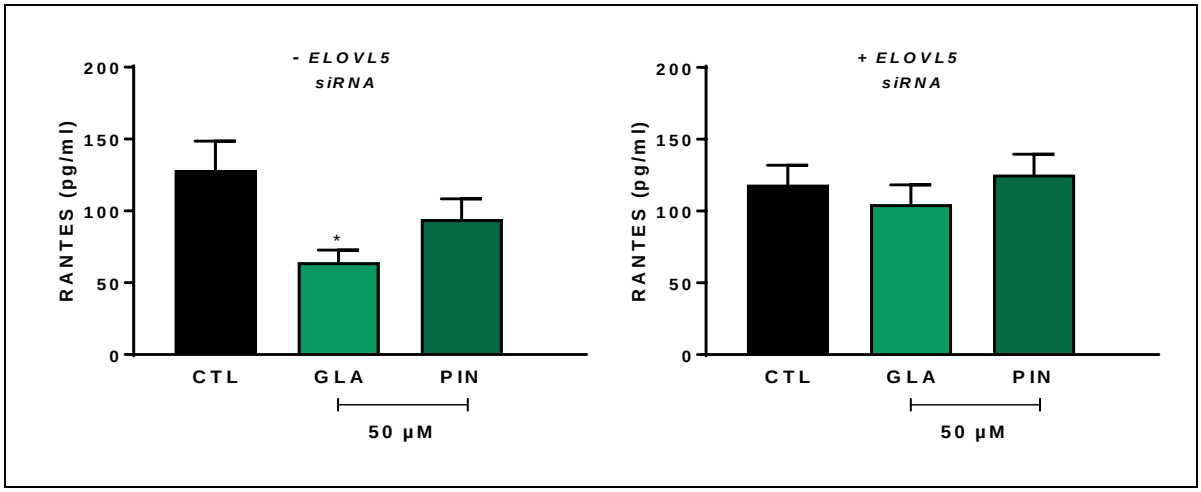

Figure 8.10 Effect of FA exposure on RANTES production by TNFα stimulated EA.hy926 cells, with or without ELOVL5 siRNA

Mean (±SEM) (n = 3) production of RANTES by TNFα activated EA.hy926 cells without incubation with FA (control; CTL) or with 48 hours prior exposure to GLA and PIN (50µM) followed by 24 hour TNFα (1ng/mL).

EA.hy926 were prior incubation without (-) or with (+) ELOVL5 siRNA. RANTES was measured in the supernatant of endothelial cell cultures. One way ANOVA control vs FA, ***p<0.001.

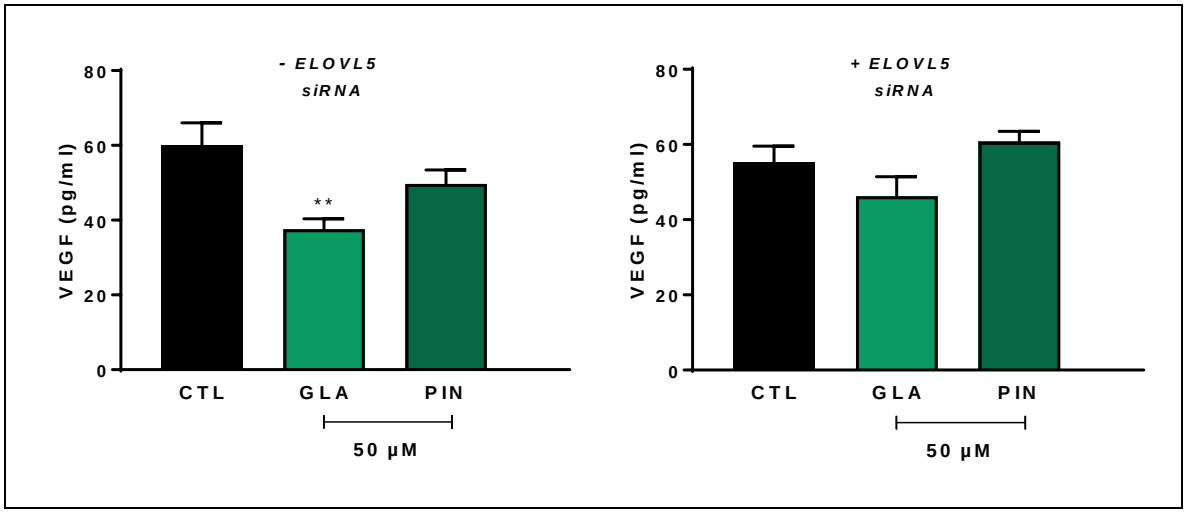

Figure 8.11 Effect of FA exposure on VEGF production by TNFα stimulated EA.hy926 cells, with or without ELOVL5 siRNA

Mean (±SEM) (n = 3) production of VEGF by TNFα activated EA.hy926 cells without incubation with FA (control; CTL) or with 48 hours prior exposure to GLA and PIN (50µM) followed by 24 hour TNFα (1ng/mL). EA.hy926 were prior incubation without (-) or with (+) ELOVL5 siRNA. VEGF was measured in the supernatant of endothelial cell cultures. One way ANOVA control vs FA, **p<0.01.

GLA and PIN both significantly decreased the secretion of ICAM-1 compared to stimulated control cells (p<0.001) (Figure 9.5). This reduction was not seen in ELOVL5 siRNA silenced cells: incubation of these cells with GLA or PIN lead to no changes in levels of ICAM-1. Similar results were observed after treatment with GLA and PIN and IL-6 production (Figure 9.6), which was significantly decreased by both FAs (P<0.01), and again this reduction was not seen in ELOVL5 siRNA treated cells.

GLA treatment also lead to the significant decrease of RANTES (p<0.05) and VEGF (p<0.01) by EA.hy926 cells compared to stimulated control cells (Figure 9.9 and Figure 9.10). These effects of GLA treatment were not seen in ELOVL5 siRNA treated cells.

IL-8 production by EA.hy926 was significantly decreased after exposure to PIN (p<0.001) (Figure 9.7), and was no longer seen in ELOVL5 siRNA treated cells.

MCP-1 production was also decreased after treatment with both FAs (Figure 9.8), with a greater reduction observed after GLA treatment compared to PIN ($p<0.001$ and $p<0.05$ respectively). ELOVL5 siRNA silencing only prevented the effects of PIN. GLA incubation in ELOVL5 silenced cells still lead to the same significant decrease in MCP-1 as seen in unsilenced cells ($p<0.001$). This may indicate a direct role for GLA and MCP-1 regulation.

8.5 Discussion

The aims of the research described in this chapter were to firstly identify the likely elongation pathway of PIN to ETrA in EA.hy926 cells and secondly to compare the anti-inflammatory effects of two plant-derived FAs (GLA and PIN) in EA.hy926 cells that have their elongase 5 activity silenced via siRNA mediated inhibition. siRNA inhibition of ELOVL5 was analysed via RT-qPCR, and the production of elongation products in EA.hy926 analysed via GC. The effects of these interventions on production of various inflammatory mediators (ICAM-1, MCP-1, VEGF, IL-6, IL-8, RANTES) by cultured ECs were analysed by Luminex. These objectives and the overarching aim were met.

siRNA was shown to successfully silence ELOVL5 in the cells as shown by 80-90% decrease in relative gene expression (RT-qPCR). This was shown to result in a reduction in elongation products after incubation of the cells with GLA and PIN in siRNA ELOVL5 silenced cells, as shown by GC analysis. This strongly suggests that the gene silencing resulted in less protein (enzyme). siRNA controls proved the specificity of the manipulation, which was shown by GC and RT-qPCR analysis which revealed similar results to control cells after incubation: elongation products were present and the relative gene expression of ELOVL5 was unaltered.

As shown previously, incubation of EA.hy926 cells with GLA and PIN lead to an increase in both DGLA and ETrA. These results have also been seen by others. Levin *et al*. saw modest increases in DGLA in LL carcinoma cells and HL-60 leukaemia cells after GLA incubation [216], and Chapkin *et al*. saw DGLA levels increase in murine macrophages incubated with GLA [176]. Chuang *et al.* describe dose-dependent increases in ETrA in RAW264.7 murine macrophages after incubation with PIN [179].

Here it was demonstrated that siRNA silencing of ELOVL5 lead to the inhibition of the ability of EA.hy926 cells to metabolise GLA and PIN to DGLA and ETrA. Although this pathway is described by others for GLA [212], the metabolic pathway of elongation of PIN has not been described previously. These results provide strong evidence for elongase 5 being directly involved in the elongation of PIN.

GLA and PIN at a concentration of 50 μM showed inhibitory effects on inflammatory mediator production by stimulated EA.hy926 cells (Figure 8.6 - Figure 8.11), confirming results from earlier (Chapter 3). Comparison of inflammatory mediator production by ELOVL5 siRNA silenced EA.hy926 cells showed a loss in some of these anti-inflammatory effects of GLA and PIN, although differential effects were seen depending on the inflammatory marker examined.

GLA had the greatest inhibitory effect on mediator production compared to control (TNFα stimulated EA.hy926 cells). PIN also significantly reduced many of the mediators examined. Observations made here are similar to previous results (Chapter 3). All decreases in production of inflammatory mediators were abolished by ELOVL5 silencing other than the effect of GLA treatment on MCP-1 production which remained unchanged in silenced cells. This suggests a direct role for GLA in controlling MCP-1 production. Dirks *et al*. described reduced MCP-1 in Sprague Dawley arts fed GLA in the form of evening primrose oil [210].

Loss of the effects of GLA and PIN in ELOVL5 silenced cells indicate that the anti-inflammatory actions of these FAs described in this model are likely exerted through their elongation products. Direct effects of DGLA or ETrA on ECs have not yet been explored.

DGLA can be metabolised to anti-inflammatory eicosanoids via the COX pathway (described section 1.4.1). This may indicate how the metabolism of GLA to DGLA can lead to anti-inflammatory effects. Chapkin *et al.* describe that a DGLA diet supressed the development of dermatitis in vivo in NC/Tnd mice through generation of prostaglandin D1 [176]. Others describe significant prevention of atherosclerosis in high cholesterol diet fed mice by feeding DGLA [217]. This effect was attenuated by naproxen a COX-2 inhibitor, suggesting that it was driven by COX-2 and 1-series PG production. Chene *et al*. also describe that DGLA induced COX-2 activity in HaCat cells [191]. Together these findings suggest that GLA may act via elongation to DGLA and subsequent production of anti-

inflammatory eicosanoids via COX-2. The possible mechanism of action of ETrA has however been less explored. Chaung *et al.* describe PIN as resulting in a decrease in both PGE_1 and PGE_2 in LPS stimulated RAW264.7 murine macrophages, but an increase in COX-2 protein levels [179].

8.5.1 Conclusion

Together these data suggest a role for the elongation products DGLA and ETrA as having anti-inflammatory actions in this model. The pathway of metabolism of PIN to ETrA has not been described before, but here inhibition of ETrA production in ELOVL5 silenced EA.hy926 was seen suggesting that PIN is metabolised by elongase 5. The effects of GLA and PIN on inflammation in this model indicate a therapeutic role for these sustainable FAs, but that they mainly act through their elongation products. Thus, there are likely to be therapeutic actions for DGLA and ETrA.

Chapter 9: Effects of altering the ratio of linoleic acid to alpha-linolenic acid on inflammatory responses of cultured endothelial cells

9.1 Introduction

LA and ALA are essential FAs in humans. LA is one of the most abundant FAs within modern Western diets. It is the most abundant PUFA in the diet and is consumed in much larger quantities than ALA. Furthermore, LA intake increased markedly in Western diets over the course of the 20th century [17]. ALA intake seems not to have increased as much [17] meaning that the ratio of LA to ALA and the ratio on n-6 to n-3 FAs in the diet has increased over time. As previously described in section 1.1.3.1, LA and ALA compete for D6D and their metabolites have different biological activities. Metabolism of LA leads to the production of AA, which is a substrate for synthesis of inflammatory eicosanoids, which have been shown to be increased in inflammatory conditions [18]. ALA is metabolised to EPA and DHA, and the eicosanoids and docosanoids derived from these have been shown to be less inflammatory or pro-resolving in nature [78, 79]. Therefore it is argued that the increase in dietary LA can lead to a more inflammatory state and predisposition to inflammatory conditions, including atherosclerosis [18].

Cell culture studies offer an ideal way to explore the effects of altering LA and ALA in a controlled manner that is difficult to achieve using other experimental models. Therefore the EA.hy926 cell model was used to investigate the effect of altering the ratio of LA to ALA on inflammatory responses. Few studies have examined the effects of different ratios of LA and ALA in ECs and how this might impact on inflammatory status [192, 218].

In the experiments described in this chapter, the ratios of LA to ALA used were based on current consumption among different countries [21], although consumption of these FAs is difficult to determine accurately as they come from many different sources. Table 9.1 shows the estimate of different average dietary ratios of LA to ALA in different countries for men and women. Based on these estimates, ratios with higher quantities of LA to ALA were chosen with the highest being 19:1 LA to ALA. However it was also chosen to examine a 1:1 ratio of LA and ALA and a ratio where by ALA was the abundant FA, 0.25:1 LA to ALA. Exploration of the effects of these ratios in the inflammatory model will help to understand the mechanism of action of competition between ALA and LA for D6D and whether an increase in ALA consumption has the potential to reduce inflammation. Plant-derived ALA may therefore help to slow atherosclerosis progression.

Table 9.1 Estimated ratios of consumption of LA:ALA across the world (Baker *et al*. [21])

Country	LA:ALA ratio	
	Men	Women
Australia/New Zealand	9.1	10.1
Austria	9.1	9.5
Belgium	9.8	9.1
Denmark	6.5	5.2
Finland	4.5	4.5
France	9.2	9
Germany	5.8	6.1
Greece	15.5	14.1
Iceland	NA	NA
Italy	18.1*	
Netherlands	9.1	9.5
Norway	7.6	7.8
Portugal	17.3*	
Spain	27*	
Sweden	6.1	6.5
UK	8.1*	
USA	9.9	10.8

The research described in this chapter compares the effects of ALA, LA and five different ratios of LA to ALA on inflammatory responses of ECs in vitro. This is an important step in understanding how the shift in the consumption of these FAs in human diets may influence inflammatory processes.

9.1.1 Aim and objectives

The aim of the research described in this chapter was to compare the effects of five different LA:ALA ratios on inflammatory responses of cultured ECs.

The specific objectives were to:

- Determine the viability of EA.hy926 cells after culture with TNFα and various ratios of LA to ALA;
- Determine the FA composition of EA.hy926 cells after culture with various ratios of LA to ALA;
- Compare the effects of ALA, LA and five LA:ALA ratios on production of inflammatory mediators (ICAM-1, MCP-1, VEGF, IL-6, IL-8, RANTES) by cultured ECs;

9.2 Methods

All experimental work carried out in the chapter was in collaboration with Christian Bork.

9.2.1 Reagents

Reagents and materials used for experiments are described in Appendix A.

9.2.2 Fatty acid dilution.

ALA was dissolved in 100% ethanol to prepare 160 mM, 100 mM, 40 mM, 20 mM and 10 mM stock solutions. LA was dissolved in 100% ethanol to prepare 190 mM, 180 mM, 160 mM, 100 mM and 40 mM stock solutions. Dilutions were made from stocks in DMEM (complete medium) to create working concentrations of each FA. This gave an ethanol concentration of 0.1% in working concentrations. FAs were diluted according to requirements for each experiment. Prior to each experimental procedure GC analysis was used to verify FA stock concentrations (data not shown).

9.2.2.1 Fatty acid concentrations and LA:ALA ratios

LA and ALA were diluted in DMEM and added to EA.hy926 cells at different concentrations to create a ratio of LA to ALA and a total overall concentration of 100 μM. For example 50 μM ALA was added to 50 μM LA giving a 1:1 ratio of LA:ALA (Ratio 1; R1) and the total concentration of LA+ALA of 100 μM. Table 9.2 shows the concentrations of individual FA for each ratio.

Table 9.2 Fatty acid ratio concentrations

FA or ratio	FA concentration (μM)		Total Concentration (μM)
	LA	ALA	
ALA	-	100	100
Ratio 0.25	20	80	100
Ratio 1	50	50	100
Ratio 4	80	20	100
Ratio 9	90	10	100
Ratio 19	95	5	100
LA	100	-	100

9.2.3 Treatment of EA.hy926 cells with fatty acids

As described previously (section 2.3.2) confluent EA.hy926 cells were scraped from flasks and resuspended at a density of 2 x 10^5 cells per mL. Cells were seeded at 1 x 10^4 cells per well in 96-well flat bottom plates, and incubated for 48 hours at 37°C with the FAs at a concentration of 100 μM followed by 24 hour incubation with TNFα at 1 ng/mL prior to further analysis.

9.2.4 MTT assay

Cell viability was measured using the MTT Cell Proliferation assay kit described (section 3.3.3.1). The assay procedure was carried out as described in section 3.3.3.1.1. Briefly EA.hy926 cells were seeded at 1 x 10^4 cells per well in 96-well flat bottom plates, incubated for 48 hours at 37°C with various FAs ratios at a total FA concentration of 100 μM followed by 24 hour incubation with either DMEM or TNFα at 1 ng/mL.

After treatment, MTT at 5 ng/mL was added to wells (100 μl/well), and incubated for 4 hours at 37°C. After incubation, 85 μl supernatant was removed and 75 μl DMSO added to each well and wells were then incubated at 37°C for a further 5 minutes. Plates were then read on the plate reader at 540 nm. Controls consisted

of cells incubated with DMEM alone and DMEM with 0.1% ethanol. Controls in DMEM alone were considered to be 100% viable; effects of treatments were determined as a % of control.

9.2.5 Analysis of cellular FA composition

Confluent cultures were incubated with either culture medium with supplements (control) or culture medium with supplements containing FAs (ALA, LA and ALA/LA ratios) at a concentration of 100 μM. Cells were incubated for 48 hours at 37°C. The GC protocol was used as described previously in section 3.3.3.3.1; briefly after FA incubation cells were scraped, centrifuged at 1200 rpm for 5 minutes, and resuspended in 0.9% NACl at a volume to achieve 1.25 x 10^6 cells/mL.

9.2.5.1 GC analysis

Total lipid was extracted from cell pellets as described in section 3.3.4.1. FAMEs were prepared (section 3.3.4.1) and analysed using a Hewlett-Packard 6890 gas chromatograph (section 3.3.4.2).

9.2.6 Production of inflammatory mediators

Cells were incubated for 48 hours at 37°C with ALA, LA or LA:ALA ratios at a total FA concentration of 100 μM followed by 24 hour incubation with either DMEM or TNFα at 1ng/mL. Supernatant was removed and stored at -20°C until analysis. Concentrations of cytokines and adhesion molecules in EC culture supernatants were determined simultaneously using Human Magnetic Luminex Screening Assay kits by R&D using the assay described in section 2.4.4.1.

9.2.7 Statistical analysis

Supernatant concentrations of cytokines and inflammatory mediators were calculated using the Luminex Bio Plex Manager 6.0 software (2.3.3). FAME histograms were analysed with Agilent ChemStation software (section 3.3.4) and FA concentrations calculated as μg of FA/1x10^6 cells.

Data are expressed as mean ± standard error; data analysis was performed in PRISM and Excel. Multiple group differences were compared using one way ANOVA.

9.3 Results

9.3.1 Viability of EA.hy926 exposed to LA and ALA different ratios

FAs were dissolved in ethanol and further diluted in DMEM before EA.hy926 cell exposure. The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30. Cells were incubated for 48 hours with FA ratios and then further exposed to TNFα at 1 ng/mL for 24 hours (see Figure 2.21 for the experimental design). Cell viability was measured using the MTT assay and viability was calculated as a % of control (DMEM). Ethanol at 0.1% did not affect cell viability. Cells exposed to 1 ng/mL TNFα alone were shown to be viable over this time course (>90%). FAs combined with TNFα exposure had no significant effect on EA.hy926 cell viability (Figure 9.1).

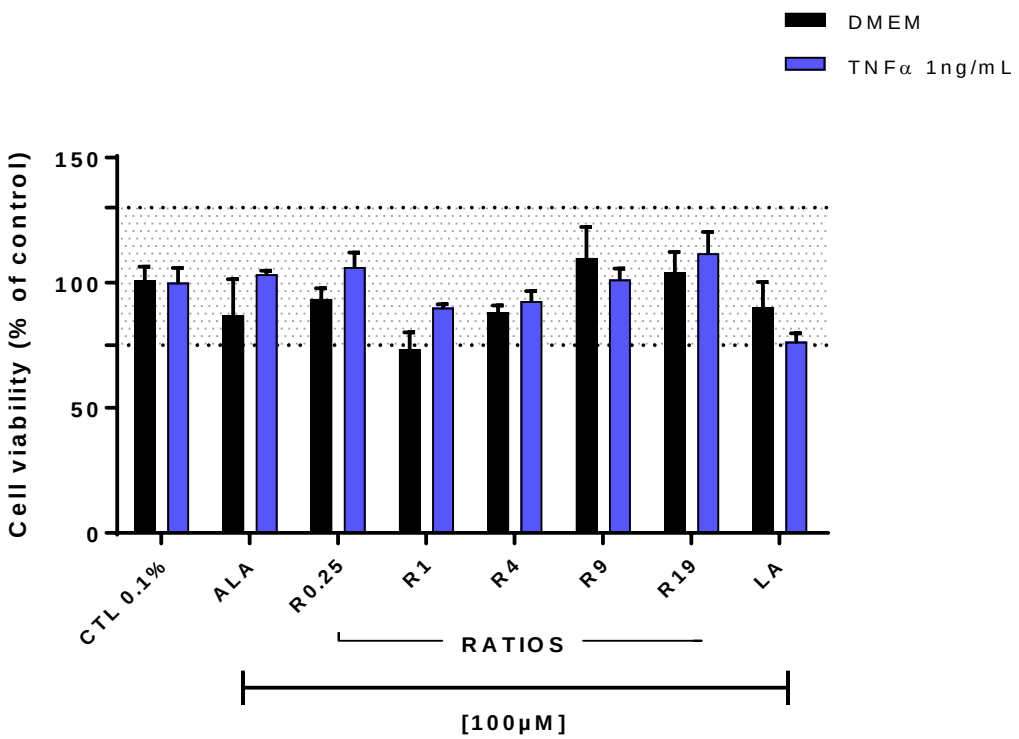

Figure 9.1 Effect of ALA, LA and different ratios of LA:ALA at 100 µM with or without TNFα on EA.hy926 cell viability

Mean (±SEM) (n = 3) % of control (EA.hy926 + DMEM) cell viability after 48 hour exposure to ALA, LA and LA:ALA ratios (100 µM) followed by 24 hour TNFα (1ng/mL). Two way ANOVA

FA treatment after 48 hour in unstimulated cells did not affect viability. EA.hy926 cell viability (Figure 9.1), although there was a tendency for ratio 1 (R1) to decrease viability although this was not significant.

FA treatment for 48 hour followed by 24 hour 1ng/mL TNFα had no significant effect on EA.hy926 cell viability (Figure 9.1). There was a tendency for LA treatment alone at 100 μM to decrease EA.hy926 cell viability, although this was not significant. Viability for these FAs was deemed acceptable (>75%) at concentrations of 100 μM.

9.3.2 Fatty acid incorporation into EA.hy926 cells

FAs were dissolved in ethanol and further diluted in DMEM, EA.hy926 cells were incubated with the ALA, LA and different FA ratios for 48 hours to allow incorporation into the cells. The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30.Figure 9.2 to Figure 9.4 show the change in FA composition after 48 hour incubation with each FA treatment at a total concentration of 100 μM.

After 48 hour FA treatment, FAs were successfully taken up by EA.hy926 cells in a dose-dependent manner. Incubation with the different FA ratios lead to different changes in cell FA composition compared to control cells (DMEM alone) and to the appearance of specific metabolic elongation products. The products being metabolised may be of importance when examining the effects these ratios have on the inflammatory status in stimulated EA.hy926 cells.

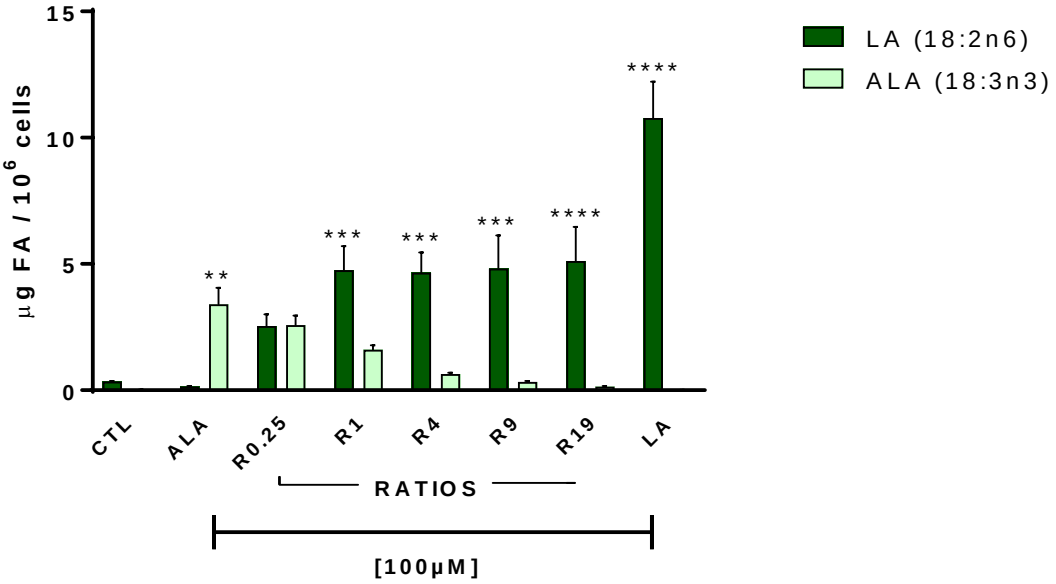

Figure 9.2 Incorporation of ALA and LA and after incubation of EA.hy926 cells with ALA, LA and ratios of LA:ALA for 48 hours

Mean (±SEM) (n = 3) µg of FA/10^6 cells compared to control (EA.hy926 +DMEM) after 48 hour exposure to ALA, LA and ratios of LA:ALA (100µM) Two way ANOVA; control vs FA, **p<0.01, ****p<0.0001

Figure 9.2 shows the incorporation of ALA and LA after incubation with ALA, LA and the different ratios. There was a significant dose-dependent increase in LA in EA.hy926 cells after incubation with ratios with increasing LA concentration. Ratios 1, 4 and 9 significantly increased LA in the cells ($p<0.001$, $p<0.001$, $p<0.001$ respectively), which was further significantly increased with ratio 19 ($p<0.0001$) and with LA alone ($p<0.0001$). ALA in EA.hy929 cells only significantly increased after incubation with ALA alone (100 µM) ($p<0.01$).

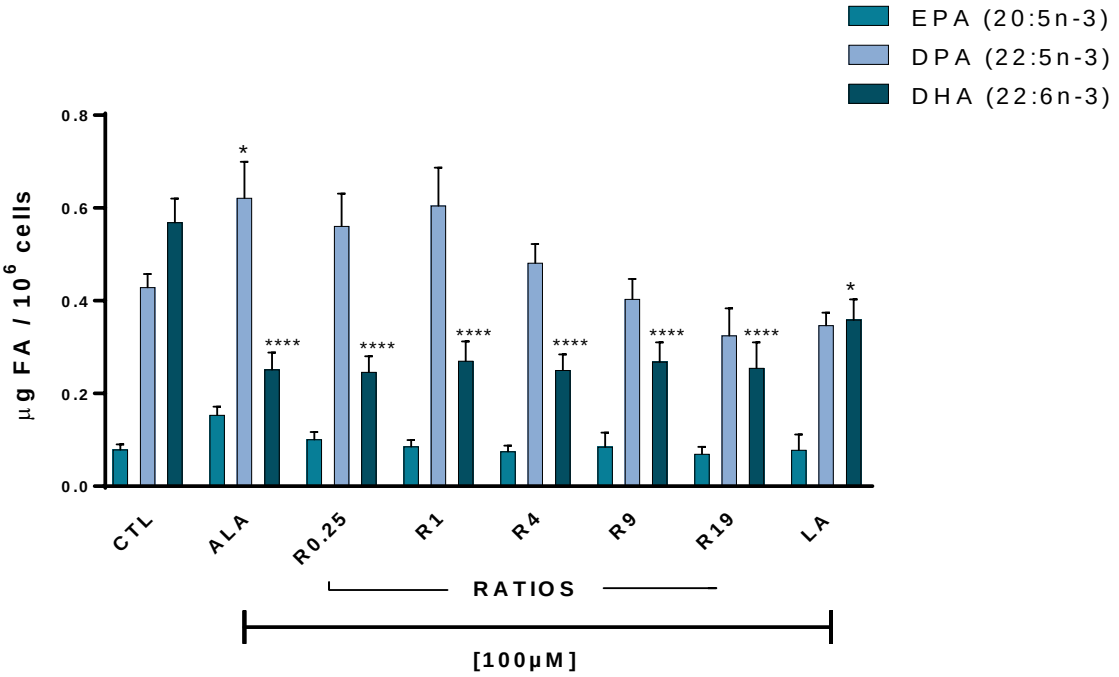

Figure 9.3 Incorporation of EPA, DPA and DHA after incubation of EA.hy926 cells with ALA, LA and ratios of LA:ALA for 48 hours

Mean (±SEM) (n = 3) µg of FA/10^6 cells compared to control (EA.hy926 +DMEM) after 48 hour exposure to ALA, LA and ratios of LA:ALA (100µM) Two way ANOVA; control vs FA, *p<0.05, ****p<0.0001

Figure 9.3 shows the presence of ALA metabolites; EPA, DPA and DHA after incubation with ALA, LA and the different ratios. Incubation with ALA alone (100 µM) significantly increased DPA in EA.hy926 cells ($p<0.05$). DHA was seen to significantly decrease across all treatments, but to a lesser extent after LA treatment; ALA ($p<0.0001$), R0.25 ($p<0.0001$), R1 ($p<0.0001$), R4 ($p<0.0001$); R9 ($p<0.0001$) and LA ($p<0.05$). No changes in EPA were observed after any treatment compared to control cells.

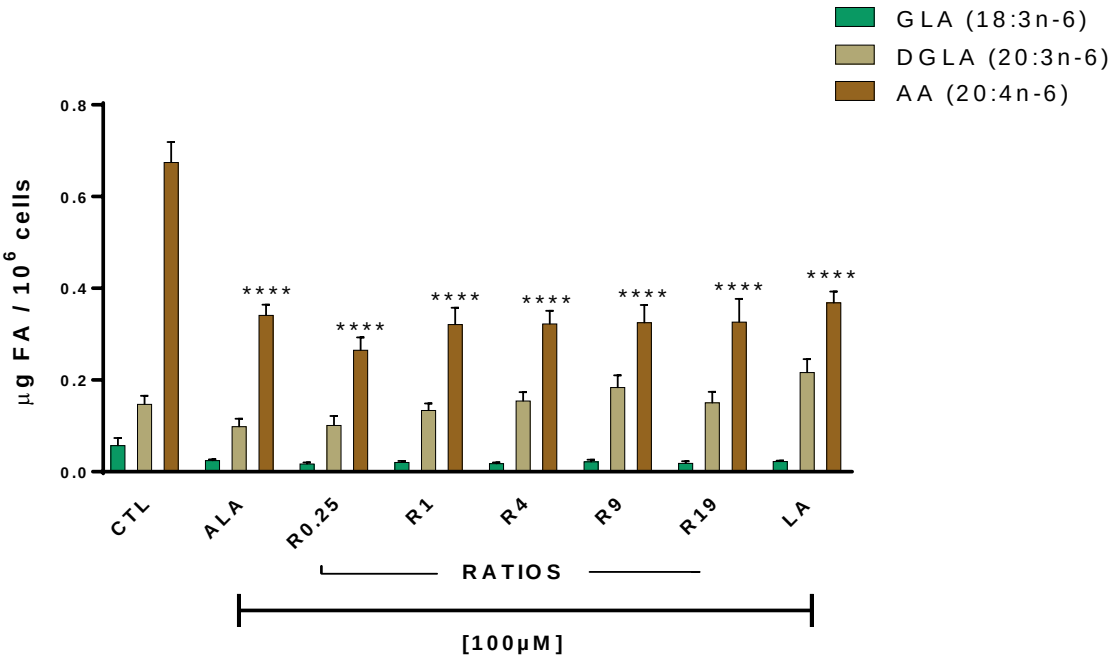

Figure 9.4 Incorporation of GLA, DGLA and AA after incubation of EA.hy926 cells with ALA, LA and ratios of LA:ALA for 48 hours

Mean (±SEM) (n = 3) µg of FA/10^6 cells compared to control (EA.hy926 +DMEM) after 48 hour exposure to ALA, LA and ratios of LA:ALA (100µM) Two way ANOVA; control vs FA, ****p<0.0001

Figure 9.4 shows incorporation of LA metabolites; GLA, DGLA and AA. No changes were observed in GLA or DGLA quantities in EA.hy926 cells after any treatment. AA was shown to significantly decrease after incubation with all treatments compared to control cells ($p<0.0001$ for all conditions).

9.3.3 Inflammatory mediators

In order to investigate the effect of different FAs on the production of inflammatory mediators, EA.hy926 cells were exposed to ALA, LA and different LA:ALA ratios at total FA concentrations of 100 µM for 48 hours followed by 24 hour TNFα stimulation at 1 ng/mL. Concentrations of mediators in the culture medium after 24 hours were evaluated by Luminex assay. The data shown are from 3 separate experiments each conducted in triplicate, EA.hy926 were used at a passage of >30.

TNFα treatment was previously shown to increase production of each of the analytes examined (

Figure 2.8 - Figure 2.13). FA exposure was shown to have differential effects depending on the individual FA and LA:ALA ratio (Figure 9.5 to Figure 9.10). ALA showed the most potent anti-inflammatory effects for all analytes measured, as did low LA:ALA ratios, however some decreases in inflammatory markers were observed after incubation with LA alone.

9.3.3.1 ICAM-1

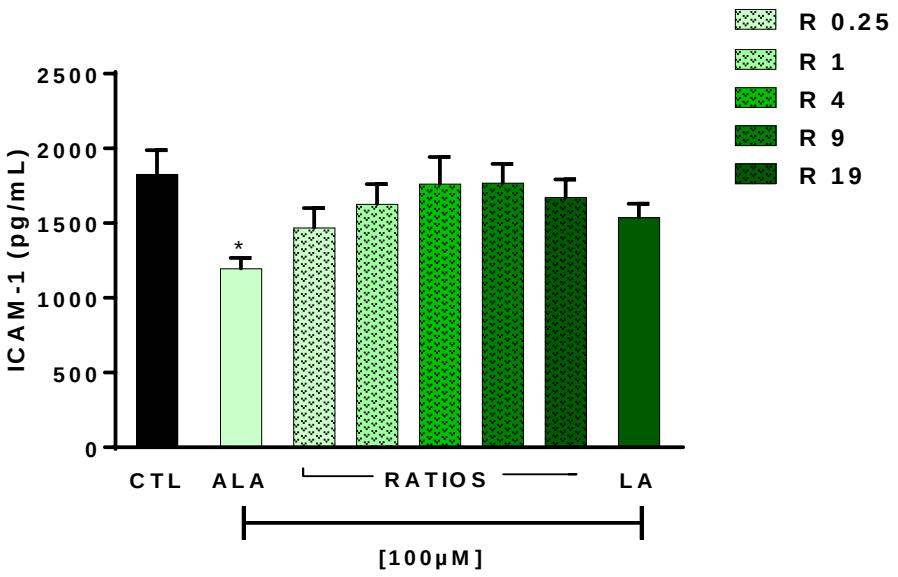

Figure 9.5 Effect of FA exposure on ICAM-1 production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) production of ICAM-1by TNFα activated EA.hy926 cells without prior incubation with FA (control; CTL) or with 48 hours prior exposure to ALA, LA or ratios of LA:ALA (100 µM) followed

by 24 hour TNFα (1ng/mL). ICAM-1 was measured in the supernatant of endothelial cell cultures. One way ANOVA: control vs FA *p<0.05

9.3.3.2 IL-6

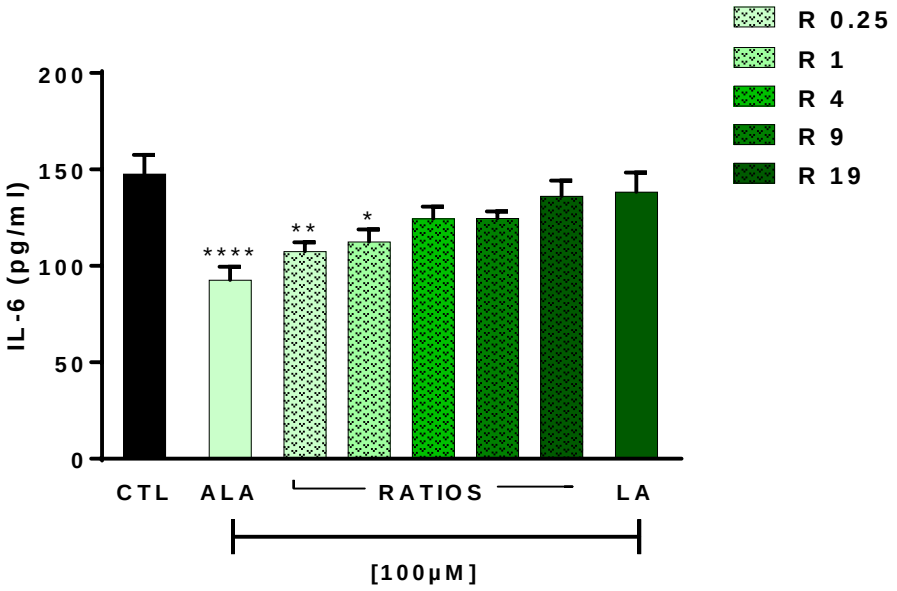

Figure 9.6 Effect of FA exposure on IL-6 production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) production of IL-6 by TNFα activated EA.hy926 cells without prior incubation with FA (control; CTL) or with 48 hours prior exposure to ALA, LA or ratios of LA:ALA (100 µM) followed by 24 hour TNFα (1ng/mL). ICAM-1 was measured in the supernatant of endothelial cell cultures. One way ANOVA: control vs FA *p<0.05, **p<0.01, ****p<0.0001

9.3.3.3 IL-8

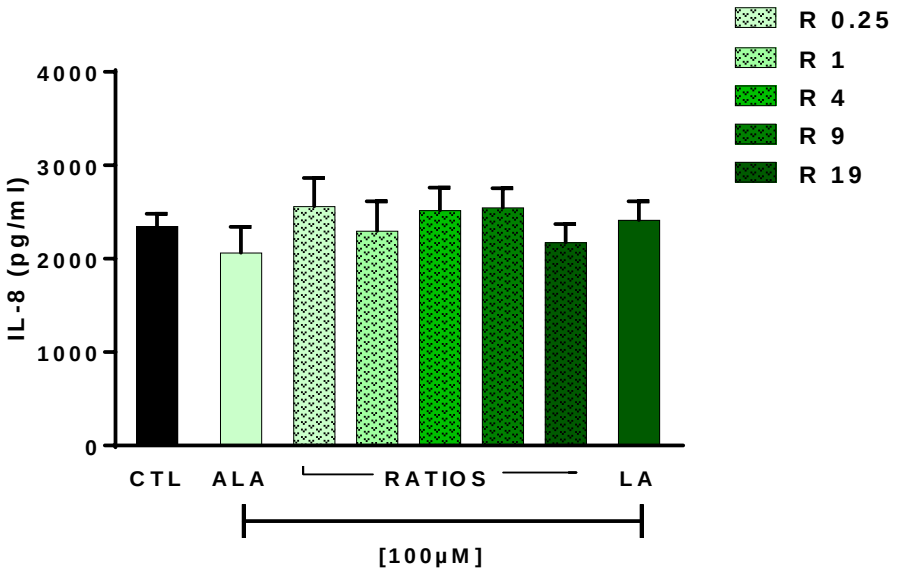

Figure 9.7 Effect of FA exposure on IL-8 production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) production of IL-8 by TNFα activated EA.hy926 cells without prior incubation with FA (control; CTL) or with 48 hours prior exposure to ALA, LA or ratios of LA:ALA (100 µM) followed by 24 hour TNFα (1ng/mL). ICAM-1 was measured in the supernatant of endothelial cell cultures. One way ANOVA: control vs FA *p<0.05, **p<0.01, ****p<0.0001

9.3.3.4 MCP-1

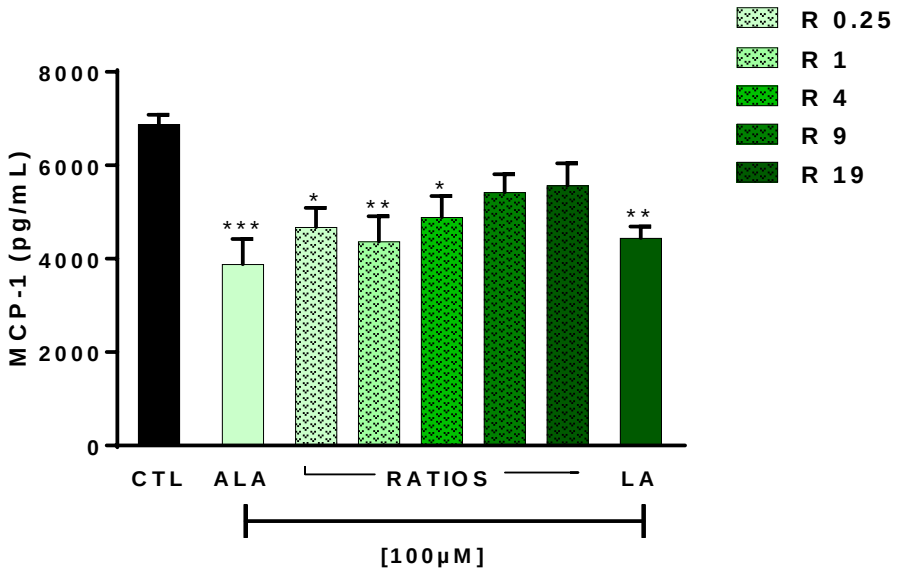

Figure 9.8 Effect of FA exposure on MCP-1 production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) production of MCP-1 by TNFα activated EA.hy926 cells without prior incubation with FA (control; CTL) or with 48 hours prior exposure to ALA, LA or ratios of LA:ALA (100 µM) followed by 24 hour TNFα (1ng/mL). ICAM-1 was measured in the supernatant of endothelial cell cultures. One way ANOVA: control vs FA *p<0.05, **p<0.01, ***p<0.001

9.3.3.5 RANTES

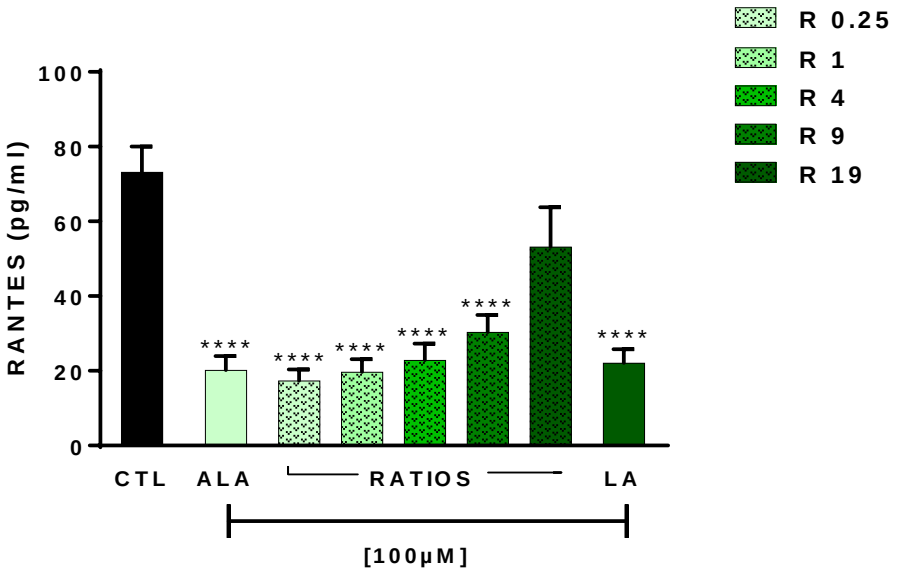

Figure 9.9 Effect of FA exposure on RANTES production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) production of RANTES by TNFα activated EA.hy926 cells without prior incubation with FA (control; CTL) or with 48 hours prior exposure to ALA, LA or ratios of LA:ALA (100 µM) followed by 24 hour TNFα (1ng/mL). ICAM-1 was measured in the supernatant of endothelial cell cultures. One way ANOVA: control vs FA, ****p<0.0001

9.3.3.6 VEGF

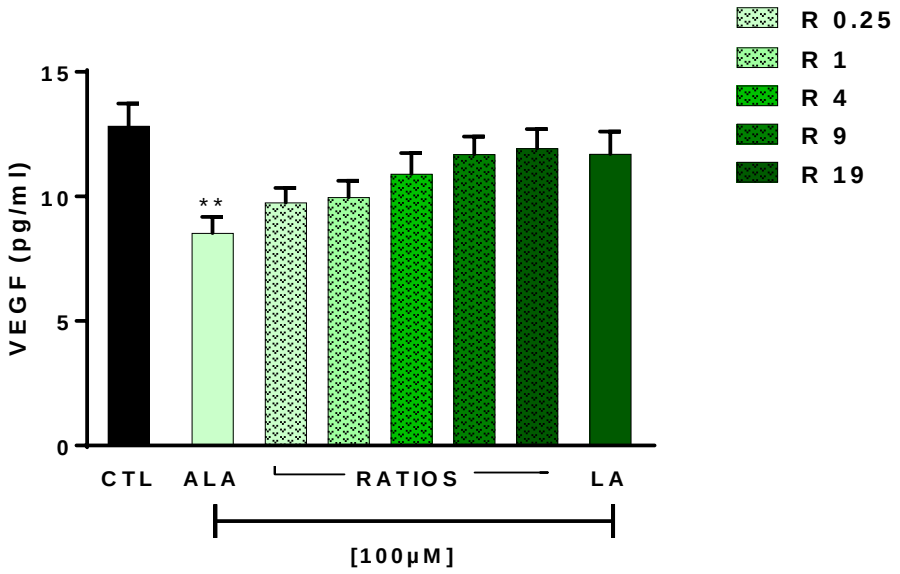

Figure 9.10 Effect of FA exposure on VEGF production by TNFα stimulated EA.hy926 cells

Mean (±SEM) (n = 3) production of VEGF by TNFα activated EA.hy926 cells without prior incubation with FA (control; CTL) or with 48 hours prior exposure to ALA, LA or ratios of LA:ALA (100 µM) followed by 24 hour TNFα (1ng/mL). ICAM-1 was measured in the supernatant of endothelial cell cultures. One way ANOVA: control vs FA, **p<0.01

ALA treatment alone at a concentration of 100 µM significantly decreased production of several of the inflammatory mediators examined in this model. ALA had a potent inhibitory effect on the secretion of both IL-6 and RANTES in EA.hy926 cells compared to stimulated control cells ($p<0.0001$). MCP-1 production was also significantly decreased after ALA treatment ($p<0.001$), as were VEGF and ICAM-1 secretion ($p<0.01$, $p<0.05$ respectively). Additionally ALA exposure had a tendency to decrease IL-8 production, however this was not significant.

Ratio 0.25 (R0.25) lead to some significant decreases in inflammatory mediators. RANTES was significantly reduced after R0.25 exposure ($p<0.0001$), as was IL-6 ($p<0.01$) and MCP-1 production ($p<0.05$). R0.25 also had a tendency to decrease ICAM-1 and VEGF production compared to stimulated EA.hy926 cells.

Ratio 1 (R1) treatment also lead to some significant decreases in mediator secretion compared to stimulated control cells. RANTES, MCP-1 and IL-6 were all

significantly reduced compared to control cells ($p<0.0001$, $p<0.01$, $p<0.05$ respectively).

Ratio 4 (R4) incubation decreased fewer of the mediators being produced by stimulated cells. Production of RANTES and MCP-1 were both significantly decreased ($p<0.0001$, $p<0.05$ respectively) after R4 treatment compared to control stimulated cells.

Neither Ratio 9 (R9) nor ratio 19 (R19) had any effect on any of the mediators analysed in this inflammatory model.

Interestingly, incubation of EA.hy926 with LA alone lead to significant decreases in the production of both RANTES and MCP-1 ($p<0.0001$, $p<0.05$ respectively).

Inflammatory mediator production was diminished in a dose-dependent manner with increasing relative ALA to LA, with greatest inhibition after treatment with R0.25 to least inhibition at R19. ALA treatment alone had the most potent effect; however LA alone also decreased some of the mediators examined.

9.4 Discussion

These results confirm that ALA is anti-inflammatory in this model and so has the potential to slow the initiation and progression of atherosclerosis. ALA had potent anti-inflammatory effects in this model at a concentration of 100 μM. Earlier findings however did not indicate such potent properties of ALA, this may be due to the higher concentration being used in these experiments.

Interestingly LA at 100 μM also showed some anti-inflammatory effects. However the highest ratio of LA to ALA, ratio 19, did not affect the inflammatory mediator production of stimulated ECs, instead mediator production by cells treated with the highest ratio remained fully responsive to the TNFα stimulation. Lowering the ratio of LA to ALA decreased the inflammatory response of ECs in a ratio dependent manner. These observations indicate that a high ratio of LA to ALA may favour inflammation (and atherosclerosis), that increased prevalence of inflammatory conditions like allergy and asthma in the late 20th century may relate to the increased ratio of the Western diet, and that those who are suffering with an inflammatory condition would not benefit from consuming much higher quantities of LA than ALA.

9.4.1 FA content of ECs according to different LA to ALA ratios

The concentrations of FAs used within this model were shown to be non-toxic in EA.hy926 cells, consistent with what others have reported [174, 183, 219]. The concentrations of ALA used here are physiologically relevant [220]. Furthermore higher plasma ALA concentrations have been reported in humans after supplementation with milled flaxseed (~130 μM) [221]. The concentrations of LA used here are generally lower than those seen in human plasma [220]. The ratios used were chosen to represent dietary consumption differences which would result in different ratios in blood. Unpublished data from our laboratory indicates total plasma LA of ~1960 μM in healthy subjects with total plasma ALA of ~50 μM ALA, giving a ratio of 40:1 LA:ALA. Similarly in the Toronto Nutrigenomics and Health Study total plasma lipids of young healthy Canadian adults had ~2200 μM LA, ~55 μM ALA -and ~40:1 LA:ALA [220].

Incubation with the different FA ratios lead to changes in cell FA composition compared to control cells (DMEM alone) and to the appearance of specific metabolic elongation products. EA.hy926 cells incorporated LA and ALA in a dose-dependent manner with increasing LA:ALA (Figure 9.2) with only significant

increases in ALA after treatment with ALA alone. Surprisingly each ratio was shown to significantly decrease AA (Figure 9.4) and DHA (Figure 9.3). Others too describe decreased DHA in phospholipids after incubation of human respiratory ECs with LA and ALA (180 μM) [219], although they observed no change in AA. Incorporation patterns of FAs after exposure of ECs to different ratios of LA:ALA, however have not been described. ALA treatment of EA.hy926 cells alone was shown to significantly increase DPA content (Figure 9.3). This indicates that cultured ECs have an intact PUFA desaturation and elongation pathway that produces DPA as the end product of ALA metabolism. This is consistent with many other studies showing that ALA is a good substrate for DPA but not for DHA synthesis [21]. The increase in DPA with increased exposure to ALA could explain some of the anti-inflammatory effects of ALA. Increases of DPA in EA.hy926 cells were also seen in Chapter 3 after 10 and 50 μM ALA treatment.

9.4.2 Effects on inflammatory mediators

In this model ALA treatment alone decreased production of the majority of the analytes measured, some decreases were also seen after LA treatment alone and different ratios of LA to ALA had anti-inflammatory effects with increasing ALA concentration. Few others have described effects of ratios of LA to ALA in ECs [192, 218]. Yang *et al*. describe no changes in IL-6 production by unstimulated EA.hy926 cells among treatments with several different ratios of LA to ALA (1:1, 5:1, 10:1, 20:1) and at two different total concentrations (20 and 100 μM) [218]. Wang *et al.* describe effects of ALA, LA and different ratios of these FAs in polychlorinated biphenyls (PCB)-induced ECs, they reported increased VCAM-1 and COX-2 expression as well as NF-kB activity in the presence of LA, which was diminished by increasing relative amounts of ALA to LA. PGE_2 levels were also decreased with increasing amounts of ALA relative to LA [192].

Others have also investigated the individual effects of ALA or LA on the inflammatory status of ECs. Erdinest *et al*. examined effects of ALA and LA at 200 μM on the production of IL-6 and IL-8 in LPS stimulated HCE cells [183]. They described decreased IL-6 protein content after incubation with both ALA and LA. They also observed a significant reduction in IL-8 protein levels after ALA treatment, but no changes after LA treatment [183]. However with the current model contrasting observations were made: ALA exposure to EC alone reduced IL-6 secretion (Figure 9.6) and neither ALA or LA treatment lead to reduction of IL-8 production in EA.hy926 cells (Figure 9.7).

Young *et al*. describe differential effects of LA exposure at 180 μM in TNFα stimulated HUVECs [222], they observed increased basal IL-8 production by HUVECs after LA treatment compared to unstimulated control cells; however LA had no effect on TNFα stimulated HUVECs. Similarly they observed upregulation of cell surface expression of ICAM-1 after exposure of LA in HUVECs, but ICAM-1 expression was decreased in LA treated TNFα stimulated HUVECs [222]. Reissig *et al*. too described LA treatment lead to reduced cell surface expression of ICAM-1 in IL-1α stimulated HCAECs, and observed a tendency for LA to diminish adhesion of monocytes [223]. However LA treatment did not decrease ICAM-1 secretion by EA.hy926 cells in the current model. Likewise Stachowska *et al.* saw no changes in ICAM-1 secretion in non-stimulated HUVECs after LA treatment at 100 μM [224]. These differences may be due to the variation of LA concentrations used in these models.

Those who have examined effects of ALA describe similar decreases in ICAM-1. ALA exposure (50 μM) attenuated ICAM-1 expression in HUVECs exposed to high glucose [168]. Shen *et al.* also describe that ALA treatment (50 and 200 μM) reduced ICAM-1 secretion in LPS stimulated HUVECs [167]; however IL-6 secretion remained unchanged after treatment with any concentration of ALA [167]. ALA treatment was shown to decrease IL-6 production by EA.hy926 cells in the current model (Figure 9.6).

Of these studies examining the effects of LA, ALA or ratios of these FAs on inflammatory markers *in vitro*, no others have reported effects on VEGF, MCP-1or RANTES. These inflammatory mediators are important in the progression of both endothelial dysfunction and atherosclerosis. ALA was shown to decrease each of these mediators and this may indicate a role for this plant-derived FAs in reducing inflammation and preventing or slowing atherosclerosis. Interestingly LA alone was also shown to reduce MCP-1 and RANTES which may also indicate a role for this FA, although concentrations of LA used within this model are sustainably less than those seen in human plasma.

9.4.3 Conclusion

ALA decreased production of most of the mediators examined. Importantly, different ratios of LA to ALA had a differential effect with the largest effects occurring at the lowest ratios (i.e. with a higher amount of ALA). This may indicate not only a potential role for ALA in controlling inflammation and in the reduction of atherosclerosis, but also may point to a need for a change in the

consumption of LA. In contrast to expectations LA alone also reduced production of several inflammatory mediators.

Chapter 10: General discussion and conclusions

10.1 Rationale and summary of the main findings

There is epidemiological evidence of an association between dietary n-3 PUFAs and protection from cardiovascular disease (1.3.1). This may in part be due to reduction in atherosclerotic plaque growth and data suggests FAs may help to modulate atherosclerosis by affecting endothelial activation. n-3 PUFAs are predominantly found in fatty fish and seafood and various health organisations recommend an intake of 2-3 portions of fish a week to sustain beneficial effects [225]. Since fish stocks are becoming increasingly low, there is a need for a more sustainable source of n-3 FAs for supporting human health; non n-3 FAs may also be relevant to this objective. Some plants provide PUFAs which have been underexplored, and the aim of this research was to assess the potential beneficial effects of plant-derived PUFAs, including reduction in production of inflammatory mediators known to be involved in atherosclerotic plaque formation and progression. The overall objective of this research is to identify a sustainable source of beneficial PUFAs defined as having an anti-inflammatory effect.

The present study compared the effects of some plant-derived PUFAs to VLC marine-derived n-3 PUFAs on the inflammatory response in EA.hy926 cells. The aims of this research were met.

An inflammatory EC model was successfully set up using EA.hy926 ECs and stimulation with TNFα. Using this model effects of plant and marine-derived FAs on the production of various inflammatory mediators, and on expression of several inflammatory genes and intracellular proteins were examined. Furthermore effects of FAs on adhesion of monocytes to EA.hy926 cells were also explored. Anti-inflammatory actions of GLA and PIN were then further examined by silencing elongase 5 in EA.hy926 cells and seeing how this altered the effects of the two FAs. Lastly different ratios of LA and ALA were examined within this model. Together these experiments set out to a) find a potential sustainable source of n-3 FAs (or other PUFAs) with anti-inflammatory effects in order to benefit human health and b) evaluate the possible impact of higher quantities of LA and different ratios of LA to ALA as seen within Western diets, all within the context of endothelial dysfunction and atherosclerosis development.

The results obtained show clear anti-inflammatory effects of the marine-derived FAs, EPA and DHA in ECs, which is in line with much of the literature. This includes reduction in production various inflammatory mediators such as IL-6, IL-8, ICAM-1 and MCP-1, as well as to reduction in cell surface ICAM-1 expression,

which have also been reported by others [110, 150, 152, 158, 182, 187, 188]. Furthermore DHA treatment lead to reduced adhesion of monocytes to EC monolayers, which has also been reported by many others [110-112, 114, 150, 164, 172, 173, 182].

EPA and DHA also altered gene expression in ECs in a variety of ways, but not necessarily in the expected anti-inflammatory manner. Again literature suggests mixed outcomes of EPA and DHA on gene expression in a variety of cell models [152, 174, 190, 191]. Examination of COX-2 and activated NF-kB after EPA and DHA exposure however did indicate anti-inflammatory actions. Mixed outcomes between gene expression data and protein concentrations are also described in the literature [190, 191].

The findings confirm anti-inflammatory actions of EPA and DHA and suggest that DHA is more potent than EPA and that EPA and DHA may sometimes act through different mechanisms.

Plant-derived FAs could also act in an anti-inflammatory way in the model used. ALA and SDA treatment of ECs had minimal effects on mediator production, gene expression and intracellular proteins at the concentrations used in the early experiments. ALA used at a higher concentrations in later experiments had larger effects. SDA had a tendency to reduce both the cell surface expression of ICAM-1 and monocyte adhesion. Few studies report their effect in ECs or other cell types [167, 168, 184], however, lack of potency of ALA and SDA has been described in human trials [21]. This may relate to the relatively low intakes often used in human studies and the likelihood that these plant n-3 FAs need to be converted to the more bioactive EPA and DHA to exert strong effects.

GLA and PIN were both shown to reduce production of several of the inflammatory mediators examined, inflammatory protein expression, and adhesion of monocytes. Few studies have described inflammatory outcomes with these FAs [137, 183, 189].

Thus, amongst the FAs used here the identified order of anti-inflammatory potency is as follows:

$$\text{ALA, SDA} < \text{GLA, PIN} < \text{EPA} < \text{DHA}$$

ECs elongated GLA and PIN to DGLA and ETrA, respectively. Silencing elongase 5 activity in EA.hy926 cells abolished elongation of GLA and PIN and resulting in loss of most anti-inflammatory activities. This indicates a role for the longer chain

FAs DGLA and ETrA in modulation of inflammation. Thus plant oils rich in GLA or PIN or in their elongation products represent promising alternatives to marine n-3 FA sources for the prevention, control and treatment of inflammation and for prevention of atherosclerosis.

Lastly the ratio of ALA to LA was explored within this inflammatory EC model. ALA at the concentration used had a potent anti-inflammatory effect and ratios with increasing abundance of ALA too showed reduction in production of inflammatory mediators. However exposure to EA.hy926 cells to LA alone also decreased production of several inflammatory mediators. Although this was unexpected, the literature suggests some anti-inflammatory actions of both ALA and LA in a variety of cell models [167, 168, 183, 222, 223].

10.2 Incorporation of fatty acids

FAs exist within the membrane of cells, mainly in phospholipids, and from here can exert many of their biological effects (section 1.2.6.2). These include changes in lipid rafts (signalling platforms) and in lipid mediator generation. Through these mechanisms FAs can modulate how the endothelium interacts with inflammatory cells and with external signals. It has been shown that when there are higher levels of EPA and DHA within cell membrane phospholipids there are alterations in cell signalling and increases in the production of EPA and DHA derived mediators [1] leading to less inflammation.

All FAs were shown to be incorporated into cells in a dose-dependent manner after 48 hour exposure at 10 or 50 μM (Figure 3.10 - Figure 3.15). Incubation with the different FAs also lead to the appearance of various different elongation products suggesting that ECs are able to elongate and desaturate PUFAs. These products may play a critical role in the anti-inflammatory activities of each FA investigated.

All FAs (ALA, SDA, GLA, PIN, EPA and DHA) were significantly incorporated into EA.hy926 cells, presumably in the cellular membrane, after 48 hours incubation with a concentration of 50 μM. SDA increased to a relatively low extent but there was significant incorporation of its metabolic products 20:4n-3, EPA and DPA. This may suggest that SDA is efficiently converted to its metabolic elongation products by ECs. Studies suggest conversion of SDA to be more efficient than from ALA due to it not requiring the first catalytic D6D step [12]. However ALA treatment did lead to a small increases in DPA. Livingston *et al.* observed no

significant changes in ALA content of HAECs after treatment with ALA (20 μM) and reported no changes in EPA or DHA [166]. This is similar to observations made here and may explain the lack of effect of ALA on mediator production.

GLA significantly increased the cellular content of its elongation product DGLA. DGLA has also been shown to have anti-inflammatory effects since it is a precursor of eicosanoids via COX and LOX oxidation, producing PGE_1 which has anti-inflammatory properties including inhibition of smooth muscle cell proliferation associated with atherosclerotic plaque development [134, 135].

Incubation with PIN at 50 μM lead to increases in an unknown elongation product. This product was later successfully identified using GC/MS as ETrA, an isomer of DGLA. Like PIN, ETrA is a PMI-PUFA and this unusual structure may lead to significant biological effects through action on membrane functionality through changes in lipid rafts etc. ETrA may also be a precursor to bioactive lipid mediators.

10.3 Effect of fatty acids on inflammatory processes

ECs are the main barrier of exchange and contact between blood and tissues and therefore immune mediators and molecules secreted by these cells play a major role in inflammatory processes. Inflammation caused by endothelial dysfunction and leukocyte infiltration into the blood vessel wall plays a central role in all stages of atherosclerosis development and progression. Initial lesion development begins with focal endothelial dysfunction [43] induced by chronic inflammatory processes within the arterial wall. Figure 1.7 shows the processes involved in atherosclerosis and the interaction between ECs and inflammatory cells. It has been described that marine-derived n-3 PUFAs EPA and DHA show anti-inflammatory effects on ECs [112, 150, 152, 164, 172, 173, 182, 188], as confirmed in the current study, and the literature suggests that the effect of EPA and DHA on the endothelium could in part be responsible for their health benefiting actions [26]. Plant-derived FAs may also help to lower endothelial inflammation and by doing so could have the potential to ameliorate atherosclerosis. However effects of bioactive plant-derived FAs on ECs are poorly explored [167, 168, 183].

10.3.1 Inflammatory mediator production and adhesion of THP-1 to EA.hy926 cells

It was observed that 24 hour exposure to 1 ng/mL TNFα significantly upregulated all mediators analysed in EA.hy926 cells (Chapter 2). Pre-treatment with FA showed differential effects on TNFα-induced production of mediators in EA.hy926 cells depending on FA type, FA concentration and mediator examined. Overall marine-derived FAs (EPA and DHA) demonstrated the greatest inhibitory effects across all mediators. Plant-derived FAs also demonstrated some inhibitory effects, but to a lesser extent (Figure 4.3 - Figure 4.8). Flow cytometry analysis revealed SDA, EPA and DHA to all have inhibitory effects on the cell surface expression of ICAM-1, with DHA being the most potent FA (Figure 4.9).

Marine-derived n-3 FAs showed the most potent inhibitory effect on both inflammatory mediator production by stimulated EA.hy926 cells and cell surface expression of ICAM-1 on those cells. DHA also decreased adhesion of THP-1 cells to stimulated EA.hy926 cells. Of the plant-derived FAs, GLA and PIN (at 50 μM) had the most significant anti-inflammatory effects, whereas ALA and SDA did not affect secretion of any of the inflammatory mediators examined (except when ALA was used at 100 μM). However each of the plant-derived FAs, at higher concentrations (50 μM), did decrease adhesion of THP-1 cells to EA.hy926 cells.

ALAs lack of biological activity at the lower concentrations may in part be due to less metabolic elongation products observed after incubation, which others also report [166]. Those who have investigated the effects of ALA on EC inflammation describe mixed outcomes. In agreement with the findings of the current study Shen *et al*. [167] reported lack of effect of ALA on IL-6 production by LPS stimulated HUVECs. However, others describe anti-inflammatory effects of ALA, including reduction of IL-6, IL-8 [183] and ICAM-1 [167, 168], after exposure to different concentrations of ALA and in a variety of stimulated ECs. Zhang et al. also describe reduction of neutrophil adhesion to stimulated HUVECs after ALA exposure at 50 μM. They argued this decrease was due to reduction in both ICAM-1 and P-selectin, which may suggest why despite ALA having no influence on ICAM-1 in EA.hy926 cells the FA still down regulated adhesion of THP-1 cells. When ALA was used at 100 μM it had significant effects on inflammatory mediator production.

SDA treatment at 50 μM had no significant effect on any of the mediators analysed, but SDA had the greatest inhibitory effect on ICAM-1 cell surface

expression when compared to the other plant-derived FAs. SDA also decreased adhesion of THP-1 cells to EA.hy926 cells when used at 50 μM, which may indicate a role for SDA regulating ICAM-1 activity and therefore adhesion of monocytes. Few studies report effects of SDA in vitro; however similar to these findings SDA was shown to have no effect on MCP-1 production in 3T3-L1 adipocytes [184]. No other previous research has investigated the effect of SDA exposure on monocyte binding to human ECs.

The lack of effects of these particular plant-derived FAs is likely due to the extent to which these FAs are elongated to their longer chain more bioactive metabolites.

GLA treatment at 50 μM significantly decreased soluble ICAM-1 when compared to control cells. There was a tendency for GLA to decrease MCP-1. Kim *et al*. observed reduced MCP-1 production in both rat kidney epithelial cells (NRK-52E) and mesangial cells after treatment with GLA at 10 and 100 μM [189]; however few studies report effects of GLA on ECs. Similar lack of effects of GLA on IL-6 and IL-8 were observed by Erdinest *et al*. after treatment with GLA (200 μM) in LPS-stimulated HCECs [183]. De Caterina *et al* observed little effect of GLA in human saphenous vein ECs, though they were examining VCAM-1 expression [158]. However adhesion of THP-1 cells to EA.hy926 cells was reduced after GLA treatment (50 μM). This could be due to an effect on an adhesion molecule other than ICAM-1. Again no other reports of effects of GLA on endothelial – monocyte interactions.

PIN treatment at 50 μM significantly reduced soluble ICAM-1 and MCP-1 secretion, with a tendency to reduced RANTES secretion. Others have shown PIN to reduce IL-6 production in LPS-induced murine microglial BV-2 cells [137]. However an effect of PIN on IL-6 was not observed in the current study. Treatment of EA.hy926 cells with PIN at 50 μM was also shown to reduce the adhesion of THP-1 cell to EA.hy926 cell monolayers; again no others report effects of PIN on these types of interactions.

In this study EPA and DHA (50 μM) significantly decreased production of all soluble mediators examined (ICAM-1, MCP-1, IL-6, IL-8, RANTES and VEGF), with the exception of RANTES production after EPA incubation. DHA was also shown to decrease the adhesion of THP-1 cells to stimulated EA.hy926 cells. Many others have observed anti-inflammatory effects of EPA and DHA in a variety of cell types similar to the effects seen within this inflammatory EC model. De Caterina and

Libby demonstrated a decrease in in ICAM-1, IL-6 and IL-8 after n-3 PUFA treatment of ECs [188]. Goua *et al.* saw significant reductions in TNFα-induced ICAM-1 expression in HUVECs after incubation with 25 μM EPA and DHA [150]. Another study examined the effect of EPA and DHA on ICAM-1 mRNA expression in IL-1β activated HUVECs by Northern blot and concluded that EPA and DHA at 65 μM attenuated IL-1β induced ICAM-1 expression in HUVECs [111]. Grenon *et al*. too showed an effect of EPA on EA.hy926 cell adhesion molecule expression although they described little or no effect compared to control cells (TNFα activated) but did argue inhibitory effects compared to n-6 PUFAs [152].

Several studies have also explored effects of EPA and DHA on monocyte adhesion to ECs. Similar to the findings described here, Wang *et al*. demonstrated a reduction of THP-1 cell adhesion to TNFα stimulated HAECs after DHA exposure (80 μM), with no change in monocyte adhesion after EPA exposure [112]. Similar reports were made by Yates *et al*. who describe significant reduction of adhesion of neutrophils to TNFα stimulated HUVECs after DHA treatment (5 μM) with no effects of EPA treatment (5 μM). However, much of the literature describes reduced adhesion after exposure of both EPA and DHA to ECs [164, 172, 173]. Thus an inhibitory effect of DHA treatment of ECs on adhesion of monocytes is consistently reported in the literature and is confirmed here.

The anti-inflammatory effects of EPA and DHA are consistent with data in the literature showing that they slow or prevent atherosclerosis in experimental animal models and lower the risk of mortality from CVD in humans [83].

Where cells were treated with FAs at a concentration of 50 μM there appears to be a reduction in mediator production with increasing chain length but also double bond number, position and orientation. The longer chain FAs (marine-derived) had the greatest overall anti-inflammatory effects while the simplest n-3 FA, ALA, had little effect. Some anti-inflammatory effects were seen after GLA treatment and greater potency was observed with PIN treatment.

Differences observed between the various studies in the literature may be due to FA concentration discrepancy. In this study each FA concentration was checked before use by using GC. This has not been done with studies already reported in the literature and is a significant strength of the current work.

10.3.2 The importance of elongation of GLA and PIN

Anti-inflammatory actions of GLA and PIN within this model were further explored by silencing of elongase 5 within EA.hy926 cells so blocking the production of elongation metabolites of the two FAs.

siRNA was shown to successfully silence ELOVL5 in the cells as shown by 80-90% decrease in relative gene expression. This was shown to result in a reduction in elongation products after incubation of the cells with GLA and PIN in siRNA ELOVL5 silenced cells, as shown by GC analysis. Here it was demonstrated that siRNA silencing of ELOVL5 lead to the inhibition of the ability of EA.hy926 cells to metabolise GLA and PIN to DGLA and ETrA. This provides strong evidence for elongase 5 being directly involved in the elongation of PIN, which has not been previously described.

GLA and PIN at a concentration of 50 μM showed inhibitory effects on inflammatory mediator production by stimulated EA.hy926 cells, confirming previous anti-inflammatory effects of these FAs within this model. All decreases in production of inflammatory mediators were abolished by ELOVL5 silencing other than the effect of GLA treatment on MCP-1 production which remained unchanged in silenced cells. This suggests a direct role for GLA in controlling MCP-1 production. Dirks *et al*. described reduced MCP-1 in Sprague Dawley rats fed GLA in the form of evening primrose oil [210].

Loss of the effects of GLA and PIN in ELOVL5 silenced cells indicates that the anti-inflammatory actions of these FAs described in this model are likely exerted through their elongation products. Direct effects of DGLA or ETrA on ECs have not yet been explored, although DGLA has been shown to significantly prevent atherosclerosis in mice fed a high cholesterol diet [217]. This effect was attenuated by naproxen, a COX-2 inhibitor, suggesting that it was driven by COX-2 and 1-series PG production. Chene *et al*. also reported reduced COX-2 activity in HaCat cells incubated with GLA [191]. These observations indicate that GLA may act via elongation to DGLA and subsequent production of anti-inflammatory eicosanoids via COX-2. ETrA has not been explored in EC models; however Chaung *et al.* describe PIN as resulting in a decrease in both PGE_1 and PGE_2 in LPS stimulated RAW264.7 murine macrophages, but an increase in COX-2 protein levels [179].

The effects of GLA and PIN on inflammation in this model indicate a therapeutic role for these sustainable FAs, but that they mainly act through their elongation

products. Thus, there are likely to be therapeutic actions for DGLA and ETrA. These 20-carbon FAs should be further explored.

10.3.3 Effects of LA:ALA ratio on inflammation

LA is one of the most abundant FAs within current Western diets and is consumed in much larger quantities than ALA, driving an increase in the ratio of LA to ALA. As previously described in section 1.1.3.1, LA and ALA compete for D6D and their metabolites have different biological activities. Metabolism of LA leads to the production of AA, which is a substrate for synthesis of inflammatory eicosanoids, which have been shown to increase in inflammatory conditions [18]. ALA is metabolised to EPA and DHA, and the eicosanoids and docosanoids derived from these have been shown to be less inflammatory or pro-resolving in nature [78, 79]. Therefore it is argued that the increase in dietary LA can lead to a more inflammatory state and predisposition to inflammatory conditions, including atherosclerosis [18].

Altering ratios of LA to ALA within this inflammatory EC model were explored. Incubation with the different FA ratios lead to changes in cell lipid composition compared to control cells (DMEM alone) and to the appearance of specific metabolic elongation products. EA.hy926 cells incorporated LA and ALA in a dose-dependent manner with increasing LA:ALA ratio. ALA treatment alone was shown to decrease production of the majority of the analytes measured (ICAM-1, IL-6, MCP-1, RANTES and VEGF); some decreases were also seen after LA treatment alone (MCP-1 and RANTES). Different ratios of LA to ALA had anti-inflammatory effects with increasing ALA concentration. Few others have described effects of ratios of LA to ALA in ECs [192, 218]. Yang *et al*. describe no changes in IL-6 production by EA.hy926 cells among treatments with several different ratios of LA to ALA (1:1, 5:1, 10:1, 20:1) and at two different total concentrations (20 and 100 μM) [218]. Wang *et al.* describe effects of ALA, LA and different ratios of these FAs in polychlorinated biphenyls (PCB)-induced ECs, they reported increased VCAM-1 and COX-2 expression as well as NF-kB activity in the presence of LA, which was diminished by increasing relative amounts of ALA to LA. PGE_2 levels were also down regulated with increasing amounts of ALA to LA [192].

The inflammatory mediators studied here are important in the progression of endothelial dysfunction and atherosclerosis development. When used at a concentration of 100 μM, ALA was shown to decrease production of many of

these mediators and this indicates a potential role for this plant-derived FAs. Interestingly LA alone was also shown to reduce MCP-1 and RANTES production which may also indicate a role for this FA, although concentrations of LA used within this model are sustainably less than those seen in human studies. Perhaps LA can have some beneficial effects if consumed in lower quantities.

10.3.4 Fatty acid effects on gene and protein expression

Anti-inflammatory effects of the various FAs is likely through the alteration and activation of different inflammatory genes [65] in turn through altered activity of key transcription factors. FAs can regulate the activity of several transcription factors including PPARs [207] and NF-kB [162]. In some cases this can occur through direct binding to the transcription factor and in other cases this can also be achieved via binding of FA metabolites. For example DHA can bind to PPARs [207]. Furthermore FAs can act indirectly on gene expression through either their effects on pathways that involve changes in membrane lipid composition such as G-protein receptor signalling, or their effects on enzyme-mediated pathways such as COX [65, 162, 191].

A time course was conducted to assess the effect of TNFα on inflammatory gene expression and intracellular proteins and from here the effect of FAs was examined.

Treatment with EPA and DHA was shown to induce the greatest fold change on expression of various different genes examined in stimulated EA.hy926 cells. However expression of both pro and anti-inflammatory pathways was altered in EA.hy926 cells after treatment with these FAs indicating a complex role for EPA and DHA in inflammation. Effects on genes and their proteins (e.g. COX-2) were not always in the same direction. Others too have seen different effects of DHA in gene expression compared to protein expression in model systems [190, 191].

Both EPA and DHA increased the gene expression of IL-6 in EA.hy926 cells, despite both of these FAs decreasing secretion of IL-6. Others also describe similar increases in IL-6 gene expression after treatment with EPA at ~16 μM in non-activated EA.hy926 cells [152]. It is possible the IL-6 protein is not translated, or changes in membrane composition after EPA and DHA treatment may lead to the blocking of the secretion of this protein.

DHA and EPA treatment was also shown to upregulate COX-2 gene expression in EA.hy926 cells. Others also observed increased COX-2 gene expression after

treatment of HUVECs with DHA (80 μM) [190] and of human keratinocyte HaCat cells with EPA (20 μM) [191]. However the intracellular protein levels of COX-2 were significantly reduced by EPA and DHA in EA.hy926 cells. Others too report reduced protein quantities of COX-2 after EPA and DHA treatment of HUVECs [162] and HSVECs [196], although some report no changes in COX-2 levels after treatment with EPA or DHA in VEGF stimulated HUVECs [165].

DHA treatment was shown to increase gene expression of PPARα, but had no effect on NF-kB gene expression. However DHA treatment did decrease cellular proteins quantities of NF-kB. EPA treatment resulted in significantly higher ratio of pNF-kBp65/NF-kBp65 than DHA, indicating differences in how these marine-derived FAs affect inflammation. Similarly DHA has been shown to reduce NF-kB activity in HSVECs [196] and HAECs [164]. Likewise Wang *et al*. observed a reduction in phosphorylation of IkBα after DHA (80 μM) and not EPA (80 μM) treatment in TNFα stimulated HAECs [112].

Of the plant-derived FAs only pre-treatment with GLA and SDA had significant effects on inflammatory gene expression; ALA and PIN were shown to have no effect on expression of any of the genes examined. Pre-treatment with PIN was shown to decrease protein levels of COX-2, whereas no other plant-derived FA had effect on COX-2 or NF-kB protein quantities.

GLA significantly increased gene expression of IL-6, with a tendency to increase COX-2 at both 25 and 50 μM. GLA was also seen to significantly reduce PPARα at 25 μM compared to control cells. SDA at 25 μM significantly increased relative IKKB gene expression compared to control cells. There is no previous research on the effects of these plant-derived FAs on gene expression in ECs. Some researchers have reported differences after treatment with ALA and GLA in other cell types. Similar to the effects of GLA in Ea.hy926 cells, others observed increases in COX-2 expression in human keratinocyte HaCat cells [191]. Similar to findings made here, ALA (20 μM) had no effect on COX-2 gene expression in oxidative stress induced porcine ECs [192]. However Erdinest *et al.* described ALA to significantly decrease IkBα and IL-6 expression in LPS-stimulated human corneal epithelial cells [183].

Effects of plant-derived FAs in on COX-2 and NF-kB are not well described. Similar to the findings described here, others describe no changes in COX-2 levels after treatment with either ALA or SDA in HUVECs [165], or ALA treatment of porcine ECs [192]. There are no studies describing effects of GLA or PIN on COX-2 or NF-

kB activity in human ECs. Cao *et al.* describe reduced NF-kB activity in LPS-stimulated primary goat mammary gland epithelial cells after GLA treatment (100 μM) [186].

Together these data suggest complex modulation of endothelial inflammation through FAs. Marine-derived DHA was shown to have potent anti-inflammatory actions, through reduction of inflammatory mediators, cellular inflammatory protein levels as well as cell surface expression of ICAM-1 and adhesion of THP-1 monocytes to EA.hy926 cells. EPA too showed significant anti-inflammatory effects. However gene expression data indicated these FAs to be involved in both pro and anti-inflammatory responses. Interestingly DHA and EPA acted differently on monocyte adhesion, indicating different roles for these FAs in inflammation.

10.4 Final conclusions

The findings of this study show the potency of marine-derived EPA and DHA to control inflammatory processes, which is in line with the literature. Many RCTs demonstrate EPA and DHA supplementation as reducing CVD morbidity and mortality (section 1.3.1), which in turn is likely mediated through changes in cellular membrane compassion that will influence cell and tissue responses. Furthermore changes in endothelial composition can change endothelial-monocyte interactions and thus help to reduce development of atherosclerosis. Results seen here with EPA and DHA support this notion, by reducing inflammatory responses in EA.hy926 cells.

Some of the plant-derived FAs tested have anti-inflammatory effects but they are less potent than EPA and DHA. For example EA.hy926 cells treated with SDA showed a small reduction in ICAM-1 cell surface expression. ICAM-1 plays a critical role in adhesion of monocytes to the surface of the endothelium and may be upregulated in dysfunctional endothelium which may lead to the development of atherosclerosis. Therefore SDA may provide a sustainable source to help to inhibit atherosclerotic progression. Treatment with PIN and GLA also decreased soluble ICAM-1 and MCP-1, both important in inflammation and atherosclerosis. MCP-1 has been described as being responsible for the transmigration of monocytes into the intima at sites of lesion formation. Reduction in these inflammatory mediators could help to alleviate plaque formation. Actions of GLA and PIN on decreasing inflammatory mediators were abolished after blocking EA.hy926 cells ability to create the elongation products DGLA and ETrA from GLA

and PIN, thus indicting a role for these longer chain PUFAs. PIN treatment was also shown to decrease COX-2 protein, which may in turn reduce inflammatory mediator production from AA. Furthermore all plant-derived FAs decreased the adhesion of THP-1 cells to Ea.hy926 cells, again providing evidence for these plant-derived FAs as an alternative source to EPA and DHA in controlling inflammation and perhaps inhibiting atherosclerosis. Furthermore increasing ALA abundance over LA in EA.hy926 cells lead to decreased secretion of many of the inflammatory mediators measured.

Taken together these data indicate that plant-derived FAs could help to reduce the inflammation involved in atherosclerosis. From these findings it is clear that plant-derived PUFAs still need further examination and the mechanisms behind anti-inflammatory properties of GLA and PIN need to be explored.

10.5 Study limitations and future work

Like all pieces of scientific research, the current study has some limitations.

The inflammatory EC model was set up using EA.hy926 cells, an EC line. Use of a cell line may help to limit problems with biological variation which may occur when using primary cells taken from multiple samples/donors, and can in turn lead to experimental variation. EA.hy926 cells showed consistent results when treated with TNFα which allowed for robust exploration of the mechanisms of FAs in stimulated ECs. Whilst these cells do have many of the innate properties of primary ECs [154, 155], they may not act in exactly the same way for all processes. Therefore it would be of interest to repeat these sets of experiments in other cell types including primary HUVECs.

Although effects of FAs on pNF-KBp65 were identified and are likely to be involved in many of the inflammatory outcomes measured, the NF-kB signalling pathway was not fully explored. In particular the levels of different components involved in the NF-kB cascade could be assessed in future research: IKB kinases, IKB, phosphorylated IKB and nuclear NF-kB protein. Gene expression and protein concentrations of these molecules may be assessed by western blotting or RT-qPCR. The effect of these FAs on the PPARα and PPARγ system within ECs could also be further explored again, by western blot.

The intracellular IL-6 protein concentration should be assessed particularly after EPA and DHA treatments, since these FAs were shown to increase IL-6 gene

expression but to decrease secretion of IL-6. It is possible that IL-6 protein is prevented from leaving the cell due to the altered membrane. This may also be explored using western blot and intracellular flow cytometry, in order to examine whether altered membrane composition, after FA treatment, could inhibit the movement of IL-6 out of the cell.

Effects on COX-2 protein were seen but lipid mediators were not assessed. Future work could examine a panel of COX and LOX metabolites, such as prostaglandins, in order to identify effects of plant and marine FAs on these.

The ability of FAs to affect adhesive interactions between THP-1 monocytes and ECs was identified in the current research. The adhesion assay that was developed used static conditions which are not physiological. Thus examining adhesion under conditions of flow will be important in taking this research forward. This could be combined with investigating the ability of MAbs to ICAM-1 and other adhesion molecules to interfere with the adhesive interaction.

It would also be interesting to explore the effects of other stimuli such as LPS or IL-6, on inflammatory mediator production in EA.hy926 cells, and if FA treatment in EA.hy926 cells exposed to other stimuli results in similar outcome as after TNFα stimulation.

Ultimately other cell lines, animal studies and human trials will be needed to clarify the role of plant-derived FAs to control inflammation and atherosclerosis. In this regard GLA, PIN and their elongated derivatives have good potential.

Initially animal studies may be used to examine effects of the different FAs. The plant derived FAs could be incorporated into diets of animals prone to develop atherosclerotic plaques or cardiovascular disease such as apoE or LDL receptor knockout mice, in order to study the potential therapeutic use of these FAs. Furthermore human clinical trials could be developed which may include plant derived FAs as part of a healthy diet.

Inflammation and endothelial dysfunction play a critical role in the development of atherosclerosis and cardiovascular disease. FAs studied in this model show potential to reduce inflammation and restore functionality of EA.hy926 cells, indicating a potential therapeutic role for FAs in slowing atherosclerosis. Marine derived FAs replicated similar anti-inflammatory actions in EA.hy926 as seen in various other studies, whilst novel plant-derived FAs also showed some anti-

inflammatory effects but are less studied, indicating a need for future research into these particular FA.

Appendices

Appendix A

Methods

Flow cytometer set up for BD FACSCalibur

Detector	Voltage	Amp	Mode	Compensation	
FSC	E00	5.24	Lin	FL1	0.7
SSC	427	3.00	Lin	FL2	0.0
FL1	505	1.00	Log	FL2	0.0
FL2	628	1.00	Log	FL3	0.0
FL3	150	1.00	Log		

Reagents used and their sources

Section	Reagent/Material	Supplier	Cat. No.
Cell culture	EA.hy926 ATCC CRL-2922 - Human, somatic cell hybrid, endothelial	LGC standards	CRL-2922
	THP-1 ECACC – THP-1, human monocytic leukaemia	ECACC	88081201
	Dulbecco's Modified Eagle's Medium (DMEM) - high glucose	Sigma	D6546
	Foetal bovine serum	Sigma	F7524
	Penicillin/ Streptomycin/ Glutamine	Sigma	G6784
	HAT media supplement	Sigma	H0262
	Recombinant human TNF-a,	PeproTech EC	300-01A

Flow Cytometry	Foetal bovine serum	Sigma	F7524
	Phosphate Buffer Saline (PBS)	Sigma	
	PE Mouse Anti-Human CD54	BD Biosciences	555511
	BD CellFIX	BD Biosciences	340181
	BD Sheath Solution (FACS Flow)	BD Biosciences	
Luminex Kits	Human Magnetic Luminex Screening Assay (IL-6, IL-8, RANTES, MCP-1, VEGF and ICAM-1)	R&D Systems	LXSAHM-06
PCR	ReliaPrep™ RNA Cell Miniprep System	Promega	Z6011
	GoScript™ Reverse Transcriptase	Promega	A5003
	TaqMan® Gene Expression primers ;- NF-kB (subunit 1) (Hs00765730_m1) PPARα (Hs00947536_m1) PPARg (Hs00234592_m1) MCP1 (Hs00234140_m1) B2M (Hs00187842_m1) GAPDH (Hs02786624_g1)	Thermo Fisher Scientific	4331182

	RPL13A (Hs04194366_g1)		
	TaqMan® Gene Expression Master Mix	Thermo Fisher Scientific	4369016
Western blotting	Optiblot SDS Gel 10% (10 x 10 cm) - 12 Well	Abcam	ab119202
	Anti-COX-2 / Cyclooxygenase 2 antibody [EP1978Y] (1/500 dilution)	Abcam	ab62331
	Anti-GAPDH antibody [EPR16891] (1/10,000 dilution)	Abcam	ab181602
	Anti-NF-kB p65 antibody [E379] (1/10,000 dilution)	Abcam	ab32536
	Anti - NFKB p65 (Phospho s536) antibody [EP2294Y] (1/2000 dilution)	Abcam	ab76302
	Goat Anti-Rabbit IgG H&L (HRP) (1/10,000 dilution)	Abcam	ab205718
	Prism Ultra Protein Ladder (10-245 kDa)	Abcam	ab116028
	SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Fisher Scientific	34580
	4x Laemmli protein sample buffer for SDS-PAGE	Bio-Rad	1610747
Fatty Acids	Linolenic acid (ALA)	Sigma	L2376
	Stearidonic acid (SDA)	Larodan	10-1840

	Y-linolenic acid (GLA)	Sigma	L2378
	Pinolenic acid (PIN)	Cayman Chemical	10008654
	Eicosapentaenoic acid (EPA)	Sigma	E2011
	Docosahexaenoic acid (DHA)	Sigma	D2534
	Linoleic Acid	Cayman	90150
siRNA			
	siRNA dilution buffer	Santa Cruz biotechnology	sc-29527
	siRNA transfection reagent	Santa Cruz biotechnology	sc-29528
	siRNA transfection medium	Santa Cruz biotechnology	sc-36868
	ELOVL5 siRNA	Santa Cruz biotechnology	sc-62269
	Control siRNA-A	Santa Cruz biotechnology	sc-37007
Static adhesion			
	Vybrant™ Cell Adhesion Assay Kit	Fisher Scientific	VXV13181

Appendix B

Results

GC analysis - Cell culture fatty acid composition after 48 hours incubation with various fatty acids

µg/1x10^6 cells	10µM						
	Control	ALA	SDA	GLA	PIN	EPA	DHA
16:0	4.607	4.556	3.900	3.035***	3.565*	3.717*	3.531***
16:1n7	0.334	0.259	0.330	0.220	0.217	0.263	0.236
18:00	4.540	3.452*	3.052***	3.286**	3.268**	3.157****	3.202****
18:1n-9	3.163	1.608***	1.555****	2.330	1.860*	2.496	2.100**
18:2n-6	0.453	0.526	0.459	0.352	0.374	0.400	0.373
PIN	0.000	0.000	0.000	0.000	0.513	0.000	0.000
GLA 18:3n-6	0.101	0.051	0.057	0.269	0.055	0.058	0.026
ALA 18:3n-3	0.056	1.585***	0.013	0.019	0.016	0.017	0.027
SDA 18:4n-3	0.000	0.000	0.356	0.000	0.000	0.000	0.000
ETrA	0.000	0.000	0.000	0.464	2.247****	0.000	0.000
DGLA 20:3n--6	0.276	0.396	0.251	1.583**	0.014	0.276	0.181
20:4n-6	0.760	0.637	0.729	1.091	0.298	0.403	0.381
20:3n-3	0.105	0.750	0.745	1.146	0.527	0.465	0.547
20:4n-3	0.054	0.170	0.366	0.138	0.197	0.120	0.117
EPA 20:5n-3	0.156	0.293	0.951	0.037	0.023	0.637	0.157
DPA 22:5n-3	0.000	1.237*	2.246****	0.516	0.584	3.009****	0.561
DHA 22:6n-3	0.667	0.909	0.789	0.533	0.705	0.594	2.704****

Mean (n=3) Control *vs* FA at 10 µM *p<0.01; **p<0.01; ***p<0.001; ****p<0.0001

µg/1x10^6 cells	50µM						
	Control	ALA	SDA	GLA	PIN	EPA	DHA
16:0	4.607	3.774	3.887	4.063	3.703*	2.936****	2.561****
16:1n-7	0.334	0.164	0.264	0.176	0.191	0.185	0.148
18:00	4.540	3.911	3.560**	3.718*	3.473**	2.868****	2.528****
18:1n-9	3.163	1.262****	1.370****	1.177****	1.429****	1.058****	0.429****
18:2n-6	0.453	0.294	0.357	0.115	0.201	0.263	0.328
PIN	0.000	0.000	0.008	0.000	3.302****	0.000	0.000
GLA 18:3n-6	0.101	0.120	0.097	1.806****	0.000	0.020	0.023
ALA 18:3n-3	0.056	4.485****	0.030	0.073	0.097	0.029	0.000
SDA 18:4n-3	0.000	0.000	0.709	0.000	0.000	0.000	0.000
ETrA	0.000	0.000	0.000	0.000	8.613****	0.000	0.000
DGLA 20:3n-6	0.276	0.352	0.295	7.299****	0.000	0.248	0.245
20:4n-6	0.760	0.887	0.244	1.355	0.279	0.199	0.048*
20:3n-3	0.105	0.776	0.309	0.130	0.118	0.350	0.619
20:4n-3	0.054	0.339	4.107****	0.000	0.000	0.066	0.000
EPA 20:5n-3	0.156	0.293	1.616****	0.458	0.620	2.135****	0.000
DPA 22:5n-3	0.000	0.835	1.786****	0.175	0.155	4.149****	0.594
DHA 22:6n-3	0.667	0.409	0.361	0.632	0.565	0.220	2.807****

Mean (n=3) Control *vs* FA at 50 µM; **p<0.01; ***p<0.001; ****p<0.0001

Appendix C

Results

GC analysis - Fatty acid composition of complete culture medium

µg/mL	DMEM	DMEM	DMEM	Ave.
16:00	8.186	6.236	5.478	6.633
16:1n7	0.514	0.398	0.433	0.449
18:00	6.387	3.955	3.464	4.602
18:1n-9	6.205	5.185	4.519	5.303
18:2n-6	1.111	1.046	0.933	1.030
PIN	0.000	0.000	0.000	0.000
GLA 18:3n-6	0.035	0.041	0.025	0.034
ALA 18:3n-3	0.103	0.058	0.050	0.070
SDA 18:4n-3	0.000	0.000	0.000	0.000
ETA	0.000	0.000	0.000	0.000
DGLA 20:3n--6	0.571	0.506	0.440	0.506
20:4n-6	1.413	1.469	1.307	1.396
20:3n-3	0.000	0.000	0.000	0.000
20:4n-3	0.050	0.063	0.059	0.057
EPA 20:5n-3	0.247	0.239	0.210	0.232
DPA 22:5n-3	0.000	0.000	0.000	0.000
DHA 22:6n-3	1.029	1.062	0.868	0.987

Example FA compositions from complete culture medium (DMEM) and mean (n=3)

GC analysis - Fatty acid stock concentrations and calculations

Area	ALA	ALA	SDA	SDA	GLA	GLA	PIN	PIN	EPA	EPA	DHA	DHA
x	298.046	258.151	204.966	200.265	190.971	204.836	189.609	201.719	171.951	181.435	211.807	196.663
21:0 Standard	392.392	357.375	296.635	309.69	337.197	337.736	318.126	332.238	285.318	300.05	347.65	318.97
21.0 std	30	30	30	30	30	30	30	30	30	30	30	30
21.0 area/added	13.07973	11.9125	9.887833	10.323	11.2399	11.25787	10.6042	11.0746	9.5106	10.00167	11.58833	10.63233
µg per 800 µL	ALA	ALA	SDA	SDA	GLA	GLA	PIN	PIN	EPA	EPA	DHA	DHA
x	22.78686	21.6706	20.72911	19.39988	16.99045	18.19492	17.88056	18.21456	18.07993	18.14048	18.27761	18.49669
µg per mL	ALA	ALA	SDA	SDA	GLA	GLA	PIN	PIN	EPA	EPA	DHA	DHA
x	28.48357	27.08825	25.91139	24.24985	21.23807	22.74365	22.3507	22.7682	22.59991	22.6756	22.84701	23.12087

Vials	µg/Ml	mg/Ml	MW	mM	µM	Average	SD
ALA	28.48357	0.028484	278.43	0.102301	102.3007		
ALA	27.08825	0.027088	278.43	0.097289	97.28926	99.79495	3.543591
SDA	25.91139	0.025911	276.41	0.093743	93.74259		
SDA	24.24985	0.02425	276.41	0.087731	87.73147	90.73703	4.250506
GLA	21.23807	0.021238	278.43	0.076278	76.27794		
GLA	22.74365	0.022744	278.43	0.081685	81.68535	78.98165	3.823615
PIN	22.3507	0.022351	278.43	0.080274	80.27402		
PIN	22.7682	0.022768	278.43	0.081774	81.77353	81.02378	1.060312
EPA	22.59991	0.0226	302.49	0.074713	74.71293		
EPA	22.6756	0.022676	302.49	0.074963	74.96313	74.83803	0.176913
DHA	22.84701	0.022847	328.49	0.069552	69.55161		
DHA	23.12087	0.023121	328.49	0.070385	70.3853	69.96845	0.589504

FAs were then diluted to appropriate stock concentrations based on GC analysis.

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