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OXIDISED FATTY ACID DERIVATIVES AND THEIR
PUTATIVE RECEPTORS IN ATHEROSCLEROSIS AND
DIABETES

Thesis submitted by
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FEBRUARY 2011

for the degree of Doctor of Philosophy
in the School of Medicine and Dentistry
James Cook University, QLD
AUSTRALIA

Declaration

I, the undersigned declare that this research investigation was carried out on my own and it has not been previously submitted anywhere for another degree or diploma at any university or institution of tertiary education in or out of Australia. Information derived from the published or unpublished works of others has been acknowledged in the text and a list of references is given.

Venkat N. Vangaveti

February 2011

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Declaration on Ethics

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human* (1999), the *joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimental Ethics. Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimental Ethics Review Committee approval number **H3992**

Venkat N. Vangaveti

February 2011

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Abstract

Atherosclerosis, a disease of the arteries is one of the leading causes of mortality throughout the world. A variety of risk factors contribute to this cardiovascular disease including genetic factors, diet, lack of exercise and other lifestyle changes that have become prevalent during the last few decades. These risk factors contribute to two important characteristic features; increased levels of lipids in the arterial wall and oxidative stress – both leading to accumulation of oxidised lipids. Once modified, lipids are reactive with arterial endothelial cells, vascular smooth muscle cells and macrophages. The latter are one of the primary cell types that are attracted to sites of inflammation in the arterial wall, and are derived from circulating monocytes. Macrophages have immune functions and phagocytic properties enabling them to take up oxidised lipids. These latter molecules are known to be key regulators of atherosclerosis. Understanding the fundamental mechanisms of interaction between oxidised lipids and macrophages might enable us to modulate metabolic processes involved in atherosclerosis. Two components of oxidised lipids are the hydroxy-derivatives of linoleic acid, derived by action of the enzyme lipoxygenase during early stages, and non-enzymatically during the later stages of atherosclerosis. The products of this oxidation are 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE). The nature of their interaction with macrophages and how they influence key processes involved in disease progression are not completely understood. We therefore, tested the hypothesis, in this study, that 9-HODE and 13-HODE would have differing effects on monocytes and macrophages in regulating atherosclerosis. We aimed to test the effects of HODEs and their parent fatty acid linoleic acid and the n-3 fatty acid α -linolenic acid on cell proliferation, differentiation, genes of lipid metabolism and generation of inflammatory mediators in THP-1 monocyte-macrophage cells. We wished to establish whether HODEs might act through the recently described G protein-coupled receptors for long-chain fatty acids GPR120 and GPR132. This study investigates regulation of expression of these receptors in THP1 cells and in primary monocyte cultures from diabetic patients where they were studied alongside serum markers for cardiovascular risk.

HODEs decreased cell viability in both monocytes and macrophages in a dose-dependent manner. This was found to be a result of apoptosis, an effect not seen with α -

linolenic and linoleic acids. Gene expression studies revealed increased expression of the intracellular lipid transporter FABP4 in both monocytes and macrophages with HODEs. Also, an increase in expression of scavenger receptor B (decreasing lipid burden) with HODEs in macrophages was observed. These processes were accompanied by an increase in expression of the transcription factor peroxisome proliferated-activated receptor- γ (PPAR- γ), which regulates inflammation. GPR132 was highly up-regulated by HODEs in monocytes, while HODEs decreased expression of GPR120 in macrophages. None of these effects were observed with α -linolenic acid or linoleic acid. Similar effects of HODEs on lipid regulatory genes were documented to occur during macrophage differentiation.

Overall, studies with Oil Red O and gene expression suggested that HODEs enhance processes that are associated with reduction of lipid burden. Further studies with HODEs demonstrated that they are involved in regulating secretion of pro-inflammatory cytokines with effects mediated through both PPAR- γ and GPR132. By decreasing both lipid accumulation and vascular inflammation, HODEs may thus have an overall protective effect as far as atherosclerosis is concerned. The mechanisms underlying these protective effects was investigated using specific PPAR- γ antagonists and by silencing the GPR132 gene. Here, successfully for the first time GPR132 was silenced in THP-1 macrophages. Experiments with HODEs revealed a partial role for PPAR- γ in regulating expression of FABP4, and also that regulation of inflammatory mediators by HODEs was largely GPR132 independent. Expression of GPR132 was also investigated in primary monocytes from diabetic subjects. Mononuclear cells from diabetic subjects expressed higher levels of differentiation markers CD14, CD54 and CD36 (identified using flow cytometry) compared with normal controls. Gene expression studies revealed increased expression of GR132 in monocytes from diabetic subjects. Furthermore, this correlated with increased serum levels of inflammatory proteins, indicating that increased GPR132 might be associated with increased vascular risk.

This study shows that components of the oxidised lipid in atheroma, the hydroxy-octadecadienoic acids, may be involved in regulating disease progression by promoting development of macrophages with protective phenotype. Effects of the two HODEs were broadly similar in THP1 cells, but findings of this study were consistent with the notion that 13-HODE might exert protective effects in early atherosclerosis while the

combination of 9-HODE and 13-HODE in later stages may promote atheroma formation. The receptors GPR120 and GPR132 were shown, for the first time, to be expressed on THP-1 cells using immunohistochemistry. In this work, we have examined the role of GPR132 in the control of inflammation mediated by HODEs. These oxidised lipids appear to regulate the differentiation of monocytes into a protective M2 phenotype. HODEs are abundant in atherosclerotic plaque, and have important regulatory functions. This work suggests that measurement of HODEs or expression of the receptors for long-chain fatty acids may be significant biomarkers for the atherosclerotic process. Perhaps more important, the effects of HODEs through cell surface G protein-coupled receptors and nuclear transcription factors may represent novel therapeutic targets leading to development of treatments that retard the process of atheroma formation.

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Chapter 1

Introduction and Literature Review

Overall Aim of the Thesis

Lipid accumulation in the vascular wall and oxidative stress are two key processes in atherosclerosis. They lead to accumulation of oxidized lipids at sites of vascular damage and inflammation. Oxidized lipids, particularly oxidized low-density lipoprotein (oxLDL), have long been known to regulate many of the cellular processes involved in atheroma formation. However, relatively little is known about which components of the oxidized lipid fraction are involved in regulating atherosclerosis, and which signalling processes mediate these functions. Hydroxyoctadecadienoic acids (HODEs) are stable oxidation products of the omega-6 (n-6) fatty acid linoleic acid (LA). HODEs are abundant in atherosclerotic plaque and, indeed are the most abundant fatty acids at sites of vascular damage. In this thesis, I examined the effects of HODEs (9-HODE and 13-HODE) on monocytes and macrophages. My interest in this area was stimulated by two recent developments:

1. Fatty acids have been shown to have specific signalling functions. Cao et al.¹ used a lipidomic approach to show that palmitoleate regulated insulin sensitivity in mice. This paved the way for study of a new class of hormone that they termed “lipokines”.
2. A series of G protein-coupled receptors (GPCR) for which fatty acids are the natural ligands has been identified and characterized.^{2, 3} These GPCR have specificities for fatty acids of differing chain length and degree of saturation. GPR120 and GPR132 are receptors for long-chain fatty acids.

Macrophages in the arterial wall are derived from circulating monocytes, and regulate the responses to lipid accumulation and oxidative stress. In this thesis, I have examined the effect of HODEs on macrophage cell number, apoptosis, genes involved in lipid trafficking, and generation of inflammatory mediators. Previous studies^{4, 5} have shown that HODEs can affect some of these processes in cells of monocyte/macrophage lineage. I set out to examine whether the two major HODEs, 9-HODE and 13-HODE, had similar or differing effects. GPR132 has been identified as a receptor for 9-HODE in keratinocytes but this receptor does not interact with 13-HODE. It is therefore possible that 9-HODE and 13-HODE have distinct regulatory functions. I have also

compared the effect of HODEs with that of other C18 fatty acids – the n-6 fatty acid LA and the n-3 fatty acid α -linolenic acid (ALA). The previous literature has not considered possible differences between monocytes and cells that have been differentiated into macrophages. In this study, I have examined these differences using the human monocytic cell line THP-1. I have also investigated whether the receptors GPR120 and GPR132 could be useful markers of monocyte activation both in THP-1 cells and in primary cultures of monocytes from patients with diabetes and obesity. These studies add to the body of knowledge on how the processes involved in atherosclerosis are regulated. They may ultimately help identify new drug targets and novel markers of susceptibility in high-risk groups such as patients with diabetes.

Atherosclerosis and Clinical Background

Cardiovascular disease is a leading cause of death in the world, ⁶ and a major health concern for Australia. Heart, stroke, and vascular disease accounted for 50,294 deaths (37.6% of all deaths) in Australia in 2002 – more than any other disease group. ⁷ Atherosclerotic vascular diseases affect nearly 4 million Australians, and more than a million are considered to be disabled because of these conditions. Although life expectancy is improving for some cardiovascular disorders because of advances in medical care, the prevalence of heart, and vascular diseases has increased. This increase amounted to 18.2% in the decade 1994 – 2004, and the prevalence of cardiovascular disorders will continue to increase because of the aging population and the high prevalence of cardiovascular risk factors. Indigenous Australians are particularly vulnerable to heart, stroke and vascular diseases. ⁸ Compared with non-Indigenous Australians, those of Aboriginal or Torres Strait Islander descent are 2 – 3 times more likely to die from, or be hospitalised because of, coronary heart disease, cardiac failure, or stroke. ^{7, 9} The reasons for this increased risk in Indigenous Australians include increased prevalence of risk factors such as obesity, diabetes, harmful levels of alcohol intake, and renal disease. Health-care expenditure on cardiovascular diseases was \$5.9 billion in Australia in the year 2004-2005, amounting to 11% of total expenditure of the health system. ^{10, 11}

The classic risk factors for atherosclerosis are dyslipidaemia, diabetes, obesity, smoking, hypertension, lack of exercise and a positive family history. In addition, psychosocial factors such as depression, stress, social isolation and lack of social support contribute to increased cardiovascular disorders.¹² The clustering of dyslipidaemia, hypertension, abdominal obesity and glucose intolerance is known as “the metabolic syndrome” and now affects one in four adults. The prevalence of cardiovascular risk factors is high in Australia: 60% of adults is overweight (7.4 million); 51% have high blood cholesterol (6.4 million) and 30% have high blood pressure (3.7 million); Diabetes now affects 8% of adults, and the prevalence continues to increase; Less than half of the population are sufficiently active to protect their health, 20% of the population continue to smoke, and 10% drink alcohol to a degree that is harmful to health. Smoking has decreased slightly over the past decade, and the present government is taking active steps to reduce this even further. Control of hypertension has improved through wider use of effective prescription drugs. However, other risk factors have not improved. In particular, the prevalence of obesity, physical inactivity, and diabetes continues to increase while there has been no improvement in prevalence of hyperlipidaemia.⁷

The American Heart Association and Centers for Disease Control have very recently published their 2011 review of the prevalence of cardiovascular disease in the United States.¹³ Heart, stroke or other vascular disorders accounted for 1 of every 2.9 deaths. More than 2,200 Americans die from cardiovascular disease each day – one death every 39 seconds. Most of these deaths are premature. Coronary heart disease accounts for one in six deaths in the United States. Each year, an estimated 785,000 Americans have a new coronary event while 470,000 suffer a recurrent event. On average, there will be one coronary event affecting an American citizen every 25 seconds. Each year, approximately 795,000 Americans will suffer a stroke – one every 40 seconds. As in Australia, the prevalence of cardiovascular risk factors remains high with obesity and diabetes on the increase. Hypertension is particularly common, and poorly controlled, in African Americans. Diabetes affects 8% of the American population with African Americans, Mexican Americans, those of Hispanic or Latino descent, and other minority ethnics groups being particularly susceptible. Many Americans (23.1% of men and 18.3% of women) choose to continue smoking. Cardiovascular disease places an

enormous burden on the health and social care systems. Annual direct and indirect costs in the United States are estimated at \$286 billion.

A key feature in the pathogenesis of atherosclerosis is accumulation of lipids. This and oxidative stress in the arterial wall gives rise to a complex interaction between oxidized lipids, inflammatory secreted factors, endothelial cells, vascular smooth muscle cells (VSMC) and macrophages. Although atherosclerosis is known to be a lipid accumulative disorder, its pathogenesis is not fully understood. Of particular interest is the role of macrophages which are involved in mature complex plaque that may rupture or erode, and which may lead to an acute coronary syndrome or ischaemic stroke. Their role in intracellular accumulation of oxidised lipids and foam cell formation has been studied extensively, but the nature of their interactions and signalling mechanisms involved remain to be fully elucidated.¹⁴ This study aimed to investigate the effects of two oxidized lipids, namely the hydroxyoctadecadienoic acids, which are stable oxidative products of linoleic acid. Work on monocytes and macrophages included studies on cell proliferation, transmembrane signalling receptors, transcription factors, and fatty acid metabolism. This Chapter begins with the pathogenesis of atherosclerosis and associated lipid abnormalities. This is followed by classification of fatty acids, generation of oxidised lipids, and the effect of different classes of fatty acid on human health. Interaction between various cell types and oxidised lipids with specific reference to macrophages is reviewed in the latter half of the Chapter followed by detailed aims of the work carried out for this thesis.

Atherosclerosis is a disease of hardening of arteries due to accumulation of fat deposits and inflammation. Lipid accumulation initially as fatty streaks is a characteristic feature observed in intima of arteries, leading to its degeneration.^{15 16} Multiple factors contribute to the development of fatty streaks including interaction with circulating oxidized lipoproteins, hemodynamic factors and interactions with various cell types including macrophages, vascular smooth muscle cells (VSMC), endothelial cells and T lymphocytes. Other classic risk factors include hypertension, insulin resistance, obesity and dyslipidemia.¹⁷ This changes the endothelial permeability leading to migration of monocytes into the subendothelial space thereby increasing retention of low-density lipoprotein (LDL) (which is subsequently transformed into minimally modified LDL and eventually into oxLDL). Normally, LDL which enters the subendothelial space is

dynamically transported back out. However, in developing atherosclerotic lesions, it becomes adherent to proteoglycans and is then taken up by macrophages.⁶ (Figure 1.1). The role of the macrophage in pathogenesis of atherosclerosis is a complex area of study. They have been implicated during the initial and progressive stages. Monocyte-derived macrophages take up lipids (oxLDL) via a family of transmembrane scavenger receptors. Release of monocyte chemoattractant protein-1 (MCP-1), further increases attraction of monocytes to sites of inflammation.¹⁸ Lipid uptake in the form of oxidized and acetylated lipoproteins results in formation of foam cells. These cells have a role in eliminating excess lipids through ATP-binding cassette transporters favouring binding of high-density lipoproteins (HDL). Increased levels of oxLDL in the circulation of high-risk individuals result in increased inflammation leading to migration of VSMC from the tunica media to the intima of arteries, along with altered secretion of extracellular matrix. This is the basis for the atherosclerotic plaque, which subsequently undergoes calcification and renders the artery walls both rigid and fragile. When plaque ruptures, there is formation of thrombus, the artery becomes blocked and the tissue supplied by the artery becomes ischaemic.^{15, 19}

It is vital to understand the role of oxLDL (which contains oxidized forms of long chain fatty acids) and their interactions with the various cell types involved in atherogenesis. Of the above cell types, monocytes and macrophages are of considerable interest because of their extensive involvement throughout the process of atherogenesis. Modifying the activity of pathways regulated by fatty acid derivatives may lead to development of novel therapeutic approaches. Such a strategy is supported by the recent description of a lipokine (palmitoleic acid) that enhances insulin sensitivity.¹ Furthermore, measurement of oxidised fatty acids or receptors for long-chain fatty acids (LCFA) may be useful as a marker for high risk of vascular disease – including in patients with diabetes.

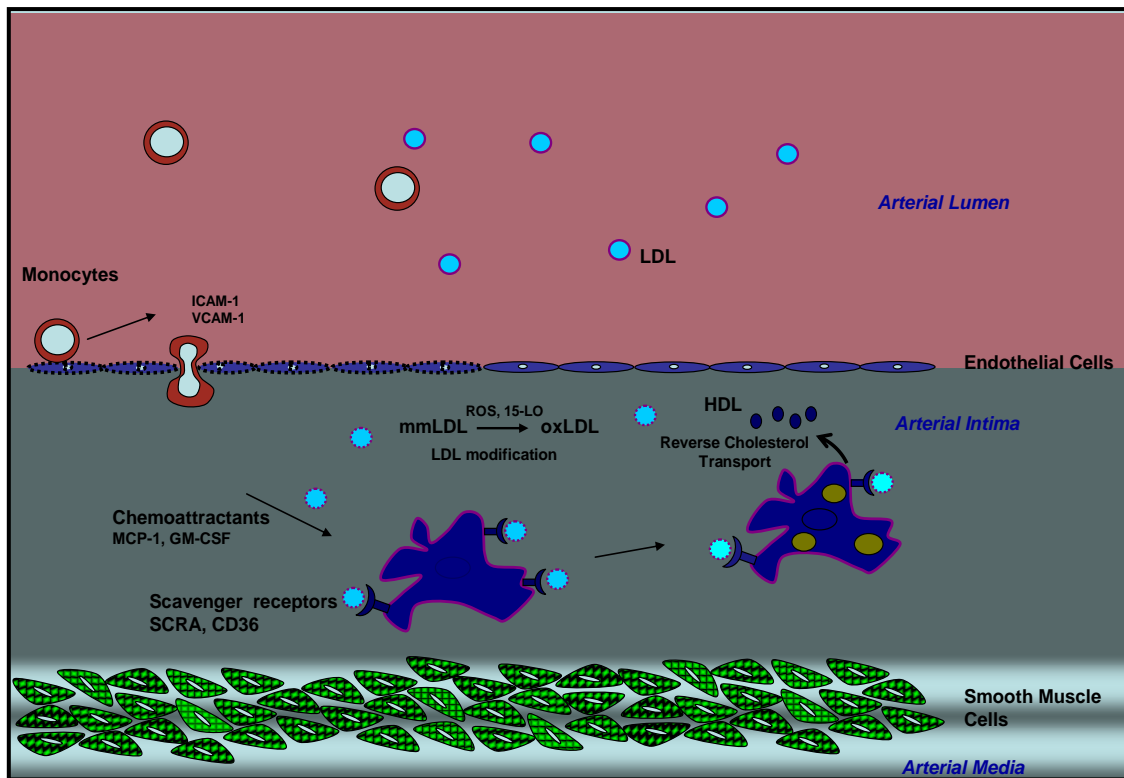


Figure 1.1 Foam Cell Formation

Changes in the intimal region of the artery during foam cell formation. Decreased levels of antioxidants and increased levels of LDL could trigger damage to endothelium. Increase in adhesion molecules (ICAM-1 and VCAM-1) allow the circulating cells to adhere and enter into the intimal layer. When activated monocytes differentiate into macrophages, they release chemoattractant factors (e.g. MCP-1) thereby recruiting more leucocytes. Macrophages in the arterial wall then take up modified lipids through scavenger receptors. Excess lipids are effluxed to enhance formation of HDL. When unable to process the increased levels of LDL these cells accumulate lipids and turn into foam cells.^{15 6, 19, 20}

Modification of LDL and Components of Atherosclerotic Lesions

Under normal circumstances, cholesteryl ester carrying LDL pass through the intimal layer. Antioxidants such as α -tocopherol (vitamin E),²¹ help to prevent oxidation of LDL in the circulation.²² Other important antioxidants include γ -tocopherol, α and β -carotene, lycopene, ubiquinol-10.²¹ In a complex process, which is not clearly understood (but which involves macrophages, VSMC and endothelial cells), LDL-cholesterol undergoes oxidation resulting in minimally modified low-density lipoprotein (mm-LDL) and subsequently further oxidation to ox-LDL. Two major factors that promote these processes are increased lipid accumulation and oxidative stress. Low antioxidant status leads to modification of native LDL into the oxidised form. A high saturated fatty acid rich diet contributes to higher levels of LDL in the circulation. High levels of free radicals arise due to various factors including smoking, diabetes and hypertension.²³ Lipids modified under conditions of oxidative stress contribute to attraction of monocytes (which differentiate into macrophages) and T lymphocytes to sites of inflammation. At later stages, increased smooth muscle cells also containing lipid droplets are observed where there is vascular damage (Figure 1.2). The resulting yellow lipid deposit is described as a “fatty streak” – the precursor to an atherosclerotic plaque.²⁴

Studies conducted to determine the composition of fatty streak in human aorta have shown that the major component is cholesterol with relatively lower amounts of phospholipids and triglycerides. Most of the cholesterol is esterified. Adjacent normal tissues have far less cholesterol, but similar levels of triglycerides to those present in fatty streak.²⁵ Lang and Insull²⁶ showed that lipid droplets isolated from fatty streaks contained up to 95% cholesteryl esters, and less than 2% of phospholipids, triglycerides and free cholesterol. Further examining the percentage of individual fatty acids in the lipid droplets using gas-liquid chromatography, the same study revealed high amounts of oleic acid, and 15% linoleic acid. Studying composition of atherosclerotic plaque and examining fatty acids and their derivatives, it was observed that principal products were oxidised derivatives of arachidonic acid (AA) – hydroxyeicosatetraenoic acids (HETEs), and LA derivatives - HODEs and oxo-octadecaenoic acids (oxoODEs). HODEs in particular varied in amounts depending on the condition of the plaque (Table 1.1).²⁷

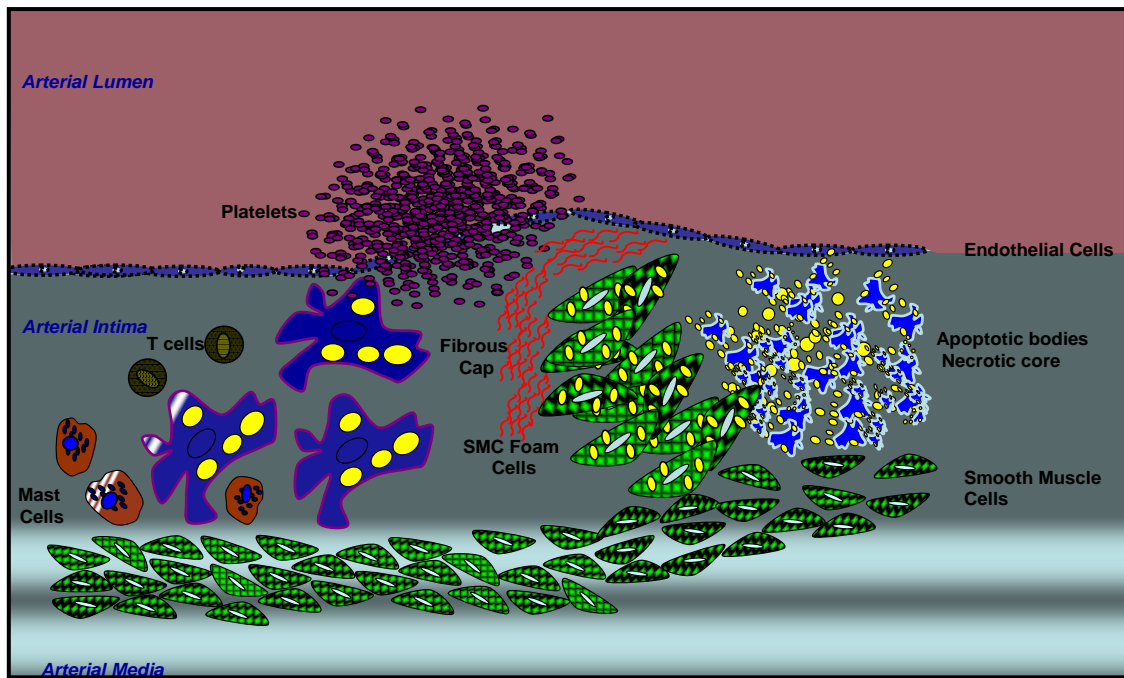


Figure 1.2 Advanced Stages of Atherogenesis

With progression of atherogenesis, increased foam cells and a variety of cells including mast cells, T cells and platelets are observed at the site of the lesion. The smooth muscle cells also contain lipid droplets. The necrotic core consists of apoptotic bodies surrounded by VSMCs and formation of a fibrous cap.^{6, 15, 19, 20}

<i>Composition of Fatty Streaks</i>	
Total cholesterol	23 mg/g
Percentage esterified cholesterol	65 %
Phospholipids	5 mg/g
Triglycerides	1.2 mg/g
<i>Components of Lipid droplets of fatty streak (%)</i>	
Total cholesterol	95
Percentage esterified cholesterol	1.7
Phospholipids	1
Triglycerides	2.5
<i>Fatty acids in Lipid droplets of fatty streak (%)</i>	
Oleic acid	50
Linoleic acid	15
Palmitic acid	9
Palmitoleic acid	6
Eicosatrienoic acid	7
Arachidonic acid	7
Myristic acid	2
Stearic acid	2
<i>Composition of oxidised products</i>	
Total HODEs and oxoODEs	20 ng/μg LA
Total HETEs, oxoETEs	13 ng/μg of AA
<i>HODEs in Different Plaque (ng/μg Linoleic acid)</i>	
Lipid of advanced plaque	11
Calcified plaque	15
Plaque with Thrombosis	13
Fibrotic advanced plaque	28

Table 1.1 Compositions of Fatty Acids in Atherosclerotic Plaque

A major percentage of cholesterol in fatty streak is esterified, unlike in lipid droplets. Linoleic acid and its oxidised products HODEs are present in high concentration in fatty streak and advanced stages of atherosclerosis. ^{24, 25, 27}

n-3 and n-6 Fatty Acids

Fatty acids are composed of hydrocarbon chains with varying degrees of unsaturation and terminating with a carboxylic acid group. Saturated fatty acids do not contain any double bonds. These fatty acids form straight chains that are densely packed, assisting with easy energy storage in living organisms. The shortest fatty acid is formic acid (C1). The most studied short-chain fatty acid is butyric acid, which is a 4-carbon compound (C4). Fatty acids with double bonds are either mono- or poly-unsaturated. Broadly, fatty acids (FA) are classified according to the number of carbons present. FA with up to 8 carbon chains are short chain fatty acids (SCFA), 8-14 carbons are medium chain fatty acids (MCFA), and more than 14 are long chain fatty acids (LCFA).²⁸ Depending on the adjacent carbon on either side of double bond, they can either be in the *cis* or the *trans* configuration. When adjacent carbons are on the same side of double bond, they are said to be in the *cis* configuration, and if they are on opposite side it is said to be in the *trans* configuration. A *cis* configuration induces a bend in the chain resulting in restricted conformational freedom. FAs with *trans* configuration are also called trans fats, and they have similar properties to saturated fatty acids. This difference in the structure plays an important role when considered in complex biological systems such as cell membranes. Depending on the position of the double bond from the methyl end of chain, fatty acids can be classified as n-x or ω -x (ω = omega) for a fatty acid that has the double bond at xth carbon from methyl end of the chain. For example, α -linolenic acid is an 18 carbon n-3 polyunsaturated fatty acid with 3 double bonds.²⁹

Two main families of long chain polyunsaturated fatty acids (LC-PUFA) have been extensively studied namely the n-3 and the n-6 fatty acids. These fatty acids have been widely studied because of their distinctive biological properties. Linoleic acid (18:2n6) and α -linolenic acid (18:3n3) are parent PUFA that were collectively known as Vitamin F in the 1920s. They are now widely known as LC-PUFA. Other LC-PUFA are derived from these two parent fatty acids, although ALA is poorly metabolised in humans.³⁰ LC-PUFA are derived from ALA and LA as follows:

From α -linolenic acid (18:3n3)

1. Eicosapentaenoic acid (EPA) (20:5)
2. Docosahexaenoic acid (DHA) (22:6)

From linoleic acid (18:2n6)

1. Gamma-linolenic acid (GLA) (18:3)
2. Dihomo-gamma-linolenic acid (DGLA) (20:3)
3. Arachidonic acid (AA) (20:4)

n-6 LC-PUFA sources include safflower oil, peanuts, corn oil and soybean.³¹ A combination of both n-3 and n-6 LC-PUFA is considered important since they have an effect on eicosanoids with hormonal and local regulatory activity. The ideal ratio of intake of n-3 and n-6 has been under considerable debate since studies show that both these fatty acids compete for metabolic enzymes required for production of hormones. In addition, a higher ratio of n-3 to n-6 is beneficial to the body as high consumption of n-6 leads to changes that favour inflammation, tissue damage, thrombus, cancer, osteoporosis and high mortality due to coronary heart disease.^{32,33} Over the years, there has been a general increase in consumption of n-6: n-3 ratio from 1:1 in primitive diets to 17:1 in modern diets. Studies show that lower n-6 consumption in certain regions - e.g. Mediterranean diet is associated with lower mortality rates from heart disease.³⁴ In Israel, increased consumption n-6 in preference to saturated fats resulted in a higher incidence of cardiac complications – this was known as “Israeli paradox”.³⁵ Saturated fatty acid consumption increases LDL cholesterol in plasma and is therefore pro-atherogenic,³⁶ while monounsaturated fatty acids (MUFA) have been found to decrease LDL, and are therefore considered beneficial.³⁷ A recent position statement by the American Heart Association has considered the available clinical and epidemiological evidence in this area.³⁸ It is recommended that 5- 10% of energy intake should be from n-6 fatty acids, but this intake must be combined with other lifestyle measure to decrease cardiovascular risk including adequate intake of n-3 fatty acids.

Fish living in cold water (oily fish) are the major source of n-3 PUFA, with docosapentaenoic acid (DPA), docosahexaenoic acid (DHA), and eicosahexaenoic acid (EHA) being the major FAs associated with health benefits.^{39,40} ALA is derived from

plants including walnut, flaxseed, and soybean and also has health benefits but is not as protective against cardiovascular disease as are the marine-derived n-3 PUFA. It is currently recommended that all adults consume 500 mg per day of DHA/EPA either as oily fish (2 – 3 serves per week) or as supplements. A daily intake of ALA of 2 grams is also recommended. 1000 mg per of marine PUFA is recommended for those with established coronary heart disease, while those with hypertriglyceridaemia may require up to 4,000 mg per day. The beneficial effects of n-3, and to an extent n-6, FAs on plasma lipid profile have been extensively documented.⁴¹⁻⁴³ Marine n-3 PUFAs decrease serum triglycerides by 20 – 30%, modestly increase HDL-cholesterol but do not decrease LDL-cholesterol. The benefit is largely from improved metabolism of VLDL to IDL and LDL.⁴⁴ Consumption of diets rich in marine n-3 fatty acids have been shown to decrease risk of cardiovascular events.³⁹⁻⁴³ Furthermore, the high incidence of sudden cardiac death that occurs after myocardial infarction⁴⁵ is reduced by administration of marine n-3 fatty acids. Potential benefits of n-3 fatty acids have also been reported in a range of other disorders: attention deficit/hyperactivity disorder,⁴⁹ in animal models of Parkinson's disease,⁵⁰ Alzheimer's disease,⁵¹ depression,^{52 53} and hypertension.⁵⁴ They have also been shown to enhance maturation of the immune system in infants.⁵⁵ At the cellular level, n-3 FAs alter the lipid composition of cells membranes, affect the activity of a number of enzymes involved in carbohydrate and lipid metabolism, and increase mitochondrial beta oxidation. The cardiovascular benefits of n-3 supplementation are not only through their effect on plasma lipid profile – they also improve endothelial function, modulate inflammatory responses, decrease plasma leptin, lower blood pressure, and increase the threshold for ventricular arrhythmias through effects on cardiac ion channels. The biological and clinical properties of n-3 fatty acids has recently been reviewed.⁴⁴

Consumption of diets rich in n-3 fatty acids e.g. fish oil, which contains EPA and DHA decrease cardiovascular risk.⁴⁵⁻⁴⁹ Omega-3 fatty acid consumption reduces the serum tryglyderide levels by 20-30% and increases the LDL-cholesterol levels by 5 to 10% and HDL-Cholesterol by 1 to 3%. The benefit is largely from improved metabolism of VLDL to IDL and LDL.⁵⁰ A high incidence of sudden cardiac death is known to occur in patients who survive myocardial infarction.⁵¹ Post-infarction management includes increased intake of fish oils, which has shown to benefit patients reducing risk from sudden cardiac death.⁵²⁻⁵⁴

n-3 fatty acids may also be beneficial in managing attention-deficit/hyperactivity disorder.⁵⁵ Treatment with n-3 PUFA has been reported to have favourable effects in animal models of Parkinson's disease⁵⁶ and Alzheimer's disease,⁵⁷ in reducing depression,^{58, 59} managing hypertension,⁶⁰ and in the maturation of the immune system in infants.⁶¹

The principal aim of this study was to investigate the effects of ALA (n-3), LA (n-6) and the LA derivatives HODEs (9-HODE; n-6 and 13-HODE; n-7) on monocytes and macrophages, along with their mechanism of action. Understanding the mechanism may help us design therapeutics that could be used for managing risks associated with cardiovascular diseases. Some of the structures of fatty acids are shown in Figure 1.3.

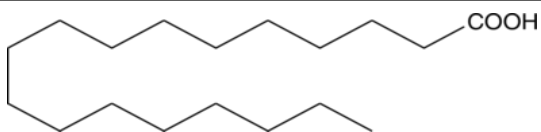
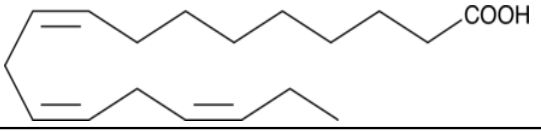
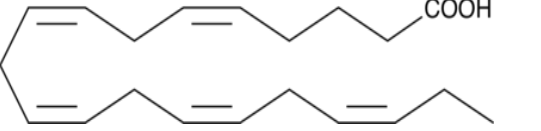
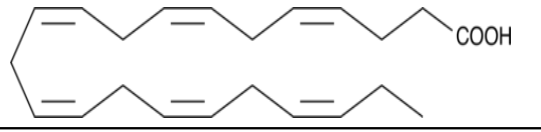
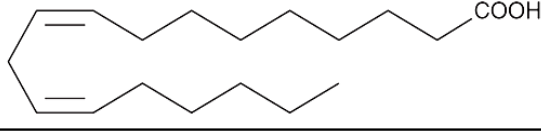
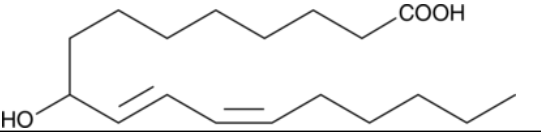
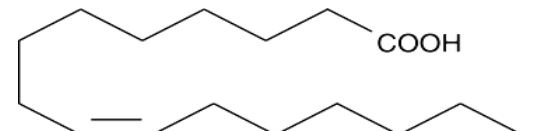
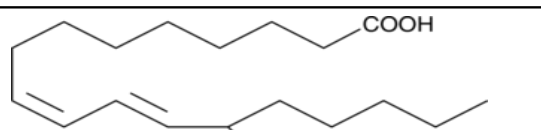
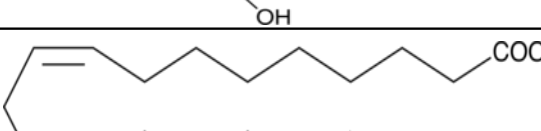
Fatty Acid	Structure
Stearic Acid Octadecanoic Acid $C_{18}H_{36}O_2$	
α-Linolenic Acid (n-3) 9c,12c,15c-Octadecatrienoic Acid $C_{18}H_{30}O_2$	
EPA (n-3) 5,8,11,14,17-Eicosapentaenoic Acid $C_{20}H_{30}O_2$	
DHA (n-3) 4,7,10,13,16,19-Decosahexaenoic Acid $C_{22}H_{32}O_2$	
Linoleic Acid (n-6) 9c,12c-Octadecadienoic Acid $C_{18}H_{32}O_2$	
9-HODE (n-6) 9-Hydroxy-10,12-Octadecadienoic Acid $C_{18}H_{32}O_3$	
Palmitoleic Acid (n-7) (9Z)-hexadecenoic acid $C_{16}H_{30}O_2$	
13-HODE (n-7) 13-Hydroxy-9,11-Octadecadienoic Acid $C_{18}H_{32}O_3$	
Oleic Acid (n-9) 9-Octadecenoic Acid $C_{18}H_{34}O_2$	

Figure 1.3 Structures of Some Long Chain Fatty Acids. ^{29, 62}

Lipoproteins and Reverse Cholesterol Transport

Dietary fatty acids are absorbed by the body and are transported in the circulation as lipoproteins. The action of enzyme lipoprotein lipase (LpL) is to cleave lipids from lipoproteins, allowing them to enter cells that require these lipids for structural and signalling functions. Lipoproteins can be defined as, ‘macromolecular complexes composed of various amounts of triglycerides, free and esterified cholesterol, phospholipids, and proteins’. Their main role is in transportation of hydrophobic triglycerides and cholesteryl esters from the intestine or liver to peripheral muscle and adipose tissues. Based on the density of lipids they transport they can be broadly classified into:

- i) Chylomicrons.
- ii) Very low-density lipoproteins (VLDL).
- iii) Intermediate-density lipoproteins (IDL).
- iv) Low-density lipoproteins.
- v) High-density lipoproteins.

The surface proteins of lipoproteins are called “apolipoproteins”, and these play important role in lipoprotein metabolism by acting as ligands for lipoprotein receptors. Lipoproteins can also be described according to the apolipoprotein present on their surface, including apoA-I and apo-B. The latter has two types namely apoB100 which is made in liver and apoB48 which is truncated in size, and is made in intestine. Table 1.2 shows the various apolipoproteins and their properties. ⁶³

<i>Apolipoprotein</i>	<i>Type of Lipoprotein</i>	<i>Atherogenic</i>
Apo-A I	HDL and Chylomicrons	Anti-atherogenic
Apo-B Apo B -48 Apo B-100	Chylomicrons VLDL, IDL, HDL	Except Chylomicrons, all these lipoproteins have potential to initiate atherogenesis

Table 1.2 Properties of Apolipoproteins

In general, low levels of LDL cholesterol and higher levels of HDL cholesterol in the circulation are considered components of a good lipid profile. Circulating levels of LDL- and HDL- cholesterol are used in clinical practice to estimate the risk of coronary heart disease.⁶⁴ HDL is involved in transporting excess cholesterol from extra-hepatic tissues to the liver. The cholesterol is later excreted, involving the pathway known as reverse cholesterol transport.⁶⁵ The liver has a unique ability to metabolise excess cholesterol, resulting in decreased cholesterol accumulation in the body, thereby maintaining cholesterol homeostasis.⁶⁶ In the present study, the effect of 9-HODE and 13-HODE in modulating genes involved in reverse cholesterol transport was determined. The two keys involved are scavenger receptor B (SCRB) and ATP binding cassette transporter-A1 (ABCA1), both of which aid in reducing lipid burden on cells.⁶⁷

Diabetic dyslipidaemia is a condition in which there is an excess of VLDL and apo-B, along with lower levels of HDL-cholesterol and apo-AI. It is associated with considerably increased risk of macrovascular complications.^{68, 69} Defects in lipid metabolism in both type 1 and type 2 diabetes have been reported. In type 1 diabetes, increased levels of VLDL are due to reduced activity of lipoprotein lipase. This is an insulin-regulated enzyme secreted by adipose and muscle cells, and which hydrolyses triglycerides of VLDL and chylomicrons.⁷⁰ Lipoprotein abnormalities are also observed in type 2 diabetes, and characterized by higher levels of triglycerides and LDL and low levels of HDL cholesterol.^{71, 72}

In the early stages of atherosclerosis, accumulation of lipoprotein particles in the sub-endothelial space is observed. Decreased circulating antioxidants is one factor that leads to oxidative stress. This, in turn, induces oxidation of LDL and formation of oxidized lipids. Oxidized lipids are harmful and, as a protective mechanism, differentiated macrophages take up these lipids and assimilate them. Excessive accumulation of these oxidised lipids leads to uncontrolled uptake of these lipids by macrophages resulting in foam cell formation.

Eicosanoids and Generation of HODEs

Oxidation of fatty acids occurs as a consequence of various metabolic pathways. The enzymes involved depend on the cell type and fatty acid involved. The enzymes include lipoxygenase, myeloperoxidase, xanthine synthase, NADPH oxidases. All of these enzymes produce reactive oxygen species (ROS) that then oxidise LDL.⁷³ Polyunsaturated fatty acids in cholesteryl esters, triglycerides also react with ROS, giving rise to other lipid products, which are lipid hydroperoxides and cyclic peroxides.⁷⁴ Oxidation of fatty acids enzymatically results in formation of eicosanoids. These are signalling molecules that have major influences on immunity and inflammation. They are derived as a result of enzymatic action on the n-3 and n-6 EFA series. The principal eicosanoids include prostaglandins and leukotrienes from which various other eicosanoids are derived. Eicosanoids are derived from EPA, which belongs to the n-3 series, as well as from arachidonic acid and DGLA from the n-6 FA series. These lipids are acted upon by two classes of enzymes: cyclooxygenase (COX) and lipoxygenase (LO) resulting in formation of prostanoids and leukotrienes (LT) respectively.^{75, 76} Types of prostanoids include prostaglandins (PG), prostacyclins (PGI) and thromboxanes (TX).

Synthesis of eicosanoids takes place as and when required. Fatty acids are present in the cell membrane or nuclear membrane and are stored in glycerol-esterified form in phospholipids. When activated by G protein-coupled receptors (GPCR), the ligands for which are cytokines or other messengers, FA are released into the cytoplasm where they are acted on by enzymes (including the phospholipases). Different types of phospholipase differ in their capacity to free FA from the phospholipid backbone. Free FA could either diffuse outside the cell and undergo metabolism or reincorporate to phospholipids. They are acted upon by COX and LO resulting in production of prostanoids and LT (Figure 1.4).⁷⁷

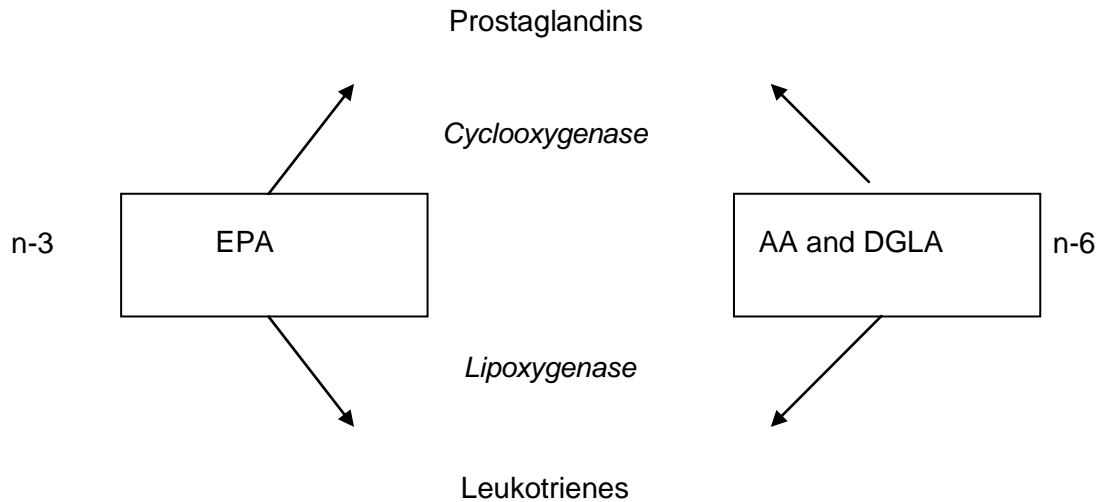


Figure 1.4 Generation of Leukotrienes and Prostaglandins

n-3 and n-6 fatty acids are acted upon by enzymes, cyclooxygenase and lipoxygenase resulting in the formation of eicosanoids, namely prostaglandins, prostacyclins and leukotrienes.⁷⁶

Lipoxygenases are non-haem dioxygenases that oxidise PUFA. They are thought to be involved in pathological processes including atherosclerosis, cancer and inflammation. Their exact role in atherogenesis is not fully understood. Two isoforms, 15-LOX-1 (the human orthologue of LO) and 15-LOX-2 have been isolated from human macrophages. These oxidise AA esterified in phospholipids and cholesteryl esters resulting in formation of 15-*S*-hydroperoxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic acid (15*S*-HpETE) and 12-*S*-hydroperoxy-5Z, 8Z, 10E, 14Z-eicosatetraenoic acid (12*S*-HpETE).⁷⁸ LA is the substrate for 15-LOX-1 which oxidises at C9 or C13 resulting in formation of 9-*S*-hydroperoxy-10E, 12Z-octadecadienoic acid (9*S*-HpODE) and 13-*S*-hydroperoxy-9Z, 11E-octadecadienoic acid (13*S*-HpODE). These peroxy products are unstable and are reduced to the hydroxyl form by glutathione peroxidase forming 12*S*- or 15*S*-hydroxyeicosatetraenoic acid (HETE). In the case of LA, the products are 9*S*- or 13*S*-hydroxyoctadecadienoic acid (HODE).

Non-enzymatically, HODEs are derived from LDL by lipid-peroxidation induced by free radicals present in the circulation or extracellular fluids.⁷⁹ It has also been suggested that the peroxy products act in signalling via cell surface receptors and hydroxyl products in signalling through nuclear receptors.⁸⁰ Hydroxyoctadecadienoic

acids are stable oxidized products of LA. The latter is present abundantly in atherosclerotic plaque and is oxidised by lipoxygenases resulting in formation of HODEs.

Oxidised lipids are pathologically important since they have been found at the site of tissue injury, atherosclerotic plaque and myocardial infarction. A direct correlation between the amount of aortic lipid peroxides (later reduced to HODEs) ⁸¹ and atherosclerosis has been shown in animal models and in humans. ⁸² *In vivo* generation of HODES as a result of action of lipoxygenase present in plasma membranes of reticulocytes has been reported. Culturing LDL isolated from plasma with monocytes and analysing the resulting oxidised LDL by HPLC revealed a high concentration of esterified hydroperoxyoctadecadienoic acid (HPODE). There were also relatively small amounts of hydroxyoctadecadienoic acid. ⁸³ HODEs can be generated from LA in endothelial cells, resulting in predominantly 9-HODE rather than 13-HODE. ⁸⁴ Neutrophils have been shown to have omega-6-LOX which catalyses formation of 13-HODE from LA. ⁸⁵ When the amount of HODEs in LDL of young patients with atherosclerosis was quantified, it was found that they had levels of HODEs up to twenty-fold higher than those found in normal controls. ⁸⁶ Patients with rheumatoid arthritis are at increased risk of developing atherosclerosis, and have been shown to have higher levels of HODEs in circulating LDL. ⁸⁷

HODEs - Are They Pro- or Anti- atherogenic?

It is important to understand what effects HODEs have on atherogenesis. Structurally, the two isomers 9-HODE (n-6) and 13-HODE (n-7) differ very little but they could have distinct biological effects. High quantities both these oxidised FA have been found in atherosclerotic plaque. ²⁷ HPLC and gas chromatography-mass spectroscopy (GC-MS) studies have been used to measure HODEs qualitatively and quantitatively. It has been shown that when LA is the substrate for 15-LOX-1, the predominant product is 13-HODE while a mixture of 12 and 15-HETE are produced when AA is the substrate. It is interesting to note that non-enzymatic oxidation using copper sulphate (CuSO₄; an oxidising agent) of LA results in equal mixture of 9-HODE and 13-HODE (and equal amounts of S and R enantiomers). ^{88, 89} There is considerable debate as to whether

lipoxygenases and their products are protective or not in relation to atherogenesis. There is published evidence reporting either protective or pro-atherogenic effects.

5-lipoxygenase (5-LOX) and 15-LOX-1 are present in the initial stages of atherogenesis in animal models, and in early atherosclerotic lesions in humans where they are co-localised with LDL at macrophage rich areas of lesions.⁹⁰ Zhu et al.⁹¹ showed that LDL particles are oxidised due to translocation of 12/15 LO from cytoplasm of macrophages to the plasma membrane. LDL particles then attach to low-density receptor related protein (LRP) on the macrophage membrane and are then oxidised by the lipoxygenase. Evidence suggests that 15-LOX increases the expression of CD36 in macrophages thereby increasing foam cell formation through peroxisome proliferator-activated receptors- γ (PPAR- γ).⁹² HDL particles have also been shown to be oxidised by 15-LOX, thus decreasing their capability in reverse cholesterol transport.⁹³

By contrast, it has also been noted that 15-LOX metabolite 13-HODE increases the expression of ABCA1 involved in reverse cholesterol transport through PPAR- α and PPAR- γ .⁹⁴ Studying the presence and involvement of HODEs during disease progression of atherosclerosis in high cholesterol fed rabbits, it was found that enzymatically derived HODEs predominated in the early stages, while at later stages had non-enzymatically derived HODEs predominated.⁷⁰ The presence of non-enzymatically derived oxidized products during the later stages of lesion could be due to the sensitive nature of LOX as it is known to undergo suicidal inactivation on oxidising lipids. With increase in the levels of LDL, the momentum shifts to non-enzymatic derivation of oxLDL.⁹⁵ Whether HODEs are generated enzymatically or non-enzymatically, the peroxy products are formed first and then, being unstable, are reduced to hydroxy products. For example, 13-hydroperoxy-oxydecadienoic acid (13-HPODE) is formed first and is quickly reduced to 13-HODE. A similar mechanism applies to the formation of 9-HODE. Recently, the gasotransmitter hydrogen sulphide (H₂S) has been suggested to be an anti-atherogenic agent. H₂S might be involved in converting peroxy products to hydroxy products. The latter are less reactive compounds than are peroxy products.⁹⁶ With the presence of LOX and HODEs at lesion sites recognized, we need to understand what role they play in lesion formation.

The metabolite products of 15-LOX namely 13-HODE and 15-HETE have been considered to be anti-atherogenic. Over-expression of 15-LO has a variety of consequences in animal models of atherosclerosis. In a study in which 15-LO was over-expressed in LDL^{-/-} transgenic mice, more lesions were observed in the aorta than in wild-type animals.⁹⁰ By contrast, 15-LO was suggested to be protective in a transgenic rabbit model where 15-LO was expressed in macrophages. The macrophage lesion areas were significantly decreased compared to controls and this macrophage specific 15-LO expression was protective at early stages of atherosclerosis.⁹⁷ Human 15-LOX-1 has been reported to be pro-atherogenic since its activity is increased in atheromatous arteries and the enzyme increases oxidation of lipids and thus enhances lesion formation.⁹⁸

HODEs are known to occur in barley, malt and adlay seeds.^{99, 100} It is not known whether dietary HODEs are of any benefit or whether they influence bodily processes such as atherosclerosis. LA is known to decrease platelet adhesion and thrombogenicity. It is quite possible that some of the beneficial effects ascribed to LA are due to its oxidised products.¹⁰¹ HODEs are also used as an indicator of oxidative stress.^{102, 103} When exogenous antioxidant supplements are administered their effect can be measured using HODE radioimmunoassay.¹⁰⁴ Compared to the F₂-isoprostanes, which are conventionally used as a measure of oxidative stress, HODEs are twenty-fold higher during conditions of oxidative stress.¹⁰⁵ It would be useful to understand the role of HODEs, which are abundantly present in different cell types involved in atherogenesis.

Effects of HODEs on Different Cell Types

Endothelial cells (EC), vascular smooth muscle cells and macrophages are the principal cell types involved in the pathogenesis of atherosclerosis. The endothelium of artery consists of monolayer of cells, which acts as a barrier between blood flow and sub-endothelial space. Damage to this layer leads to a state of inflammation and a series of events take place which include activation of EC, release of inflammatory, pro-thrombotic and chemotactic mediators.

Endothelial Cells

EC are known to generate 9-HODE and 13-HODE enzymatically via lipoxygenase.¹⁰⁶ ¹⁰⁷ oxLDL but not LDL has been shown to induce apoptosis in these cells. Incubation of oxLDL with human umbilical venous endothelial cells (HUVEC) leads to apoptosis, and this is dose-related.¹⁰⁸ HODEs decrease the adhesion of platelets to EC; however, HODEs do not bind to the EC but directly affect platelet adhesion.⁷⁹ On the other hand, HODEs have shown to increase the expression of plasminogen activator inhibitor type-1 (PAI-1) in EC. Increased plasma levels of PAI-1 have been linked to MI and venous thrombosis. Human EC, when treated with HODEs, showed increased PAI-1 mRNA expression possibly via PPAR- γ . PAI-1 expression has been also been reported to be regulated by LDL in adipocytes suggesting involvement of common regulatory mechanisms between obesity, insulin resistance and vascular risk.¹⁰⁹ 13-HODE has been reported to increase synthesis of vasodilator prostacyclins¹¹⁰ in fetal bovine EC. 13-HODE also increased expression of intercellular adhesion molecule (ICAM-1). This mediator helps monocytes adhere to the endothelial surface, a process that is thought to be regulated by PPAR- γ .¹¹¹

Vascular Smooth Muscle Cells

In the later stages of atherosclerosis, VSMC migrate to the intimal portion of the arterial wall. They then proliferate and secrete proteins of the extracellular matrix, which form a fibrous cap protecting against plaque rupture and thrombosis. VSMC are known to synthesise HODEs - predominantly 13-HODE with lesser amounts of 9-HODE.¹¹² 15-HETE and 13-HODE promote differentiation of VSMC, while 12-HETE increases prostacyclin synthesis and motility of VSMC,¹¹³ and also has been shown to increase intracellular.¹¹⁴ Free fatty acids also induce apoptosis in VSMC, possibly weakening the fibrous cap.¹¹⁵ oxLDL has also been shown to induce apoptosis in VSMC.¹¹⁶ 13-HODE, 12- and 15-HETE increased the expression of PPAR- γ in these cells. Rosiglitazone also increased PPAR γ -2 expression along with increase in 12-LO and 15-LO. The increased expression of PPAR γ -2 with rosiglitazone could be prevented by adding the LO inhibitor baicalein. This suggests that the increase in expression of PPAR γ -2 was regulated by lipoxygenase metabolites.¹¹⁷ HODEs may also increase

expression of chemokine receptor CXCR1 (regulated by PPAR- γ in macrophages), thus making it pro-adhesive to coronary VSMC.¹¹⁸

Platelets

Thrombus formation is another critical process in vascular disease with initial accumulation of platelets and their interaction with EC. Once adhered to EC, they increase inflammation by releasing cytokines and attracting other cells. Platelets also produce HODEs; ability of human platelets to convert LA to predominantly 13-HODE (85%) and to a lesser extent 9-HODE was reported by Daret et al.¹¹⁹ HODEs might be anti-thrombotic by inhibiting cyclooxygenase and by regulating cytoplasmic calcium levels.¹²⁰ Incubation with HODEs has been reported to decrease platelet adherence to EC.¹²¹ Contrasting effects have been reported by another group, which reported no effect of 13-HODE on platelet-endothelial interaction.¹²² The peroxy product 13-HPODE may inhibit cyclooxygenase selectively, an effect that may not be apparent with its hydroxyl product 13-HODE.¹²³

Monocytes and Macrophages

Monocytes and macrophages are of considerable importance in atherogenesis: Monocytes are one of the earliest cell types to migrate to sites of inflammation, and during the process of this they differentiate into macrophages. A clearer understanding of their role and how they interact with oxidised lipids and regulate various aspects of metabolism is one of the primary aims of this work. HODEs are known to effect viability and apoptosis in macrophages. Ox-LDL increases macrophage proliferation and promotes secretion of pro-inflammatory cytokines.¹²⁴ Culturing bone marrow derived macrophages with ox-LDL resulted in increase in cell survival and DNA synthesis. When supplemented with GM-CSF and colony stimulating factor (CSF), oxLDL had a significant effect on cell proliferation and survival suggesting that oxLDL could increase macrophage numbers in atheromatous lesions. oxLDL, but not native LDL, was reported to promote monocyte-macrophage differentiation using CD11b as maturation marker (flow cytometry) and this maturation required activation of receptor

for GM-CSF possibly by components of oxLDL. Maturation of monocytes occurred with uptake of oxLDL followed by its degradation in cells. This suggests that oxLDL, which is taken up by cells, is processed by intracellular enzymes.¹²⁵ Studying the effects of HODES on monocytes, Nagy et al. (1998)¹²⁶ demonstrated that oxLDL and HODEs could help trigger monocyte maturation. Using flow cytometry, they reported that expression of the maturation marker CD14 increases with oxLDL interaction. There is no published detailed study of the effect of HODEs on cell viability, apoptosis and cytotoxicity in THP-1 cells.

Treatment of human monocyte-macrophages with AA, DHA and EPA, ALA and LA was undertaken to investigate whether they had effects on apoptosis.¹²⁷ AA was the most potent promoter of apoptosis, followed by DHA and EPA. ALA had a lower effect and LA did not affect the cells. oxLDL but not LDL has been shown to induce apoptosis in macrophages similar to what has been observed with EC and VSMC. Hardwick et al.¹²⁸ demonstrated that oxLDL induces apoptosis in human macrophages that was concentration dependent. They also showed that with increase in incubation time, apoptosis induction increased. 13-HODE was shown to induce apoptosis in monocytes. This also accompanied by an increase in expression of CD36. When 13-HODE was added along with pro-caspase inhibitor, it suppressed apoptosis.⁵ Another study⁴ examined the effects of both HODES on the U937 monocytic cell line and demonstrated that 9-HODE selectively induced apoptosis. Based on the nature of synthesis of HODEs, wherein predominantly 13-HODE is derived enzymatically during early stages of atherogenesis and equal amounts of both 9-HODE and 13-HODE derived non-enzymatically during late stages.^{88, 90} It would be appropriate to study the effects of 13-HODE on monocytes (early atherosclerosis) and effects of both HODEs with macrophages (late stages of atherosclerosis) would help understand the role of oxidised lipids.

Foam cell formation is a characteristic feature of atherosclerosis. Macrophages under normal circumstances have down-regulated genes for LDL receptor and are therefore unable to take up circulating LDL-cholesterol. However, they are able to take up oxLDL, acetyl-LDL through scavenger receptors (SCRA) (in oxLDL environment).¹²⁹ Fatty acids from oxidised LDL could be released by action of various endogenous macrophage enzymes. Phospholipase A2 and lysosomal esterases released from

macrophages due to oxidative stress could trigger fatty acid release from oxLDL.¹³⁰ The role of cytokines in homing cells derived from bone marrow is another crucial step. MCP-1 is most potent of chemoattractant factors^{131, 132} produced by EC, T lymphocytes, fibroblasts and tissue macrophages. Other important chemoattractants include MCP-2, MCP-3, MCP-4, macrophage colony-stimulating factor (M-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), TNF- α , TGF- β and RANTES, and inflammatory cytokines IFN- γ , IL- β , IL-6, and TNF- α .¹³³ Under unstimulated conditions, monocytes undergo apoptosis and thus do not enter peripheral tissues.¹³⁴ Once OxLDL are taken up by monocytes and macrophages many different effects have been observed. They influence viability, induce apoptosis, and also regulate genes involved in inflammatory responses and lipid metabolism. It is important to understand how the FA and oxidised lipids interact since they have been known to exert varied effects on cells (Figure 1.5).

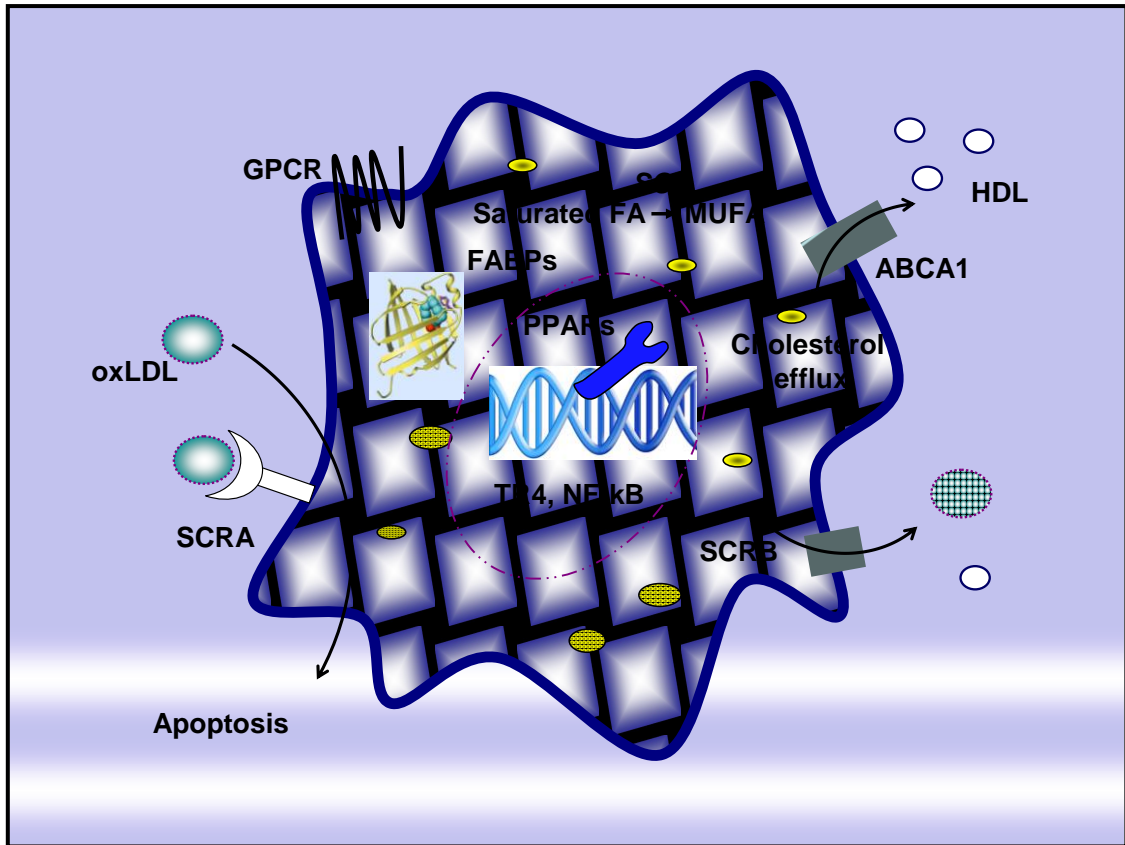


Figure 1.5 Oxidised Lipids and Macrophages

Oxidised lipids have a variety of effects on macrophages. Some of these may involve their signalling pathways that include the GPCRs for long-chain fatty acids. Lipids are taken up by scavenger receptors. Inter-cellular transport of lipids involves the lipid transporter FABP that could be regulated by SCD. Other processes that are altered when oxidised lipids are internalised by macrophages are induction of apoptosis and reverse cholesterol transport. Intracellular transcription factors have been implicated in these processes.

Fatty Acid Binding Proteins and Stearoyl-CoA Desaturase

Fatty acid binding proteins (FABPs) are intracellular proteins, which bind to wide range of hydrophobic compounds.¹³⁵ Adipocyte lipid binding protein (ALBP/aP2/FABP4) belongs to family of FABP. This protein is highly expressed in cytoplasm and nucleus of adipocytes, and is involved with transport of lipids by binding to hydrophobic regions of long chain fatty acids. These proteins are well known to be regulation of lipid accumulation in macrophages and formation of foam cells.¹³⁶⁻¹³⁸ They have been implicated in protecting PUFA against peroxidation.¹³⁹

Expression of FABP4 was reported in THP-1 macrophages when they were treated with oxidised LDL. This included expression of mRNA and aP2 protein determined by northern blot assays and immunochemical analysis respectively. In addition, activation of NF- κ B pathway was observed.¹⁴⁰ The same authors also suggested that increase in aP2 could promote foam cell formation. They observed an increase in this gene when THP-1 macrophages were treated with 9-HODE, 13-HODE and 15-dPGJ₂ and when aP2 is over-expressed by adenovirus construct studies.¹⁴¹ A decrease in the expression of ABCA1 was also reported in these aP2 over-expressing cells.¹⁴² Macrophages from aP2 deficient mice displayed defects in cholesterol accumulation. Increased PPAR- γ and CD36 expression and decreased expression of NF- κ B was observed with increased uptake of modified resulting in repression of proinflammatory cytokines.¹⁴³ Inhibiting aP2 expression using an inhibitor to suppress the activity of aP2 was an effective therapeutic agent in mouse models. It inhibited the expression of MCP-1 thereby preventing atherosclerosis and was quite effective when administered during late stages of disease.¹⁴⁴ The effect of both HODEs on aP2 expression has not been studied in detail. Their role in foam cell formation could be studied with the help of oil-red O stain studies.¹⁴⁵ The Oil Red O stain is a red dye specifically binds to the lipid droplets and in the cells.

FABP5 (mal1) is also expressed in macrophages.¹⁴⁶ Both FABP4 and FABP5 are considered important as aP2^{-/-} mice do not develop insulin resistance and diabetes when induced obesity with diet and do not express pro-inflammatory TNF- α .¹⁴⁷ Mice deficient in FABP5 exhibit higher insulin sensitivity.¹⁴⁸ When both these FABP are deficient the mice they displayed better lipid metabolism, insulin sensitivity and

reduced atherosclerosis. Interestingly, these mice also have very low expression of stearoyl-CoA desaturase-1 (SCD-1) in the liver.¹⁴⁹

SCD-1, an enzyme primarily involved in catalysing Δ^9 -cis desaturation of fatty acid-CoA for e.g. palmitoyl-CoA and stearoyl-CoA to palmitoleoyl-CoA and oleoyl-CoA respectively.¹⁵⁰ The ratio of saturated and unsaturated fatty acids in cell membrane is critical in maintaining membrane fluidity. Abnormality in this ratio has been correlated with disease states including diabetes and vascular disease.¹⁵¹ Treatment with AA and LA have shown to decrease the expression of hepatic SCD.¹⁵² Cao et al.,¹ studying double deficient *aP2-mal1*^{-/-} mice, found higher levels of circulating palmitoleate and in adipose tissue. They demonstrated that increased levels of palmitoleate from adipose tissue suppresses the expression SCD-1 in the liver, an example of enzymatic negative feedback mechanism since SCD-1 is involved in generation of palmitoleate from palmitate increasing insulin sensitivity in cells. On the other hand palmitate reduced insulin sensitivity. They suggested that palmitoleate plays an important role in regulating lipid metabolism. They coined the term “lipokine”, with hormonal functions. This study is significant since 13-HODE is also an omega-7 fatty acid like palmitoleate and it would be interesting to study the effects of 13-HODE on FABPs and SCD-1.

Macrophages – Role in Complications of Diabetes and Obesity

Macrophages are a population of cells that are widely distributed and perform a variety of functions.¹⁵³ The term “macrophage” was coined by Metchnikoff in 1882 to denote a population of cells that were large and phagocytic, and were present in all invertebrates and vertebrates.¹⁵⁴ In 1924, Aschoff described the reticuloendothelial system (RES) which comprised reticulum cells, reticuloendothelial cells and histiocytes, and which were positive for lithium carmine stain. In the 1960s, advances in histochemistry, microscopy and immunology revealed that macrophages were distinct from reticulum and reticuloendothelial cells with respect to function, morphology and origin.¹⁵³ It was also reported that macrophages were derived from monocytes, which originated from progenitor cells in bone marrow.¹⁵⁵ This was first suggested by Amano et al. in 1948 in studies which used supravital stains. The sequential development was illustrated as follows: Progenitor cells- monoblasts- promonocytes – monocytes to macrophages.¹⁵⁶

In 1972, Van Furth introduced the concept of mononuclear phagocytes in which reticulum cells, reticuloendothelial cells and fibroblasts were considered separate from macrophages thus, replacing the concept of RES. One of the main drawbacks of this theory was that macrophages were claimed to lack proliferative capacity,^{72, 157, 158} while others reported that macrophages divide and proliferate.¹⁵⁹

During inflammation, monocytes from bone marrow migrate in the blood stream to sites of inflammation and differentiate into macrophages. Monocyte chemoattractant protein-1 produced by endothelial cells, T lymphocytes, fibroblasts and tissue macrophages help attract monocytes to sites of inflammation. Other chemoattractants include MCP-2, MCP-3, MCP-4, macrophage colony-stimulating factor (M-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β) and RANTES (Regulated on Activation, Normal T cell Expresses and Secreted). MCP-1 is the most potent of these chemoattractant factors.^{131, 132} In unstimulated conditions, the monocytes undergo apoptosis and thus do not enter peripheral tissues.¹³⁴ Recent studies have provided abundant evidence that macrophages are involved in atherosclerosis, diabetes and other complications of obesity (Figure 1.6).

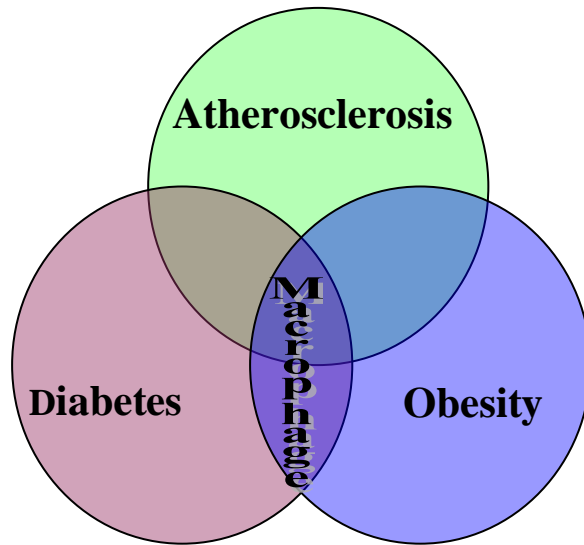


Figure 1.6 Macrophage Involvement in Metabolic Syndrome

Diabetic Complications

Diabetes mellitus is characterized by hyperglycemia and is due to defective insulin action, defective insulin secretion, or both. Symptoms include polyuria (excessive urination), polydipsia (excessive thirst), and polyphagia (excessive hunger). Insulin deficiency results in a catabolic state, evidenced by weight loss. Long-term complications include microvascular (eye, kidney and nerves) and macrovascular (coronary artery disease, stroke and peripheral vascular disease) conditions. Diabetes can be classified into two major types: A) Type 1 diabetes is associated with absolute deficiency of insulin production by the β cells in the pancreatic islets of Langerhans. It accounts for 5-10 % cases of diabetes, and results from autoimmune destruction of insulin the β cells by autoantibodies^{160, 161} and autoreactive T cells.¹⁶² Unless treated with insulin, this disease is fatal. The autoimmune destruction of the β cells is due a combination of genetic and environmental factors. Patients with type 1 diabetes are prone to other autoimmune diseases including Hashimoto's thyroiditis, Grave's disease, Addison's disease, and myasthenia gravis.^{163, 164} B) Type 2 diabetes is due to a combination of insulin resistance and defective insulin secretion. This accounts for 90-95% of cases. Patients with type 2 diabetes are usually overweight or obese, and this is

a major factor in insulin resistance. They are initially treated with diet, although many go on to require oral hypoglycaemic drugs and ultimately insulin treatment. Another type of diabetes is gestational diabetes, which arises from insulin resistance in pregnancy.

Recent studies suggest that macrophages play an important role in complications of diabetes. In diabetic nephropathy, accumulation of macrophages in the glomeruli and renal interstitium occurs early¹⁶⁵⁻¹⁶⁷ Macrophage accumulation has also been observed in the db/db mouse model and the degree of infiltration correlates with severity of renal damage.¹⁶⁸ Diabetic neuropathy is associated with loss of sensation and some motor functions mainly affecting the feet.¹⁶⁹ Macrophage infiltration has been observed in the initial stages, and this may play a role in causing nerve damage.^{170, 171} Diabetic retinopathy is a common cause of visual impairment.¹⁷² Immunohistochemical studies of the iris in patients with early diabetic retinopathy showed increased macrophage infiltration.¹⁷³ Patients with diabetes develop atherosclerosis prematurely, suffer from widespread atherosclerotic disease, and 80% of those with type 2 diabetes ultimately die from macrovascular complications.¹⁷⁴ Macrophage accumulation has been well documented in atherosclerotic plaques in patients with diabetes.¹⁷⁵ Although the exact pathogenesis of these complications is not yet fully understood, it is clear that macrophage plays a significant role in promoting inflammation within the lesions that lead to vascular complications.

Obesity

A combination of obesity and diabetes increases the risk of cardiovascular disease.¹⁷⁶ Obesity is a major risk factor for type 2 diabetes.¹⁷⁷ Treatment with insulin, sulphonylureas or thiazolidinediones promotes weight gain, and this may offset some of the potential benefits of these drugs.^{178, 179} There is a direct correlation between weight loss and improved insulin sensitivity. With weight loss, there is improved insulin-mediated glucose uptake by tissues.^{163, 180, 181} Adipose tissues from obese individuals release more pro-inflammatory adipokines. These have direct effects on metabolism and promote insulin resistance. The molecules involved include TNF- α ,¹⁸² MCP-1,¹⁸³ IL-6,¹⁸⁴ and TGF- β .¹⁸⁵ An increase in adipose tissue, particularly visceral adipose tissue, has

direct effects on metabolic and endocrine functions. For example, leptin, which is involved in regulating energy intake, energy storage and insulin sensitivity, is secreted in direct proportion to the amount of adipose tissue.¹⁸⁶

Association between macrophages and obesity has been emphasized by Weisberg et al.¹⁸⁷ Subcutaneous adipose tissue samples from humans given a high-fat diet for eight weeks were examined. Immunohistochemical studies revealed macrophage accumulation. Reverse transcriptase-polymerase chain reaction (RT-PCR) studies also revealed higher expression of CD-68 (macrophage identification marker) compared to non-obese subjects. Macrophages comprised around 10% of the cells of adipose tissues in non-obese subjects, but 40 – 50% in adipose tissues of obese subjects. Interaction between the macrophages and adipocytes leads to increased adipokine secretion. This leads to a low-grade systemic inflammatory state, which contributes to insulin resistance and macrovascular complications.¹⁸⁸ *In vivo* studies investigated the interaction between mouse macrophages and adipocytes using the 3T3-L1 adipocyte line and RAW264 macrophage line. Co-cultured cells expressed high levels of TNF- α , MCP-1 and IL-6. In addition, the free fatty acids released by 3T3-L1 cells promote release of TNF- α by macrophages. The interaction between these two cell types in adipose tissue depots is at the heart of the low-grade inflammatory response that accompanies obesity.¹⁸⁹

Recently, Takayoshi et al.¹⁹⁰ reported that the free fatty acids (FFA) released by adipocytes might be natural ligands for the toll-like receptor-4 (TLR-4) which is expressed on both adipocytes and macrophages. The interaction between FFA and TLR-4 may enhance secretion of TNF- α which, in turn, could trigger the NF- κ B pathway, leading to release of pro-inflammatory cytokines.¹⁹⁰ Co-culture medium contained markedly increased IL-6 compared to individual cultures of either adipocytes or macrophages.¹⁹¹ Carey et al.¹⁹² reported that macrophage-secreted factors impair insulin-mediated GLUT4 translocation to plasma membrane thereby causing insulin resistance and a pro-inflammatory state. This was reversed by addition of TNF- α neutralising antibody to macrophage-conditioned medium before treating the adipocytes. *In vivo* studies have demonstrated that growth of adipocytes is promoted by macrophage colony-stimulating factor (MCSF).¹⁹³ Culturing 3T3-L1 pre-adipocytes and human abdominal pre-adipocytes with conditioned medium (CM) from THP-1 cells

revealed that factors in the CM decreased adipogenesis. Triglycerol accumulation was decreased, along with which there was increased expression of fatty acid synthase (FASN), PPAR- γ and adiponectin.¹⁹⁴ Similar results were reported by Lacasa et al.¹⁹⁵ who cultured human primary pre-adipocytes in the presence of CM from macrophages differentiated from blood monocytes.

Lipopolysaccharide-treated macrophages were shown to release pro-inflammatory cytokines that induced preadipocyte proliferation. CM from macrophages derived from adipose tissue also decreased differentiation of preadipocytes. Treating 3T3-L1 adipocytes with CM obtained from LPS-stimulated RAW264.7 cells led to increased expression of TNF- α . When K-111, a PPAR- γ agonist, was added to 3T3-L1 adipocytes, TNF- α expression was decreased and this correlated with increased IL-6 expression. siRNA transfection experiments were performed to silence IL-6 in RAW264.7 cells. CM from these cells treated with K-111 failed to decrease TNF- α production by 3T3-L1 adipocytes, suggesting that activation of PPAR- γ induces production of IL-6, which inhibits TNF- α expression.¹⁹⁴

The THP-1 Cell Line

This cell line has monocytic characteristics, and was established by Tsuchiya and his colleagues in 1980.¹⁹⁶ The cells were derived from the blood of a young male suffering from monocytic leukaemia, and its characteristics include lysozyme secretion and phagocytotic properties. The cell line can maintain monocyte characteristics for more than a year of continuous culture. On treatment with phorbol diester, THP-1 cells were observed to become adherent to the culture plate. They also retained their phagocytotic properties, demonstrated when yeast and IgG-coated sheep erythrocytes were added to culture.¹⁹⁷ This cell line can be converted to mature cells with phorbol diester treatment, and these cells are well known to exhibit many of the functions of macrophages. A wide range of methodologies has been adopted to study the effects of long chain fatty acids on monocytes and macrophages. Cell lines are suitable models to study some of the fundamental effects elucidated by stimulants and fatty acids. A range of cell lines including HL-60¹⁹⁸, K562¹⁹⁹, THP-1¹⁹⁶, MM6⁵ and U-937⁴ monocytic cell lines have been used a model for studying monocytes and macrophages. THP-1 cell

line is widely used for studying macrophages, and has been chosen as the initial cell model for the work of this thesis.

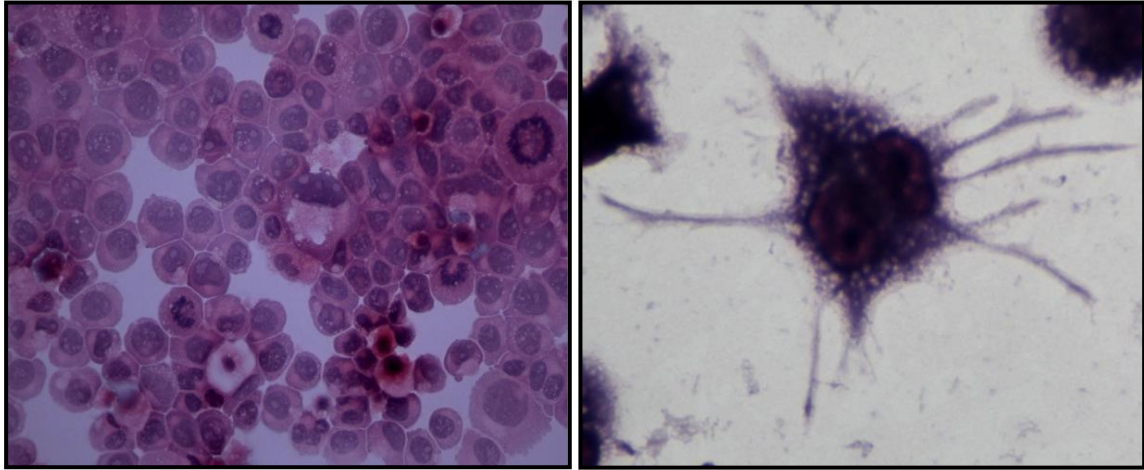


Figure 1.7 Monocyte and Macrophage

THP-1 cells were stained using hematoxylin (left) and differential quick stain on the right. Macrophages were differentiated using PMA

Receptors Involved in Fatty Acid Signalling

Fatty acids could act through various receptors present on the cell surface, thus triggering intracellular pathways. The mechanisms by which fatty acids regulate metabolic and immune responses could be directly via receptors present on cell surface, which include:

- i) Toll like receptors (TLR)
- ii) Scavenger receptors
- iii) G-protein coupling receptors (GPCR)

Apart from this FA also modulate cellular processes by effecting intracellular signalling pathways, generating active metabolites. In addition they also induce changes in cell membrane and lipid rafts and in production of eicosanoids. Intracellular signalling by FA results in regulation of immune responses and cell proliferation.²⁰⁰ n-3 FA affects the extracellular signal regulated kinase 1/2 (ERK 1/2) which belongs to the mitogen-

activated protein kinase (MAPK) pathway and which is involved in proliferation and differentiation of cells. Macrophage studies have shown that FA inhibited ERK1/2 and JNK phosphorylation, which is involved in regulating activator protein-1 (AP-1), a transcription factor for proinflammatory cytokines.²⁰¹ THP-1 cell studies showed that FA had an effect on GM-CSF by increasing its secretion by activating protein kinase.²⁰² FA have also been reported to change the biophysical properties of plasma membrane, by affecting lipid rafts which cholesterol laden micro domains in plasma membrane known to play an important role in T cell signalling.²⁰⁰

Toll-like Receptors

TLRs are a group of transmembrane receptors involved in recognition and induction of inflammatory responses.²⁰³ In total, eleven members of the family have been identified,²⁰⁴ of which TLR4 has been reported to be induced by saturated FA, resulting in activation of COX-2 (cyclooxygenase-2) in macrophages.²⁰⁵ Unsaturated FA inhibit the effect induced by TLRs. n-3 PUFA are more effective than n-6 PUFA in inhibiting the TLR-induced activation of NF- κ B and COX-2.²⁰⁶ The TLR4 ligand lipopolysaccharide (LPS) has been shown to promote foam cell formation in RAW264 macrophages.¹⁹¹

Scavenger Receptors

Principal receptors responsible for uptake of modified lipids are cell surface transmembrane proteins known as scavenger receptors.²⁰⁷ These receptors were identified in resident macrophages which are characteristic of atherosclerotic lesions and arise when monocytes are attracted to sites of inflammation caused by damage to the endothelium of the artery. When macrophages are unable to excrete high amounts of cholesterol, excess cholesterol is stored in the cytoplasm as lipid droplets, in the form of neutral lipids (triglycerides, cholesteryl esters and phospholipids). These cells, when observed under microscope, have a foamy appearance - hence their description as foam cells.²⁰⁸ Resident macrophages ingest and degrade cholesterol-carrying lipoproteins, which are then released into blood stream because of damaged endothelium. Steinbrecher et al.²⁰⁹ reported that the oxidised form of LDL is up taken by macrophages. The acetyl LDL receptor was later named as a scavenger receptor.²¹⁰

Scavenger receptors are of two types, Type I and Type II, and have a broad range of ligands apart from LDL. Both types of receptor are expressed in macrophages. They were earlier identified as acetyl LDL receptors, but later renamed due to the receptors ability to bind with various ligands including polyribonucleotide, polysaccharides and phospholipids. Later, with cloning studies it was known that there are more groups of them. Based on the tertiary structure of the receptor these receptors are now classified into six groups (Table 1.3):

Type	Sub types	Ligands
SR-A	SR-AI, SR-AII, SR-AIII, MARCO	oxLDL (SR-A I and II), acLDL, LPS, AGE modified proteins
SR-B	SR-BI, CD36	oxLDL, acLDL, LDL, HDL, vLDL, collagen (CD36)
SR-C	dSR-CI	acLDL, β -glucan
SR-D	CD68	oxLDL
SR-E	LOX-I	oxLDL
SR-F	SREC	oxLDL, acLDL

Table 1.3 Types of Scavenger Receptors

Scavenger receptors are classified into six groups with two principal ones SCRA and SCRB which are primarily involved in lipid accumulation in macrophages.^{211, 212}

Oxidized LDL has been studied extensively due to its importance in the development of microvascular and macrovascular complications by foam cells. These cause release of MCPs, MCSF, and MG-CSF by the endothelial cells. This could affect the differentiation, migration and proliferation of macrophages, which could further increase the process of atherogenesis.^{213, 214} Scavenger receptor class A-I/II are the principal receptors for uptake of oxidized LDL. SR-BI, CD36 (fatty acid translocase), along with ABCA1 are considered important in process of reverse cholesterol transport.

Mice lacking SCRA receptor and CD36 had extremely reduced uptake of oxidized lipids and these two receptors account for close to 90% of uptake of oxidized and acetylated LDL.²⁰⁷ Polyinosinic acid and fucoidan ligands (which bind to scavenger receptors) induced interleukin-1 (IL-1) production when THP-1 macrophages were treated with these ligands.²¹⁵ IL-1 has been reported to be pro-inflammatory and to

promote atherogenesis by increasing ICAM-1 expression on endothelial cell wall thus augmenting adherence of cells.²¹⁶ CD36, another receptor for oxLDL²¹⁷ is expressed on macrophages, adipocytes, platelets, endothelial cells and muscle cells. Macrophages of CD36^{-/-} mouse have been shown to accumulate lesser lipid and phospholipids. These mice do not develop advanced atherosclerosis features including thrombus formation. Though SCRA and CD36 are considered important in lipid uptake by macrophages, Moore et al.²¹⁸ have shown that in mice lacking SCRA or CD36 had lower macrophage lipid accumulation but an increase in aortic sinus lesions was observed suggesting involvement of other pathways apart from scavenger receptors for lipid accumulation. Increased expression of CD36 with PPAR agonists has been reported,^{92, 219} although different pathways including the retinoic acid pathway²²⁰ and testicular orphan nuclear receptor 4 (TR4)²²¹ might be involved in regulating this receptor.

SR-BI has been recognised as a unique receptor for HDL,²²² and has anti-atherogenic properties. Studies with the SR-B1/ApoE double mutant mouse model for studying coronary heart disease studies showed increase in lipid and fibrin in the artery, defective muscular system, MI and premature death.²²³ This demonstrates the protective role of SR-BI in helping to promote reverse cholesterol transport, preventing accumulation of lipoproteins in plasma and efflux of cholesterol from sub-endothelial macrophages to HDL. Along with SCRB-I, ABCA1 is considered important in efflux of excess cholesterol from peripheral tissues to HDL. oxLDL was shown to increase the expression of ABCA1 in THP-1 macrophages, a process involving PPAR- γ pathway.⁶⁷

Advanced glycosylation end products (AGE) are formed in increased amounts with diabetes due to the excessive glucose the circulation reacting with proteins.²²⁴ Receptors present on macrophage take up AGE to degrade them. Interaction of AGE with their receptors on macrophages results in secretion of TNF- α and IL-1. AGEs are known to influence THP-1 cells resulting in increased scavenger receptors particularly MSR-A, CD36 and MSR-B I. Increase in PPAR- γ and NF- κ B was also observed. Morphological studies showed increased LDL uptake and enhanced foam cell formation after AGE treatment.²²⁵ An increase in gene expression of CD36, ABCA1,⁶⁷ and SRB1 was observed when incubated with oxLDL in a time-dependent manner. Gene expression studies revealed increase in scavenger receptors namely SR-A and CD36 (by flow cytometry), a member of SR-B receptor family.¹²⁵ HODEs have been shown to

increase the expression of CD36 in monocytes while not sharing common pathway in oxLDL inducing apoptosis.¹²⁶

Lectin-like oxidised LDL receptor-1 (LOX-1) was identified as receptor for oxLDL in endothelial cells, and the up-regulation of this receptor promoted atherosclerosis.²²⁶ LOX-1 expression was also reported in macrophages, and could be functioning as scavenger receptors but not found to be expressed in monocytes. Its expression was not observed in either THP-1 monocytes or human monocytes, but was observed in differentiated THP-1 macrophages. Immunofluorescence and flow cytometry studies showed that this receptor was present on the plasma membrane.²²⁷

G Protein-coupled Receptors

GPCR are a ubiquitous and complex group of receptors with seven transmembrane domains, and form the largest family of cell surface receptors. Common cellular and physiological processes signalled through them include, sense of smell, pain, taste, vision, neurotransmission and digestion and cardiac regulation. They also play an important role in innate and adaptive immunity.²²⁸

GPCR can be classified to three main families. The rhodopsin family is the largest consisting rhodopsin, olfactory and chemokine receptors. Around twenty-five different receptors have been classified as secretin type, including the receptors for secretin, glucagon, intestinal peptides and calcitonin. The metabotropic family is smallest family, examples of which include receptors involved in taste, as well as the GABA_B and calcium-sensing receptors.²²⁹ Basic signalling via GPCRs involves heterotrimeric G-proteins, a $\beta\gamma$ - subunit and a GDP- bound α -subunit. The latter disassociates when an agonist triggers the signalling leading to structural changes in the GPCR and allowing the subunits to bind and activate. Activated subunits then regulate enzyme cascades that may have positive or negative effects within the cell.²²⁸ Feedback mechanisms result in impeding of the signal involving desensitisation by GPCR kinases (GRKs), secondary messenger protein kinases and arrestins. They are also involved in regulation of GPCR trafficking, recycling of receptors, and in downstream signalling.^{230, 231}

The two principal receptors examined in this thesis were GPR120 and GPR132. Recent literature is increasingly suggestive of their role in lipid metabolisms. In macrophages, GPCR are involved in regulating chemotaxis, cell contact, activation and survival.²²⁸ GPR43²³² and GPR120²³³ have been identified as receptors for short- and long- chain FA respectively. LA, DHA and palmitoleic acid were shown to increase Ca^{2++} in the cells in a dose-dependent manner while they increased the expression of fluorescent tagged GPR120. GPR120 is highly expressed in the intestine. When activated by long chain FA, it was reported to have an anti-apoptotic in the enteroendocrine STC-1 cell line.²³⁴ Its role in pancreatic-beta cells has been proposed but its exact role remains unclear²³⁵. GPR120 was postulated to be important in adipocyte differentiation and development, processes that correlated with increased levels of GPR120 mRNA expression in mouse models. However, a specific role of GPR120 was confirmed in adipocyte differentiation when siRNA for GPR120 inhibited adipocyte differentiation.²³⁶ Recently, a clear role for GPR120 in RAW264.7 macrophages while treated with n-3 fatty acids was shown by Da et al.²³⁷ by silencing GPR120 using siRNA they observed abrogation of anti-inflammatory effects elucidated by n-3 fatty acids.

G2A, another GPCR is expressed in lymphocytes. It is thought to be accumulated during G2 phase of cell cycle acting as anti-proliferative regulator hence the name G2A (A-Accumulation). On activation, this receptor triggers signalling cascade of MAP kinases. Its exact pathophysiological role is not understood while its natural ligands are yet to be identified. It was suggested that this receptor is expressed when there is any damage to DNA in lymphocytes until the cell machinery undergoes repair.²³⁸ G2A or GPR132 was identified as a receptor for 9-HODE²³⁹ and was reported to be involved in preventing inflammation of vasculature and atherosclerosis. A role of G2A mediated proinflammatory effects (increased intracellular calcium and inhibition of cell proliferation) by 9-HODE has been recently suggested in skin keratinocytes under oxidative stress conditions, while 13-HODE did not exhibit these effects.²⁴⁰

Expression of GPR132 in murine and human macrophages in atherosclerotic regions was reported by Rikitake et al.²⁴¹ Absence of GPR132 in mouse models led to increased interaction between monocytes and endothelial cells thus promoting plaque formation. Using a G2A knockout mouse model, interaction between monocytes and EC were examined. The EC from G2A deficient mice had increased ICAM-1 and E-

selectin expression along with increased secretion of IL-6 and MCP-1 compared with controls. With reversal of G2A expression, secretions reversed thereby decreased interaction between monocytes and EC were observed, suggesting a protective role of G2A against atherosclerosis.²⁴² The same group recently²⁴³ also studied the role of macrophages from double deficient mouse model for G2A^{-/-} apoE^{-/-} mice and found them to have lower levels of apoptosis with M1 proinflammatory phenotype with lower phagocytic activity. The G2A deficiencies also lead to increased lesion size possibly due to decreased apoptosis in aorta. Another study by Parks et al.²⁴⁴ investigating at the role of G2A in LDLR^{-/-} mice fed with high fat diet had increased atherosclerotic lesions. However in G2A deficient model, decreased numbers of intimal macrophages and higher HDL-cholesterol were observed with lower incidence atherosclerosis. The same authors also demonstrated that G2A^{-/-}LDLR^{-/-} mice secrete more Apo1 and ApoE and HDL-cholesterol fraction compared against controls with. They suggested that atheroprotective role in G2A deficient mice were dependent on ApoE and double deficient mice for G2A^{-/-}ApoE^{-/-} and mice deficient for ApoE^{-/-}LDLR^{-/-} had higher levels of atherosclerosis and decreased HDL.²⁴⁵ Signal transduction of GPR132 involves subunits-G_{12/13} and G_{q/11}.^{246, 247}

Peroxisome Proliferator-activated Receptors

PPARs constitute a family of nuclear receptors which, when activated, regulate gene expression.²⁴⁸ Three types of PPAR - PPAR- α , - β , and - γ - have been characterised,²⁴⁹ each activating expression of target genes by forming heterodimers with the 9-cis-retinoic acid receptor (RXR).²⁵⁰ Once bound, the PPAR/RXR heterodimers activate transcription by binding to peroxisome proliferator response elements (PPRE) which are located at 5' end of target genes.²⁵¹ PPAR- γ has two isoforms namely γ 1 and γ 2. The former is expressed in spleen, liver, heart, skeletal muscle, and intestine. The latter is mainly expressed in adipocytes.²⁵² Fatty acids and eicosanoids have been identified as natural ligands for PPAR activation and through this interaction regulate lipid metabolism. Synthetic ligands of PPAR, thiazolidinediones, are widely used in treatment of diabetes, although there is controversy regarding whether they promote or protect atherosclerosis.²⁴⁹

PPAR- γ has a number of roles in atherogenesis regulating inflammatory responses, in apoptosis, vascular adhesion, up-regulation of receptors for lipid uptake, intracellular fatty acid transport, and cholesterol efflux. Up-regulation of PPAR- γ has been observed when monocytes differentiate into macrophages,²⁵³ and this is involved in regulation of NO (nitrous oxide) production in rodent macrophages.²⁵⁴ LC-PUFA have been recognised to be high-affinity ligands for PPAR, with binding leading to activation of PPAR-regulated pathways.²⁵⁵ Clearly, preventing the inflammatory responses of macrophages could help in reducing macrovascular complications. Studying the effects of polyunsaturated fatty acids on THP-1 cells, it was shown that LA, ALA and DHA had anti-inflammatory properties, in contrast to the effect of palmitic acid. When THP-1 cells were incubated with lipopolysaccharide, expression of IL-6, IL-1 β and TNF- α all decreased with ALA and DHA compared to treatment with palmitic acid, suggesting anti-inflammatory effect due to reduction in NF- κ B activation through increased activity of PPAR- γ .²⁵⁶ NF- κ B, transcription factor nuclear factor is associated with promotion of proinflammatory cytokines in atherosclerosis.^{257, 258} PPAR- γ , on the other hand, is associated with inflammation control,²⁵⁹⁻²⁶¹ and activation results in decreased IL-6, IL-1 and TNF- α .^{254, 262} PPAR- α activator fenofibrate has been shown to decrease the VCAM-1 and adhesion of U-937 monocytic cells to human endothelial cells.¹⁶⁵

Atheroprotective effects of PPAR- δ agonist GW0742X has been shown in LDLR^{-/-} mice in which treatment led to decrease in ICAM-1, MCP-1 and TNF- α expression.²⁶³ In adipocyte differentiation, PPAR- γ is involved in regulation of expression of number of proteins in adipose tissues including FABP4,²⁶⁴ CD36,²⁶⁵ acyl-coA synthetase that are required for lipid catabolism.²⁶⁶ Additionally, it suppresses secretion of TNF- α .²⁶⁷ PPARs play an important role in fatty acid transportation, oxidation and storage by regulating various genes. PPAR- γ has been shown to regulate expression of lipoprotein lipase that is required for release of fatty acids from lipoproteins. Once released fatty acids can be taken up by cells (including adipocytes and hepatocytes).²⁶⁸ PPAR- γ also regulates the expression of fatty acid translocase CD36, in macrophages. This serves as a receptor for uptake of oxidized low-density lipoproteins and in promotion of fatty acid and cholesterol uptake,²⁶⁹ as well as in transport of fatty acids by up regulating fatty acid transport proteins. Once into the cell, fatty acids undergo acylation with acyl CoA

which is also regulated by PPAR- γ by regulating expression of acyl-CoA synthetase (ACS).²⁷⁰

An increase in PPAR- γ has also shown to decrease the expression of SCRA in macrophages when treated with anti-diabetic thiazolidinedione drugs suggesting an anti-foam cell role by PPAR- γ .²⁷¹ PPAR- α and PPAR- γ agonists reduce the expression of apoB-48R in THP-1 and human macrophages thereby conferring anti-atherogenic effects.²⁷² As mentioned above, aP2 is important in adipocyte differentiation, a process thought to be regulated by PPAR- γ .²⁶⁹ This appears to be true in case of macrophages as well.¹⁴¹ PPAR agonists have also shown to increase the expression of hepatic SCD.²⁷³ PPAR- α/γ has shown to regulate increase in expression of SCRB and apoA-I and HDL mediated cholesterol efflux involving ABCA1 in macrophages, resulting in increased reverse cholesterol transport.^{274, 275}

PPAR- γ (but not PPAR- α/δ) influences primary monocytes to differentiate into M2 macrophages (anti-inflammatory macrophage phenotype) rather than into pro-atherogenic M1 macrophage phenotype. PPAR- γ does not increase M2 markers on resting macrophage in atherosclerotic lesions.^{276, 277} HODEs have been shown to induce apoptosis in macrophages. Hampel et al.⁴ studied the U-937 monocytic cell line and the effect of HODEs in cell proliferation and apoptosis. On incubating monocytes with 9 and 13-HODE, they demonstrated that 9-HODE inhibited cell proliferation and induced apoptosis and increased PPAR- γ 2 transcription while no significant changes in expression of PPAR- γ was observed with both HODEs, which was to be expected since these cells express very low levels of PPAR- γ at basal state.

Studies on THP-1 cell on effects of HODEs showed an increase in PPAR- γ and vascular endothelial growth factor (VEGF) responsible for inducing vascular permeability and in promoting macrophage migration. VEGF, a growth factor which promotes vascular permeability and attracts monocytes^{278, 279} is expressed when human monocyte derived macrophages were treated with oxidised LDL.²⁸⁰ Expression of VEGF with an increase in concentration of ox-LDL was suggested to be a consequence partly by the activation of PPAR- γ .²⁸¹

Both 9- and 13- HODE are ligands of PPAR- γ , which could in turn induce lipid uptake via scavenger receptor. PPAR- γ is activated when THP-1 monocytes are treated with oxidised LDL and this in turn results in up-regulation of scavenger receptor CD36, and increased uptake of oxidised LDL, demonstrating that PPAR- γ is important in regulation of lipid metabolism in macrophages.¹²⁶ It would be relevant to investigate whether HODEs influence M1 or M2 type phenotype in circulating monocytes, and also their effect on other PPARs since PPAR- γ in particular has been in general found to be protective in controlling lipid metabolism.

Other Transcription Factors

Even though PPARs are one of the major transcription factors studied, it would be worthwhile to investigate whether other nuclear receptors could be involved in macrophages when treated with oxLDL. One such receptor of interest is testicular nuclear transcription factor (TR4). TR4 involvement has been reported in myeloid progenitor cell proliferation,²⁸² regulating apoptosis induced by retinoic acid in carcinoma cells²⁸³ and in regulating oxidative stress. The natural ligands for TR4 have yet to be identified.²⁸⁴ Recently, Xie et al.²²¹ demonstrated that mice lacking TR4 had decreased expression of CD36, which correlated with lower foam cell formation. They suggested that HODEs might be a ligand for TR4 as treatment of HODES led to increased expression of CD36 and TR4.

NF- κ B (nuclear factor kappa B) is another transcription factor, which is important in regulation of genes involved in inflammation. It exists as heterodimer complex with an inhibitory complex protein I κ B (inhibitor of NF- κ B) in the cytosol of cells. Activation of NF- κ B by various ligands which include TNF- α , IL-1, UV radiation and LPS, results in phosphorylation of I κ B by I κ B kinase, which then translocates to nucleus regulating target genes involved in inflammation and cell growth control.²⁸⁵ The effect of PUFA on activation of NF- κ B is to inhibit the binding of NF- κ B to DNA, and its downstream gene regulation in macrophages. Weldon et al.²⁸⁶ studied the effect of LC-PUFA (EPA and DHA) on LPS-stimulated THP-1 macrophages, examining the expression of NF- κ B. The two FA inhibited expression of proinflammatory cytokines TNF- α , IL-6, and IL-1 β , as well as decreasing expression of NF- κ B itself.²⁸⁶ Similar results were reported

by Novak et al. ²⁸⁷ who showed that FA inactivated NF- κ B and inhibited I κ B phosphorylation. Documented effects of oxidised lipids on monocytes and macrophages are tabled below (Table 1.5).

Cell Type	Fatty acid/ Oxidised Lipids	Gene and effects	Reference
Murine macrophages	n-3 Fatty acid emulsion	Reduction ap-1 (secreting pro-inflammatory cytokines)	²⁰¹
Macrophages	Non esterified FA	Increased GM-CSF expression, protein kinase C	²⁰²
Murine enteroendocrine STC-1 cells	Linolenic acid	GPR120 receptor activates ERK and PI3K-Akt pathway inhibiting apoptosis.	²³⁴
Macrophages	Lysophosphatidylcholine component of oxLDL	GPR132, role in atherosclerosis	²⁴¹
Monocytes	n-3 FA	Inhibited TLR induced activation of NF-kb and COX-2	²⁰⁶
Macrophages	oxLDL with GM-CSF	Macrophage survival	¹²⁴
Monocytes	oxLDL	Increased CD11b (cell differentiation), activation of GM-CSF	¹²⁵
Macrophages	LCPUFA, oxLDL	Induces Apoptosis	^{127 128}
Monocytes	9 and 13-HODE	Induces apoptosis, increase in CD14 (maturation marker) and expression of CD36 via PPAR	^{4, 5 126}
Macrophages	oxLDL	Increase in SCRA and CD36	^{207 218}
Macrophages	PMA	LOX-1	²²⁷
Macrophages	oxLDL	Increase in ABCA1	⁶⁷
Macrophages	oxLDL, HODE	aP2 expression increasing NF-kB, foam cell formation and decrease in ABCA1	^{141 140}
Macrophages	LCPUFA	Increase in PPAR and reduction in NF-kB leading reduction in IL-6, IL-1 β and TNF- α (pro-inflammatory cytokines)	^{256 286} ²⁸⁷

Table 1.4 Effects of oxLDL Monocytes and Macrophages

Hypothesis and Aims

Although studies have been carried out to investigate the effects of oxidised lipids on macrophages, the role of HODEs on monocytes and macrophages, specifically on the widely used THP-1 cell line, have not been studied in detail. Since 13-HODE is predominantly produced at initial stages of atherogenesis, it would be ideal to study its effect on monocytes and the effect of 9-HODE, on macrophages. Very few studies have investigated changes in monocytes while they differentiate into macrophages. A study involving the HODEs with or without PMA would help us understand the effects the oxidised lipids have while the cells are differentiating. Differentiation markers and also pro- or anti-inflammatory cytokines released by them (conditioned medium or serum) can be studied using flow cytometry. Specific inhibitors of transcription factors and the recent technique of silencing genes of interest using RNA interference (RNAi) was employed in this study to understand fatty acid signalling. Differentiation and activation markers on human monocytic cells and serum levels of obesity markers were also studied.

It was hypothesized that 9-HODE and 13-HODE would have differing effects in regulating macrophage differentiation and in inducing expression of adipocyte molecules in macrophages compared with ALA and LA. We further hypothesised that HODEs would mediate inflammatory functions signalling through GPCRs for long-chain fatty acids.

General Aim:

To study the effect of α -linolenic, linoleic, 9-hydroxyoctadecadienoic and 13-hydroxydecadienoic acid on cell proliferation and fundamental mechanisms involved in modulating lipid metabolisms in monocytes and macrophages using THP-1 cell line and extending the studies to clinical study.

Specific Aims:

Aim 1: To study the effect of LCFA on the key processes viability, apoptosis and cytotoxicity on THP-1 monocytes and macrophages.

Aim 2: To study the effect of LCFA on lipid metabolism genes in monocytes and macrophages.

Aim 3: To study the effect of LCFA at different stages during the differentiation of monocytes to macrophages

Aim 4: To determine the role of transcription factors and long chain GPCR using specific antagonists and gene silencing techniques.

Aim 5: To understand the role of activation and differentiation markers on monocytes derived from diabetic and obese patients with cardiovascular risk factors.

Summary

Lipid accumulation and oxidative stress are characteristic features observed in atherosclerosis. Not much is known about the interaction between components of oxidised lipids HODEs abundantly present in vascular wall during atherogenesis with monocytes and macrophages. This study attempted to fill some of the knowledge gaps relating to how oxidised lipids influence these cells by examining their role in cell proliferation, apoptosis, lipid accumulation, and genes involved in regulating lipid metabolism in macrophages. It is vital to consider the role of transmembrane receptors involved in HODEs signalling and the putative receptor for 9-HODE, GPR132. Effects of HODEs on the regulation of transcription factor PPAR- γ involved in control of inflammation needs to be examined in detail due to its involvement in controlling inflammation. One of the major challenges has been to understand the heterogeneity of monocytes and the circumstances leading to monocytes being differentiated into M2-macrophages (known to have protective functions in controlling inflammation). Diabetic patients are predisposed to developing cardiovascular problems. This study also examined the role of GRP132 as a candidate marker of monocyte activation in these patients. GPR132 could serve as a diagnostic marker in risk stratification, and also may be a potential candidate for modulating monocytes thereby having therapeutic application as drug targets.

The outline for the thesis is as follows:

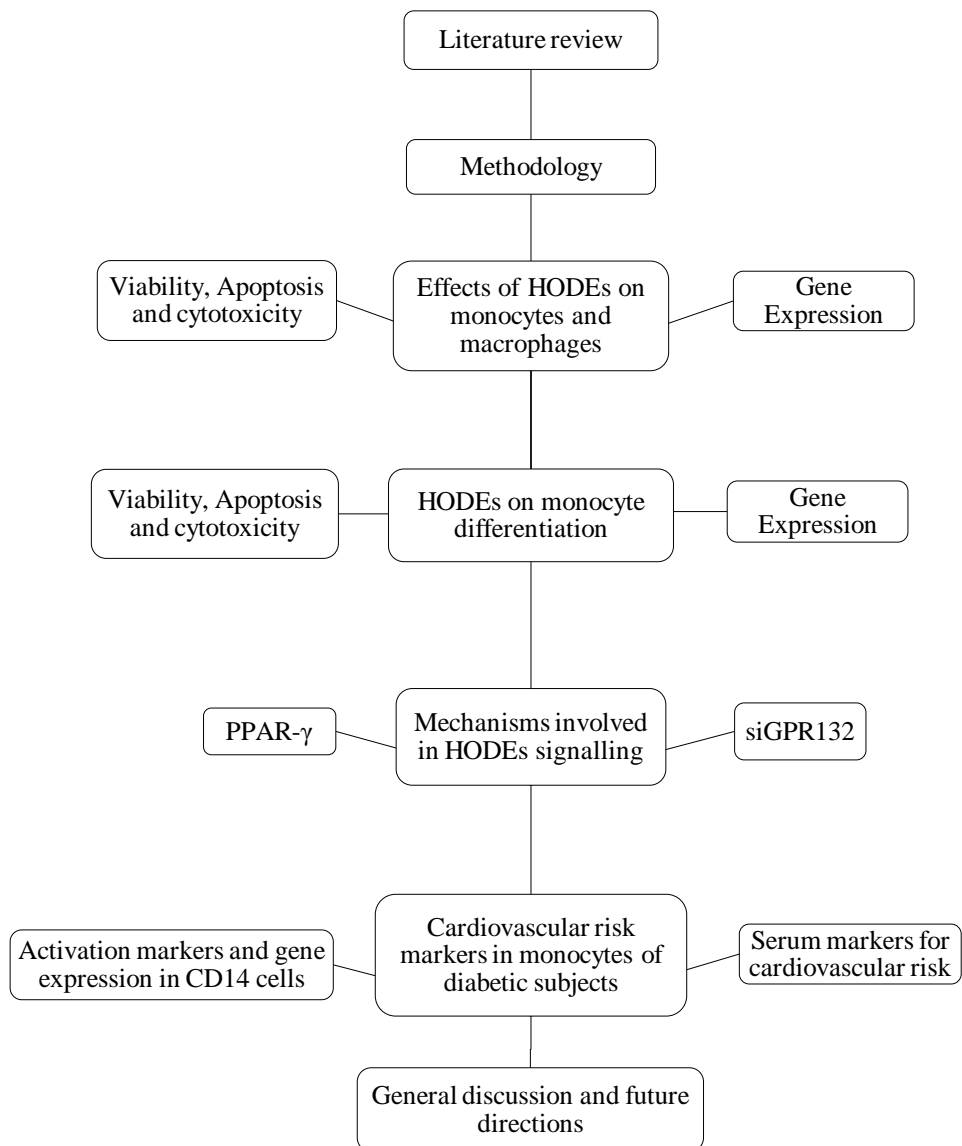


Figure 1.8 Outline of Thesis

Chapter 2

Materials and methods

Materials

Materials and reagents were purchased as follows: Tissue culture materials including serological pipettes (5, 10 and 25 mL), transfer pipettes (3.5mL) and centrifuge tubes (15 and 50mL) from Sarstedt (Ingle Farm, South Australia, Australia); Cell culture plates (6, 12, 24 and 96 well) from Nunc (Thermo Fischer Scientific, Scoresby, VIC, Australia); THP-1 cells, (PMA), trypan blue, ethanol, chloroform, Ficoll-Hypaque (Histopaque), ALA and LA from Sigma-Aldrich (Castle Hill, NSW, Australia); 9- and 13-hydroxyoctadecadienoic acid (HODEs), rosiglitazone, PPAR- γ antagonists 2-chloro-5-nitro-N-4-pyridinyl-benzamide (T0070907) and 2-chloro-5-nitrobenzanilide (GW9662) from Cayman Chemicals (Sapphire Biosciences, Redfern, NSW, Australia address); Antibodies (all rabbit polyclonal) to GPR120, GPR132 and FABP4 from Abcam (Sapphire Biosciences, Redfern, NSW, Australia); RPMI medium (without L-Glutamine), fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, Dulbecco's Phosphate Buffered Saline (DPBS), trypsin, lipofectamine RNAiMAX transfection reagent from Invitrogen (Mulgrave, VIC, Australia); Anti-Rabbit IgG, 3,3'-Diaminobenzidine (DAB) and VectaMount from Vector Labs (Abacus ALS, Brisbane, QLD, Australia); Faramount Aqueous mounting medium from Dako (Campbellfield, VIC, Australia); Streptavidin conjugated horseradish peroxidase (HRP) from Perkin Elmer (Melbourne, VIC, Australia); Glass slides, saline glass slides, Oil Red O dye and coverslips from Starfrost (Proscitech, Kirwan, QLD, Australia); Adipogenesis assay kit from Millipore (North Ryde, NSW, Australia); 4',6-Diamidino-2-phenylindole (DAPI) from Thermo Fischer Scientific (Scoresby, VIC, Australia); CellTiter-Glo luminescent cell viability assay, Cyto Tox-Glo assay and Apo-ONE Homogeneous Caspase-3/7 assay from Promega (Alexandria, NSW, Australia); 96 well sterile white culture plates for assay measurements were purchased from Perkin Elmer (Melbourne, VIC, Australia); RNeasy Mini extraction kit, DNase, QIAzol and QuantiTect SYBR Green RT-PCR kit, 0.1 ml PCR tubes, RNAi starter kit, siRNA for GPR132 (G2A) from Qiagen Pty Ltd (Doncaster, VIC, Australia); SABiosciences RT² PCR Primer sets for human GPR120, PPAR- γ , MSR1, SCARB1, SCD, TR4, FABP4, FABP5, ABCA1, ITGAM, CD36, PPIA, RBP-4 and RETN, human multiplex cytokine kits Th1/Th2 11plex and Obesity 9plex kit from Bender Medsystems from Jomar Diagnostics Pty Ltd (SA, Australia); GPR132 (Sense primer ACGAGATGAGACGGAACTG and anti-sense CTGCCTCTGTGCCTTAGC) primer from Sigma-Aldrich (Castle Hill, NSW,

Australia); Flow cytometry antibodies, CD36 fluorescein isothiocyanate (FITC), CD11b phycoerythrin (PE), CD14 phycoerythrin-cyanin7 (PECY7), CD54 allophycocyanin (APC), isotype controls and flow cytometry round bottom tubes from BD biosciences (North Ryde, NSW, Australia); FcR blocking reagent, CD14 magnetic beads and MS columns from Miltenyi Biotec (North Ryde, NSW, Australia); Flow cytometry sheath fluid from Beckman Coulter (Gladsville, NSW, Australia); Electrophoresis materials - acrylamide, electrophoresis setup, gel cast, western blot apparatus (blot papers and filter papers), from BIO-RAD (Gladsville, NSW, Australia).

Cell culture and differentiation

THP-1 Human monocytic leukemia cell line was obtained from ATCC Sigma-Aldrich (Castle Hill, NSW, Australia) The stock cells were maintained in liquid nitrogen. An aliquot of cells were taken out and cultured in RPMI medium with additives containing 10% decompimented FBS (heat-inactivated), 4mM L-glutamine and antibiotics (100U/mL penicillin and 100µg/mL streptomycin) in T75 cm² flasks at 37⁰C in a 5% CO₂ and 95% air humidified atmosphere. ¹⁴¹ The cell line was regularly maintained at a density of 2×10⁵ to 1×10⁶ cells/mL. The cells were kept well supplemented and passaged regularly by adding fresh medium after centrifugation at 500g for 5minutes, following which the supernatant was discarded. All cell culture work was carried out under sterile conditions.

Differentiation of Monocytes into Macrophages

For the purpose of experiments, one million monocytes (in triplicate) were differentiated into macrophages by treating THP-1 cells with 2mL 100nM PMA for 36 hours in a six-well plate in RPMI medium with additives. Cells were then washed with DPBS three times and rested for 24 hours in 2mL of low serum RPMI media (0.4% FBS) with antibiotics and L-glutamine.

Fatty Acid Treatment

A concentration of 30 μ M of long chain fatty acids (LCFA) ALA, LA, 9- and 13-HODE, was used to study their effects on monocytes and macrophages. The cells were incubated for 24 – 48 hours. Following experimental time point micrographs were captured using a NIKON Eclipse 50i camera (Coherent Scientific, Hilton, SA, Australia). Macrophages were washed with DPBS after removing the conditioned medium (stored for protein studies), treated with 0.05% trypsin solution made from 0.25% trypsin stock solution (2.5g/L trypsin and 0.38g/L ethylenediaminetetraacetic acid), and incubated for 5 minutes at 37°C. Action of enzyme was stopped by adding RPMI medium and displaced cells were resuspended. A sterile cell scraper was used to ensure maximal yield of cells. Monocytes and macrophages were then centrifuged at 400g for 5 minutes, after which conditioned medium (from monocytic culture) and cell pellet were collected separately and stored at -80°C for ELISA and RNA extraction respectively.

Differentiation Experiments

For these experiments, the effects of fatty acids were studied at a stage during which the cells were differentiating into macrophages. One million THP-1 cells were treated with 1 - 100nM PMA with or without LCFA; ALA, LA, 9-HODE and 13-HODE for 24 hours. Micrographs were recorded using the NIKON Eclipse 50i camera. Conditioned medium and cell pellet were stored at -80°C for ELISA and RNA extraction.

Cell Counts

Trypan blue exclusion and cell counting were performed after the monocytes and macrophages were treated with LCFA. After completion of experiments, 10 μ L of cell suspension was added to 10 μ L of trypan blue and counted using a haemocytometer (Hausser Scientific, Horsham, PA, USA). The total cell count was carried out using the formula:

$$\text{Total count} = \text{cell count of 10 chambers} \times \text{dilution factor} \times 1000 \text{ cells/mL.}$$

Viability, Apoptosis and Cytotoxicity Assays

The protocols for these assays were similar to the above experiments but with the number of cells scaled down. 24,000 THP-1 monocytes or macrophages (differentiated using PMA) were cultured in 50 μ L RPMI medium (0.4% FBS with additives) in a white 96 well opaque plate. They were treated for 24 – 48 hours incubated at 37°C with 5% CO₂. LCFA with increasing concentrations 0, 30, 70 and 100 μ M was used.

A luminescence based assay, the Celltiter-Glo Luminescent cell viability assay was used. This produces luminescence in proportion to the ATP present in viable cells. Using a recombinant luciferase system that is stable against ATPases, this in the presence of ATP is catalysed and emits a luminescent signal proportional to the ATP present.

The apoptosis assay (Apo-ONE Homogeneous Caspase-3/7 Assay) is based on the presence of caspase 3/7 activity and the resulting fluorescence due to cleavage of pro fluorescent reagent rhodamine-110 (bis-(N-CBZL- aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110) by caspase, releasing DEVD peptides. The rhodamine group released is intensely fluorescent. This was measured at an excitation wavelength 499nm and emission wavelength 521nm.

The cytotoxicity assay is a luminescent-based assay measuring the dead cells protease activity. Using a luminogenic peptide substrate (alanyl-alanylphenylalanyl-Aminoluciferin) that reacts with proteases released from dead cells resulting in generation of aminoluciferin, emitting a luminescent signal.

Briefly, as per the manufacturers recommended protocol for each assay, an equal amount of assay reagent (a homogeneous mixture of substrate and buffer) was added to the cell culture after LCFA treatment and incubated for 30 minutes at room temperature on an orbital shaker (350 rpm). The plate was pre-equilibrated to room temperature for 30 minutes for the viability assay after which reaction reagent was added and incubated. Luminescence (viability and cytotoxicity) and fluorescence (apoptosis) was then measured using Wallac 1420 multi-label reader (Perkin Elmer, Australia). Measurements were taken using the default settings for luminescence and fluorescence

for a 96 well plate. An additional step for a shake and settling of 10 seconds each was included before the measurements were taken.

Real-time RT-PCR analysis of mRNA expression

Total RNA was extracted from frozen cells (-80°C) using RNeasy extraction kits on a dedicated RNA free bench. Briefly, the samples were thawed to room temperature after which, 200µL of Qiazol (this method for extraction was adopted since using the conventional method of RLT buffer for these cells resulted in high DNA contamination) was added and vortexed. 800µL of Qiazol was then added, vortexed and incubated at room temperature for 5 minutes. 200µL of chloroform was then added, shaken vigorously for 10 – 15 seconds and incubated at room temperature for 2 - 3 minutes. After centrifuging at 12,000g for 15 minutes (4°C), the clear aqueous layer was removed carefully. An equal amount of 70% ethanol (EtOH) was added, mixed well, and added to a new spin column and centrifuged immediately at 8000g for 25 seconds. The filtrate was discarded and columns were washed with 350µL RW1 buffer twice. After this, the column was washed with 250µL of 100% EtOH leaving the column dry before DNase treatment (10µL+70µL RDD buffer) for 30 minutes at 37°C. Columns were washed twice with RW1 and RPE buffer (8000g, 25 seconds). The columns were then centrifuged for 2 minutes at 16,000g to remove any residual RPE. Columns were placed in new collection tubes and 80µL of RNase-free water was added (40µL, twice) and centrifuged for 1 minute at 8000g. The resultant eluant contained RNA.

RNA was quantified using Nanodrop spectrophotometer (Thermoscientific, Australia). The samples were checked for any DNA contamination by running a no RT (reverse transcriptase) reaction on the PCR. Real-time RT-PCR was performed on Corbett Roto-gene 6000 (Qiagen Pty Ltd, Doncaster, VIC, Australia) acquired at SYBR green channel. The reactions were performed in duplicates in a volume of 15µL containing 40-100ng/µL RNA, 7.5µl SYBR green master mix, 0.15µl RT mix and 0.6µl of appropriate primer in a 0.1ml RNase-DNase free PCR tubes. Some of the runs were performed as a 10 µl reaction with 5 µl of master mix, 0.1µl RT mix and 0.4µl of appropriate primer. Samples were normalized and quantified against one of the reference genes (internal control) peptidylpropyl isomerase A (PPIA), β-actin or

glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Thermal cycling program used was 50°C for 30 minutes for cDNA synthesis, 95°C for 10 minutes (enzyme activation), 40 cycles of 94°C, 15 seconds (denaturation), 55°C for 30 seconds (annealing), 72°C for 30 seconds (extension) for GPR120, GPR132, PPAR- γ and PPIA. Cycling program for MSR1, SCARB1, FABP4, FABP5, SCD, TR4, ITGAM, CD36 and ABCA1 was a two-step cycling of 95°C for 15 seconds and 60°C for 60 seconds (recommended by SA Biosciences).

Immunocytochemistry

GPR120, GPR132 and FABP4 rabbit polyclonal antibody expression on monocytes and macrophages was performed. Briefly, 100,000 monocytes were differentiated (PMA) into macrophages by adding 200 μ L cells on the sterile coverslip carefully not allowing it to flow out to the wells on a 24-well plate. After 10 - 15 minutes, an additional 300 μ L of PMA medium was added to the wells and incubated for 36 hours at 37°C with 5% CO₂. Coverslips were then washed with DPBS and fresh medium added (0.4% FBS) followed by incubation for another 24 hours. Once the cells had differentiated, they were then fixed in 75% EtOH in DPBS for 10 minutes. Coverslips were then mounted on a slide, left to dry at 37°C, and stained with specific antibodies.

Monocytes (100,000 cells in 200 μ L medium) were added onto the cytopspin cuvette, which was placed on the slide holder under which blotting paper was placed on a sialinised slide. The slides were then cytopspin (Cytospin 4 centrifuge, Thermo Fischer Scientific, Scoresby, VIC, Australia) for 5 minutes at 500rpm. The blotting paper was carefully removed and slides were air-dried for 10 minutes and fixed in 75% EtOH in DPBS for 10 minutes and air dried at 37°C. Two methods were adopted for staining namely, DAB-based immunoreaction product and indirect immunofluorescent-tagged product.

DAB Method

3,3'-Diaminobenzidine (DAB) reacts with peroxidase to give a brown colour. The method used was as follows: Slides were rehydrated with DPBS and incubated with

0.5% H₂O₂ for 10 minutes (for monocytes it was changed every 5 minutes). Slides were then washed with Tris-buffered Saline Tween-20; 0.05% Tween-20 (TBST buffer). This was followed by Tris Neutral Buffer (TNB) buffer blocking non-specific antigens for 30 minutes followed by addition of anti-rabbit GPR120 or GPR132 or isotype control (1:300; 45 minutes), biotinylated rabbit IgG-G (1:750; 30 minutes), HRP-streptavidin (1:100; 30 minutes) with TBST wash (1 minute) in between each step followed by addition of DAB chromogen. Slides were subjected to dehydration steps using an alcohol and xylene series, after staining for the nucleus using Mayer's haematoxylin. They were then mounted with coverslips (VectaMount) and photographed using the NIKON Eclipse 50i camera. The slides were then analysed and the staining intensity was scored independently by a pathologist.

Immunofluorescence

The second and more sensitive method employed was using indirect-immunofluorescent tagging. FABP4 was also stained with this method along with GPR120 and GPR132. Essentially, the protocol adopted was similar to the DAB method. Additional steps of avidin and biotin block (30 minutes), and streptavidin-FITC (1:200; 30 minutes) stain was added after biotinylated-anti-rabbit IgG incubation step and the wash steps were increased to 5 minutes between each wash step. DAPI was used to mount the slides. The fluorescent micrographs captured using the state of the art Zeiss Imager Z.1 (Carl Zeiss, North Ryde, NSW, Australia).

Oil Red O studies-foam cell assay

Slides

Macrophages were cultured on coverslips using a similar protocol adopted for immunocytochemistry. They (in triplicate) were treated with fatty acids ALA, LA, 9- and 13- HODE for 24 hours. Cells were fixed in neutral buffered formalin (NBF) for 10 minutes. Dried coverslips were then mounted on slides and treated with 50µL of Oil Red O stain for 20 minutes and carefully washed with distilled water for 2 minutes. Nuclear staining was performed using Mayer's haematoxylin and slides were mounted

using aqueous mounting media. Micrographs were captured using the Zeiss imager. Accumulation of lipids was scored by an independent observer on blinded slides based on droplets accumulated per cell.^{288, 289}

Quantification

Accumulation of lipids was quantified for the experiments were carried out for treatment of fatty acids ALA and LA on a six-well plate. One million THP-1 cells were differentiated into macrophages using 100nM PMA for 36 hours. After 24 hours incubation in low serum medium, the macrophages were treated with ALA, LA, 9-HODE or 13-HODE. Following 48 hours of treatment, medium was removed and the cells were washed with DPBS twice. 0.5 ml of Oil Red O solution was added to each well and incubated for 15 minutes at room temperature on an orbital shaker. Staining solution was removed and the wells were washed with wash solution (3 x 1mL). Micrographs were captured and 0.5ml of dye extraction solution was added to each well, following which plates were placed on orbital shaker for 15 minutes. The extracted dye was then pipetted out into a 96-well plate and absorbance was read on a spectrophotometer (TECAN, Sunrise) at 492nm with plate shaken for 5 seconds and allowed to settle for 10 seconds before measurements. The measurements were also taken using spectrophotometer at 520nm.

Antagonist studies

A total of 5×10^5 THP-1 monocytes (in triplicate) were differentiated into macrophages (100nM PMA) for 36 hours in 1mL of RPMI medium containing 10% FBS, L-glutamine and antibiotics in a 12-well plate. The cells were washed with DPBS and incubated for 24 hours in 0.4% FBS medium with additives. Cells were then treated with or without PPAR- γ antagonist T0070907 (10 μ M) for 2 hours, washed with DPBS three times and treated with or without 9-HODE/ 13-HODE (30 μ M) or the PPAR- γ agonist rosiglitazone (0.1 μ M) for 24 hours. Conditioned medium and cell pellet were collected and stored at -80°C as described previously.

siRNA studies

Macrophages were obtained by differentiating 5×10^5 THP-1 monocytes (in triplicate) by treatment with 100nM PMA in 1mL RPMI medium containing 10% FBS and L-glutamine but no antibiotics for 36 hours in a 12-well plate. Cells were washed with DPBS and incubated for another 24 hours with RPMI medium containing 0.4% FBS without antibiotics. The medium was removed and 900 μ L of fresh medium was added. siRNA treatment was carried out using a combination of the four sequences derived from the GPR132 gene structure:

CAGGATTGCCGGGTACTACTA
ACGGACCATTCCCGCCAAGAA
CTGGGTCACCATCGAGATCAA
TACCAATTTCTCGTTCCTGAA

A mixture was prepared by incubating 1 μ L of 10 μ M siRNA and 2 μ L/sample of transfection reagent lipofectamine RNAiMAX for 15 minutes at room temperature in 97 μ L/sample RPMI medium devoid of serum and antibiotics. Control groups included transfection reagent, negative control (all stars negative control supplied by manufacturer containing scrambled sequences which doesn't bind to any mRNA) and positive control siRNA for silencing MAPK (validated sequence). Transfection complex was added drop-by-drop into treatment wells and thoroughly mixed giving a final concentration of 10nM. After six hours treatment, the medium was removed and 1mL of RPMI medium containing 10% FBS and L-glutamine without antibiotics was added for another 18 hours. The cells were then treated with 9-HODE/13-HODE (30 μ M) or rosiglitazone (0.1 μ M) for 24 hours. Conditioned medium and cell pellet were collected and stored at -80°C for protein and gene expression studies respectively.

Human Peripheral Mononuclear Cells

Isolation of leucocytes

A total of 24mL of venous blood was collected from consented donors in heparinised tubes from diabetic/control subjects. Equal amount of DPBS (room temperature) was added and mixed well. 10mL of Ficoll-Hypaque (room temperature) was added to a 50mL Falcon tube and 40mL of DPBS- blood was gently added over Ficoll-hypaque layer. The tubes were centrifuged at 550g for 12 minutes at room temperature with brakes off. The top aqueous layer containing plasma and platelets was aspirated and discarded. The buffy coat was carefully pipetted out and added collectively (pooling the tubes from same sample) into a 50mL tube. Granulocytes and erythrocytes formed a red pellet which was discarded. The tube was filled with DPBS and centrifuged at 600g for 10 minutes at room temperature. Once supernatant (which contains remnant Ficoll-hypaque and plasma) was discarded, DPBS was added (up to 50mL) and centrifuged at 300g for 8 minutes, which aided in removal of platelets. Cells were then resuspended in 5mL of DPBS and a cell count was performed using a haemocytometer. A total of 1 million cells was used for flow cytometry studies. Magnetic bead separation was employed for separation of monocytes.

Magnetic bead separation

Monocytes and macrophages were separated by positive selection of CD14 micro beads. Cells were resuspended into a concentration of 10^7 total cells in 80 μ L buffer (DPBS containing 0.5% FBS and 2mM EDTA). To this 20 μ L of CD14 micro beads were added and incubated for 15 minutes in the refrigerator (2 - 4°C). Cells were then washed by adding 2mL of buffer, and centrifuged at 300g for 5 minutes at 4°C. Supernatant was then discarded and pellet was resuspended in 500 μ L of buffer. Magnetic separation was carried out on a MS column. An MS MACS column was placed on the MACS separator, which was placed on stand. The column was prepared by adding 500 μ L of buffer. The cell suspension was then added to the column. Once the flow through was completed, the column was removed from the separator and placed on a new collection tube. 1mL of buffer was added to the column, and flushed by pushing

the plunger into the column. The cells were counted and stored at -80°C for RNA extraction and gene expression studies.

Flow Cytometry

Human mononuclear cells examined for differentiation markers using flow cytometry studies.

Human Mononuclear cells

One million cells were stained for flow cytometry studies. Cells were first washed with cold flow cytometry buffer (DPBS, containing 0.5% BSA and 2mL of 0.5M EDTA in 500mL of DPBS) and centrifuged at 300g for 5 minutes at 4°C. The supernatant was discarded and cells were incubated with FcR block (1:50 dilution) for 15 minutes at 4°C. The antibody cocktail was then added directly to the cells (CD14-PE-CY7, CD11b-PE, CD36-FITC and CD54-APC or isotype controls) and incubated for 30 minutes at 4°C. Cells were then washed by adding 500µL of flow cytometry buffer and centrifuged at 300g for 5 minutes at 4°C. 300µL of buffer was then added and samples were run on the Fluorescence Activated Cell Sorter (FACS) Calibur (BD Australia, North Ryde, NSW, Australia).

Protein Studies

SDS-PAGE

Conditioned medium collected from experiments were examined for the presence of FABP4. 30µL of sample was loaded with equal amount of loading buffer, heated for 5 minutes at 95°C. Samples were then run on a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel. A mini gel consisting resolving gel and stacking gel was prepared in duplicate in the following quantities:

<i>Ingredient</i>	<i>Resolving gel</i>	<i>Stacking gel</i>
Acrylamide (30%) (mL)	4.04 mL	375 μ L
Running gel buffer (mL)	2.5 mL	625 μ L
ddH ₂ O(mL)	3.51 mL	1500 μ L
Ammonium persulphate 10% (μ L)	37.5 μ L	12.5 μ L
TEMED (μ L)	10 μ L	3.75 μ L
<i>Total (mL)</i>	<i>10.1mL</i>	<i>2.51mL</i>

Table 2.1 SDS-PAGE Gel Preparation

Electrophoresis plates were arranged as per manufacturer's instructions, resolving gel was poured and a thin layer of isopropanol was added, and gel allowed to set for 30 minutes. The isopropanol was washed with water and excess water removed. Stacking gel was poured, and comb was placed. This layer was also allowed to set while the samples were prepared. Samples were run for 10 minutes at 70V followed by 120V for 30 minutes. The gels were removed and one was stained for coomassie blue for 30 minutes followed by destaining solution for 1hour and pictures taken using Biorad Chemidoc System. The other gel was used for western blots.

Western Blot

Gel and nitrocellulose membrane and filter papers were equilibrated in transfer buffer (tris-glycine-methanol) for 30 minutes. The gel was then placed in the following manner from anode to cathode polarity: Pre-wet filter paper, nitrocellulose membrane, gel and filter paper. This was subjected to electroblotting for 30 minutes at 10V. The gel was rinsed with water, rinse any particles of gel off the blot then incubate it in Ponceau S solution for 1 minute. The gel was then rinsed in water until the background is reduced. The membrane was placed on a transparency sheet and photocopied for a permanent transfer record. The blot was rinsed several times in TBS-Tween to remove the stain. After transfer, the membrane was washed for 5 minutes in TBST-T before blocking on a rocker. A 5% block solution was used to incubate the membrane for 1 hour at room temperature followed by addition of primary antibody (FABP4) 1:2000 dilution after tipping off excess blocking solution for 2 hours. Membrane was washed with TBS-T for 10 minutes three times. Secondary antibody was added and incubated for 1 hour

followed by a wash with TBS-T three times as in the previous step, and a 5 minute wash with TBS twice. Pictures were taken using Biorad Chemidoc System.

Cytokine studies

Cytokine were analysed using the multiplex flowcytomix kit from Bender Medsystems. This multiplex it works on the basis of beads that are coated with antibodies, which are differentiated by their variable sizes and spectral addresses. A mixture of antibody-beads of interest is incubated. The analytes from the sample bind to these fluorescent beads. Biotin-conjugated secondary antibodies added then bind to the first captured antibodies. Streptavidin-PE is added next which binds to biotin emitting fluorescent signal when excited by flow cytometer. The secreted proteins were detected in cell culture supernatants and serum. The analysis was performed using the software provided by manufacturer (FlowCytomix 2.3, Bender Medsystems).

Cell culture supernatants were stored as described earlier after the LCFA treatment at -80°C. Human Th1/Th2 cytokine multiplex kit (IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), TNF- α , TNF- β and IFN- γ) was used to determine the cytokines secreted into the medium by after treatment. The protocol recommended by the manufacturer was followed. Briefly, bead mixture, biotin-conjugate mixture, and standard mixture and dilutions were prepared. Samples were stained using flow cytometry tubes. Blank control had 25 μ l assay buffer, standard dilution mixture for cytometer setup, standard dilutions (S1-S7 dilutions) and samples (25 μ L) were incubated with bead mixture and biotin-conjugate mixture at room temperature for 2 hours in the dark. Samples were then washed using 1mL of assay buffer twice carefully discarding the supernatant after spins leaving 100 μ L of liquid (200g for 5 minutes). Streptavidin-PE solution (50 μ L/sample) was then added and samples incubated for 1 hour at room temperature in the dark. Samples were then washed twice as in the previous step. 350 μ L of assay buffer was added and run in the flow cytometer (FACS Calibur, BD biosciences). Setup beads (500 μ L) were first run determining the bead populations as per manufacturer's instruction.

Blood was collected in sterile tubes without anti-coagulant and allowed to clot for 20 mins. The serum was collected by centrifuging the tubes for 10 minutes at 1500g at room temperature. Serum was collected and stored at -80°C for cytokine analysis. A similar protocol was followed for measuring cytokines in serum. The cytokines measured tabled in Figure 2.1.

TH1/TH2 cytokine panel	Obesity Marker Kit
IL-1 β	sCD40L
IL-2	sICAM-1
IL-4	IL-6
IL-5	Leptin
IL-6	MCP-1
IL-8	MPO
IL-10	OPG
IL-12 (p70)	resistin
TNF- α	sTNF-R
TNF- β	
IFN- γ	

Table 2.2 Cytokine Panels

Statistical Analysis

Data were analysed using Graphpad Prism software. All the experiments were performed at least three times and the results from representative experiment have been presented. Cytokine studies samples were assayed in duplicate. In some cases pooling of data was considered and appropriately presented. Results are expressed as mean \pm standard error of the mean (SEM) unless otherwise stated. Data was then normalised with considering the control expression as maximum expression and data from other groups represented as percentage of control.

For cell counts, cell proliferation assays and Oil Red O quantification, the data exported into Microsoft excel and entered in Graphpad Prism.

For analysing real time RT-PCR, the samples were run in duplicate, the results from sample and its duplicate were tabled in excel. The calculated concentration data for each of the genes was exported from the Corbett rotor gene run-analysis software. Two columns were prepared with reference gene on one and the gene of interest on the other. The relative expression was obtained by using the formula;

The calculations were carried out for both options wherein the relative concentration was obtained for calculated concentrations of;

- 1) Gene of interest / reference gene and duplicate of gene of interest / duplicate.
- 2) Duplicate of gene of interest / reference gene and gene of interest / duplicate of reference gene.

An average of total four values obtained was then plotted thus eliminating any errors that could arise by considering one option by calculating only the samples and duplicates separately.

Flow cytometry analysis was performed using the cell quest acquisition / analysis software. The appropriate gates were set and markers were set on the histograms of interest. The corresponding values for each marker was then plotted on excel sheet and Graphpad. The cytokine bead array data was generated using the Flowcytomix Pro software by Bender Medsystems for data analysis. The resulting data was then plotted on to Graphpad.

Depending on the sample size appropriate statistical tests were performed using Graphpad Prism. When the sample size was more than five in each group, normality test was performed and depending on the outcome, a non-parametric or parametric test was

performed. $p < 0.05$ was deemed statistically significant. Statistical comparisons between treatments were calculated with one-way analysis of variance (ANOVA) using Tukey's multiple comparison test. Unpaired student t test was used for direct comparison of two sets of data.

Chapter 3

Specific Effects of Hydroxyoctadecadienoic Acids on Apoptosis and Genes of Lipid Signalling in Monocytes and Macrophages

Introduction

Monocytes and macrophages are subjected to the presence of fatty acids and their derivatives present in the circulation and extracellular fluids. These cells are attracted to the sites of inflammation and their exact role in the pathogenesis of arterial disease is not clearly understood. The documented beneficial effects of dietary polyunsaturated fatty acids on cardiovascular health has stimulated considerable interest in studies on how fatty acids affect monocytes, macrophages, and the other cells of the arterial wall (endothelial and vascular smooth muscle cells). The fatty acids that are of interest include dietary components LA, palmitic acid (PA), ALA, and DHA, as well as components of oxLDL, LDL, VLDL and prostaglandins that are present in circulation.^{125, 256} Fatty acids AA, LA, ALA, oxLDL and HODEs have been shown to affect cell viability of monocytes in a dose-dependent manner. Decrease in cell viability has been shown to be at least partly due to apoptosis induced by these fatty acids and their derivatives.^{5, 126, 128, 290}

Apoptosis of macrophages is known to be an important process in atherogenesis though there is still much to be learned about the precise contribution it makes to atherogenesis.^{291, 292} During early atherosclerosis, there is increased monocyte recruitment and differentiation into macrophages and inhibition of apoptosis. In the later stages, enhanced apoptosis is observed with increased clearance of lipid-laden cells. This increased apoptosis results in formation of a necrotic core and a rupture prone plaque. Increased oxidative stress in animal models of atherosclerosis, and in humans prone to atherosclerosis, leads to generation of oxLDL (which are important mediators in atherogenesis). oxLDL generated non-enzymatically is involved in modulating various processes in the arterial wall including apoptosis.²⁹³ oxLDL has been reported to induce apoptosis in macrophages in some studies,^{294, 295} while other studies have suggested that it inhibits the process.²⁹⁶⁻²⁹⁸ 13-HODE, the predominant product derived from enzymatic action of 15-LOX on LA is abundant during the initial stages of atherosclerosis. 9-HODE and 13-HODE, derived non-enzymatically, are present during the later stages of the disease process, and are present in equal amounts. Published work to date has not extensively investigated the effects of HODEs on THP-1 cells. In this study, I have investigated the hypothesis that HODEs might exert different effects on activation and differentiation of cells depending on the stage of development of the

atheromatous plaque.^{91,95} Both HODEs are known to be PPAR- γ ligands and the latter, when activated, is involved in regulating apoptosis in macrophages.^{4, 299} Pro-apoptotic effects of HODEs have been reported in human monocytic cells.^{4,5}

Some of the key receptors expressed during foam cell formation belong to the family of scavenger receptors, some of which are involved in uptake of lipids by cells, while others are involved in reverse cholesterol transport.²⁰⁷ The effect of HODEs on lipid scavenger receptors on THP-1 cells has not been documented to date. These processes involve a wide range of gene products including transcription factors, genes involved in producing unsaturated fatty acids, and intracellular lipid transporters. The roles of the putative long-chain fatty acid receptors GPR120 and GPR132 in atherogenesis have not been documented. 9-HODE is a ligand for GPR132, activation of which leads to pro-inflammatory signals although it is not clear whether the activation of this receptor leads to atherosclerosis or not.²⁴³

Many processes involved in lipid metabolism are regulated by exposure of cells to oxidised lipids, and these processes are thought to be regulated by PPAR- γ , activation of which increases levels of FABP4 and generates anti-inflammatory signals.^{141, 142, 300} Increased FABP4 expression has been associated with insulin resistance and increased risk of atherosclerosis, and is reported to be induced by oxLDL.¹⁴¹ It is not certain, therefore, whether the effects of HODEs and other oxidised lipids are protective in relation to atherosclerosis or whether they might indeed promote the processes that lead to plaque formation.

The aim of this Chapter was to investigate the regulation of apoptosis and key genes involved in lipid metabolism by long-chain fatty acids (ALA, LA, 9-HODE and 13-HODE) on monocytes and macrophages. It was hypothesised that 9-HODE would have pro-atherosclerotic effects, while 13-HODE would be protective. In general, HODEs (as major components of oxLDL) may be involved in mediating pro-atherosclerotic effect of oxidative stress. The studies presented here include those on cell viability, apoptosis, cytotoxicity, cytokines secreted and lipid accumulation. Regulation of the expression of genes involved in fatty acid signalling (GPCRs and transcription factors) and fatty acid metabolism were also studied.

Results

Monocytes: Cell Viability

One million cells were treated with 30 μ M ALA, LA, 9-HODE and 13-HODE for 24 to 48 hours. The experimental conditions used to study cell viability were determined from the previous literature and from preliminary experiments. Cells were treated with ALA and LA for 48 hours and with HODEs for 24 hours. This was because ALA and LA showed no effect in 24 hours while treatment with HODEs for greater than 24 hours led to a decrease in the viability of cells in culture. A simple cell count using a haemocytometer was performed following treatment with the various fatty acids. No significant change in cell viability was observed after 48 hours treatment with ALA. The n-6 fatty acid LA significantly decreased ($p < 0.05$) the cell number at 48 hours. The oxidised lipid 9-HODE significantly decreased the cell numbers after 24 hours of treatment (Figure 3.1). By contrast, 13-HODE had no significant effect on cell number after 24 hours.

The above experiments were carried out in low serum medium (0.5% FBS). Similar experiments were carried out with 10% FBS (Figure 3.2). In this case, neither ALA nor LA affected cell viability at 48 hours. There was no difference in the effects of HODEs on cell viability comparing low serum with high serum medium (data not shown). Accurate and reproducible data could only be obtained with THP1 monocytes. Following differentiation into macrophage-like cells with PMA, the cells became adherent and trypsinisation to count cells yielded variable results.

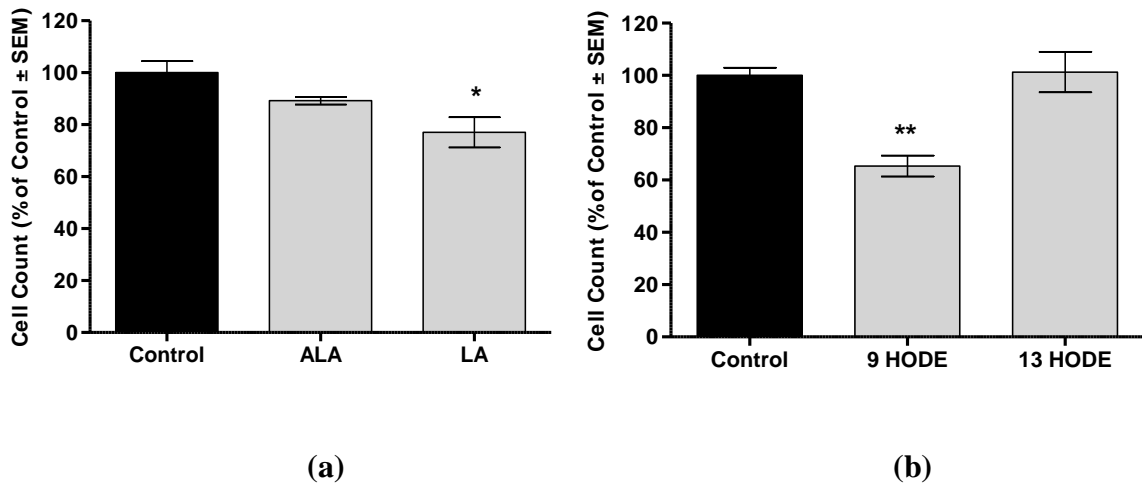


Figure 3.1 Effect of Fatty Acids on Cell Count

Cell count is expressed as a percentage of control

(a) Cell count of THP-1 cells after 48 hours treatment with 30 μ M linolenic and linoleic acid ($p < 0.05$) compared with control.

(b) Cell count after 24 hours treatment with 9-HODE ($p < 0.01$) and 13-HODE (NS) compared with control.

Data shown are mean \pm SEM from groups treated in triplicate and normalised as percentage of control (one way ANOVA, Tukey's post test) * = $p < 0.05$ and ** = $p < 0.01$.

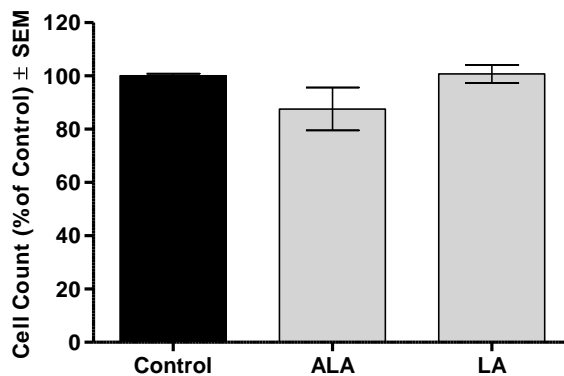


Figure 3.2 Cell Count in High FBS Medium

Cell number with 48 hours ALA ($p > 0.05$) and LA ($p > 0.05$) treatment with 10% FBS medium.

Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test).

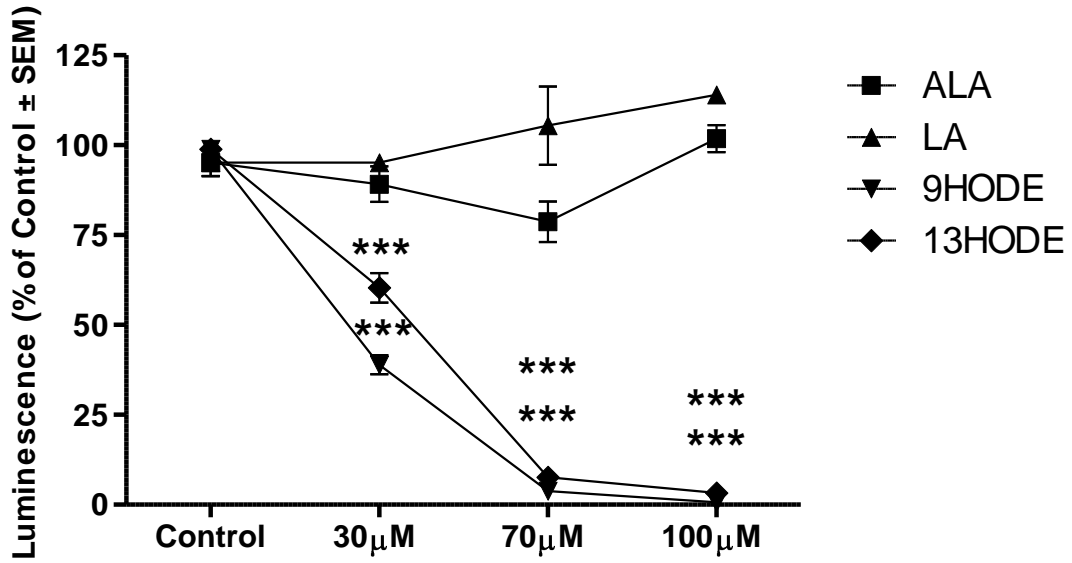
Cell viability, Apoptosis and Cytotoxicity

Luminescence- and fluorescence- based assays were employed to determine if the decrease in cell viability observed with 9-HODE was due to apoptosis or to cytotoxicity. Also, use of these assays could overcome the technical difficulties experienced in experiments counting macrophage cell number. Identifying the most appropriate concentration of fatty acid is important, as there have been inconsistencies in the literature with the treatment concentration employed in experiments. A dose response to determine the effect of these LCFA on cell viability was performed with concentrations of 0, 30, 70 and 100 μ M. Parallel experiments were set up to study if the effects were due to apoptosis or cytotoxicity. Initial experiments investigated two time points, namely 24 and 48 hours (data not shown). Significant changes were not observed with ALA after 24 hours and hence it was decided therefore to investigate changes associated with cell proliferation at 48-hour time point with ALA and LA. When the effect of 9-HODE and 13-HODE was studied, it was quite clear that at 24-hour time point there was a significant decrease in cell viability with increase in dose. Hence, the 24-hour time point was chosen for investigating the effects of HODEs.

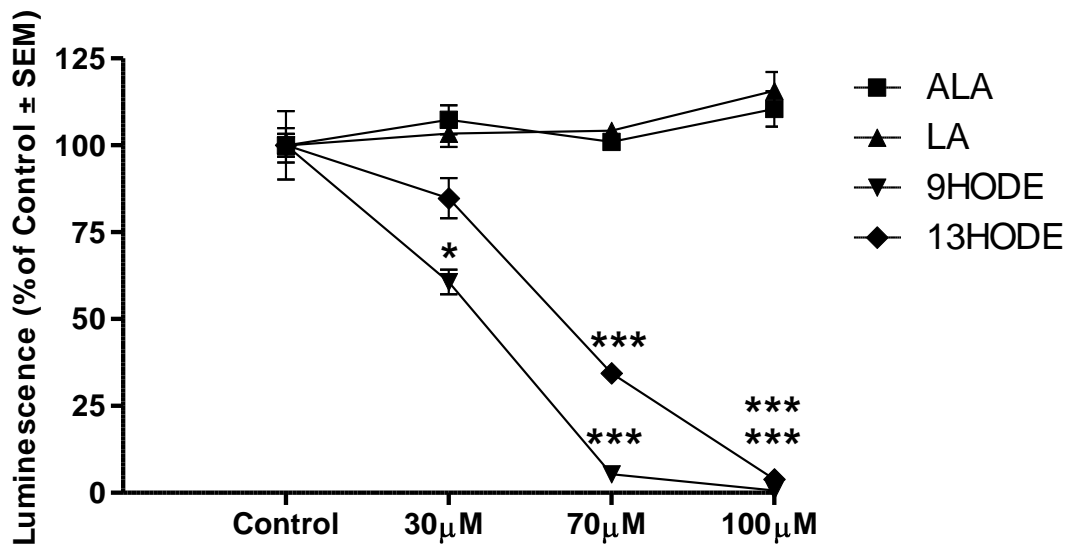
The assays were performed on opaque sterile 96-well plates. 24,000 THP-1 cells or PMA differentiated macrophages were treated with or without C-18 LCFA \pm 1-100nM PMA for 24 to 48 hours in 50 μ L of culture medium. After the experimental time points were reached, cell culture plates were taken out of incubator and equal amount of assay reagent was added. Manufacturer's recommended protocol was followed. After 30 minutes of incubation time, luminescence or fluorescence measurements were taken. The parent fatty acids ALA and LA, at 48-hour time point, did not significantly decrease cell numbers. HODEs on the other hand significantly decreased the cell numbers progressively with increase in concentration ($p < 0.001$, ANOVA, Tukey's multiple comparison) with 9-HODE being more potent than 13-HODE at a concentration of 30 μ M. This was also found to be true in case of macrophages with parent fatty acids having no apparent effect on cell viability. 9-HODE was more potent in case of macrophages as well at concentrations 30 and 70 μ M (Figure 3.3). Experiments with the Caspase 3/7 apoptosis assay suggested that the decrease in cell number and viability is apoptosis induced (Figure 3.4). It was important to consider the dose of fatty acids for future experimental studies. An appropriate dose that was not

cytotoxic to cells is important for experimental design to study, for example, expression of genes involved in lipid metabolism.

As expected, there were no changes observed with treatment of monocytes with ALA or LA. Cytotoxic assays revealed that a 13-HODE dose of 30 μ M and 70 μ M (both $p < 0.05$, ANOVA) had no significant effect, but the higher dose of 100 μ M was found to be cytotoxic ($p < 0.001$). 9-HODE, on other hand, was cytotoxic at 30 μ M ($p < 0.05$) with higher doses being more cytotoxic ($p < 0.001$). Macrophages showed cytotoxic effects with a 13-HODE dose of 30 μ M ($p < 0.05$, ANOVA) but not with 9-HODE at that concentration. With increase in dose, cytotoxicity was observed with both oxidised lipids with increasing effect with increasing concentration of HODE - 70 μ M and 100 μ M 9-HODE (both $p < 0.001$), and 13-HODE being cytotoxic in a more dose-dependent manner - 30 μ M ($p < 0.05$), 70 μ M ($p < 0.01$) and 100 μ M ($p < 0.001$). As with monocytes, ALA and LA did not significantly alter cytotoxicity in macrophages (Figure 3.5).



(a)



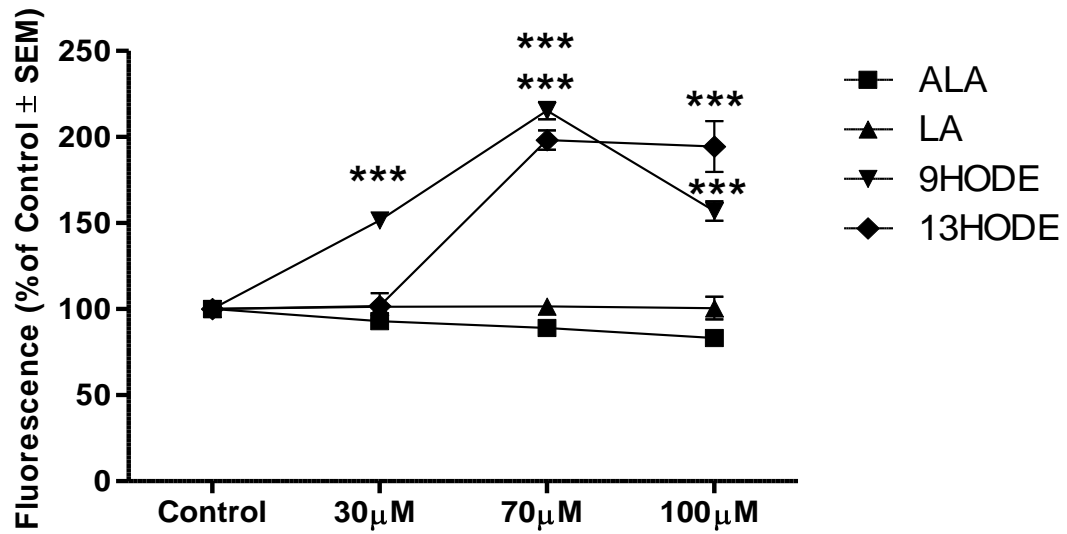
(b)

Figure 3.3 Cell Viability Assay

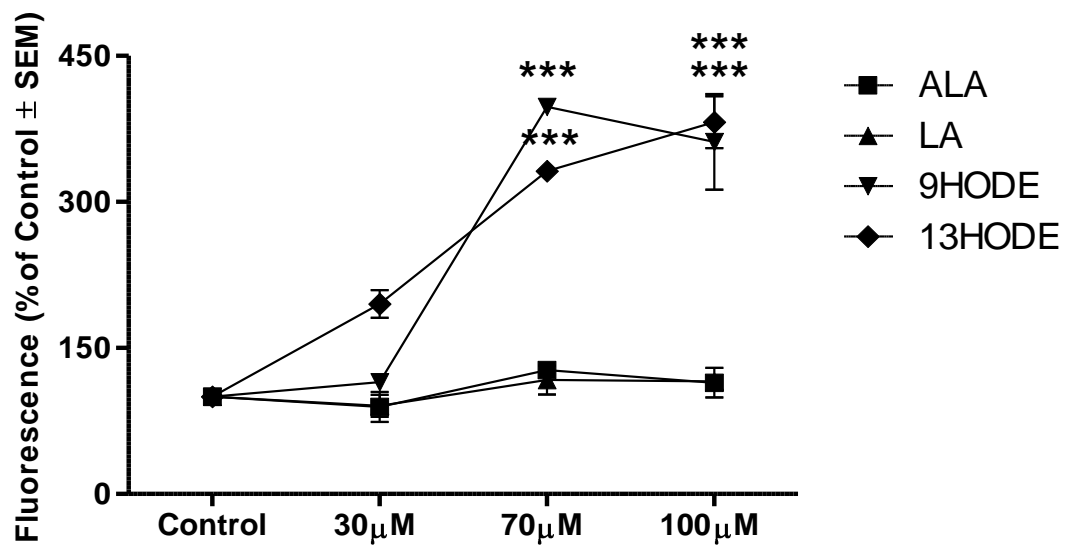
(a) Monocytes: Effect of ALA (48 hours, 30-100μM – $p > 0.05$), LA (48 hours, 30-100μM – $p > 0.05$), 9-HODE (24 hours, 30-100μM – $p < 0.001$), and 13-HODE (24 hours, 30-100μM – $p < 0.001$).

(b) Macrophages: Effect of ALA (48 hours, 30-100μM – $p > 0.05$), LA (48 hours, 30-100μM – $p > 0.05$), 9-HODE (24 hours, 30-100μM – $p < 0.001$), and 13-HODE (24 hours, 30μM $p > 0.05$, 70-100μM – $p < 0.001$).

All expressed as a percentage of control (normalised). Data shown are mean \pm SEM of groups treated in triplicate and normalised as percentage of control (one way ANOVA, Tukey's Post Test). * = $p < 0.05$ and *** = $p < 0.001$.



(a)



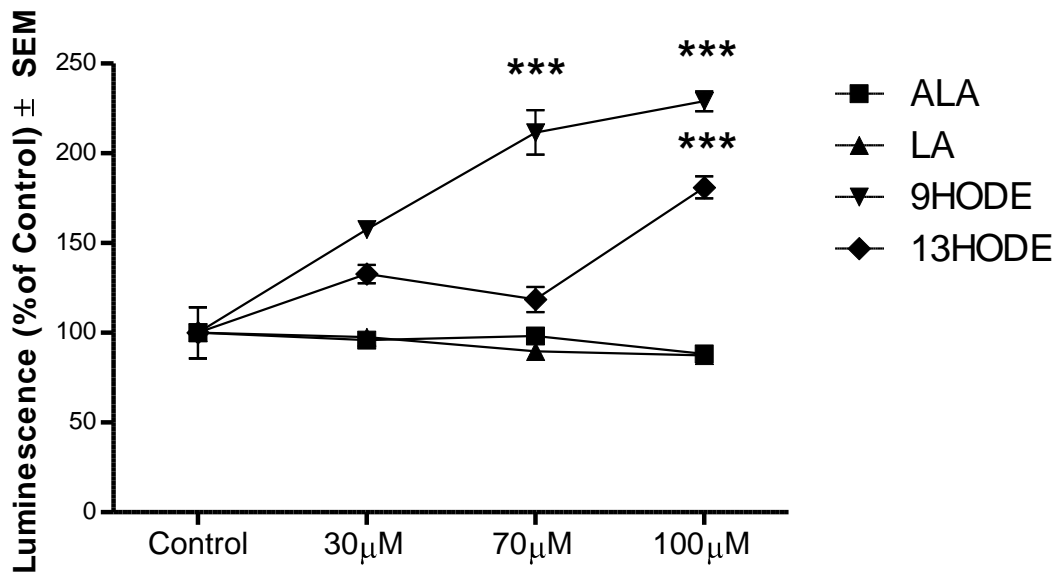
(b)

Figure 3.4 Apoptosis Assay

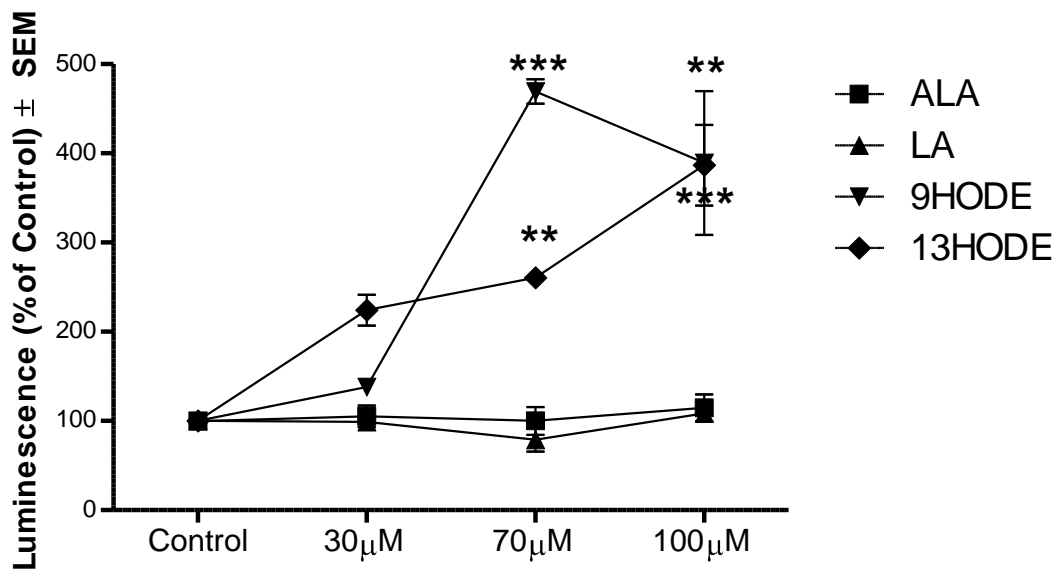
(a) Monocytes: Effect of ALA (48 hours, 30-100μM – $p > 0.05$), LA (48 hours, 30-100μM – $p > 0.05$), 9-HODE (24 hours, 30-100μM – $p < 0.001$), and 13-HODE (24 hours, 30 μM – $p > 0.05$, 70 - 100μM – $p < 0.001$).

(b) Macrophages, effect of ALA (48 hours, 30 - 100μM – $p > 0.05$), LA (48 hours, 30 - 100μM – $p > 0.05$), 9-HODE (24 hours, 30μM – $p > 0.05$, 70 - 100μM – $p < 0.001$), and 13-HODE (24 hours, 30μM – $p > 0.05$, 70 - 100μM – $p < 0.001$).

All expressed as a percentage of control (normalised). Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's Post Test). *** = $p < 0.001$.



(a)



(b)

Figure 3.5 Cytotoxicity Assay

(a) Monocytes: Effect of ALA (48 hours, 30 - 100µM – $p > 0.05$), LA (48 hours, 30 - 100µM – $p > 0.05$), 9-HODE (24 hours, 30 µM – $p > 0.05$, 70 - 100µM – $p < 0.001$), and 13-HODE (24 hours 30 - 70µM – $p > 0.05$, 100µM – $p < 0.001$).

(b) Macrophages: Effect of ALA (48 hours, 30 - 100µM – $p > 0.05$), LA (48 hours, 30 - 100µM – $p > 0.05$), 9-HODE (24 hours, 30 µM – $p > 0.05$, 70µM – $p < 0.001$, 100µM – $p < 0.01$), and 13-HODE (24 hours, 30µM – $p > 0.05$, 70µM – $p < 0.01$, 100µM – $p < 0.001$).

All expressed as a percentage of control (normalised). Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's Post Test). * $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$

Oil Red O studies

Quantification

Though the parent fatty acids ALA and LA did not have any effect on cell proliferation when cell numbers were counted (in contrast to the effect of HODEs), it was important to investigate whether treatment of these cells with the four fatty acids also showed differential effects on lipid accumulation. This investigation was performed using the foam cell assay with Oil red O to indicate the degree of lipid accumulation. Cells were exposed to control medium or to medium containing the test fatty acid for 48 hours. Parallel experiments were set up with Oil Red O experiments to investigate effects of fatty acids on genes involved in lipid transport and metabolism.

At the end of 48 hours incubation with control or test medium, 0.5mL of Oil Red O stain was added after removing the medium and washing the cells with DPBS three times. After the culture plates were incubated at room temperature for 15 minutes on an orbital shaker, excess stain was removed, and the cells were rinsed with wash solution. Dye extraction solution was then added (0.5mL) and incubated for 15 minutes. Extracted dye was removed and was quantified using the Nanodrop at 520nm. There were no significant increases in lipid accumulation with either ALA or LA (Figure 3.6). The effect of HODEs proved to be inconsistent and data are not therefore shown here. However, in later studies (Chapter 4), lipid droplets were counted following addition of Oil Red O. These studies showed that the effect of HODEs was clearly different to that of ALA and LA.

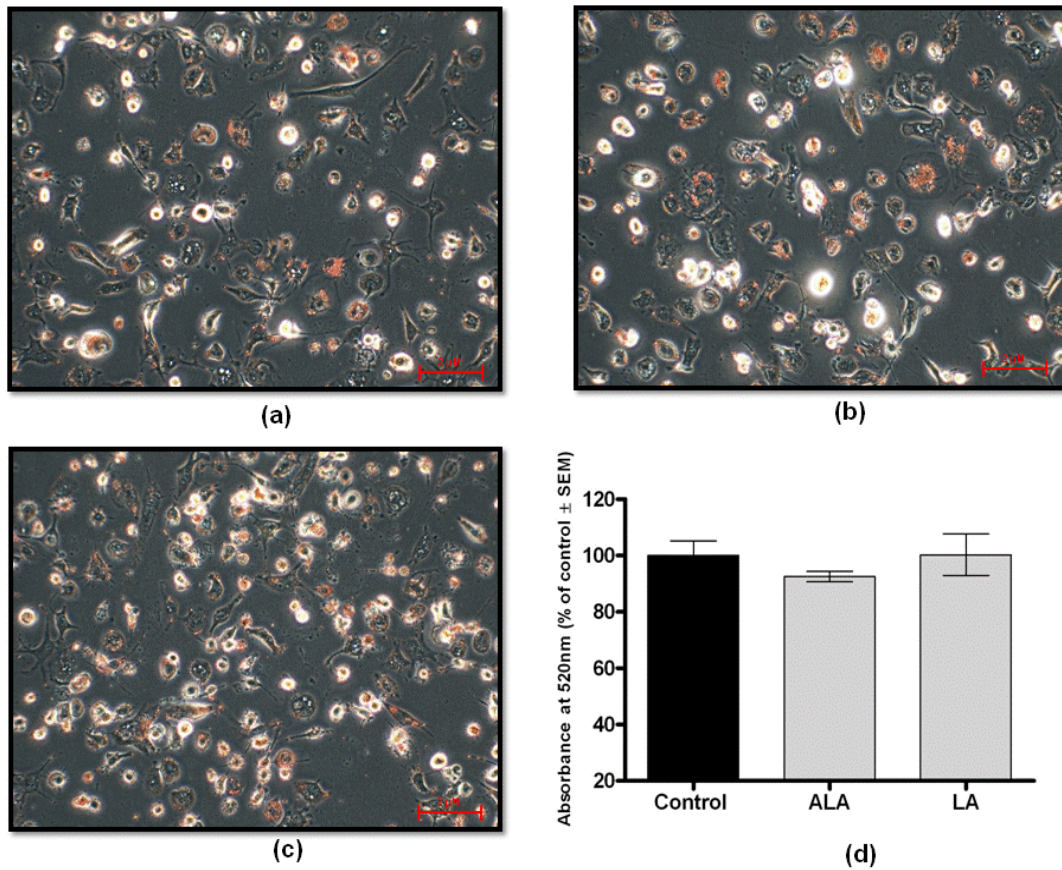


Figure 3.6 Oil Red O Quantification

Micrographs of cells exposed to (a) control medium (b) 30µM linolenic acid (c) 30µM linoleic acid for 48 hours.

(d) Quantification of Oil Red O stain by the cells after the dye extraction step.

Data shown are mean ± SEM of triplicates in each group normalised against control ($p > 0.05$, one way ANOVA, Tukey's Post test).

Gene Expression Studies

Viability and apoptosis studies showed that there is a significant decrease in cell number with 9-HODE treatment. Measurement of mRNA expression of genes involved in fatty acid signalling was carried out after LCFA treatment. Briefly, one million THP-1 cells or PMA differentiated macrophages were incubated with 30 μ M ALA, LA, 9-HODE or 13-HODE. Time points were based on apoptosis and cell viability studies. Cells were treated with ALA and LA for 48 hours and with HODEs for 24 hours. After the experimental period, conditioned medium was removed and stored at -80°C for protein studies. Cells were trypsinised with reaction stopped by adding media and cells were harvested using a cell scraper. RNA was extracted using the Qiagen mini kit, quantified using Nanodrop, and were tested for any DNA contamination using a no reverse transcriptase reaction and ensured that they were clean before setting up assays to quantify mRNA levels. The genes possibly involved in fatty acid signalling GPR120 and GPR132 and those involved in lipid metabolism and signalling were studied – including SCRA, reverse cholesterol transport SCRB, the transcription factor PPAR- γ , the lipid chaperone FABP4, and SCD.

Fatty Acid Signalling Receptors

In monocytes, significant differences with GPR120 were not observed in with HODE treatment compared with controls. Indeed, no changes in GPR120 expression were observed with any of the four fatty acids. ALA and LA did not affect GPR132 expression in monocytes, while HODEs increased the expression. More specifically, with 9-HODE expression of GPR132 was increased four-fold ($p < 0.001$, ANOVA). 13-HODE also had a significant impact on GPR132 with expression being increased two-fold compared with controls ($p < 0.05$, Figure 3.7).

In macrophages, similar effects were observed, with ALA and LA not affecting GPR120 expression. Interestingly, HODEs decreased the expression of GPR120 ($p < 0.05$) - with 9-HODE and 13-HODE having similar effects. Unlike in monocytes, GPR132 expression was not affected by any of the four LCFA in macrophages (Figure 3.8). None of the four fatty acids affected expression of the gene for scavenger receptor

A (SRA). 13-HODE decreased the expression of this receptor for lipid uptake in macrophages ($p < 0.05$), while 9-HODE, ALA and LA were without effect ($p > 0.05$, Figure 3.9).

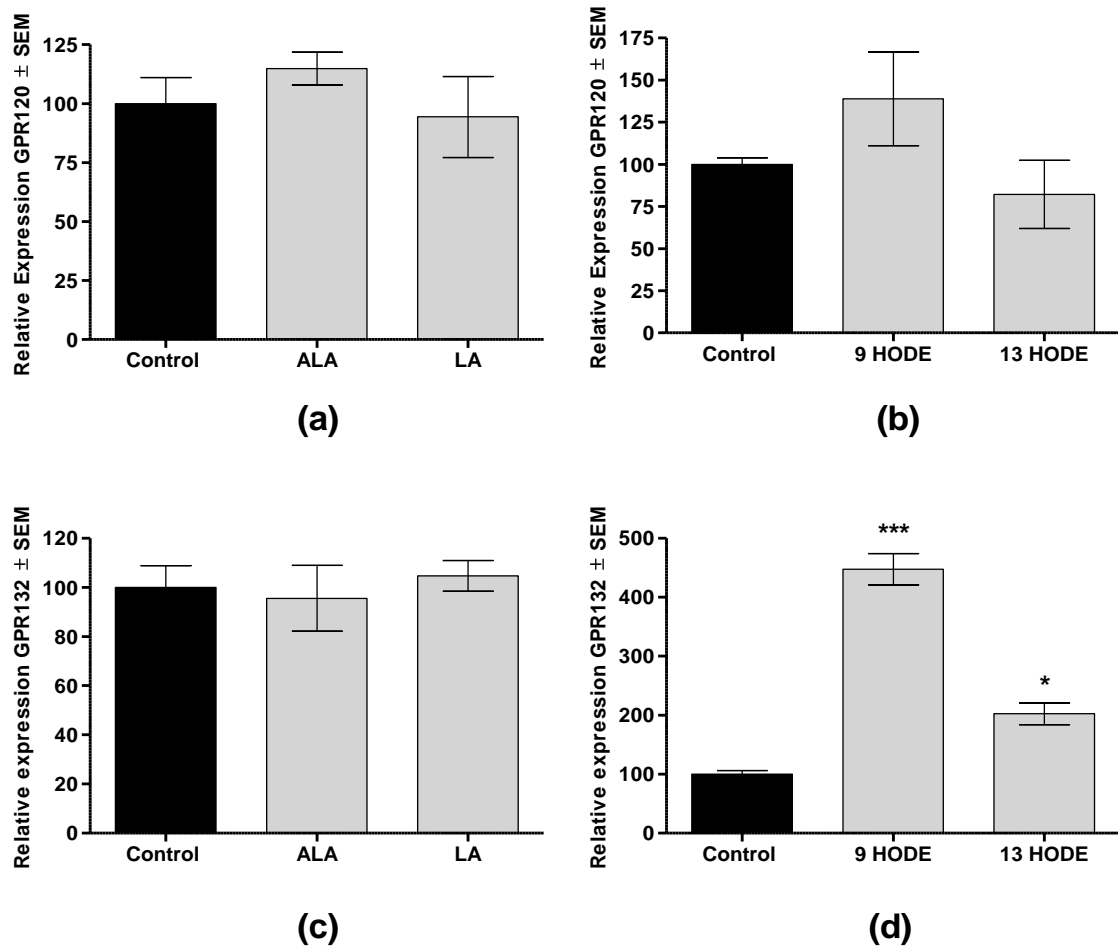


Figure 3.7 GPR120 and GPR132 Expression in Monocytes

GPR120 expression: (a) ALA ($p > 0.05$) and LA ($p > 0.05$) (b) 9-HODE ($p > 0.05$) and 13-HODE ($p > 0.05$)

GPR132 expression: (c) ALA ($p > 0.05$) and LA ($p > 0.05$) (d) 9-HODE ($p < 0.001$) and 13-HODE ($p < 0.05$).

Data shown are mean \pm SEM of triplicates in each group normalised against control (one way ANOVA, Tukey's post test). * = $p < 0.05$ and *** = $p < 0.001$.

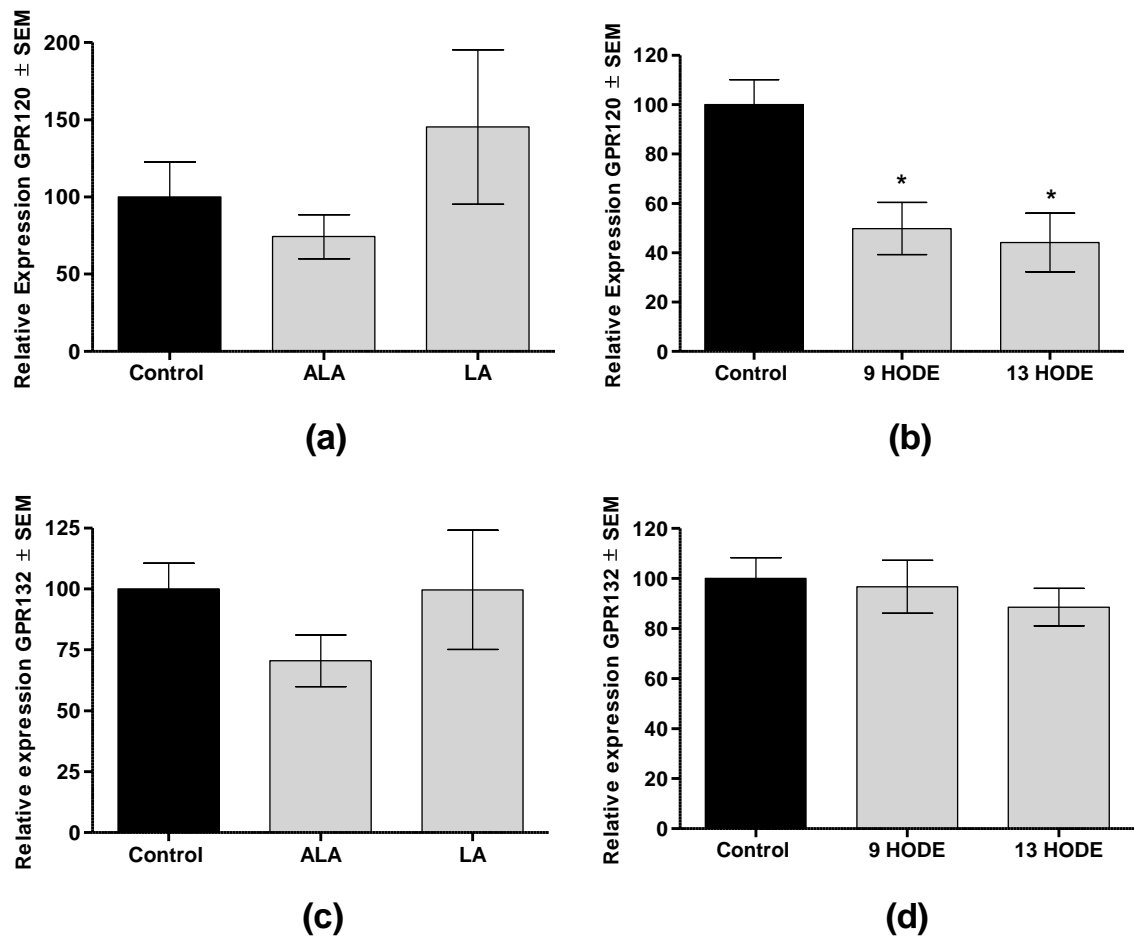


Figure 3.8 GPR120 and GPR132 Expression in Macrophages

GPR120 expression: **(a)** ALA ($p > 0.05$) and LA ($p > 0.05$) **(b)** 9-HODE ($p < 0.05$) and 13-HODE ($p < 0.05$)

GPR132 expression: **(c)** ALA ($p > 0.05$) and LA ($p > 0.05$) **(d)** 9-HODE ($p > 0.05$) and 13-HODE ($p > 0.05$).

Data shown are mean ± SEM of triplicates in each group normalised against control (one way ANOVA, Tukey's post test). * = $p < 0.05$.

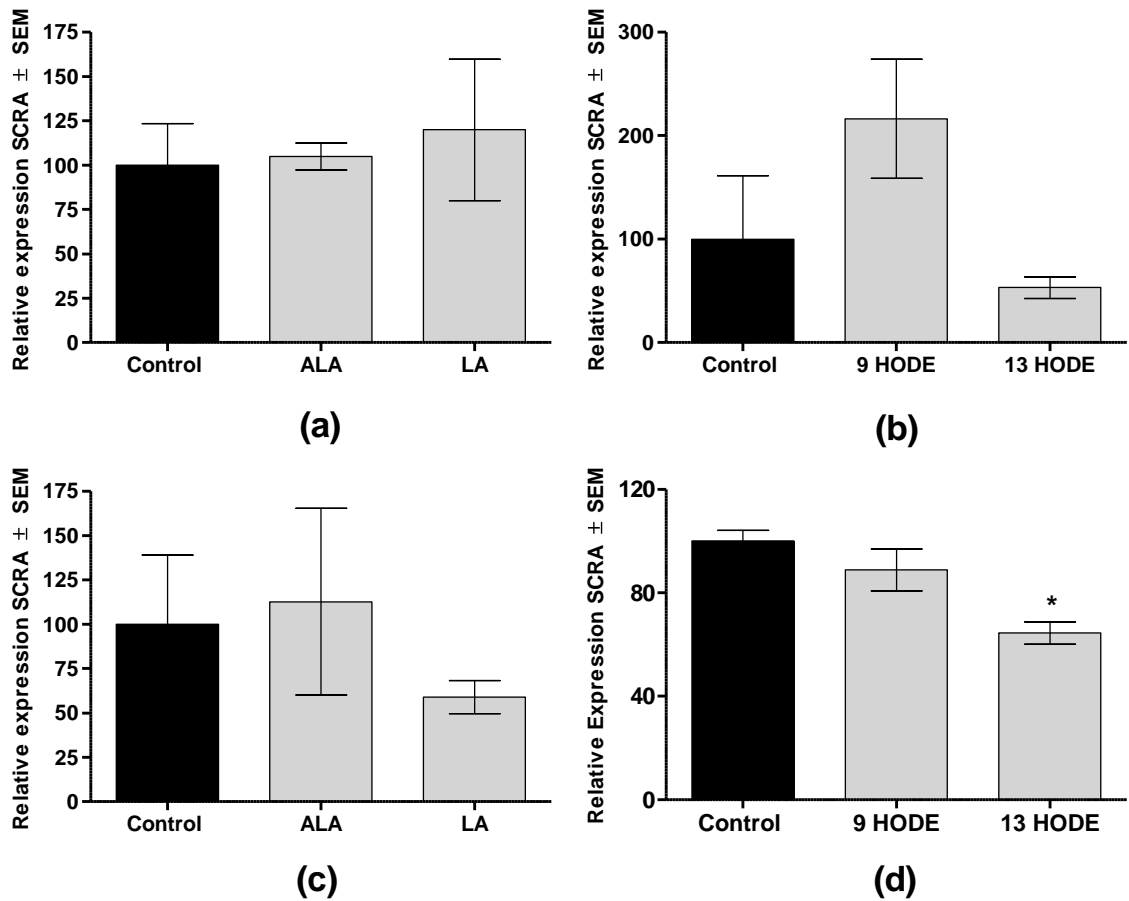


Figure 3.9 SCRA Expression in Monocytes and Macrophages

Monocytes: **(a)** ALA ($p > 0.05$) and LA ($p > 0.05$) **(b)** 9-HODE ($p > 0.05$) and 13-HODE ($p > 0.05$).

Macrophages: **(c)** ALA ($p > 0.05$) and LA ($p > 0.05$) **(d)** 9-HODE ($p > 0.05$) and 13-HODE ($p < 0.05$).

Data shown are mean \pm SEM of triplicates in each group normalised against control (one way ANOVA, Tukey's post test). * = $p < 0.05$.

SCRB is a gene involved in reverse cholesterol transport. Expression of the gene was not significantly affected by ALA, LA, or by either HODE in monocytes. In macrophages, 9-HODE and 13-HODE increased expression of the gene significantly. 9-HODE increased expression two-fold, ($p < 0.01$) and 13-HODE, increased expression by 1.5 times compared with control ($p < 0.05$, Figure 3.10). ABCA1, another protein involved in reverse cholesterol transport was also studied in macrophages. Unlike with SCRB, a significant increase was not detected with either of the HODEs (Figure 3.11).

Expression of the transcription factor PPAR- γ was markedly increased in monocytes with HODEs, but not with the parent fatty acids ALA and LA. 9-HODE increased expression by more than ten-fold ($p < 0.01$), while there was an eight-fold increase with 13-HODE ($p < 0.05$) in monocytes. By contrast, none of the four fatty acids altered PPAR- γ expression when the cells were differentiated into macrophages (Figure 3.12). Expression levels of the transcription factor TR4 were not affected by HODE treatment in macrophages (Figure 3.13).

Fatty acid binding protein-4 (FABP4) was very significantly increased with HODE treatment in monocytes and macrophages. Again, the parent ALA and LA did not alter expression of this gene in monocytes ($p > 0.05$), while both 9-HODE and 13-HODE increased its expression by around five-fold compared with control (Figure 3.14). In macrophages, as in monocytes, ALA and LA did not alter expression of this gene ($p > 0.05$). Both 9-HODE and 13-HODE increased FABP4 by more eight-fold ($p < 0.001$, Figure 3.14). Furthermore and an increase in FABP5 was also observed on incubation of macrophages with 9-HODE ($p < 0.05$, Figure 3.13). Interestingly, SCD expression was increased with 9-HODE treatment in monocytes ($p < 0.05$) and in macrophages ($p < 0.001$), an effect that was not apparent with 13-HODE in either monocytes or macrophages ($p > 0.05$, Figure 3.15).

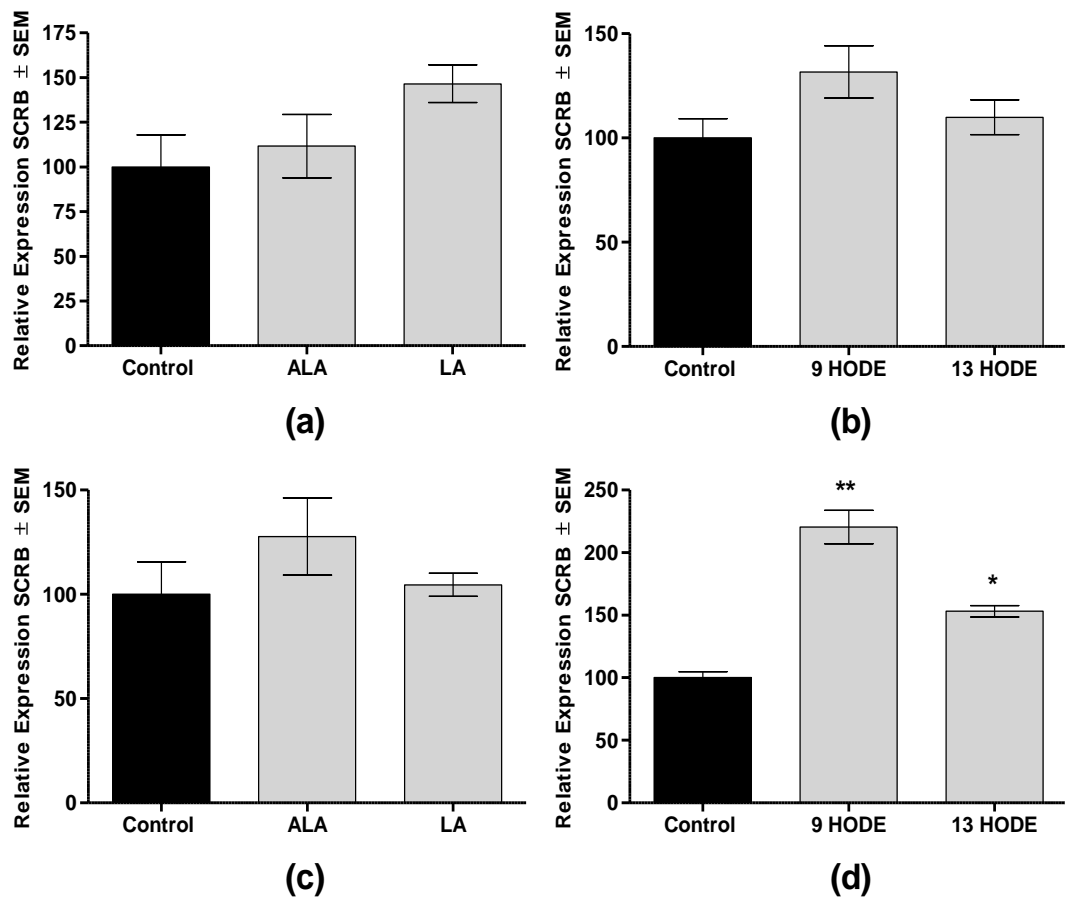


Figure 3.10 SCR B Expression in Monocytes and Macrophages

Monocytes: (a) ALA ($p > 0.05$) and LA ($p > 0.05$) (b) 9-HODE ($p > 0.05$) and 13-HODE ($p > 0.05$).

Macrophages: (c) ALA ($p > 0.05$) and LA ($p > 0.05$) (d) 9-HODE ($p < 0.01$) and 13-HODE ($p < 0.05$).

Data shown are mean \pm SEM of triplicates in each group normalised against control (one way ANOVA, Tukey's post test). * = $p < 0.05$ and ** = $p < 0.01$.

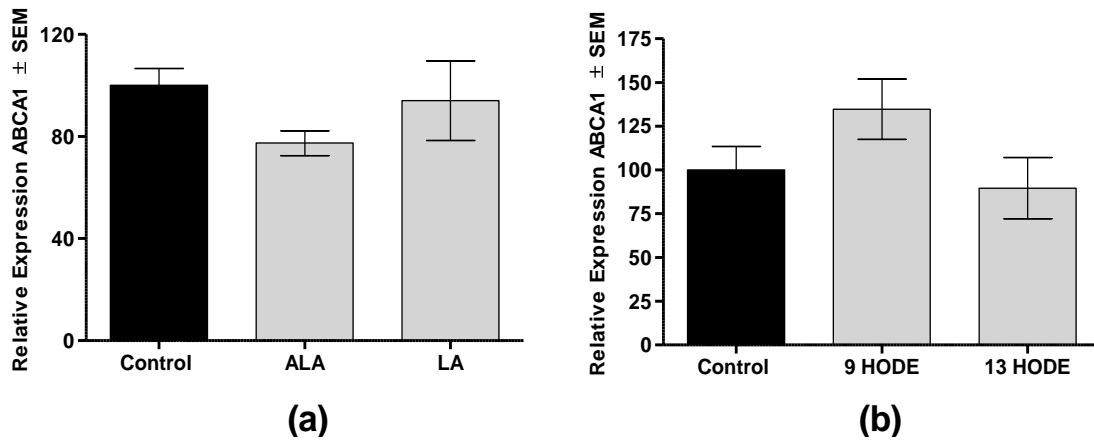


Figure 3.11 ABCA1 Expression in Macrophages

(a) ALA ($p > 0.05$) and LA ($p > 0.05$) and (b) 9-HODE ($p > 0.05$) and 13-HODE ($p > 0.05$)

Data shown are mean \pm SEM of triplicates in each group normalised against control (one way ANOVA, Tukey's post test).

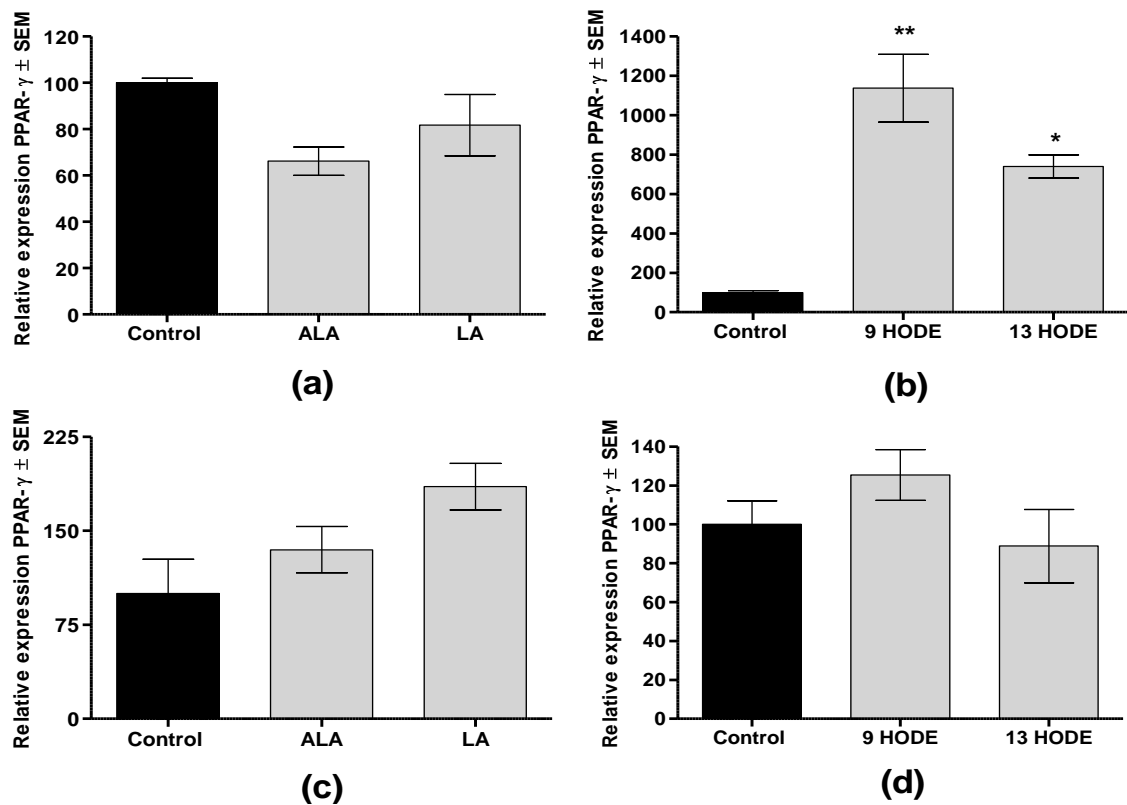


Figure 3.12 PPAR- γ Expression in Monocytes and Macrophages

Monocytes: (a) ALA ($p > 0.05$) and LA ($p > 0.05$) (b) 9-HODE ($p < 0.01$) and 13-HODE ($p < 0.05$).

Macrophages: (c) ALA ($p > 0.05$) and LA ($p > 0.05$) (d) 9-HODE ($p > 0.05$) and 13-HODE ($p > 0.05$).

Data shown are mean \pm SEM of triplicates in each group normalised against control (one way ANOVA, Tukey's post test). * = $p < 0.05$ and ** = $p < 0.01$.

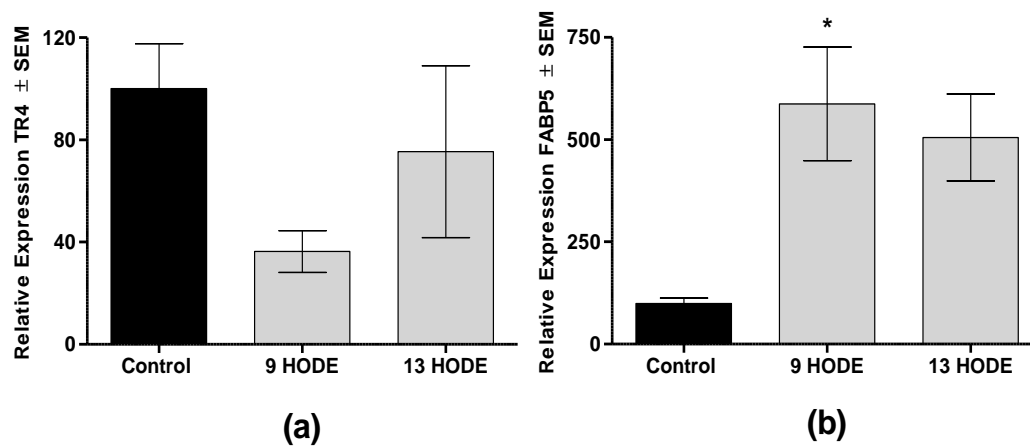


Figure 3.13 TR4 and FABP5 Expression in Macrophages

TR4: (a) 9-HODE ($p > 0.05$) and 13-HODE ($p > 0.05$).

FABP5: (b) 9-HODE ($p < 0.05$) and 13-HODE ($p > 0.05$).

Data shown are mean \pm SEM of triplicates in each group normalised against control (one way ANOVA, Tukey's post test). * = $p < 0.05$.

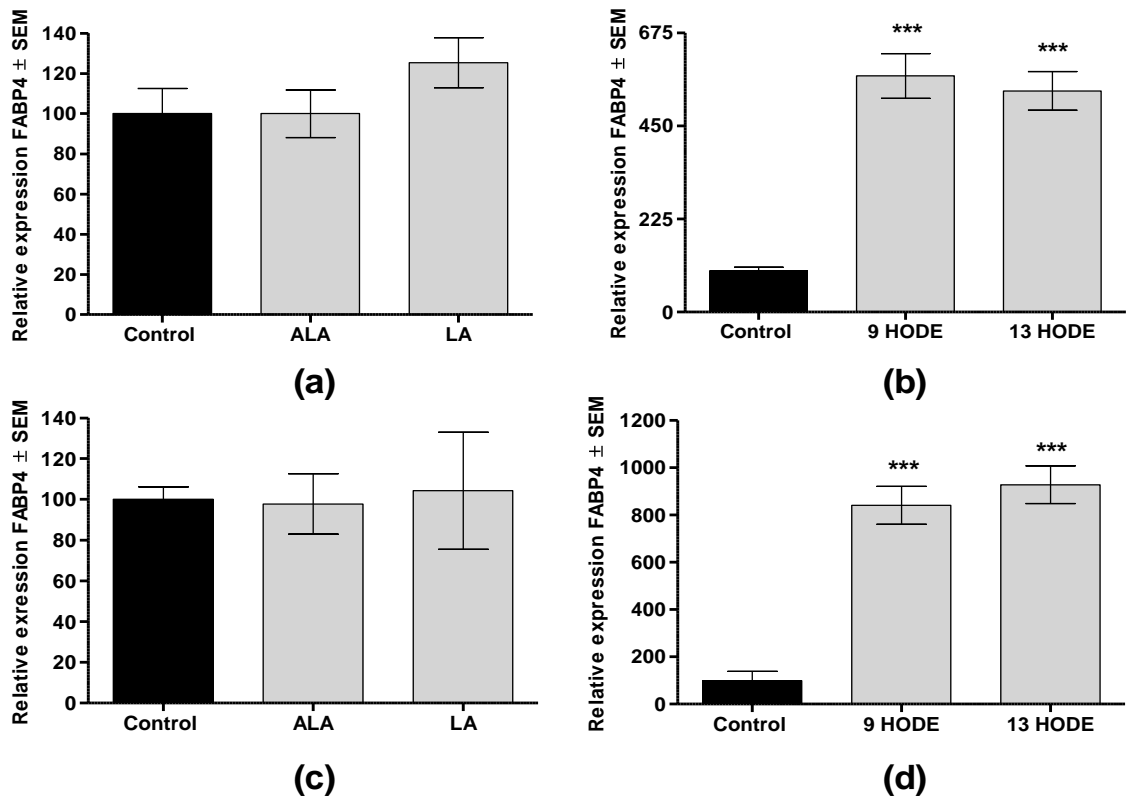


Figure 3.14 FABP4 Expression in Monocytes and Macrophages

Monocytes: (a) ALA ($p > 0.05$) and LA ($p > 0.05$) (b) 9-HODE ($p < 0.001$) and 13-HODE ($p < 0.001$).

Macrophages: (c) ALA ($p > 0.05$) and LA ($p > 0.05$) (d) 9-HODE ($p < 0.001$) and 13-HODE ($p < 0.001$).

Data shown are mean \pm SEM of triplicates in each group normalised against control (one way ANOVA, Tukey's post test). *** = $p < 0.001$.

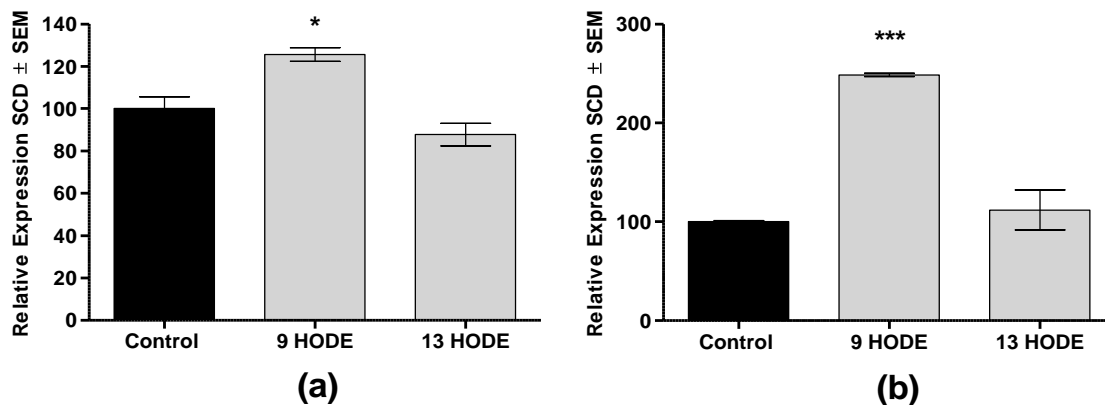


Figure 3.15 SCD Expression in Monocytes and Macrophages

(a) Monocytes: 9-HODE ($p < 0.05$) and 13-HODE ($p > 0.05$).

(b) Macrophages: 9-HODE ($p < 0.001$) and 13-HODE ($p > 0.05$).

Data shown are mean \pm SEM of triplicates in each group normalised against control (one way ANOVA, Tukey's post test). * = $p < 0.05$ and *** = $p < 0.001$.

Immunocytochemistry

The conventional DAB chromogen method was employed to stain monocytes and macrophages for GPR120 and GPR132. 100,000 THP-1 cells were cytospun and fixed on a salianised slide. Macrophages were differentiated using 100nM PMA for 36 hours and then rested for 24 hours in low serum medium. Cells were then fixed and stained for GPR120 and GPR132. Briefly, the protocol steps included, rehydration using DPBS, incubation with 0.5% H_2O_2 for 10 minutes, washing with TBS-T followed by TNB buffer blocking step for 30 minutes, addition of anti-rabbit GPR120 or GPR132 or isotype control (45 minutes), biotinylated rabbit IgG-G (30 minutes), HRP-streptavidin (30 minutes) with TBS-T wash (1 minute) in between each step, followed by addition of DAB chromogen. Slides were subjected to dehydration steps using an alcohol and xylene series after staining for the nucleus using Mayer's haematoxylin. They were then mounted with coverslips (VectaMount) and photographed.

Monocytes expressed lower levels of GPR120 and GPR132 with 10-30% of cells staining positive for each of either GPR120 or GPR132. By contrast, macrophages displayed higher levels of both the G-protein receptors (Figure 3.16).

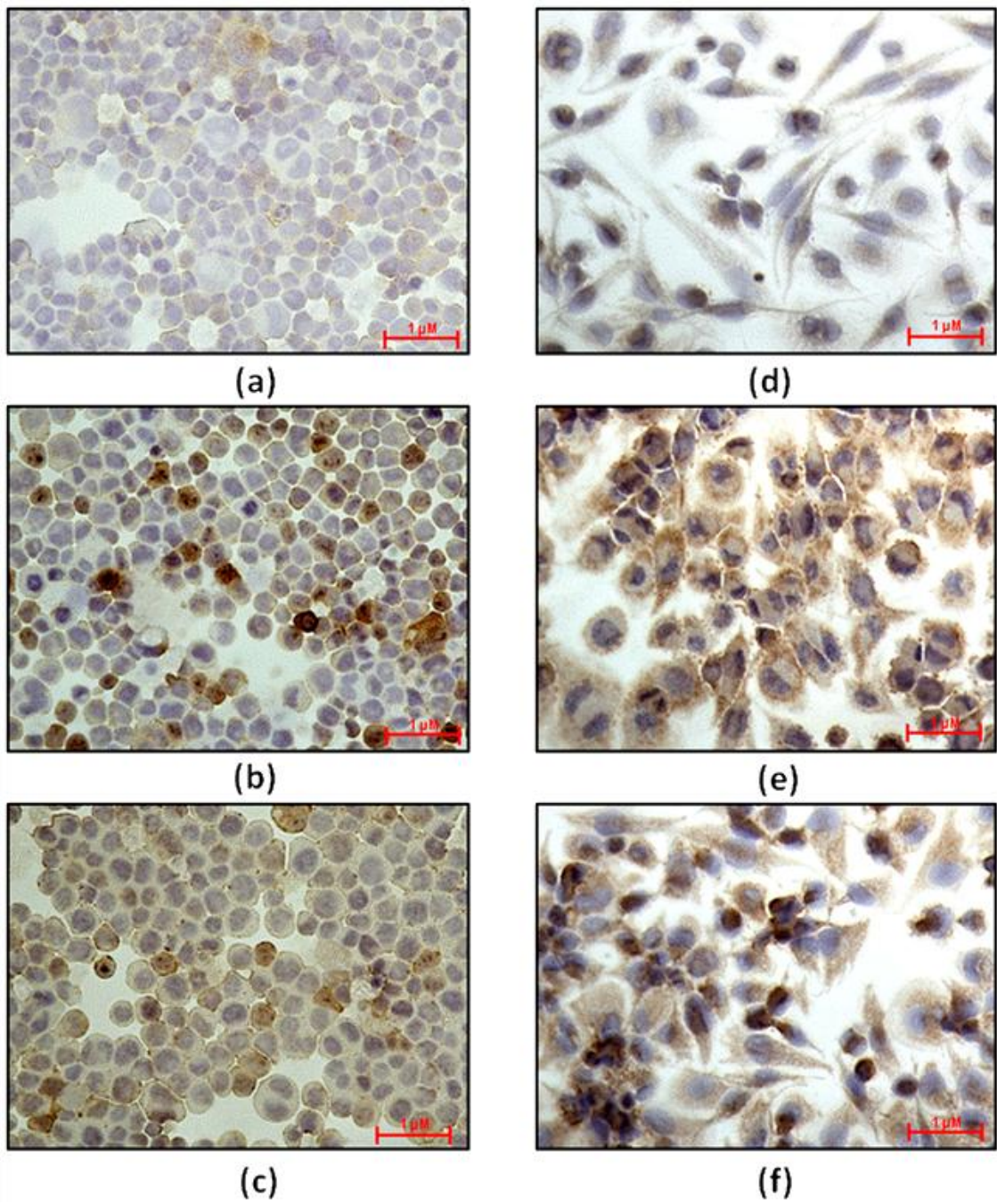


Figure 3.16 Immunocytochemistry for GPR120 and GPR132

Monocytes: (a) Isotype control (b) GPR120 (c) GPR132

Macrophages: (d) Isotype control (e) GPR120 (f) GPR132

Cytokine Assays

Conditioned medium collected from the above experiments was stored at -80°C pending cytokine analysis. The conditioned media samples were tested for the TH1 and TH2 cytokines (details of cytokine panel in Chapter 2). The assay system from Bendormedsystems was employed, with $25\mu\text{L}$ of sample being used to analyse the inflammatory cytokines. As per manufacturer's instructions, samples, standards were incubated with a mixture of anti- beads and anti-bead-biotin followed by incubation with streptavidin PE. The samples were then run on FACS Calibur once the instrumental settings were optimised as per protocol provided with set up beads.

Down- regulation of Pro-inflammatory Cytokine in Macrophage CM by HODEs

The conditioned media from monocytes were also tested, but levels of cytokines released from monocytes were generally very low compared with comparable media from macrophage cultures. Pro-inflammatory cytokine secretion was markedly decreased when cells were exposed to HODEs (Figure 3.17). 9-HODE and 13-HODE exposure both decreased secretion of IL-8 (both $p < 0.001$). Both HODEs also decreased secretion of IL- 1β (both $p < 0.05$). There was a particularly marked decrease in secretion of TNF- α when cells were exposed to either 9-HODE or 13-HODE (both $p < 0.001$, Figure 3.17).

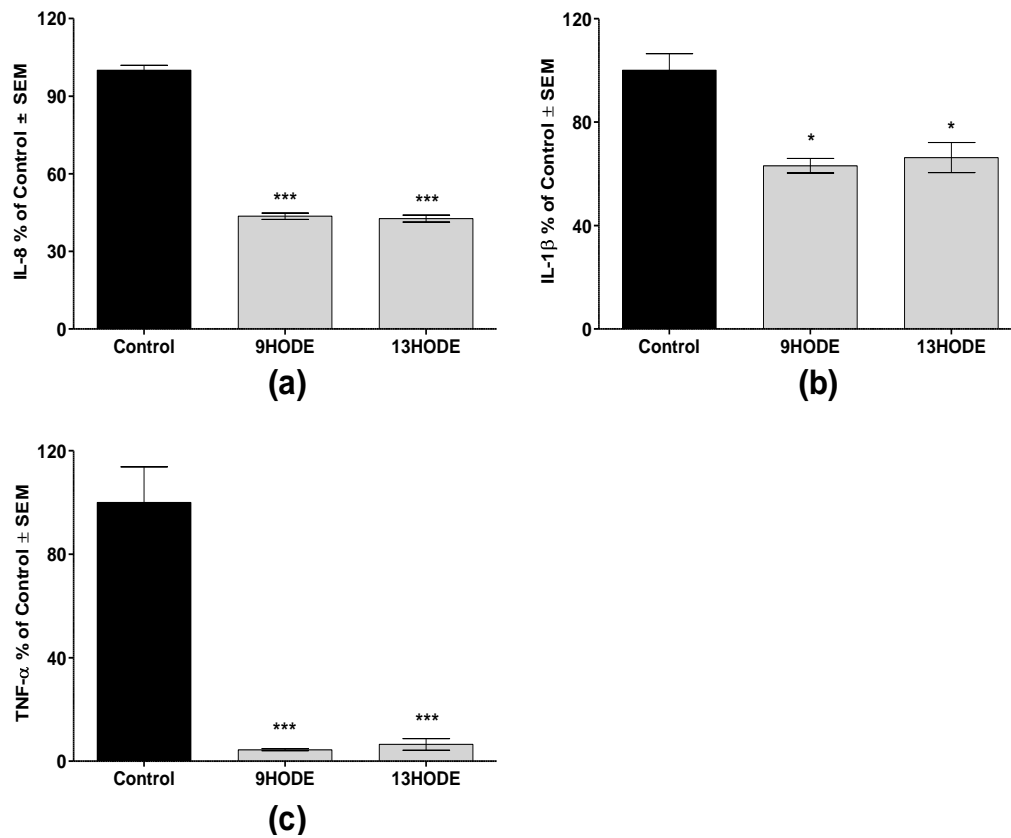


Figure 3.17 Cytokine Secretion from Macrophages

Cytokines were measured in conditioned media from THP1 macrophages exposed to HODEs (or control medium) for 24 hours.

(a) IL-8: 9-HODE and 13-HODE (both $p < 0.001$).

(b) IL-1 β : 9-HODE and 13-HODE (both $p < 0.05$).

(c) TNF- α : 9-HODE and 13-HODE (both $p < 0.001$).

Data shown are mean \pm SEM from groups treated in triplicates and normalised as percentage of control (one way ANOVA, Tukey's post test). * = $p < 0.05$ and *** = $p < 0.001$.

Discussion

Varying levels of FBS have been tested in the experimental design, ranging from serum free medium up to 10% as previously described.³⁰¹ In general, a low percentage of serum is used for experiments to avoid misinterpretation of results from experiments with higher levels of serum. The latter have been found to increase adhesive properties of cells and may also provide higher levels of fatty acids, thus potentially confounding results.^{302, 303} A concentration of 50 - 200µM is generally considered to be non-toxic for some fatty acids such as conjugated linoleic acid.³⁰⁴ Levels of HODEs (structurally related to conjugated linoleic acids) used here are comfortably within this range.

Monocytes and macrophages are involved in the arterial wall in regulating atherogenesis. They are exposed to oxidised lipids, which potentially regulate some of the processes of lipid metabolism, increasing lipid accumulation, and possibly enhancing apoptosis.^{294, 295} Of the four fatty acids examined in this study, both 9-HODE and 13-HODE increased apoptosis determined using caspase 3/7 activity in THP-1 monocytes and macrophages after 24 hour incubations. LA and ALA did not affect the caspase activity even at higher concentrations. Only 9-HODE decreased the cell number in 24-hour cultures, even though both HODEs had similar effects on caspase 3/7 activity and similar cytotoxic effects. 9-HODE was found to be more potent in decreasing cell viability. This was in accordance with the hypothesis that 9-HODE being more potent regulator of apoptosis than 13-HODE. This effect of 9-HODE may contribute to producing an acellular, rupture-prone plaque in the later stages of atherosclerosis where 9-HODE (produced non-enzymatically) is most abundant.

Specific effects of HODEs (compared with ALA or LA) at low concentrations suggest that they may have important signalling functions mediating the atherosclerosis-enhancing effect of oxLDL. Macrophage apoptosis is a feature of advance of atherosclerosis, although it may also have a role in early lesions contributing to the clearance of damaged or dying cells.^{294, 295, 305} It is not clear from this study so far which pathways 9-HODE is acting through but it does appear to have a selective effect on decreasing cell viability despite having a similar effect to 13-HODE on caspase activity. HODEs, derived from linoleic acid, are known to be present in atherosclerotic plaque, possibly mediating some of the effects of oxidative stress.⁴ Between the two

forms of HODEs, 13-HODE is predominant product during the initial stages of atherogenesis, when it is derived enzymatically. Equal amounts of 9-HODE and 13-HODE are present in the later stages of atherosclerosis, and are formed non-enzymatically.^{91,95} The recently identified receptors for long chain fatty acids may well be important in regulating key genes involved in atherogenesis, although this has not been systematically studied to date.³⁰⁶

Hampel et al.⁴ reported that 9-HODE but not 13-HODE or PPAR- γ agonist ciglitazone increased apoptosis in U937 monocytic cells with the effects not being abolished when an antagonist for PPAR- γ (GW9662) was used. These cells express very low PPAR- γ in their basal state thus making it unlikely that the pro-apoptotic effect induced by 9-HODE is PPAR- γ mediated. Jostarndt et al.⁵ described the pro-apoptotic effects of 13-HODE but did not study 9-HODE. This study used a different method to assess apoptosis, and this could account for an apparent difference with study of Hampel et al.⁴ which suggested that 9-HODE selectively increases apoptosis. It is possible that the effects mediated by HODEs are PPAR- γ mediated. Although natural ligands for PPAR- γ are not yet known, HODEs are leading candidates.³⁰⁷ TR4, another transcription factor recently described has been regarded as another candidate for HODE signalling.²²¹ It was suggested to be involved in CD36 signalling and foam cell formation by 13-HODE. In our experiments, there was no significant change in expression of TR4 when cells were exposed to HODEs. This does not, of course, exclude the possibility that some of the effects of HODEs on macrophages are mediated through TR4.

GPR120 and GPR132 are putative long-chain fatty acid receptors that have been recently described. They have been implicated in nutrient sensing, and are highly expressed in cells of monocytic lineage, as well as in nutrient sensing cells of the gut.³⁰⁸ Natural ligands for these receptors are not known, although GPR132 has been previously been described as a receptor for 9-HODE.³⁰⁹ Activation of this receptor in keratinocytes has been shown to promote pro-inflammatory signals²⁴⁰ and to promote secretion of molecules that enhance chemotaxis of phagocytic cells.³¹⁰ Despite this, GPR132 has been proposed to have protective effects in atherosclerosis.²⁴³ The present study examined expression of GPR120 and GPR132 by RT-PCR and by immunocytochemistry. mRNA expression of GPR132 in monocytes was increased, particularly with 9-HODE while both HODEs decreased the expression of GPR120 in

macrophages. This implies that these two G-protein coupled receptors may have a significant role in mediating signalling functions of HODEs. This work also suggests that expression of G-protein coupled receptors for long-chain fatty acids are increased with macrophage differentiation, and that GPR132 especially may mediate some of the regulatory effects of 9-HODE in atherosclerosis.

This study also demonstrated that both 9-HODE and 13-HODE markedly increases the mRNA expression of mRNA for the lipid chaperone FABP4. This molecule is involved in foam cell formation, and FABP4 over-expression has been shown to increase accumulation of cholesterol and triglycerides in THP-1 cells.^{141, 142} FABP4 expression is regulated by liver X receptor agonists and PPAR- γ agonists in THP-1 cells.³¹¹ In FABP4 deficient mice, increased PPAR- γ expression is accompanied by trans-activation of CD36.^{146, 312} Plasma levels of FABP4 are increased in humans with obesity,³¹³ insulin resistance,³¹⁴ or coronary heart disease.³¹⁵ It is possible that tissue resident macrophages in the arterial wall are a major source of circulating FABP4. While circulating FABP4 may be a marker for active disease processes such as atherosclerosis, *in vivo* effects may be protective in the early stages of atherosclerosis promoting, following activation of PPAR- γ , lipid sequestration and removal, along with anti-inflammatory effects. Western blot analysis did not detect any secreted FABP4 in the conditioned media collected after 24 hours of treatment. It would appropriate to determine the levels of FABP4 secreted at earlier time points.

HODE treatment led to decreased levels of pro-inflammatory cytokines secreted by macrophages. IL-8, TNF- α and IL-1 β are pro-inflammatory cytokines that are known to be secreted by macrophages. These secretory proteins have been shown to be involved in development of atherosclerosis.²³ In THP-1 macrophages, long-chain saturated fatty acids have shown to induce pro-inflammatory cytokines IL-8, IL-1 β and TNF- α .³¹⁶ In this study, HODEs specifically decreased the secretion of these cytokines suggesting that these derivatives of oxidised lipids stimulate these cells into a protective phenotype.

Scavenger receptor A, principally involved in uptake of lipids was significantly reduced in macrophages with 13-HODE treatment compared against 9-HODE. This transmembrane receptor was not affected with ALA and LA in monocytes or in

macrophages. With increased expression of genes involved in the process of reverse cholesterol transport, our observations further suggest that HODEs, in spite of previously demonstrated pro-inflammatory effects, may actually have protective actions – particularly in early atherosclerosis. While published data on 9-HODE and its putative receptor GPR132 might suggest otherwise, it is worth remembering that 9-HODE is not abundant in early atherosclerosis. *In vivo* studies, including the present one, show broadly similar effects of 9-HODE and 13-HODE since both are known to act as PPAR- γ agonists. Further studies in Chapter 4 examine the effects of HODEs on the process of macrophage differentiation.

In summary, we have demonstrated that 9-HODE and 13-HODE specifically enhance apoptosis and expression of genes involved in lipid signalling in THP-1 cells. These effects are consistent with a possible role for HODEs in mediating the pro-atherosclerotic effects of oxidative stress. However, HODEs also specifically increased expression of PPAR- γ and expression of genes involved in reverse cholesterol transport, and also decreased secretion of pro-inflammatory cytokines from macrophages. It is not clear at this stage, therefore, whether the effects of HODEs on cells of monocyte/macrophage lineage are predominantly protective or predominantly harmful. The fact that they may be markers for processes that are damaging does not necessarily mean that they exert entirely harmful effects at an early stage of arterial disease. At a later stage of disease, protective mechanisms may be overwhelmed and the effects of oxidised lipids then harmful to the organism.

Chapter 4

Hydroxyoctadecadienoic Acids Enhance Foam Cell Formation and Lipid Regulatory Gene Expression in Macrophages

Introduction

Foam cell formation is a key process in atherogenesis, and oxidised lipids may play an important part in regulating their formation. Circulating monocytes interact with oxidised lipids and this triggers activation and differentiation of monocytes into macrophages. Most of the processes involved in this are thought to be critically regulated by the transcription factor PPAR- γ .^{141, 317} HODEs are stable oxidation products of linoleic acid and are the predominant oxidised fatty acids that accumulate in LDL and atherosclerotic plaque.^{86, 318} In early atherosclerosis, there is increased expression of 15-LOX-1, which when it acts upon its substrate linoleic acid, results in generation of 13-HODE.^{88, 95} During late atherosclerosis, with increased oxidative stress, HODEs are generated non-enzymatically with equal amounts of 9-HODE and 13-HODE being formed.²⁷ Both isoforms of HODE have been shown to be capable of acting as signalling molecules in macrophages, mainly as agonists of PPAR- γ .^{92, 126} 13-HODE has been recently shown to promote foam cell formation acting through the transcription factor TR4.²²¹ 9-HODE but not 13-HODE has been shown to be a ligand for GPR132 (G2A),^{309, 319} which is expressed in vascular macrophages and thought to be involved in promoting atherosclerosis.^{245, 320}

Oxidised lipids increase foam cell formation from sub-endothelial macrophages. HODEs, components of oxidised lipids, may be important in regulating foam cell formation, and have been reported to increase lipid accumulation in differentiated macrophages.¹⁴¹ Furthermore, the lipid chaperone FABP4 is also known to be involved in these processes, shuttling intracellular lipids for storage and possibly having signalling functions.¹⁴¹ Other markers for monocyte differentiation include ITGAM and SCRA, the latter being the principal receptor for lipid uptake in macrophages.²¹⁸ This process of lipid intake is compensated by reverse cholesterol transport of lipids by SCRB and ABCA1 which efflux excess lipids to HDL, the lipids then being transported back to the liver and excreted.³²¹ A key regulator of synthesis of unsaturated fatty acids is the enzyme stearoyl CoA desaturase (SCD), which could possibly have a role in insulin sensitivity. When SCD expression is suppressed in macrophages, the process of atherosclerosis is accelerated.^{322, 323} HODEs are involved in regulating macrophage apoptosis,^{4, 5} a feature of advanced atherosclerosis associated with increased generation

of inflammatory mediators in the vessel wall and ultimately to formation of acellular plaque.

This chapter aimed to investigate the effects of HODEs in monocyte differentiation. It was hypothesised that exposure of monocytes to HODEs would lead to differentiation of cells and increased lipid metabolism thereby forming foam cells. This action would be augmented when the cells are treated with HODEs along with the protein kinase C activator PMA, thus increasing differentiation of cells into macrophages. Also based on the first set of experiments, we considered it important examine expression of GPR120 and GPR132 while the cells differentiated as previous experimental results show an increase in GPR132 in monocytes while GPR120 was decreased in macrophages. The effects of HODEs on apoptosis were also studied, as it is well known that macrophage foam cell formation accompanies apoptosis – and both are key features of the advanced stages of atherosclerosis. The ability to modulate the differentiating monocytes into a phenotype that is protective and anti-inflammatory is a possible approach to preventing atherosclerosis.

Results

Increased Foam Cell Formation with HODEs Treatment

THP-1 monocytes were differentiated into macrophages on sterile coverslips by treating the cells with 100nM PMA for 36 hours, and then resting them for 24 hours in low serum medium. The cells were then treated with 30 μ M ALA, LA, 9-HODE or 13-HODE in RPMI medium for 24 hours in a 24 well-plate. At the end of the incubation time, medium was removed and the cells were washed with DPBS three times, and then fixed in neutral buffered formalin for 10 minutes. Slides were then air-dried and mounted on a slide. The foam cell assay was performed using Oil Red O stain which specifically stains the lipid droplets in the cells after rehydrating the slides with DPBS. Once the Oil Red O stain was incubated for 20 minutes, excess stain was removed by washing with distilled water and cells were then incubated in Mayer's haematoxylin (nuclear staining) for 2 minutes, washed again with tap water and mounted using Aqua

mount. Slides were examined by an independent pathologist to determine the extent of foam cell formation. Compared to controls, ALA and LA treatment led to no increase in lipid droplets. 9-HODE and 13-HODE treatment increased the number of lipid droplets compared to controls and experiments with the parent fatty acids (Figure 4.1).

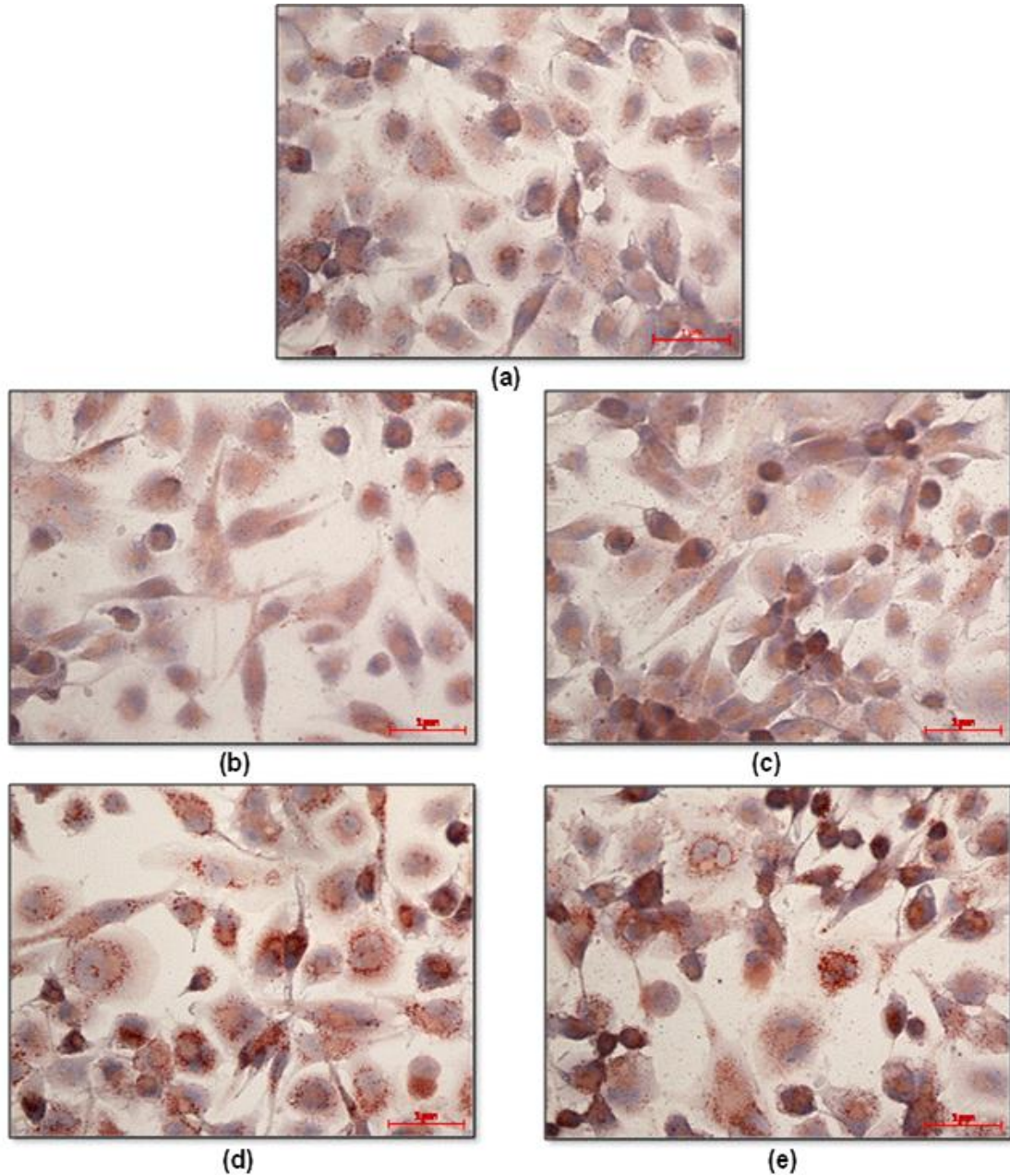


Figure 4.1 HODEs Increase Foam Cell Formation

Macrophages were incubated with 30µM LCFA for 24 hours. (a) Control (b) ALA, (c) LA, (d) 9-HODE and (e) 13-HODE.

Gene Expression Studies

One million THP-1 cells were treated with 30 μ M ALA, LA, 9-HODE or 13-HODE in 0.4% RPMI medium for 24 hours in the presence or absence of 1nM PMA in a six-well plate. After 24 hours, conditioned medium was removed and stored frozen, while cells were carefully removed using a cell scraper and stored at -80°C for gene expression studies. RNA was extracted using Qiagen RNA easy mini kit (details in Chapter 2). Real-time RT-PCR was performed for genes of interest. Data was normalized against housekeeping gene PPIA.

Determination of PMA dose

A dose response was performed to determine the effect of PMA on the macrophages. Gene expression of SCRA was chosen to be tested with PMA treatment since it is one of principal lipid accumulative markers expressed in macrophages. Doses of 1, 10 and 100nM PMA for 24 hours were tested for the purpose of determining the dose to stimulate the monocytes to differentiate (Figure 4.2).

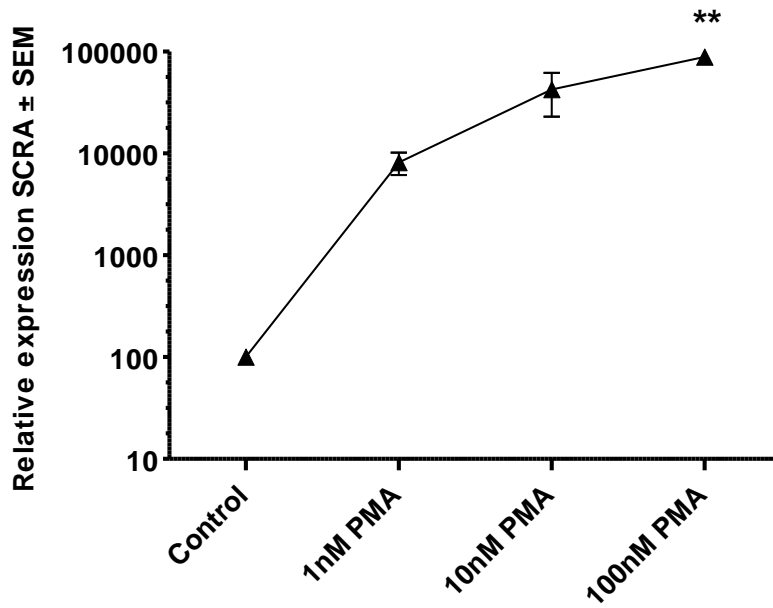


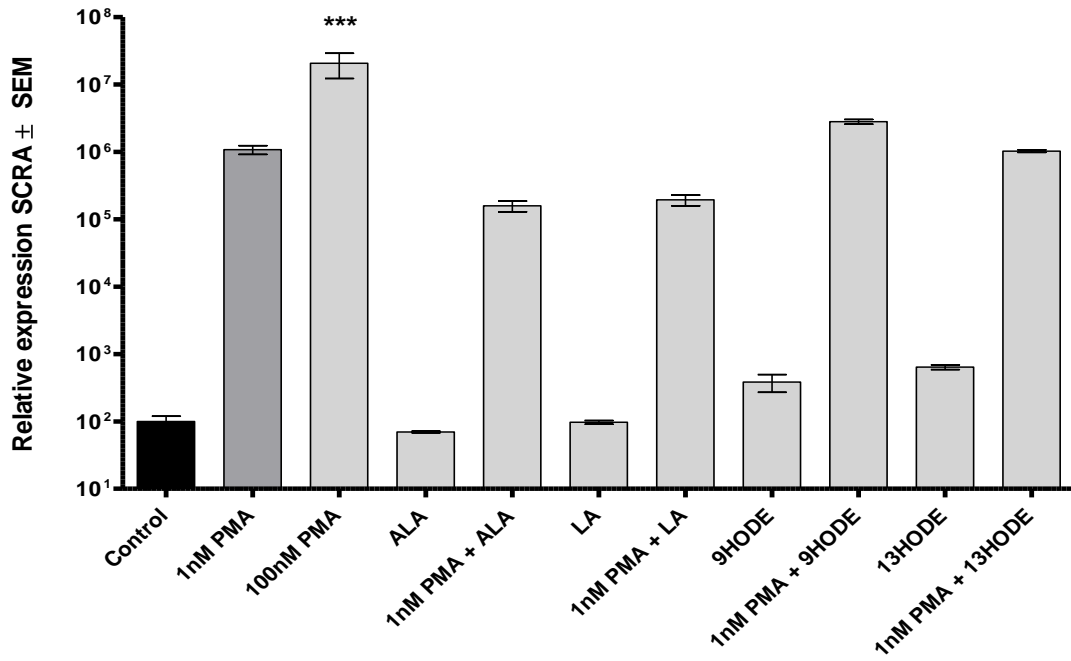
Figure 4.2 Relative Expression of SCRA

Gene expression was studied with 1 - 100nM PMA. THP-1 cells were incubated for 24 hours (100nM PMA, $p < 0.01$ compared with control).

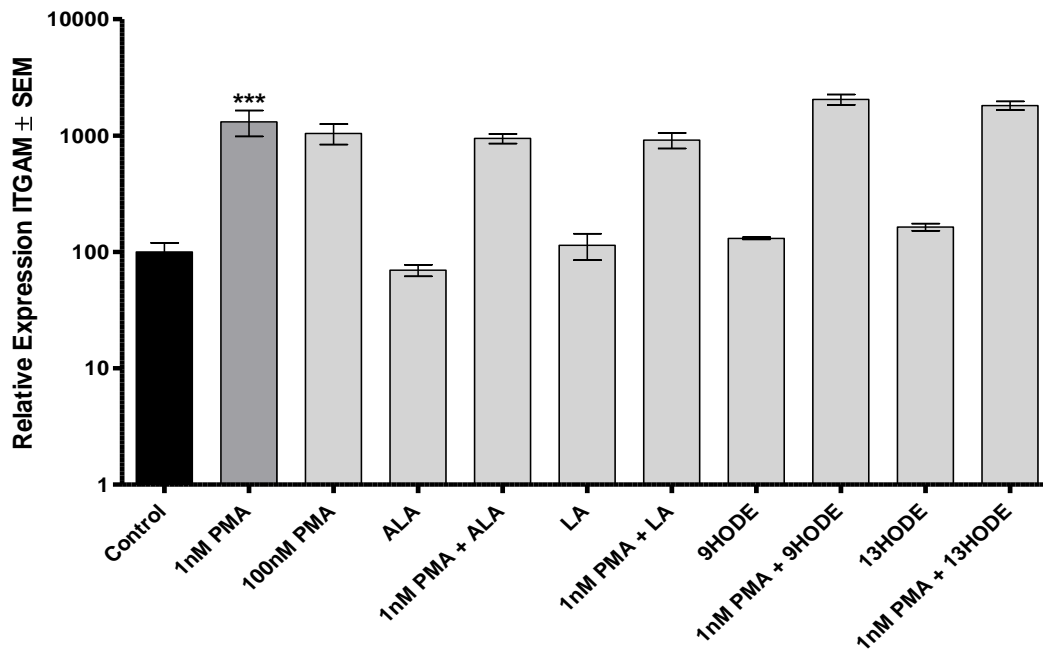
Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). ** = $p < 0.01$.

Effect of HODEs on Macrophage Differentiation Markers

The macrophage differentiation markers SCR-A and ITGAM were up-regulated with 1nM PMA treatment, while with 100nM PMA no further increase was observed. Fatty acids and their derivatives did not have affect on SCRA and ITGAM expression when added alone, and 30 μ M fatty acids when combined with 1nM PMA did not significantly alter expression when compared with 1nM PMA alone (Figure 4.3).



(a)



(b)

Figure 4.3 Gene Expression Studies

THP-1 cells following 24 hours treatment

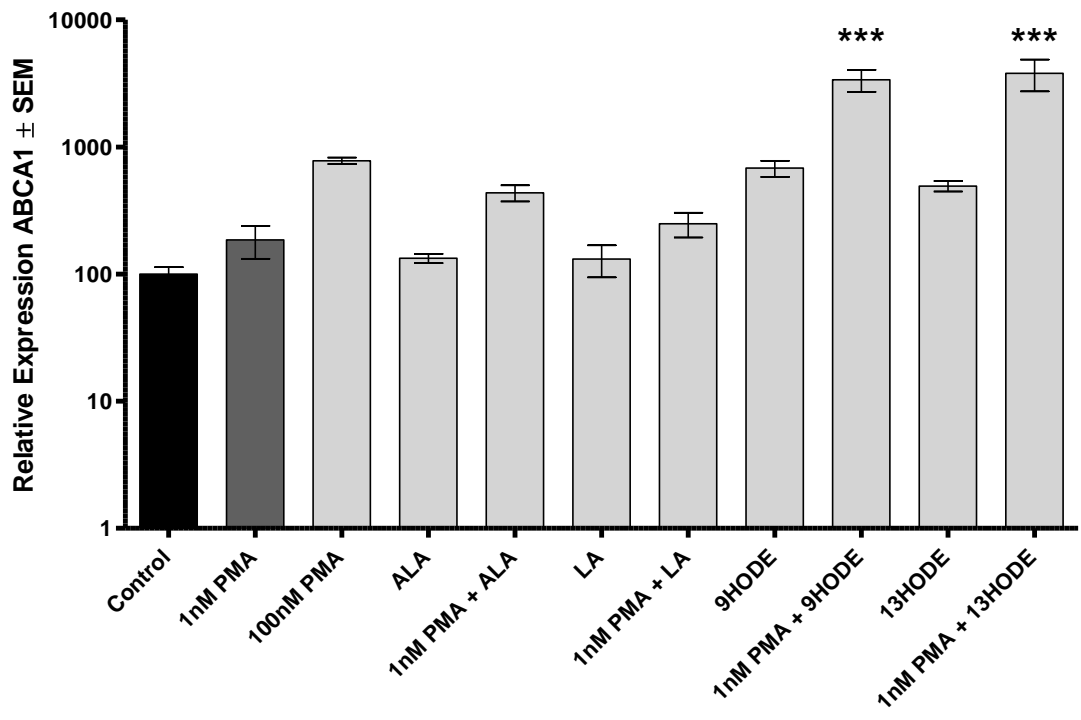
(a) SCRA: 100nM PMA, $p < 0.001$ compared with 1nM PMA.

(b) ITGAM: 1nM PMA, $p < 0.001$.

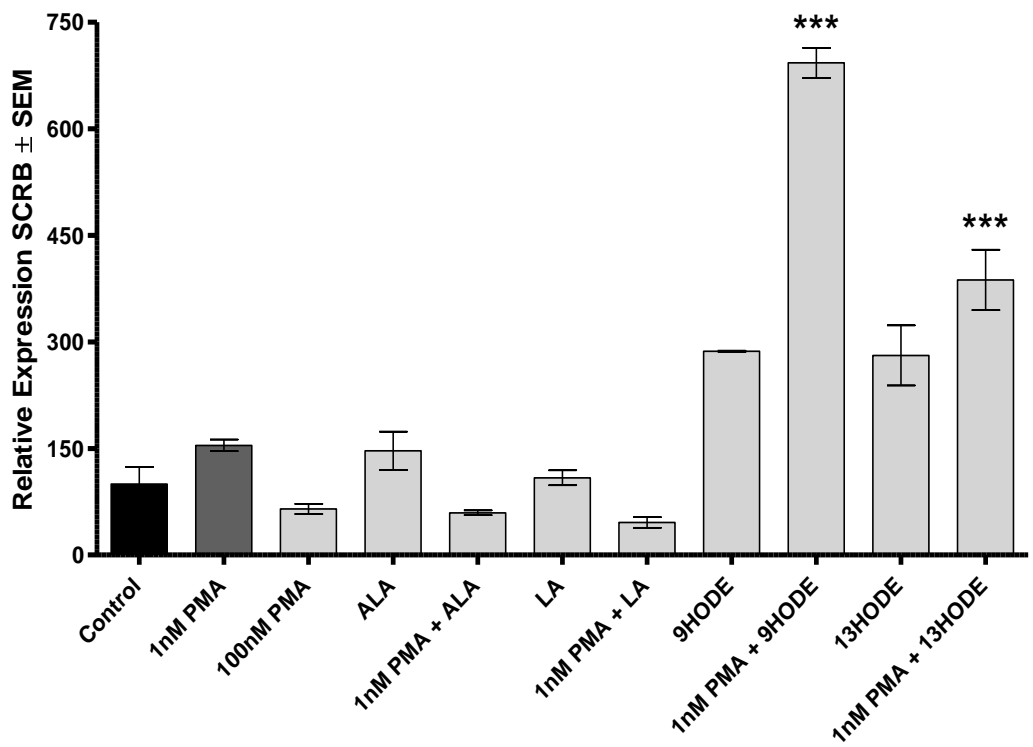
Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). *** = $p < 0.001$.

Genes of Reverse Cholesterol Transport Highly Up-regulated

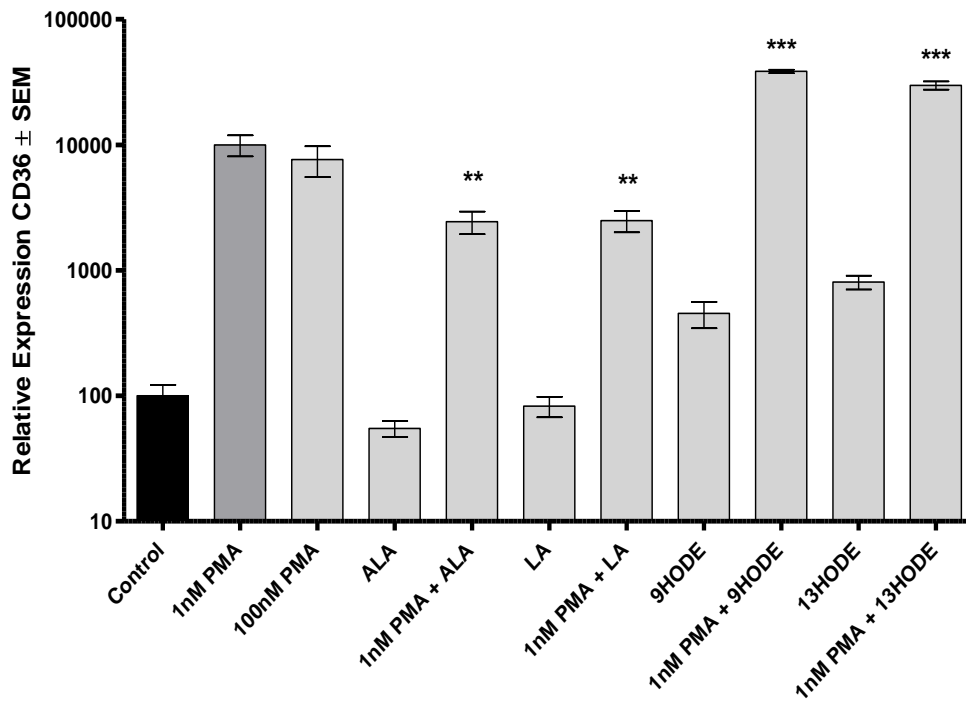
Genes involved in reverse cholesterol transport, SCR-B, ABCA-1 and CD36 were highly up-regulated when THP-1 cells were treated with HODEs (9-HODE and 13-HODE, both $p < 0.001$). 9-HODE was more potent than 13-HODE, and yielded a 7-fold increase in SCR-B expression. ABCA-1 was increased as in a dose-dependent manner with 1nM PMA and 100nM PMA. This effect was not observed when treated with PMA along with the parent fatty acids ALA and LA. Unexpectedly, CD36 was down-regulated when cells were treated with ALA and LA ($p < 0.01$ compared with 1nM PMA, Figure 4.4).



(a)



(b)



(c)

Figure 4.4 Gene Expression Studies

THP-1 cells following 24 hours treatment

(a) ABCA1: 9-HODE and 13-HODE, $p < 0.001$ compared with 1nM PMA

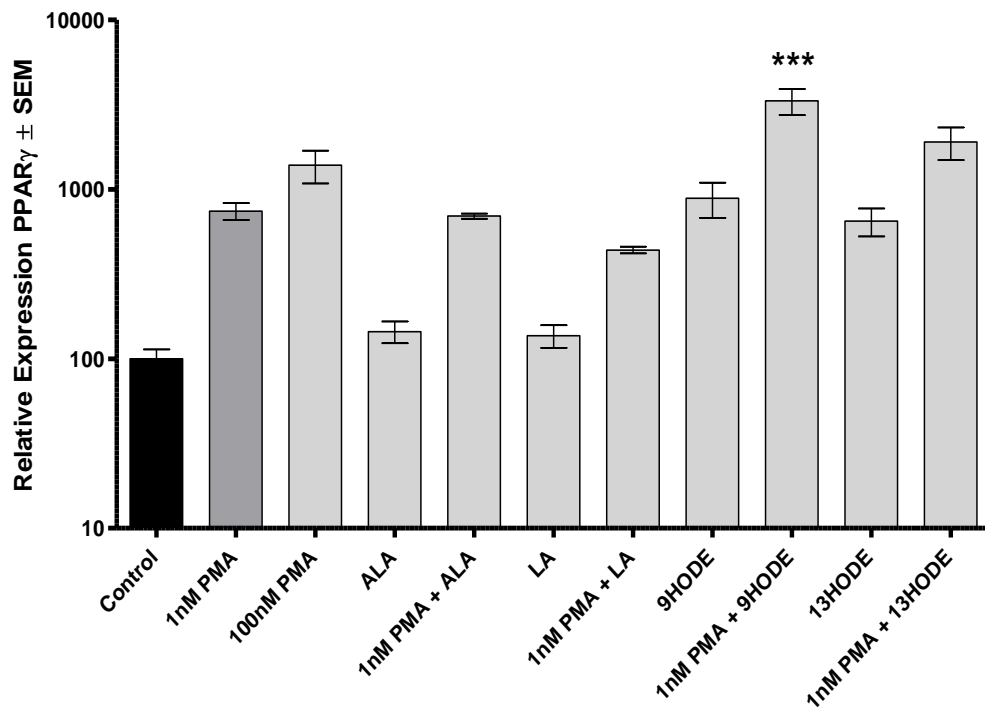
(b) SCRB: 9-HODE and 13-HODE, $p < 0.001$

(c) CD36: 9-HODE and 13-HODE, $p < 0.001$; ALA and LA, $p < 0.01$ - both compared with 1nM PMA.

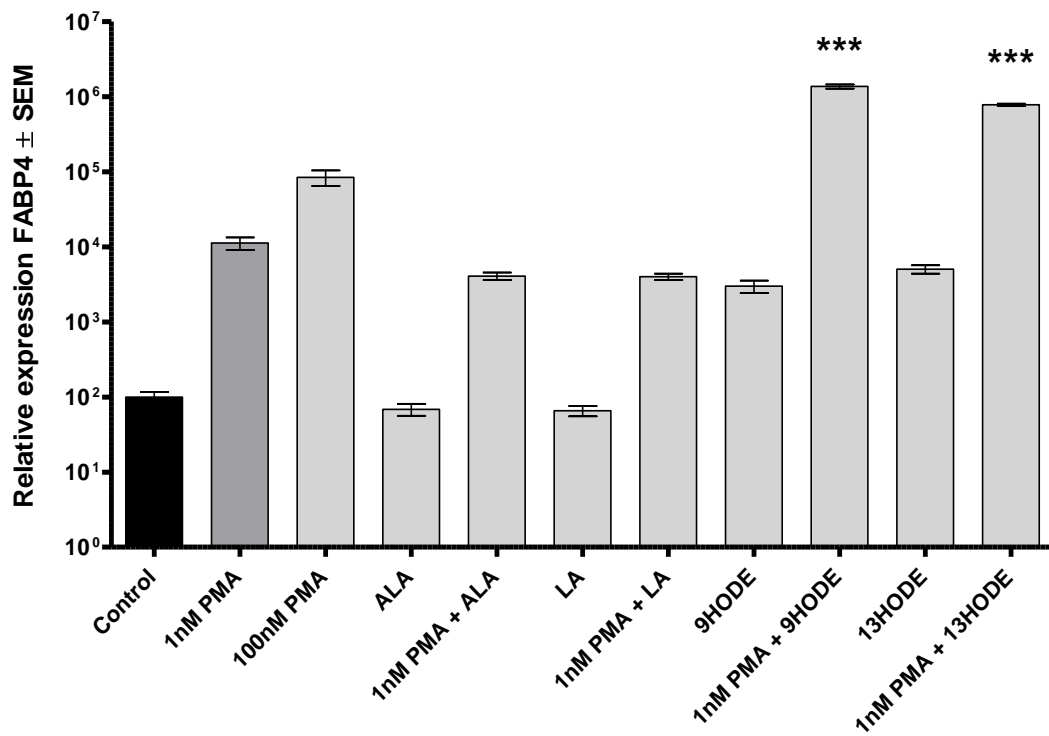
Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). ** = $p < 0.01$ and *** = $p < 0.001$.

Increased Expression of PPAR- γ , FABP4 and SCD

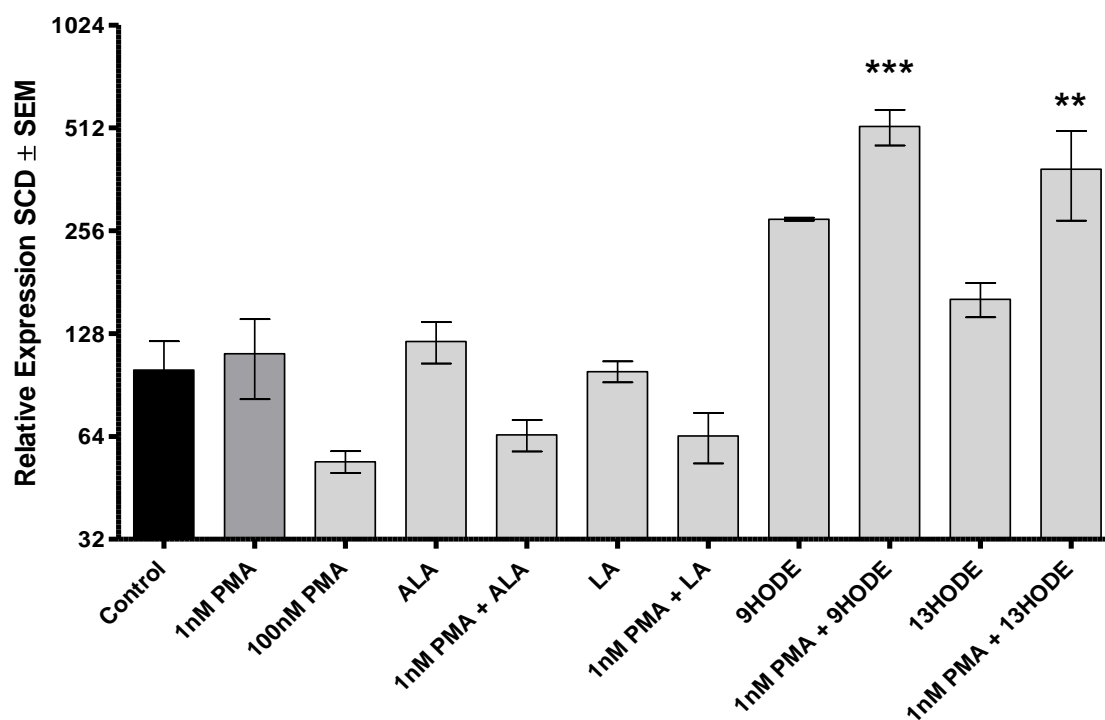
The transcription factor PPAR- γ gene expression was increased with HODE exposure, an effect not observed with ALA or LA. PMA increased the expression in a dose-dependent manner (100nM PMA vs. control $p < 0.05$). When added along with 9-HODE, PMA increased expression of the transcription factor significantly ($p < 0.001$). Fatty acid binding protein-4 (FABP4) was increased significantly as expected, with a similar dose response with PMA (not significant) and significant increase observed with 9-HODE and 13-HODE. By contrast, ALA and LA were without effect. Levels of stearoyl CoA desaturase (SCD) expression showed a non-significant decrease with 100nM PMA and when 1nM PMA was added with ALA and LA. SCD expression was increased significantly with 9-HODE and 13-HODE ($p < 0.001$ and $p < 0.01$ respectively, Figure 4.5).



(a)



(b)



(c)

Figure 4.5 Gene Expression Studies

THP-1 cells following 24 hours treatment

(a) PPAR- γ : 9-HODE, $p < 0.001$ compared with 1nM PMA

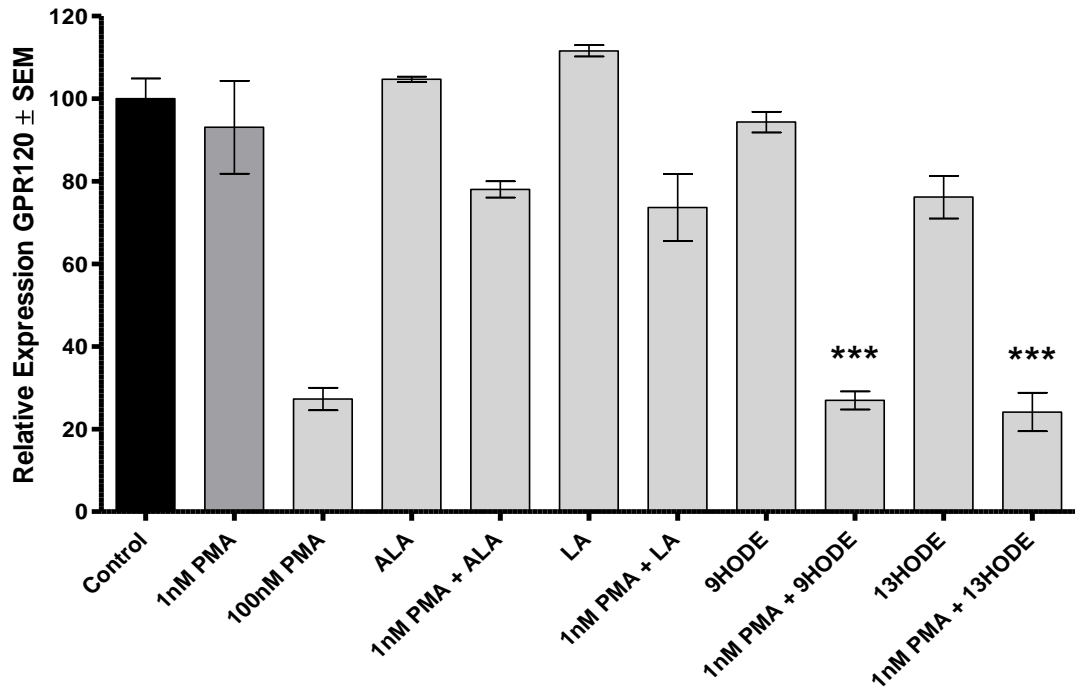
(b) FABP4: 9-HODE and 13-HODE, both $p < 0.001$

(c) CD36: 9-HODE, $p < 0.001$ and 13-HODE, $p < 0.01$ - both compared with 1nM PMA.

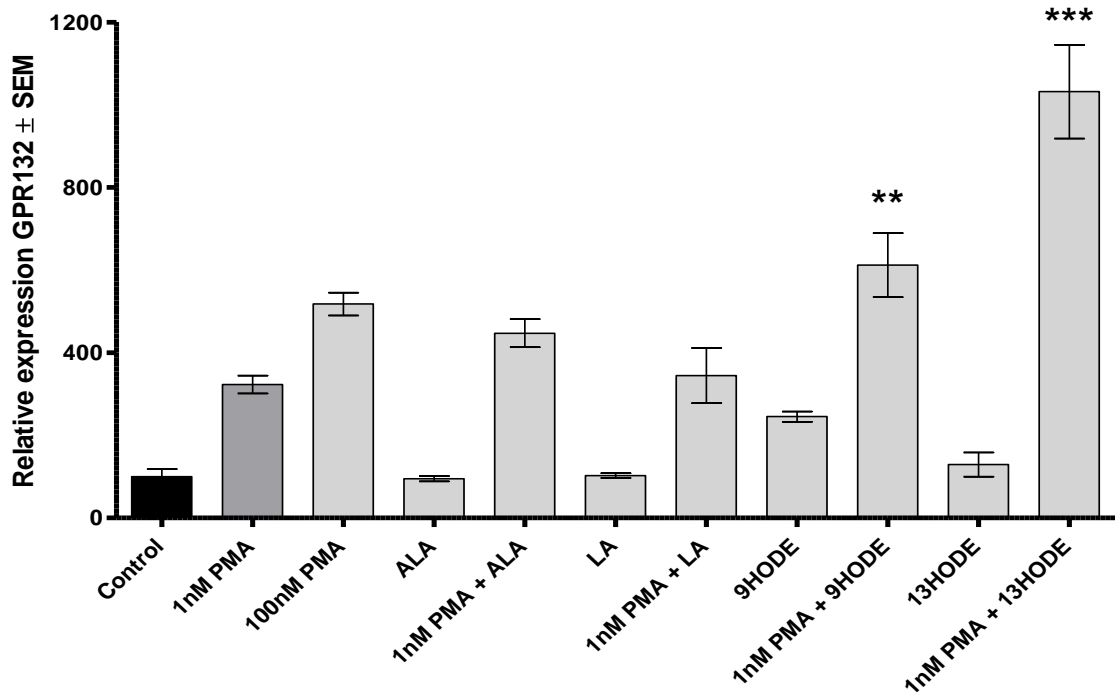
Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). ** = $p < 0.01$ and *** = $p < 0.001$.

Differential Effects on GPR120 and GPR132 During Monocyte Differentiation

The higher concentration of 100nM PMA decreased the expression of GPR120 ($p < 0.001$), while no effect was seen with 1nM PMA. When added with 1nM PMA, ALA decreased the expression of GPR120 moderately ($p < 0.05$) as observed previously with macrophages (Chapter 3). Both 9-HODE and 13-HODE decreased GPR120 expression when added along with 1nM PMA (both $p < 0.001$). Interestingly, the opposite effect was observed with GPR132 expression: PMA increased GPR132 expression in a dose-dependent manner (1nM PMA, $p < 0.05$; 100nM PMA, $p < 0.001$). When added with 1nM PMA, ALA and LA did not affect GPR132 expression. By contrast, both 9-HODE and 13-HODE when combined with 1nM PMA increased GPR132 expression significantly (both $p < 0.001$, Figure 4.6).



(a)



(b)

Figure 4.6 GPR120 and GPR132 Gene Expression

THP-1 cells following 24 hours treatment

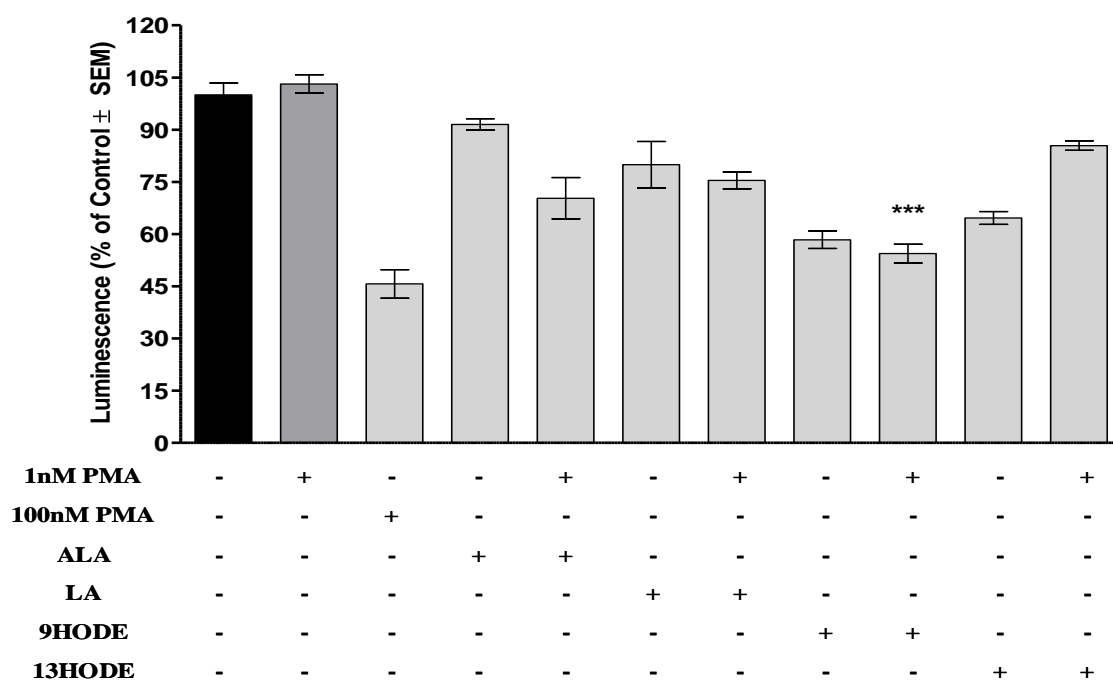
(a) GPR120: $p < 0.001$ compared with 1nM PMA alone

(b) GPR132: $p < 0.001$, $p < 0.01$ compared with 1nM PMA alone.

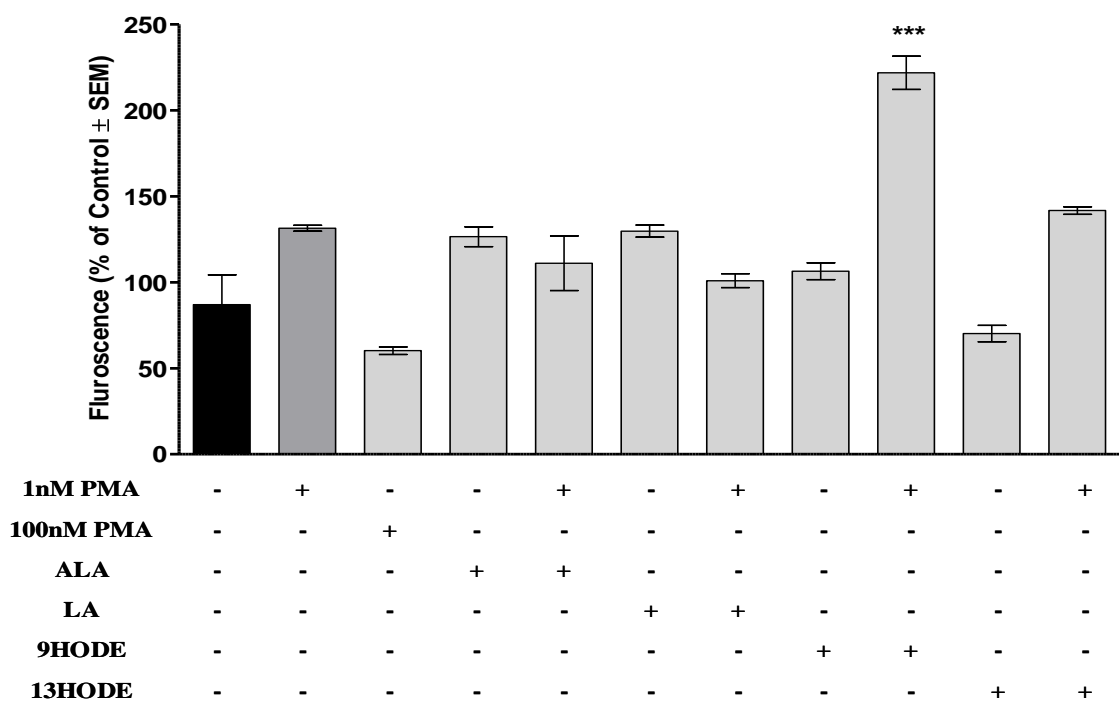
Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). ** = $p < 0.01$ and *** = $p < 0.001$.

Effects on Viability and Apoptosis during Differentiation

The assays were performed on opaque sterile 96-well plates. 24,000 THP-1 cells were treated with or without C-18 LCFA \pm 1nM PMA for 24 hours in 50 μ L of culture medium. At the end of the incubation period, cell culture plates were removed from the incubator and 50 μ L of assay reagent was added to each well. Manufacturer's protocol was followed as per the instructions. After 30 minutes of incubation time, luminescence or fluorescence measurements were taken. The parent fatty acids ALA and LA did not significantly affect cell numbers. 9-HODE, on the other hand, significantly decreased the cell number ($p < 0.001$, ANOVA, Tukey's multiple comparison). The caspase 3/7 assay data suggested that the decrease in cell number when cultures were exposed to 9-HODE was due to apoptosis ($p < 0.001$). This effect was not apparent with 13-HODE (Figure 4.7).



(a)



(b)

Figure 4.7 Cell Proliferation Assay

THP-1 cells following 24 hours treatment

(a) Viability: $p < 0.001$ compared with 1nM PMA alone

(b) Apoptosis: $p < 0.001$ compared with 1nM PMA alone.

Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). *** = $p < 0.001$.

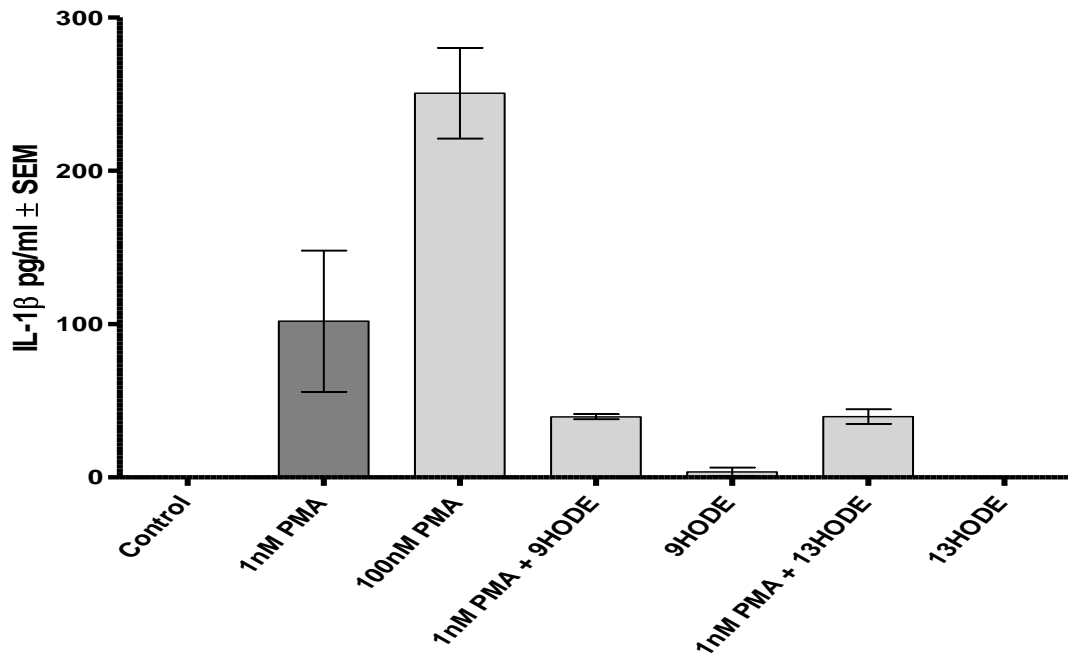
Cytokine Assays

Conditioned media collected were tested for the TH1 and TH2 cytokines. The assay system from Bendormedsystems was employed, and 25 μ L of sample were assayed for cytokines. As per manufacturer's instructions, samples and standards were incubated with anti- beads and anti-bead-biotin mixture, followed by streptavidin PE, with wash steps in-between. The samples were then run on FACS Calibur once the instrumental settings were optimised as per protocol provided with set up beads.

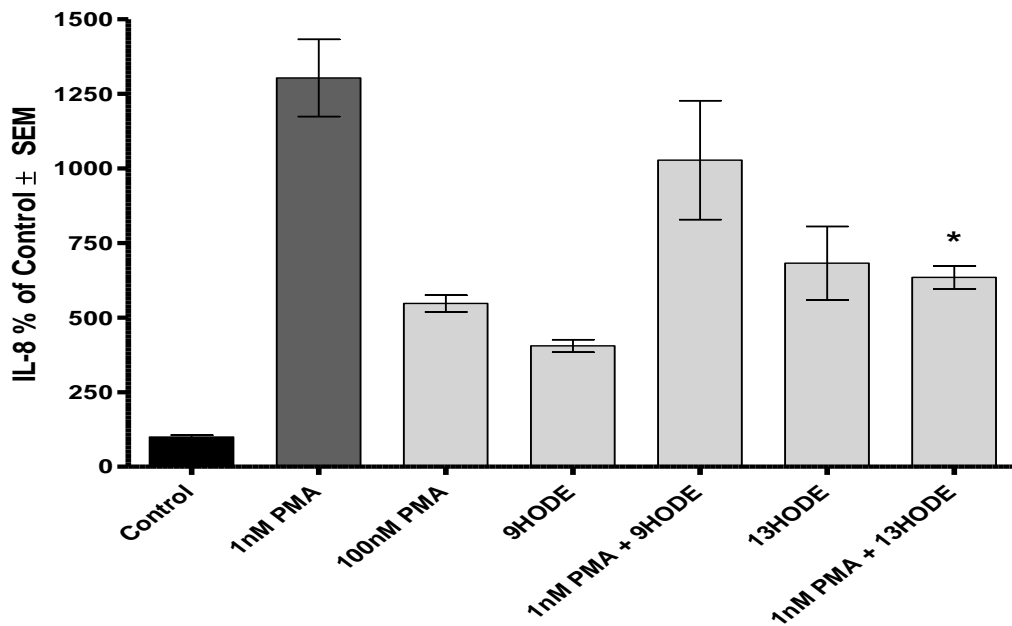
Proinflammatory cytokines IL-8 and IL-1 β suppressed by HODEs

With eleven cytokines tested, only two of them were at detectable levels in the conditioned media.

The pro-inflammatory cytokines IL-1 β and IL-8 was detected when cells were treated with PMA. IL-1 β secretion was partly decreased towards control levels when HODEs were added with 1nM PMA (not significant). An increase in IL-8 was observed when treated with 1nM PMA compared with control ($p < 0.001$). There was a significant decrease in IL-8 secretion with 1nM PMA when 13-HODE was added concurrently ($p < 0.05$, Figure 4.8).



(a)



(b)

Figure 4.8 Cytokine Assay

Conditioned medium from THP-1 cells following 24 hours treatment

(a) IL-1 β

(b) IL-8: 13-HODE, $p < 0.05$ compared with 1nM PMA alone.

Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). * $p < 0.05$.

Discussion

The human leukemic THP-1 cell line is a useful model with which to study monocytes and the processes through which they differentiate into macrophages. The cells can be differentiated using tumour-promoting phorbol esters including PMA which activate protein kinase C.^{324, 325} In this study, HODEs interact with PMA and induce several effects. They alter the molecules involved in regulating lipid transport and atherogenesis. Foam cells are lipid-laden macrophages, and are observed during the later stages of atherosclerosis. When PMA stimulated THP-1 cells are treated with HODEs, both 9-HODE and 13-HODE markedly increased foam cell formation. While oxidised lipids have been shown to increase lipid accumulation by macrophages through PPAR- γ dependent mechanisms,^{141, 317} we know of no reports where HODEs have been shown to directly increase lipid droplet accumulation in differentiated macrophages.

Differentiation markers ITGAM and SCRA were measured as part of our gene expression studies. ITGAM, also known as CD11b, is expressed in monocytes and is part of the receptor for complement component 3. SCRA on the other hand is the receptor for uptake of lipids and cell debris in macrophages.³²⁶ As predicted, these two markers were up-regulated by PMA, but there was no significant increase in their expression with HODEs (added alone or along with PMA). Both SCRB and ABCA1 are involved in reverse cholesterol transport, which decreases lipid burden in diseased arteries by effluxing the excess cholesterol to HDL which gets transported to the liver and excreted.³²¹ Both these genes were up-regulated by HODEs with further increase observed when 1nM PMA was added along with HODEs. PMA alone when added increased ABCA expression but not SCRB. Thus, the major effect of HODEs on macrophage differentiation may relate to lipid transport and the effects may be synergistic with those of PMA. If HODEs stimulate reverse cholesterol transport, their effect may be protective in relation to atherosclerosis.

As observed with adipocytes, lipid accumulation in macrophages is dependent upon increase in expression of FABP4, regulated by PPAR- γ .^{141, 142} In the present study, a dose dependent increase in expression of FABP4 was observed with PMA treatment. Added alone, HODEs did not increase FABP4 expression, but 1nM PMA synergised

with HODEs to increase FABP4 expression. Fu et al.¹⁴¹ previously reported increased FABP4 expression by HODEs but did not study the effect of phorbol ester-HODEs interaction with these cells. FABP4 is involved in the interaction between macrophages and adipocytes and its expression in both these cell types has been linked to obesity-related insulin resistance and inflammation.³²⁷ Also, plasma levels of FABP4 are increased in patients who are prone to atherosclerosis.^{315, 328} In animal models, genetic deletion or inhibition of FABP4 alleviates features of metabolic syndrome.^{1, 301} The effect of HODEs described above may help explain how increased oxidative stress in vascular wall leads to increased expression of an important mediator of atherosclerotic risk.

HODEs are ligands for PPAR- γ , which is known to regulate FABP4 expression and foam cell formation.^{92, 126} This study shows that both PMA and HODEs increase PPAR- γ expression in THP-1 cells with a higher increase observed with 1nM PMA. Though HODEs increase in PPAR- γ and can act as agonists for the transcription factor, it does not necessarily confirm that the above processes are mediated through PPAR- γ . Even without agonist activation, increased PPAR- γ has been shown to regulate expression of other genes and some of its anti-inflammatory functions mediated through transrepression.³²⁹ SCD, which converts saturated fatty acids into their corresponding monounsaturated form, when deficient in animal models, improves features of metabolic syndrome by affecting hepatic lipid metabolism.^{322, 330, 331} In macrophages, SCD activity is protective and its deficiency increases inflammation and accelerates atherosclerosis.^{323, 332} Recently, Erbay et al.³³³ reported that in mice with increased expression of SCD was involved in protection of endoplasmic reticulum stress. In this study, SCD was down regulated on exposure of THP-1 cells to PMA with 9-HODE and 13-HODE increasing its expression. With 1nM PMA the expression is further enhanced with HODEs. Factors that regulate SCD expression in human macrophages have not been previously documented. Although, HODEs arise in response to oxidative stress, they may actually have protective effects resulting in increased SCD expression in macrophages, decreased pro-inflammatory gene expression through increased PPAR- γ expression and enhanced reverse cholesterol transport.

Recently, free fatty acids haven shown act as ligands for G-protein coupled receptors forming part of body's nutrient sensing apparatus. These receptors are expressed in

circulating leukocytes.³⁰⁶ GPR120 and GPR132 are receptors for long-chain fatty acids with the former being involved in adipogenesis,³³⁴ while GPR132 is a recognised receptor for 9-HODE^{309, 319} mediating both pro-inflammatory²⁴⁰ and pro-atherosclerotic effects.^{245, 335} A decrease in expression of GPR120 was observed in this study on exposure of monocytes to PMA, while exposure to HODEs caused a modest decrease in expression alone but with addition of 1nM PMA it markedly decreased GRP120. This was similar to what was observed in macrophages described in the previous chapter. By contrast, GPR132 expression increased with addition of PMA and also when combined with 1nM PMA. HODEs markedly increased GPR132 expression with 13-HODE being more potent compared to 9-HODE. The significance of decreased expression of GPR120 during macrophage differentiation is not clear. The function of this receptor in monocytic cells is not known. In a recent study by Oh et al.,²³⁷ a GPR120 knockout mouse model displayed decreased insulin sensitivity when treated with n-3 fatty acids, suggesting that this receptor is key in controlling inflammation and increasing insulin sensitivity. A decrease in expression of GPR120 in macrophages could be suggestive of protective effects by down-regulating the inflammation induced by macrophages. Increased GPR132 on differentiation could possibly make cells more responsive to 9-HODE. This could be expected during the later stages of atherosclerosis since 9-HODE is generated non-enzymatically during the later stages. Antagonist blocking or gene silencing of these receptors would enable us to gain a clearer understanding of their roles.

Caspase 3/7 activity increased markedly when a combination of 1nM PMA and 9-HODE was added to THP-1 cells. Both HODEs decreased cell viability but the effect of 13-HODE appeared to be partly abolished when it was added with 1nM PMA. Enhanced apoptosis with oxLDL treatment has been well described,^{128, 336} with PPAR- γ playing a vital role.³³⁷ Two previous studies reported a possible role of HODEs in inducing apoptosis in human monocytic cells.^{4, 5} Neither of these studies used PMA and both studied undifferentiated cells. Jostarndt et al.⁵ reported increased apoptosis in MonoMac 6 cells mimicking the effect of enzymatically modified LDL. 9-HODE was not studied. Hampel et al.⁴ on the other hand studied 9-HODE but not 13-HODE, and showed increased apoptosis in U937 monocytic cells. The PPAR- γ antagonist GW9662 did not abrogate the effect by 9-HODE. The cells used in this study do not express substantial levels of PPAR- γ in their basal state.²⁸¹ However, in the study by Hampel et

al.⁴ these cells had increased expression of PPAR- γ_2 over 48 hours, though apoptosis was evident within 24 hours with 9-HODE. This study also suggests a selective effect of 9-HODE that is not PPAR- γ dependent since the effect was not replicated by 13-HODE. As with lipid accumulation, apoptosis of infiltrating macrophages may be protective during early atherosclerosis but the process contributes to development of advanced lesions by generating chemotactic and pro-inflammatory signals thus leading to development of an acellular and fragile plaque.^{292, 305, 338}

Cytokine studies showed a decrease in expression of pro-inflammatory cytokines IL-8 and IL-1 β . Cytokine release in general is much more apparent in cells differentiated into macrophages rather than in monocytes. In this study with incubations being 24 hours, cells incubated with a small dose of PMA the cells are not completely differentiated into macrophages, and it is thus understandable that lower levels of the cytokines are released.

In summary, HODEs have important signalling functions in macrophages that could be protective in early stages but also could stimulate processes involved in development of advanced lesions. 13-HODE predominates in early atherogenesis and could enhance lipid clearance by macrophages through increased reverse cholesterol transport and SCD activity. In later lesions, the action of 9-HODE through GPR132 predominate leading to apoptosis and pro-inflammatory responses in cells. The role of PPAR- γ and GPR132 needs to be further probed using antagonist and gene silencing studies.

Chapter 5

Role of Transcription Factor PPAR- γ and GPR132 in HODE Signalling in Macrophages

Introduction

The molecular mechanisms through which long-chain fatty acids have effects on macrophage functions are still unclear. Various pathways are thought to be involved in fatty acid signalling in cell proliferation and regulation of genes in controlling inflammation.²⁰⁰ Two key areas explored in this project are the G-protein coupled transmembrane receptors and their subsequent effects on the transcription factor peroxisomal proliferator activated receptor- γ (PPAR- γ) involved in inflammation control. Role of MAPK,²⁰¹ other protein kinases,²⁰² TLRs,²⁰⁵ and a variety of GPCRs²²⁸ have been postulated to be involved in fatty acid-macrophage signalling.

Among the transcription factors, PPAR- γ is of interest as it is involved in controlling inflammation range of cell types including adipocytes and monocytic cells.²⁵³ Fatty acids are natural ligands for this transcription factor,²⁵⁵ synthetic ligands constituting a class of drugs called thiazolidinediones that have been used extensively in treating diabetes.²⁴⁹ Blocking this transcription factor using specific antagonists would help in understanding its role when HODEs interact with macrophages, and their role in influencing PPAR- γ in inflammation control. T0070907³³⁹ and GW9662³⁴⁰ are two commercially available antagonists for the PPAR- γ receptor, with the later possibly also having antagonist effects on PPAR- δ .³⁴¹ Rosiglitazone, on the other hand, is a PPAR- γ agonist of the thiazolidine class of drugs, and is more potent than pioglitazone, the other drug of this class that has been widely used.³⁴²

GPR43²³² and GPR120²³³ have been characterised as receptors for long-chain fatty acids, with the latter having a critical role in preventing apoptosis in enteroendocrine cells.²³⁴ GPR120 has also been postulated to be important in adipocyte differentiation.²³⁶ GPR132 or G2A is thought to play a role in prevention of inflammation in vasculature, and absence of this receptor in murine macrophages increases atherosclerotic plaque formation.^{241, 343} The potential role of HODEs in GPR120 and GPR132 signalling has not been extensively studied to date. In this Chapter, the underlying mechanisms mediating the effects exhibited by HODEs documented in the previous two Chapters were probed. To do this we used a specific PPAR- γ antagonist. Antagonist studies are very important in studying the effects of specific receptor

ligands. However, specific antagonists for the GPR132 receptor are not available at present. We could have used U-73122, a phospholipase C inhibitor that is known to block G (i/o), thereby blocking effects of GPCRs signalling through that pathway.³⁴⁴ A drawback of this approach would have been that this antagonist blocks signal transmission through a range of receptors and its effect is not therefore specific for GPR132. A novel way to block a particular receptor is by using the recently described technique of RNA interference (RNAi) mediated gene silencing. GPCRs of interest can be silenced using a set of oligonucleotides that are transfected into the cells of interest and decrease expression of a particular gene. The silencing effect lasts for up to two weeks during which the experiments can be carried out.³¹⁶

Based on the results from studies on interaction between HODEs and monocytes and macrophages and while monocytes differentiate in previous two chapters, it was imperative to look at the mechanism by which HODEs were able to influence functions of these cells. Two principal candidates were the GPR132, which was highly expressed in monocytes, and PPAR- γ , which is not only known to be expressed but also has proven roles in lipid accumulation and regulation of inflammation. Work already presented suggests that HODEs have different effects on GPR120 compared with GPR132 expression. GPR120 is suppressed while the cells differentiate, and GPR132 increases while differentiating (detailed in previous Chapters).

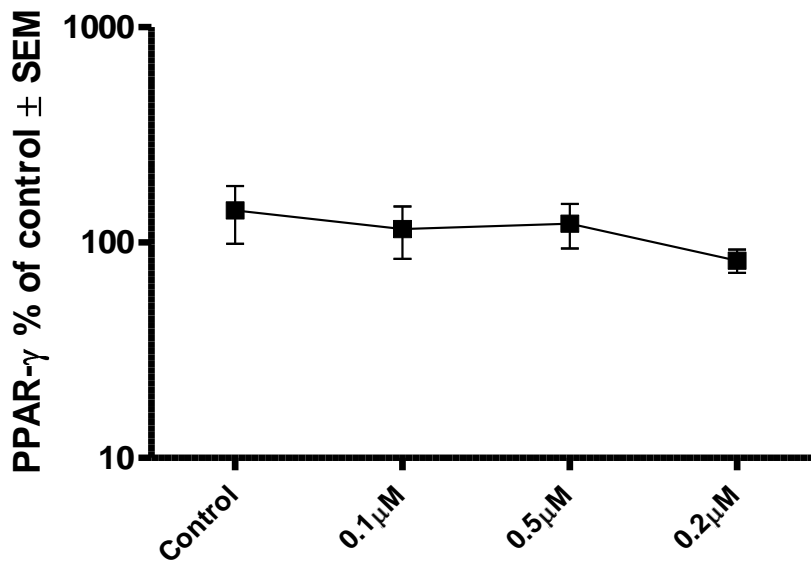
It was hypothesised that, 9-HODE might specifically signal through GPR132, while effects common to both HODEs would at least partially be mediated through PPAR- γ . To understand the fundamental effects caused by HODEs, we chose to investigate the effect of PPAR- γ antagonists and GPR132 gene silencing, examining their effects on FABP4 and SCRB expression using real time PCR. SCRB and FABP4 were chosen since they are highly expressed while the cells differentiate, and they appear to be regulated by HODEs in a very specific manner. The effects of GPR132 gene silencing and PPAR- γ antagonists on cytokine secretion were also studied.

Results

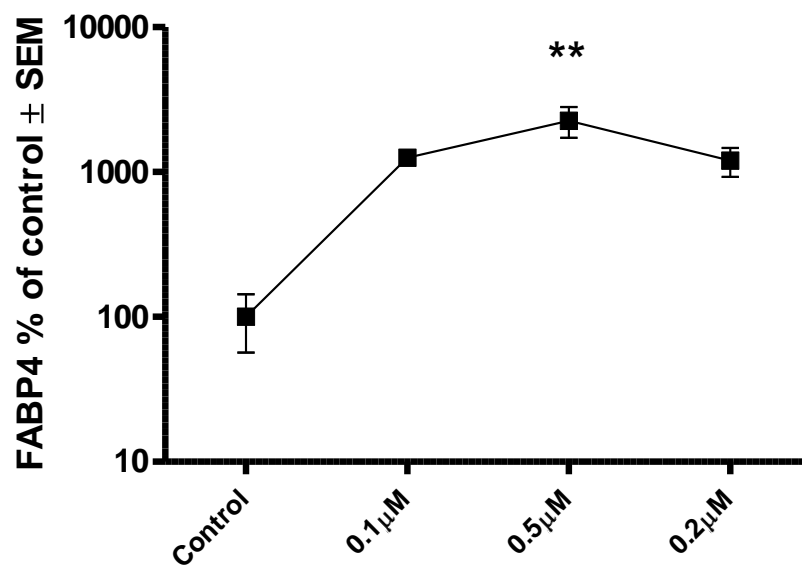
Role of PPAR- γ in HODE signalling

Preliminary studies

Rosiglitazone was used as a positive control and treated with other groups, which included 9-HODE and 13-HODE. Briefly, 500,000 THP-1 monocytes were differentiated into macrophages using 100nM PMA for 36 hours, followed by rest for 24 hours in low serum media (0.4% FBS and antibiotics). Cells were then treated with different concentrations of rosiglitazone and antagonist T0070907. Rosiglitazone was added at concentrations of 0.1, 0.5 and 2 μ M for 24 hours. Cells were then collected and stored at -80 $^{\circ}$ for RNA extraction. Real time RT PCR was performed to assess changes in expression of PPAR- γ , SCRB and FABP4 (these latter two genes are thought to be regulated by the transcription factor). Relative gene expression for the control and treated groups was calculated by using an internal control with housekeeping gene PPIA. Interestingly, there were no changes in mRNA levels of PPAR- γ . The agonist may well act through the transcription factor without affecting mRNA levels of PPAR- γ . Expression of FABP4 increased with increasing dose of rosiglitazone with significant increase in expression being apparent at 0.5 μ M being significant ($p < 0.01$, Figure 5.1).



(a)



(b)

Figure 5.1 Dose Response with Rosiglitazone

Gene expression in macrophages after 24 hours treatment with concentrations 0.1, 0.5 and 2 μM rosiglitazone, compared with control.

(a) PPAR-γ expression ($p > 0.05$)

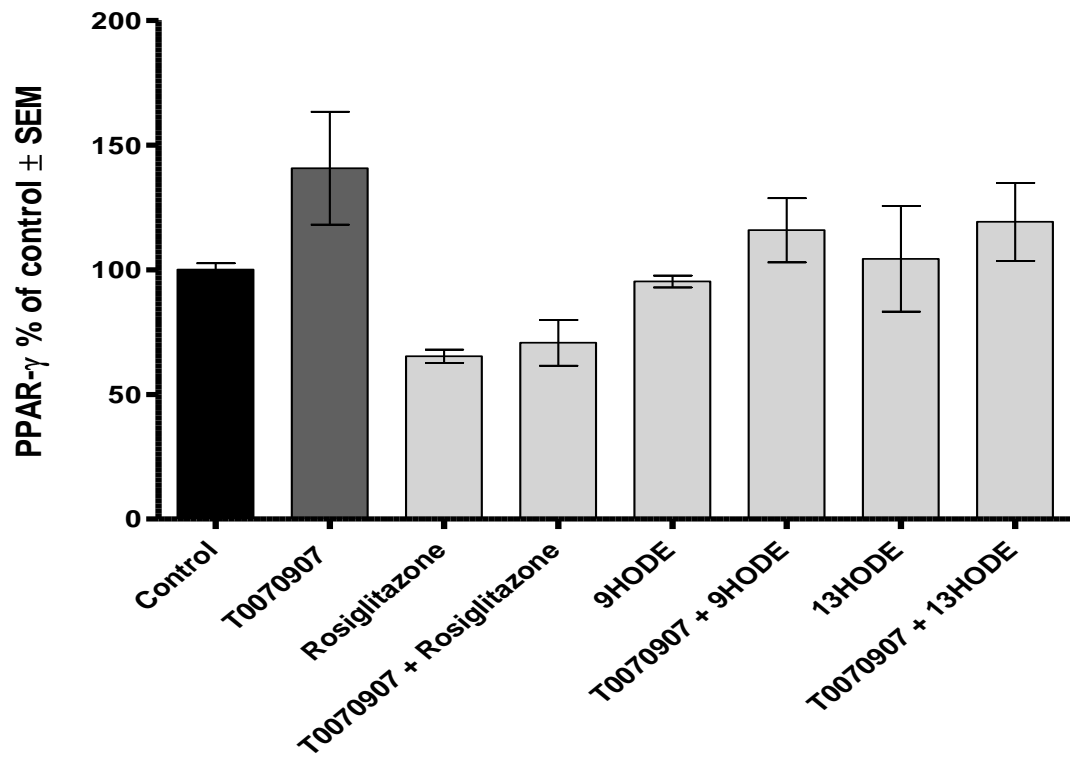
(b) FABP4: $p < 0.01$.

Data shown are mean ± SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). ** = $p < 0.01$.

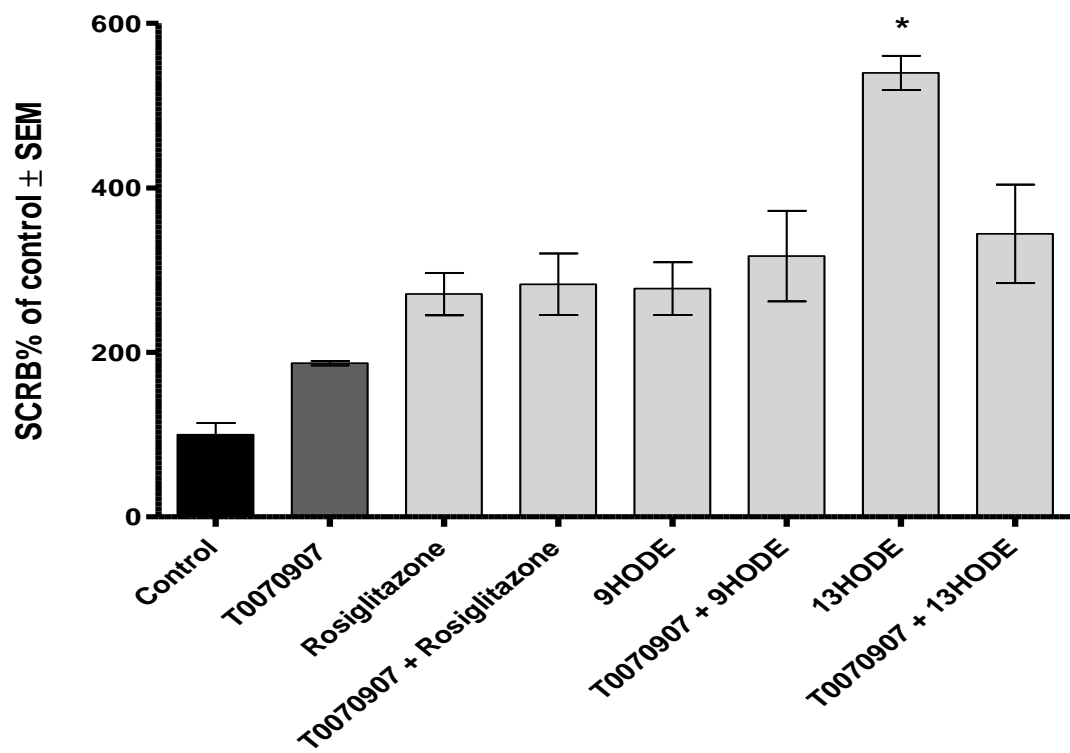
Based on the above experiment showing increased FABP4 expression with rosiglitazone, it was decided to use a concentration 0.1 μ M for experiments with or without the antagonist T0070907. The concentration of T0070907 used (10 μ M) was based on previous literature. Macrophages were incubated with 10 μ M T0070907 for 2 hours, medium was then removed, and replaced with fresh medium, and the cells were incubated for a further 18 hours.

HODEs partially signal through PPAR- γ

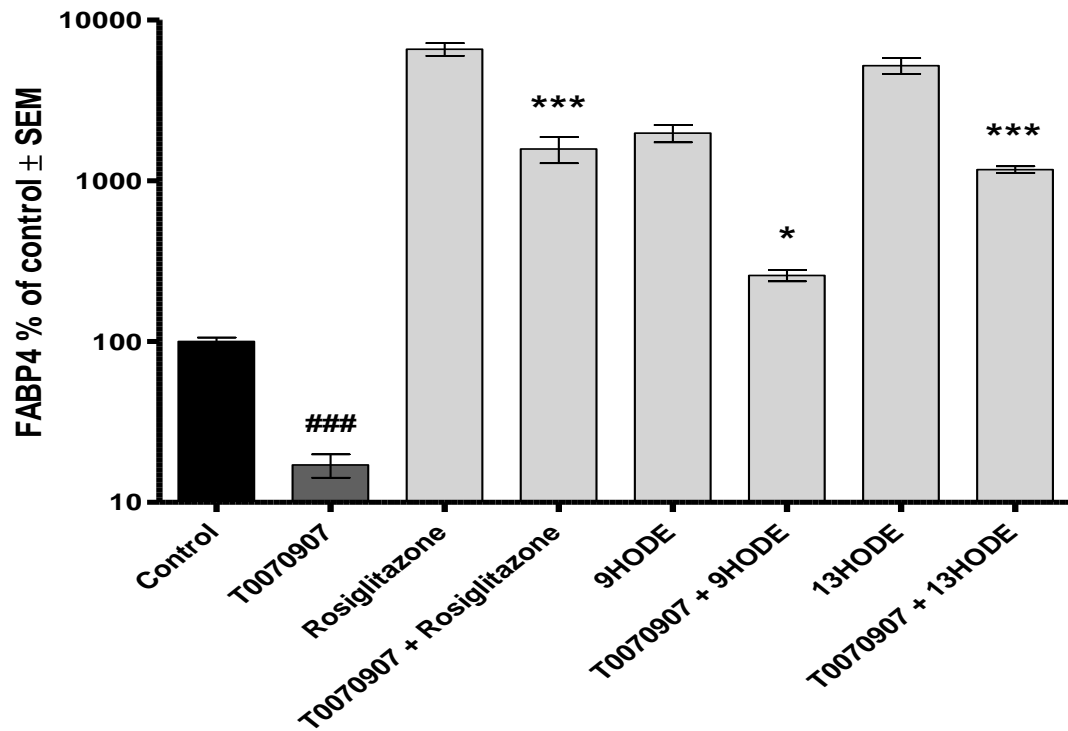
Differentiated macrophages were treated with 30 μ M 9-HODE, 13-HODE, or 0.1 μ M rosiglitazone with or without 10 μ M of the antagonist T0070907. The antagonist was incubated for 2 hours after which medium containing the long-chain fatty acids 9-HODE or 13-HODE, or rosiglitazone, were added and incubated for another 24 hours. The antagonist did not significantly affect the expression of PPAR- γ . With expression of SCRB, 13-HODE suppressed its expression significantly, but rosiglitazone and 9-HODE were without effect. FABP4 expression was suppressed significantly ($p < 0.001$) by T0070907 when compared with control. With addition of the antagonist to the three PPAR- γ agonists, expression of FABP4 was decreased in each case - rosiglitazone ($p < 0.001$), 9-HODE ($p < 0.05$) and 13-HODE ($p < 0.001$, Figure 5.2). However, this down-regulation was far from complete suggesting that each of these agonists may also be acting through pathways other than those regulated by PPAR- γ .



(a)



(b)



(c)

Figure 5. 2 Gene Expression Studies with T0070907

Gene expression in macrophages after 24 hours treatment with rosiglitazone, 9-HODE or 13-HODE, with or without antagonist T0070907

(a) PPAR- γ expression ($p > 0.05$)

(b) SCRB: 13-HODE, $p < 0.05$ compared with 13-HODE and antagonist

(c) FABP4 $p < 0.05$, $p < 0.001$, comparing the effect of agonist in the presence or absence of antagonist. $p < 0.001$ (control vs T0070907)

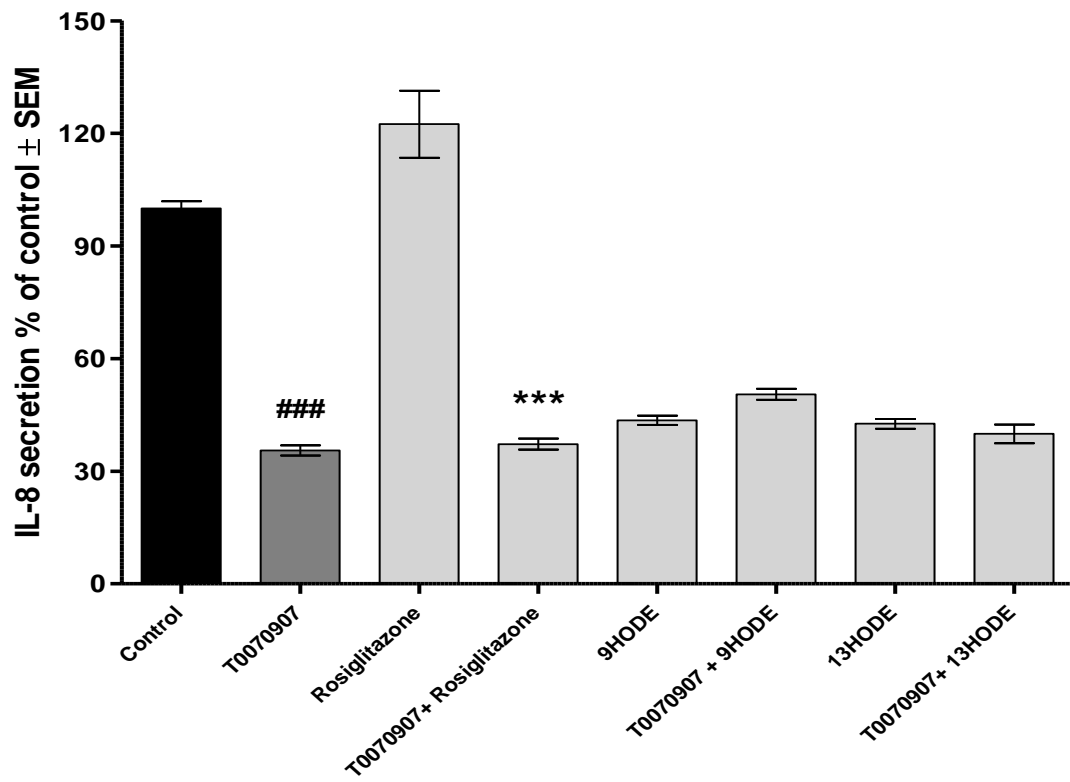
Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). * = $p < 0.05$ and ###, *** = $p < 0.001$

Cytokine Studies

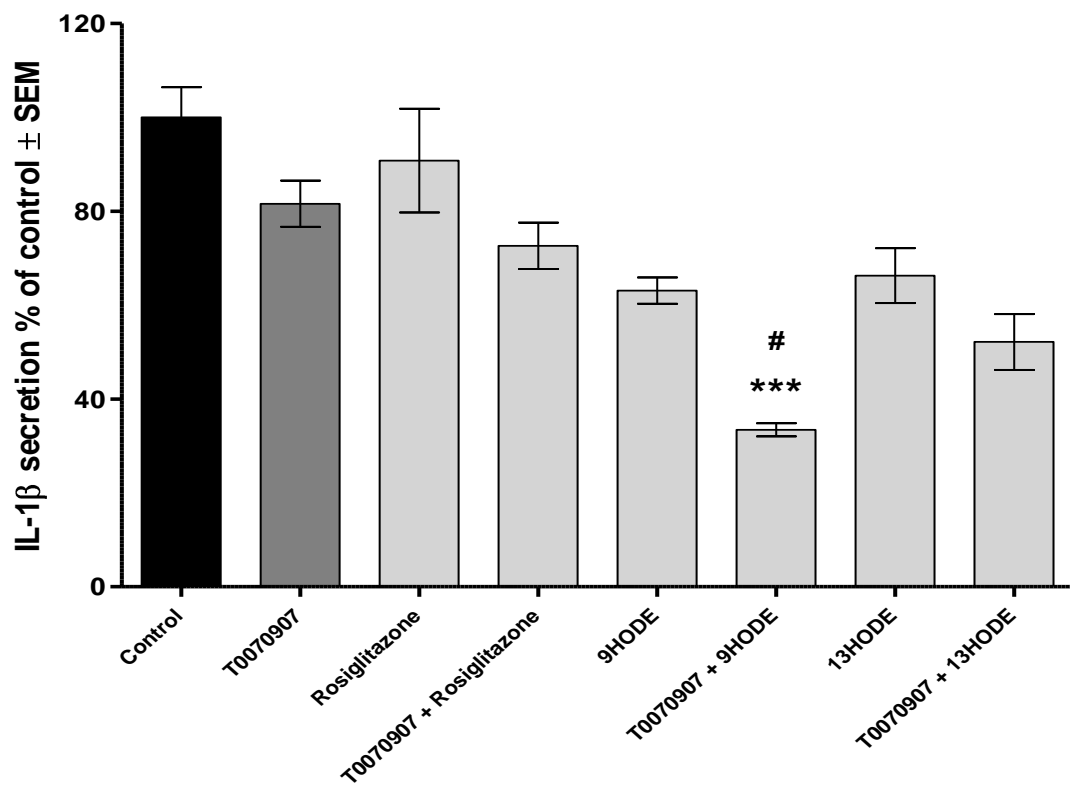
Conditioned media from the experiments were collected and stored at -80° for cytokine analysis. They were tested as described in previous chapter (detailed protocol in Materials and Methods) for TH1 and TH2 cytokines using reagents from Bendormedsystems. Briefly, 25 μ L of samples were incubated according to manufacturer's instruction in anti-bead cocktail, biotin anti-bead cocktail, followed by streptavidin PE. Samples were washed with wash buffer in between the steps. Samples and standards were then run on FACS Calibur (flow cytometer) once the conditions were optimised using the set up beads provided.

Cytokine regulation by rosiglitazone and HODEs is partly independent of PPAR- γ

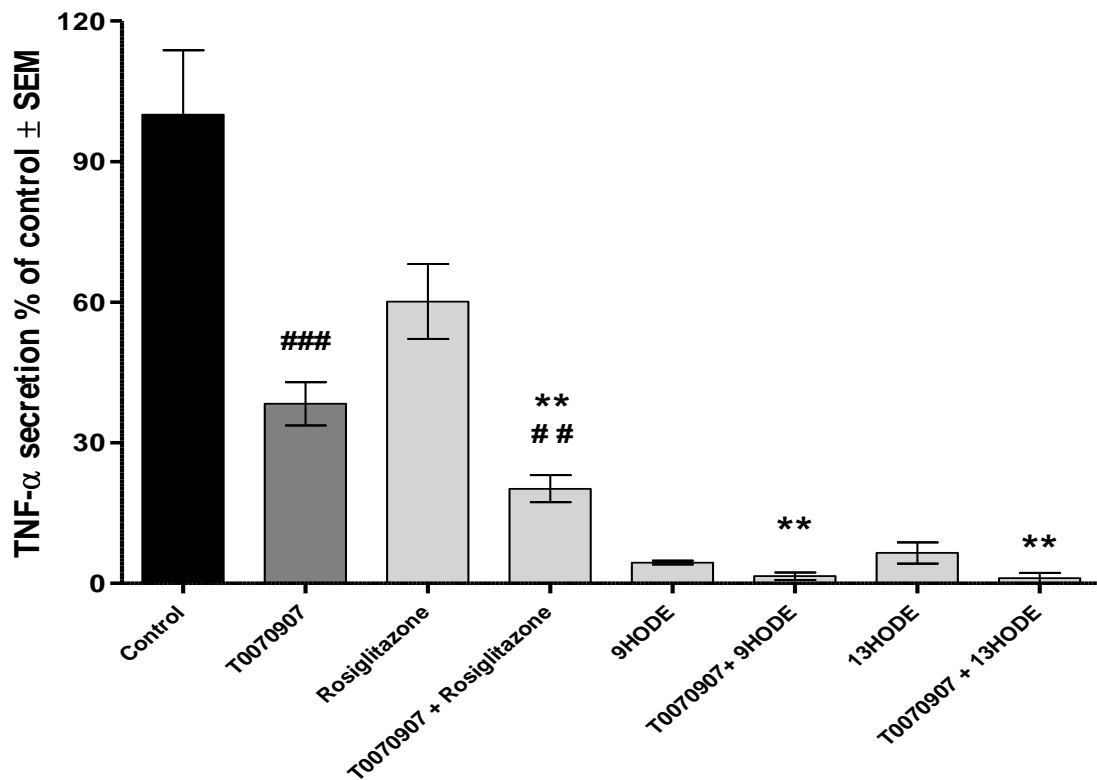
IL-8 secretion was suppressed significantly with the addition of antagonist, while rosiglitazone increased IL-8 secretion (though not statistically significant). 9-HODE and 13-HODE were without effect, and there was no effect of the combination of HODE and PPAR- γ antagonist. With the addition of antagonist and rosiglitazone, the expression of IL-8 decreased significantly compared with rosiglitazone alone. IL-1 β expression, on the other hand, was not affected by antagonist treatment. 9-HODE when added with antagonist had significantly lower levels of cytokine compared with 9-HODE alone. TNF- α secretion decreased with antagonist treatment as well as with rosiglitazone, 9-HODE, and 13-HODE. Furthermore, addition of the antagonist with each of the three agonists led to further decrease in TNF- α secretion, confirming the that effects of rosiglitazone and HODEs are at least partially PPAR- γ in relation to their effect on TNF- α secretion (Figure 5.3).



(a)



(b)



(c)

Figure 5. 3 Cytokine Studies

Conditioned medium was collected and tested for TH1 and TH2 cytokines after antagonist with or without rosiglitazone, 9-HODE or 13-HODE compared with control, antagonist or corresponding fatty acid.

(a) IL-8, T0070907 vs. control, $p < 0.001$; T0070907+ rosiglitazone vs. rosiglitazone, $p < 0.001$.

(b) IL-1 β , T0070907 vs. T0070907 + 9HODE, $p < 0.001$; 9-HODE vs. T0070907 + 9HODE, $p < 0.05$

(c) TNF- α , Control vs. T0070907, $p < 0.001$, Rosiglitazone vs. rosiglitazone + T0070907, $p < 0.01$; T0070907 vs. rosiglitazone + T0070907, $p < 0.01$ and 13-HODE vs. 13-HODE + T0070907, $p < 0.01$, 9-HODE vs. 9-HODE+ T0070907, $p < 0.01$.

Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). #, * = $p < 0.05$; ##, ** = $p < 0.01$ and ###, *** = $p < 0.001$.

siRNA Gene Silencing of GPR132

Preliminary Studies

Gene silencing of the receptor of interest has become an important technique in determining the role of a particular receptor in specific signalling pathways. A combination of specific oligonucleotides are added along with transfection reagent, and incubated with cells. The oligonucleotides degrade the mRNA of interest, and the effect lasts up to ten days during which experiments can be performed. Standardisation of the protocol was one of the major challenges of this work. No previous studies have been carried out to silence GPR132 in THP-1 cells. The oligonucleotides for GPR132 were:

CAGGATTGCCGGGTACTACTA,
ACGGACCATTCCCGCCAAGAA,
CTGGGTCACCATCGAGATCAA and
TACCAATTTCTCGTTCCTGAA.

Optimal conditions for high silencing had to be determined based on the protocol suggested by the manufacturer. The conditions that had to be standardised included, the cell numbers to each plate, amount of transfection reagent, concentration of oligonucleotides, incubation times, type of medium, addition of antibiotics or percentage of serum to use in the medium. At the end of the incubation period, RNA was extracted from cells, and real-time RT CPR was performed for the genes MAPK and GPR132 to assess the percentage knockdown. The summary of different combinations used to optimise are shown in Table 5.1.

Cell Number	Media	siRNA/Transfection	Outcome
<i>MAPK silencing</i>			
Monocyte, 1×10^5	RPMI (no L-Glutamine/Serum/Antibiotics)	25, 50 and 75nM and 6 μ L Hiperfect transfection reagent in 200 μ L for 6 hours followed by addition of 400 μ L for 18 hours. Incubation times 24, 48 and 72 hours.	Tried single samples each. Needed to try, triplicate in each group, one time point 24 hours.
Monocyte, 1×10^5	RPMI (no L-Glutamine/Serum/Antibiotics)	25, 50 and 75nM and 6 μ L Hiperfect in 200 μ L for 6 hours, followed by addition of 400 μ L for 18 hours.	50nM had highest silencing effect
<i>GPR132 silencing Monocytes</i>			
Monocyte, 1×10^5	RPMI (no L-Glutamine/Serum/Antibiotics)	50nM and 6 μ L Hiperfect, 24 hours	No silencing effect
Monocyte, 1×10^5	RPMI (no L-Glutamine/Serum/Antibiotics)	100nM and 200nM and 6 μ L Hiperfect, 24 hours	No silencing effect
Monocyte, 1×10^5	RPMI (no L-Glutamine/Serum/Antibiotics)	50nM and 6 μ L Hiperfect, 48 and 72hrs	No silencing effect
Monocyte, 1×10^5	RPMI (no L-Glutamine/Serum/Antibiotics)	100nM and 3 μ L and 6 μ L Hiperfect for 48 hours	No silencing effect and NO RNA yield with 6 μ L Hiperfect (Toxic). Had to set up with transfection control, negative and positive controls.
Monocyte, 1×10^5	RPMI (no L-Glutamine/Serum/Antibiotics)	50nM and 6 μ L Hiperfect, 48 hours with MAPK , transfection and negative control	No silencing effect, Hiperfect reagent toxic. Advised by technical support Qiagen, they have not been successful in transfecting monocytes hence started trials for macrophages.
<i>MAPK and GPR132 in Macrophages</i>			
Macrophages 2×10^4 in 24well plate	RPMI (with L-Glutamine/Serum/Antibiotics) differentiation of monocytes for 24 hours	50nM and 6 μ L Hiperfect, 48 hours	Followed manufacturer's protocol. Cells less for optimum RNA yield. Overnight PMA incubation not enough.
Cell number determination: plated 8×10^4 , 10×10^5 and 1.2×10^5 cells	RPMI (with L-Glutamine/Serum/Antibiotics) differentiation of monocytes for 36 hours		Found 130,000 to be ideal for 24well plate since 120,000 cells did not cover the well completely.
Macrophages, 1.3×10^5 in 24well plate	RPMI (with L-Glutamine/Serum/Antibiotics) differentiation of monocytes for 36 hours	50nM and 3 μ L Hiperfect, 48 hours	Good knockdown with MAPK. Cell death quite high hence tried another transfection reagent Lipofectamine RNAiMax from Invitrogen
Macrophages, 5×10^5 in 12 well plate	RPMI (with L-Glutamine and serum no antibiotics) differentiation of monocytes for 36 hours and rested for 24 hours (low serum) No antibiotics.	10nM, 50nM and 100nM siRNA with 2 μ L in no serum no antibiotic media	Good knockdown with 10nM but not with higher concentrations
Macrophages, 5×10^5 in 12 well plate	RPMI (with L-Glutamine and serum no antibiotics) differentiation of monocytes for 36 hours and rested for 24 hours (low serum) No antibiotics.	10nM, 20nM and 50nM siRNA with 2 μ L in no serum no antibiotic media	10nM found to be most effective and RNA yield good.
Monocytes Trial with :Lipofectamine 5×10^5 in 12 well plate	RPMI (with L-Glutamine and serum) devoid of Antibiotics)	10nM siRNA with 2 μ L	Only 20% knockdown achieved

Table 5.1 Optimisation of siRNA
Optimal conditions

5×10^5 THP-1 monocytes were differentiated into macrophages by treatment with 100nM PMA for 36 hours in a 12-well plate in RPMI medium with 10% FBS but devoid of antibiotics. Differentiated cells were then washed with DPBS removing excess PMA and rested for 24 hours in RPMI medium with 0.4% FBS devoid of antibiotics. Before transfection, the medium was removed and 900 μ L of RPMI medium without antibiotics and serum was added and incubated for 20 minutes. siRNA oligonucleotides for the positive control MAPK was prepared by incubating 1 μ L of 10 μ M siRNA with 2 μ L of lipofectamine RNAiMAX transfection reagent for 15 minutes at room temperature in 97 μ L of RPMI medium (without antibiotics and serum). Transfection complex was then added drop-by-drop into treatment wells and mixed well giving a final concentration of 10nM. The transfection complex medium was removed after six hours and 1mL RPMI medium (with 10%FBS without antibiotics) was added and incubated for 18 hours. The cells were then treated with rosiglitazone, 9-HODE or 13-HODE for 24 hours and conditioned media were stored for cytokine analysis, and cells were stored for gene expression studies. The positive control used was MAPK and gene expression of MAPK, GPR132 and negative control was observed compared with that of the housekeeping gene PPIA. Based on the results when optimal silencing effect was observed with MAPK, similar conditions were adopted for silencing GPR132.

Silencing of MAPK and GPR132

The above conditions were found to yield best results for MAPK. A concentration of 10nM siRNA, 2 μ L transfection reagent was found to be best suited. Gene expression studies when compared against the housekeeping gene PPIA showed a significant decrease in MAPK expression. The knockdown effect ranged from 70-80% in all the experiments (Figure 5.4). Similar conditions were adopted for silencing GPR132 and a knockdown of up to 70% was achieved with macrophages (Figure 5.5).

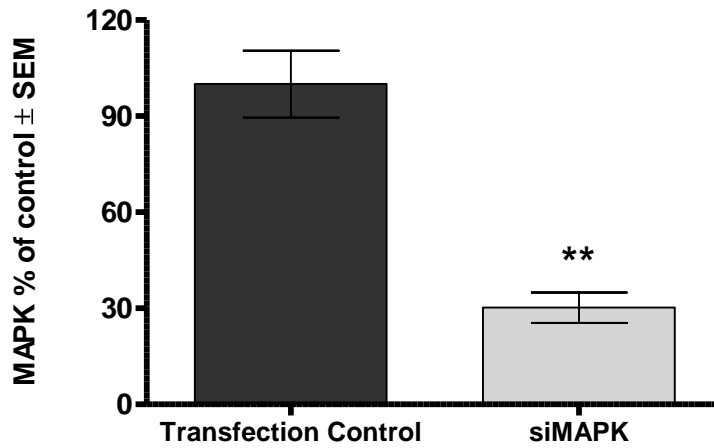


Figure 5.4 siRNA MAPK Transfection

MAPK gene expression in macrophages 24 hours after silencing of MAPK compared with control. $p < 0.01$.

Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (unpaired t test). ** = $p < 0.01$.

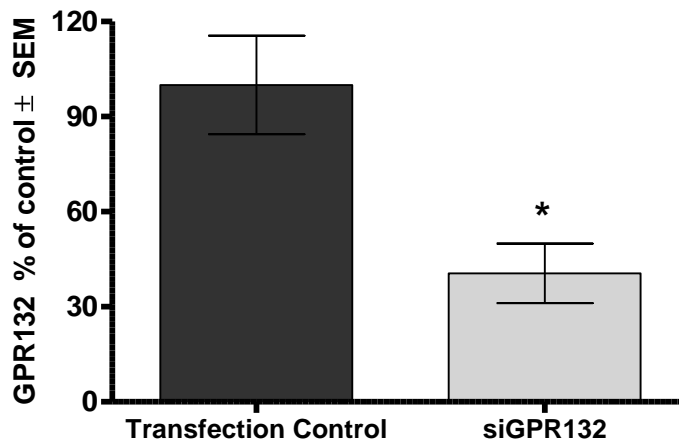


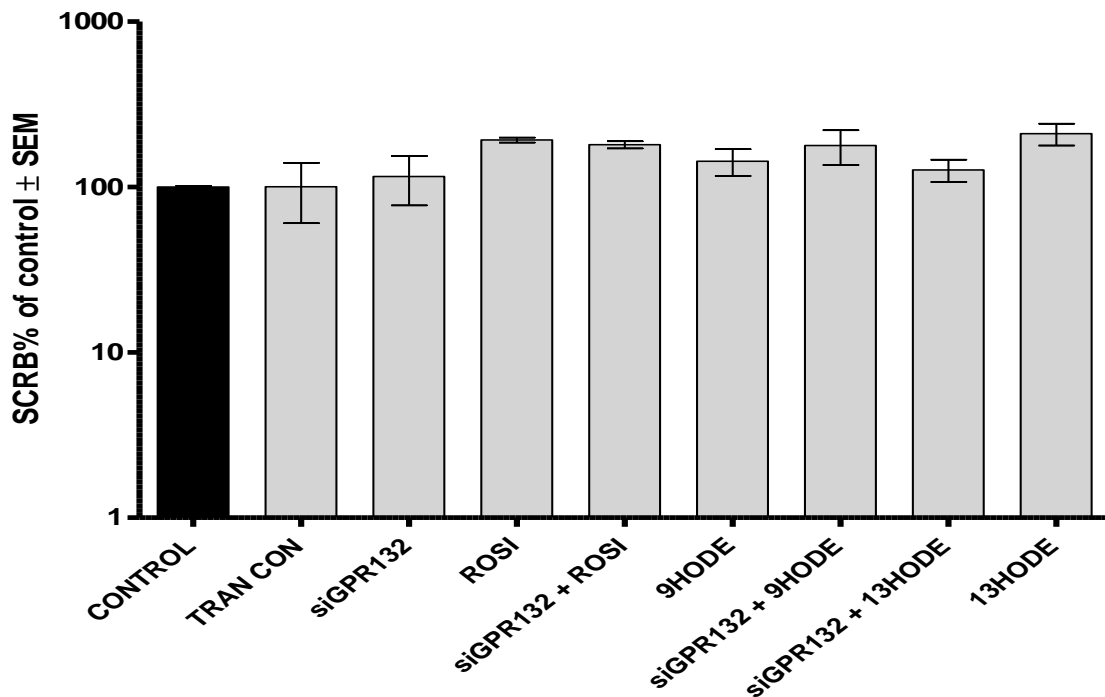
Figure 5.5 siRNA GPR132 Transfection

GPR132 gene expression in macrophages 24 hours after silencing of siGPR132 compared with control. $p < 0.05$.

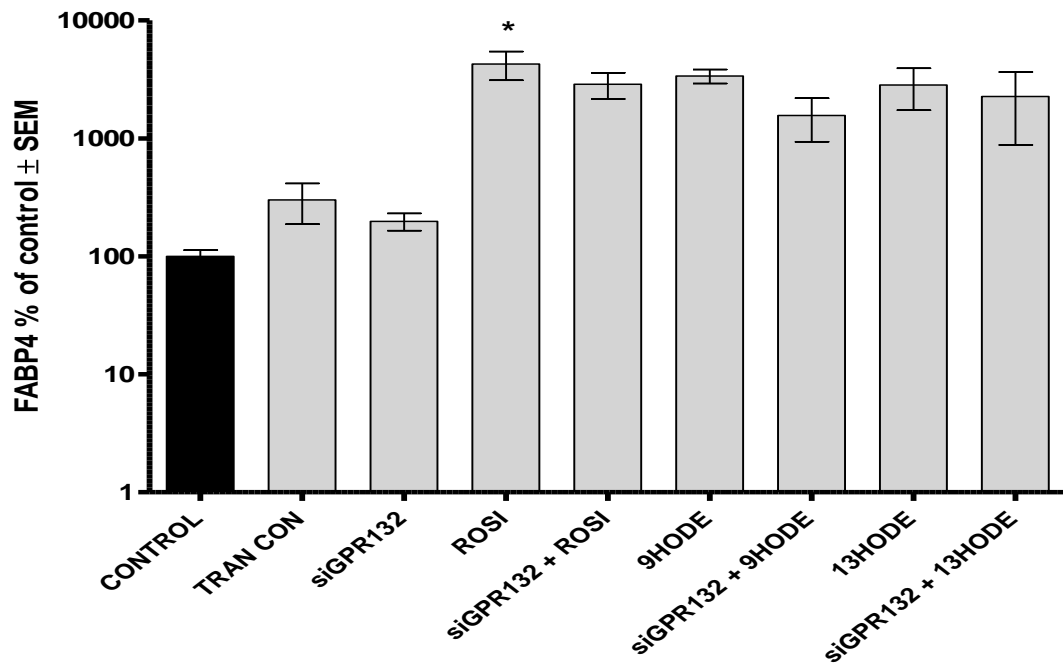
Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (unpaired t test). * = $p < 0.05$.

Effects of HODEs and Rosiglitazone on FABP4 and SCRB upon Silencing of GPR132

As significant differences in SCRB and FABP4 expression were found with HODE treatment, the role of GPR132 in their regulation was observed after gene silencing of GPR132. GPR132 appeared to have no significant role in regulating SCRB expression. Non-significant increases in SCRB expression were observed with rosiglitazone, 9-HODE and 13-HODE, and there was no change in expression with any of the agonists following GPR132 gene silencing. FABP4 expression slightly increased with silencing and as expected rosiglitazone, 9-HODE and 13-HODE increased FABP4. No differences between the HODEs treatment or rosiglitazone treatment and siGPR132 control or corresponding fatty acid were observed (Figure 5.6).



(a)



(b)

Figure 5.6 Gene Expression Studies

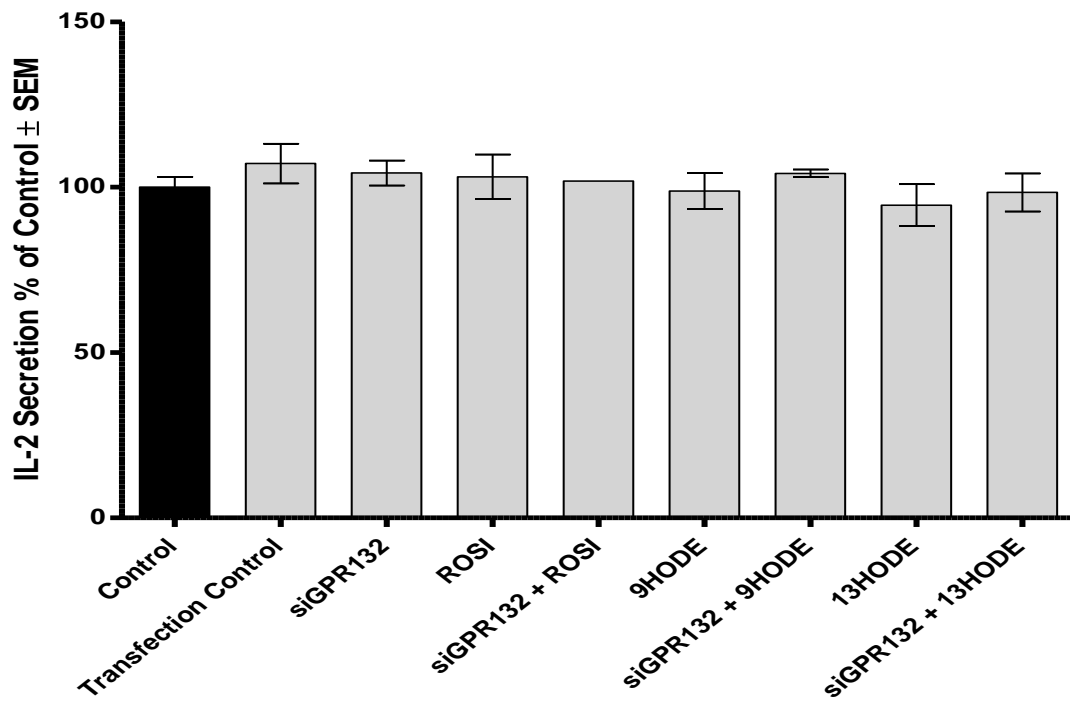
(a) SCRB gene expression in macrophages 24 hours after silencing of GPR132 compared with control.

(b) FABP4 expression in macrophages 24 hours after silencing of GPR132 compared with control, control vs. rosiglitazone ($p < 0.05$).

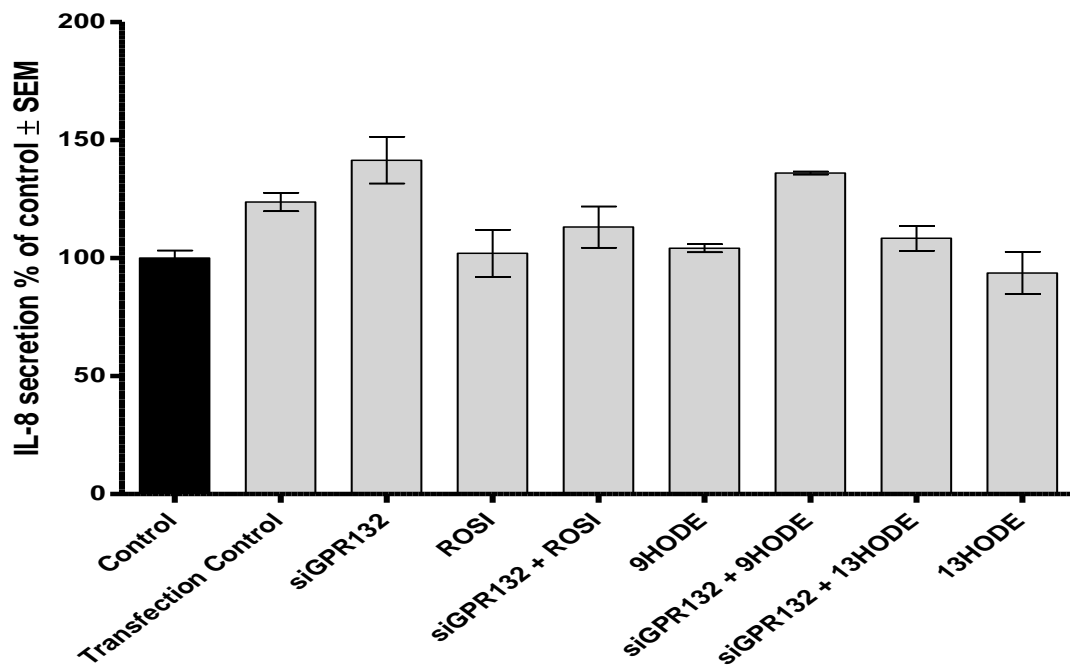
Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). * = $p < 0.05$.

Cytokine Analysis Following siGPR132 and HODEs

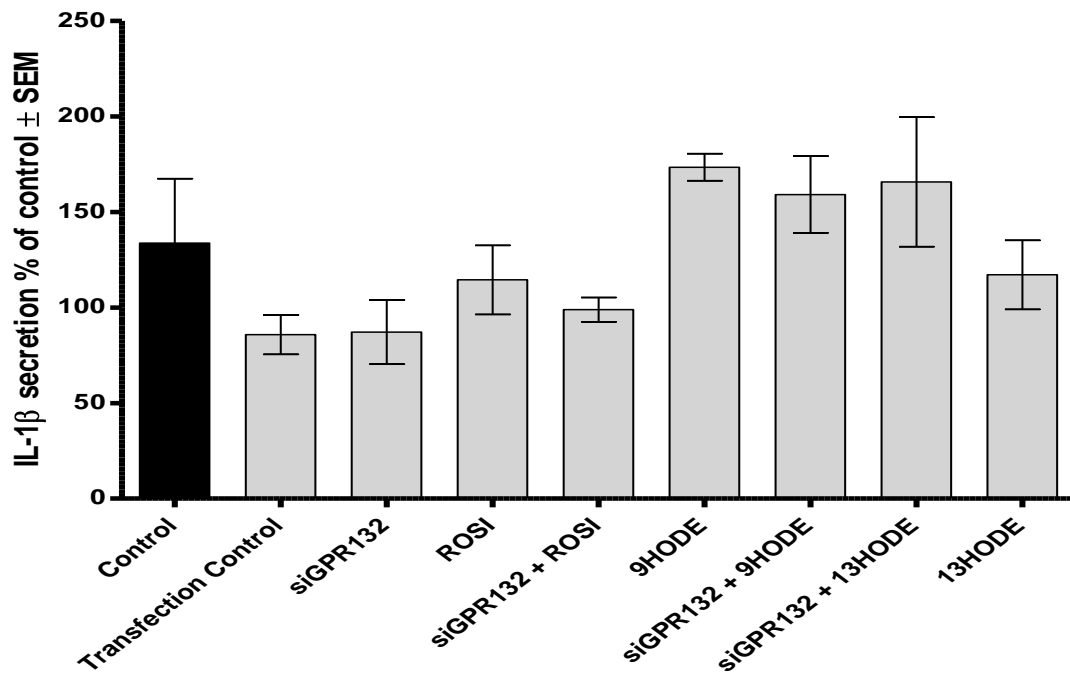
Conditioned media from the cells were stored and cytokine bead array was performed as described previously for the PPAR- γ antagonist experiment. Of the eleven TH1/TH2 cytokines tested, only four namely IL-2, IL-8, IL-1 β and TNF- α were present at detectable levels. No differences among the groups were observed with IL-2 secretion with silencing of GPR132. A moderate increase with GPR132 silencing in IL-8 secretion was observed though this was not statistically significant, while a decrease (not significant) in IL-1 β secretion was observed with silencing. Changes in the expression of TNF- α were significant between the groups. Compared with the group silenced for GPR132, the groups treated with HODEs and rosiglitazone post GPR132 silencing had significantly reduced levels of TNF- α secreted. 9-HODE ($p < 0.001$), rosiglitazone ($p < 0.001$) and 13-HODE ($p < 0.01$) secreted significantly lower levels of TNF- α (Figure 5.7) with the former two being more potent in suppressing the TNF- α secretion.



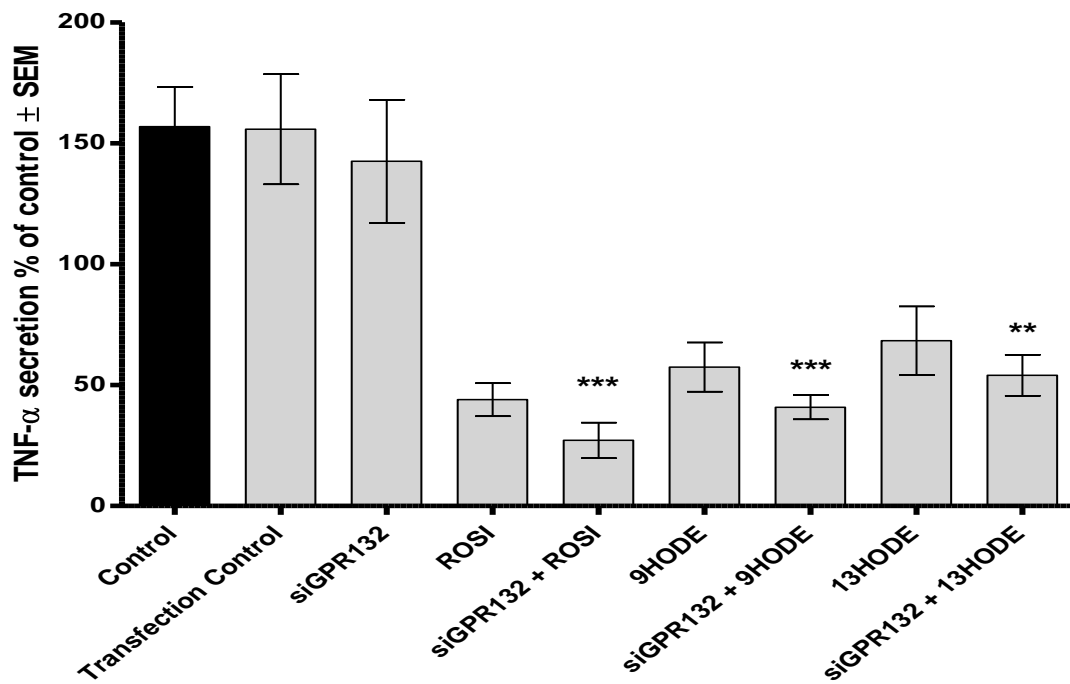
(a)



(b)



(c)



(d)

Figure 5.7 Cytokine Secretion with HODEs Treatment after GPR132 Silencing

(a) IL-2 secretion. (b) IL-8 secretion. (c) IL-1 β secretion.

(d) TNF- α secretion (siGPR132 vs. siGPR132+9-HODE, $p < 0.001$; siGPR132 vs. siGPR132+13-HODE, $p < 0.01$; siGPR132 vs. siGPR132+ rosiglitazone, $p < 0.001$).

Data shown are mean \pm SEM from groups treated in triplicate and normalised as a percentage of control (one way ANOVA, Tukey's post test). ** = $p < 0.01$ and *** = $p < 0.001$.

Discussion

THP-1 cells provide a convenient model to study the effects of fatty acids on monocytes and macrophages. They can be differentiated into macrophages using PMA. These functions on these cells can be studied using specific inhibitors for intracellular mediators, including the transcription factor PPAR- γ .³⁴⁵ Gene silencing using an RNA interference system is also a novel way to study the role of a gene of interest that has a signalling and functional role in macrophages.³¹⁶ The previous two Chapters investigated the effects of HODEs on various genes involved in lipid metabolism. HODEs specifically increased expression of FABP4 and PPAR- γ in monocytes. An increase in expression of SCRB was also observed. FABP4 expression was markedly increased in macrophages. When cells were stimulated with a low dose of PMA along with HODEs, synergy between the two regulators was observed. This experiment was performed to mimic the conditions that would be observed when the monocytes are attracted to the site of inflammation by chemoattractants and are subjected to activation.^{131, 132} An increase in expression of SCRB, ABCA1 and FABP4 was almost certainly, at least partly, through increased activity of PPAR- γ . The other major feature observed was the increase in expression of GPR132 in monocytes and when the cells were stimulated with PMA and HODEs. While increased expression of GPR132 in monocytes was noted, a reduction in expression of GPR120 was seen in macrophages, and while the cells differentiated. This study tested the hypothesis that HODEs mediate increase in expression of genes involved in lipid transport and reverse cholesterol transport acting through GPR132 and PPAR- γ .

The transcription factor PPAR- γ has been shown to be involved in controlling inflammation and in regulating metabolism in monocytes and adipocytes.²⁵³ Fatty acids are known to act as ligands for PPAR- γ .²⁵⁵ Blocking the transcription factor PPAR- γ using various antagonists have been a useful strategy to determine the role of PPAR- γ in various signalling functions.^{339, 340} T0070907 was used in the present study to block PPAR- γ by incubating the macrophages for 2 hours, followed by addition of HODEs and the known PPAR- γ agonist rosiglitazone.³⁴² Dose response studies of rosiglitazone revealed a significant increase in gene expression of FABP4 quantified using real time RT-PCR at a concentration of 0.5 μ M. A lower concentration of 0.1 μ M was used for the experiments since an increase of 10-fold was found with this concentration since with

higher dose of 0.5 μM could lead to masking of effects associated with cell differentiation and transmembrane receptors. Interestingly, no change in the mRNA expression of PPAR- γ was observed with rosiglitazone treatment. While rosiglitazone is a known agonist for the transcription factor it may not be increasing the mRNA levels but rather binding would activate the transcription factor and regulate inflammation. On the other hand it is also possible that the time point of 24 hours, at which the mRNA levels were quantified, was not the ideal time point since the mRNA levels might have normalised to basal state by this time. Blocking PPAR- γ using T0070907 did not have a significant effect on the expression of SCRB. FABP4 expression was significantly reduced with T0070907 treatment compared with control. When HODEs were added after antagonist treatment, there was a significantly lower level of expression of FABP4 compared with the corresponding group where only the fatty acid was added. Though a decrease was observed, the data suggests that FABP4 expression is regulated by other PPAR- γ independent mechanisms, and there is possibly a partial role for the transcription factor in this process.

This study also investigated the role of HODEs and PPAR- γ on the secretion of cytokines. Release of cytokines into the extracellular fluids and circulation has been shown to affect the homing of monocytes. While TH2 cytokines stimulate differentiation of monocytes into a phenotype that is called the alternative anti-inflammatory M2 macrophage, TH1 cytokines promote differentiation of these cells into pro-inflammatory M1 macrophages. During the differentiation phase, it has been shown that by activating PPAR- γ these cells are converted into M2 macrophages. Tissue resident macrophages are not stimulated to express M2 markers by activation of PPAR- γ .²⁷⁶ In the previous Chapter, the demonstrated effects of HODEs would appear to suggest that the fatty acids might contribute to monocytes differentiating into the M2 phenotype. This study would also supports the observations of Cao et al.,¹ who have suggested that fatty acids can act as hormones. These authors have coined the term 'lipokine' for such fatty acid mediators.

Pro-inflammatory cytokines IL-8, TNF- α , IL-1 β and IL-6 secreted by macrophages have been shown to be involved in development of atherosclerosis.²³ In THP-1 macrophages, long-chain saturated fatty acids have been shown to induce secretion of pro-inflammatory cytokines including IL-8, IL-1 β and TNF- α .³¹⁶ The present study

measured the cytokines released by macrophages when stimulated with HODEs and rosiglitazone, and also the role of PPAR- γ in macrophage secretion. Pro-inflammatory cytokines IL-8 and TNF- α decreased significantly with addition of the PPAR- γ antagonist T0070907 and the secretion of TNF- α was further reduced in each case when HODEs or rosiglitazone was added after T0070907 treatment (compared with antagonist control). IL-1 β secretion was also decreased, although this was not statistically significant when compared with the antagonist group. 9-HODE was more potent in reducing the secretion of this cytokine, suggesting a partial role for PPAR- γ in controlling secretion of these pro-inflammatory cytokines. Although PPAR- γ is well known to have a role in controlling inflammation, other candidates have also been suggested in regulating pro-inflammatory cytokines, including the transcription factor TLR4.³⁴⁶

GPR132 is known to be expressed in murine and human macrophages in atherosclerotic regions.²⁴¹ While absence of GPR132 in mouse models has been shown to promote plaque formation,³⁴³ other studies have shown that GPR132 may have an atheroprotective role.²⁴²⁻²⁴⁵ GPR132 was identified as a receptor for 9-HODE,²³⁹ and is possibly involved in preventing inflammation of vasculature, and may thus decrease atherosclerosis. A role for GPR132 in mediating the proinflammatory effects by 9-HODE, but not 13-HODE, in keratinocytes has been recently suggested.²⁴⁰ The present study employed the novel technique of gene silencing. THP-1 monocytes and macrophages are one of the most difficult cells to transfect.³⁴⁷ We successfully transfected macrophages, and GPR132 was silenced effectively. The role of GPR132 in expression of FABP4, SCRB and release of cytokines was investigated. No direct effect on the mRNA levels of SCRB and FABP4 was observed with silencing of GPR132. These data suggest that there are GPR132 independent mechanisms involved in regulating FABP4 and SCRB expression. A role of GPR132 in apoptosis²⁴³ is well established, but this study did not investigate the role of GPR132 silencing in the apoptosis induced by HODEs.

Cytokine secretion was also determined in this study. IL-2, which is essential for leukocyte proliferation,²³ was detected in the conditioned medium but no differences were observed with HODE incubation of cells. Of the pro-inflammatory cytokines secreted (and similar to PPAR- γ antagonist study) IL-8, TNF- α , IL-1 β were detected.

TNF- α secretion in particular was decreased (when compared to control) in the groups which were treated with HODEs or rosiglitazone following siGPR132 transfection. However, GPR132 gene silencing did not abolish TNF- α secretion, suggesting that GPR132 independent mechanisms are involved in controlling secretion of this cytokine. Secretion of cytokines is known to be regulated by multiple pathways, and the above observation is not therefore surprising.

In summary, a partial role of PPAR- γ in controlling the expression of FABP4, when stimulated by HODEs was observed. GPR132 was successfully silenced using siRNA. Although, 9-HODE is a known ligand for GPR132, no changes were observed when the receptor was silenced with respect to the secretion of FABP4. Although PPAR- γ is certainly part of the regulatory pathway, it is likely that multiple mechanisms are involved in controlling the expression of FABP4.

Chapter 6

Expression of Differentiation Markers and GPR132 in Peripheral Mononuclear Cells of Patients with Type 2 Diabetes

Introduction

Cardiovascular complications are one of the leading causes of death in diabetic subjects. Improved diagnostic markers to determine the cardiovascular risk of diabetic patients would be helpful. Although studies have investigated various activation and differentiation markers in monocytes that may be predictive for vascular risk, there are no definitive diagnostics markers. Studies have also investigated the levels of different pro-atherogenic cytokines in the circulation, as well as monocyte populations exhibiting pro-angiogenic markers in these patients.³⁴⁸⁻³⁵⁰

In general, markers associated with inflammation are increased in diabetic patients, with higher levels being particularly apparent in patients in whom blood sugar levels are poorly controlled. Increased levels of scavenger receptor CD36 in monocyte populations have been reported in diabetic and poorly controlled diabetic patients.^{349, 351-353} Augmented levels of expression of MCP-1 has also been described in diabetic patients, and expression of genes involved in controlling inflammation, PPAR- γ , receptor for LDL, and CD68 are also found to be higher in poorly controlled patients in whom monocytes show a greater affinity for endothelial cell adhesion.³⁴⁹ The fatty acid composition of macrophages is different in diabetic subjects compared against non-diabetic subjects. Higher levels of stearic acid and lower levels of linoleic acid are present in macrophages in diabetic cohorts compared with controls, while no significant differences in gene expression of PPAR- γ , SCD and ABCA1 have been reported.³⁵⁴ Changes in fatty acid composition could be used as an indicator for cardiovascular risk. Decreased levels of expression of PPAR- γ and other important proteins regulated by PPAR- γ such as CD36, FABP4 and adipoplilin was reported in patients with decreased levels of plasma HDL.³⁵⁵ These subjects also had increased levels of pro-inflammatory gene expression (IL-8, IL-1 β and TNF- α). A subset population of monocytes, CD11c⁺ cells have been shown to be increased with insulin resistance. In a genetic mouse model in which this receptor has been ablated, and these cells show increased sensitivity to insulin and decreased state of inflammation.³⁵⁶

Fatty acids have been found to have differential effects on monocytes. Sodium butyrate, the most abundant short-chain fatty acid is known to be present in concentrations as high as 30mM in the intestines, and has been shown to suppress pro-inflammatory

cytokines IL-12 and TNF- α in monocytes.³⁵⁷ While the n-3 fatty acids EPA and DHA have been shown to reduce the expression of CD36 in human peripheral monocytes, n-6 fatty acids AA and LA increase the expression of CD36 thus promoting atherogenesis.³⁵⁸ Differences among the pro-inflammatory genes and levels of circulating HODEs in diabetic patients and controls have not been studied so far. This would be helpful in understanding the correlation between derivatives of lipids and their role in regulating inflammation. Changes in various differentiation markers on mononuclear cells could be used as diagnostic marker for cardiovascular risk. The markers CD11b and CD36 are associated with macrophages when they interact with oxidised lipids,³⁵⁹ with the former being a characteristic marker to identify macrophages.¹²⁶ CD14 is another marker used for differentiating human peripheral monocytes.¹²⁵ CD54 (ICAM-1, intracellular adhesion molecule-1) is often increased during inflammation in macrophages,³⁶⁰ and this molecule is involved in promoting adhesion of leukocytes during inflammatory state.³⁶¹ The levels of GPR120 and GPR132 have not previously been studied in monocytes from diabetic patients. It is likely that from the studies on THP-1 cells detailed in the previous chapters that there could be differential expression of these GPCRs in diabetic patients who have a higher inflammatory state and are more prone to atherosclerosis.

We hypothesized that peripheral CD14⁺ monocytes in diabetic patients would have increased levels of differentiation markers CD11b, CD54 and CD36 compared to controls. In addition, there would be differences in expression of GPR120, GPR132 and resistin compared with control patients. The aim of the work in this Chapter was to investigate the levels of various differentiation markers in CD14 positive cells using flow cytometry. We also separated CD14⁺ cells from total leukocytes to study the differences in expression of GPR120 and GPR132. The serum stored from the samples was also tested for obesity markers associated with diabetes.

Results

Preparation of mononuclear cells

Patients with type 2 diabetes were recruited from diabetic clinics at the Townsville Hospital, North Queensland. Informed consent was obtained from all subjects (diabetic and controls). A total of 51 subjects were studied in all, of which 29 were diabetic and 22 control subjects. Controls were age and gender matched. Institutional approval was obtained from Human Research Ethics Committee of Townsville Health Service District. 24mL of venous blood was collected in heparinised tubes by a qualified phlebotomist. At room temperature, an equal amount of DPBS was added and mixed well in a 50mL Falcon tube. 10mL of Ficoll-hypaque was added to a fresh 50mL Falcon tube and gently the blood-DPBS mixture was added without disturbing the Ficoll layer. The tubes were then centrifuged at 550g for 12 minutes with no brakes. The white buffy coat layer was carefully aspirated and added to a new tube and filled with DPBS, and then centrifuged at 600g for 10 minutes to get rid of the excess plasma and to sediment the leukocytes. The cell pellet was then washed again with DPBS and centrifuged at 300g for 8 minutes to obtain leukocyte pellet. A total cell count was performed using a haemocytometer by adding 10 μ L of the cell suspension in counting chamber. Cells were then stained for flow cytometry and separate CD14 positive cells were obtained using magnetic bead separation. A separate tube was used to collect the blood for studying chemokine and cytokine levels in serum, and this was stored at -80°C pending assay for the various markers.

Flow cytometry studies

1,000,000 total leukocytes were stained with fluochromes for flow cytometry studies. Cells were washed with flow cytometry buffer, which was prepared by adding 0.5% BSA and 2mL of 0.5M EDTA in 500mL DPBS and centrifuged at 300g for 5 minutes at 4°C. The supernatant was removed and cells were incubated with FcR block (1:50) for 15 minutes at 4°C. An antibody cocktail was prepared which was tested for the right dilution and added to the cells. The antibody cocktail added was as follows (CD14-PE-

CY7, CD11b-PE, CD36-FITC and CD54-APC or isotype controls) and incubated for another 30 minutes at 4°C. Cells were then washed by adding 500µL flow cytometry buffer and run on FACS Calibur after adding 300µL of buffer in a new tube.

Sample acquisition and analysis

The flow cytometry machine was turned on for 30 minutes prior to sample acquisition in order to warm-up the lasers. The software Cell Quest Pro (BD) was used to acquire samples and to analyse the results. Briefly, unstained cells were run and the voltage was set for the forward scatter (obtained based on the size of cells) and side scatter (based on granularity of cells). A histogram plot was prepared and the voltage for each of the channels was set so that the cells displayed in the histogram had the unstained peak on the first quarter. Single colour stained cells were then run and appropriate compensation was performed. Once the instrument settings were finalised, samples were run and a minimum of 5×10^5 of total cells were acquired. Analysis was performed by creating a gate for CD14⁺ cells and the expression of different markers on CD14⁺ cells was calculated and tabled for analysis. The mean fluorescence intensity was used to determine the differences between the diabetic and control patients among the differentiation markers (Figure 6.1).

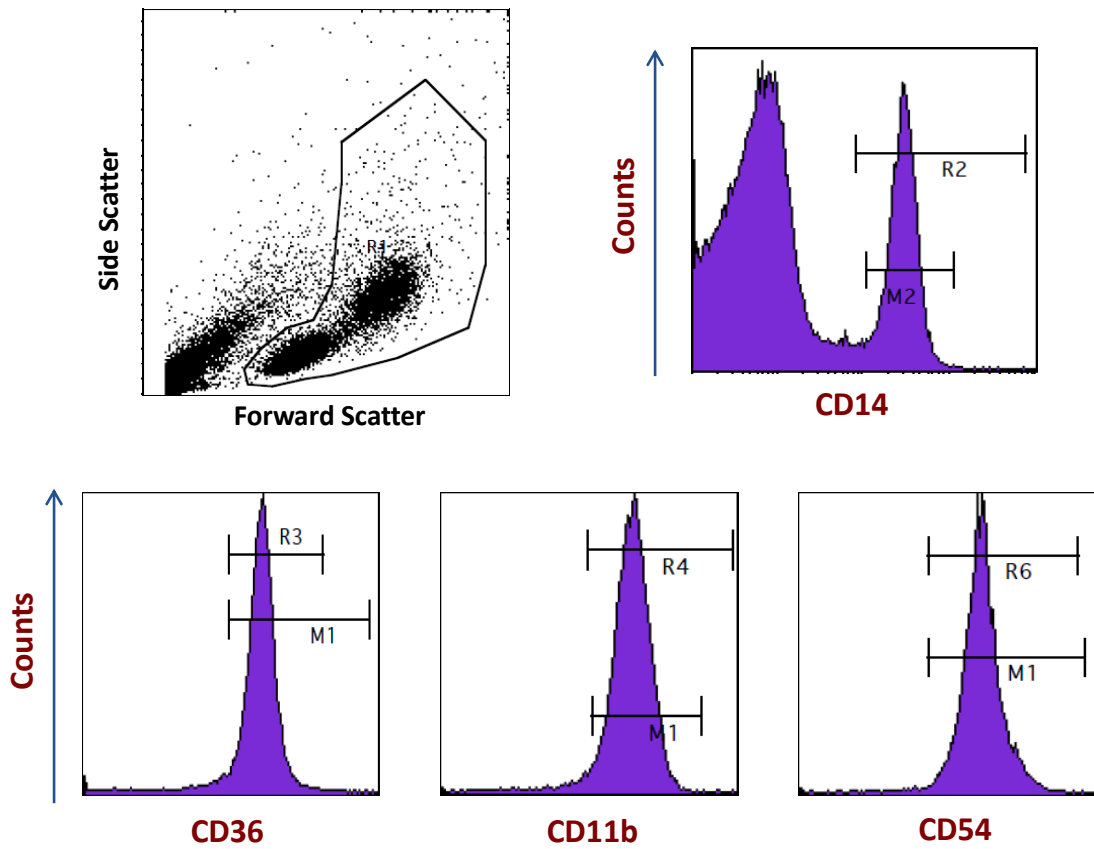


Figure 6.1 Flow Cytometry Analysis

R1 is the region gated for cells that are positive for the marker CD14 in a scatter plot. Top right shows a histogram of CD14⁺ cells. Cells positive for CD36, CD11b and CD54 are shown in histogram plots (lower three plots). Regions were drawn and the corresponding mean fluorescence intensity was tabled for analysis.

Markers of Differentiation in Peripheral Monocytes

Markers of differentiation on peripheral monocytes increased in diabetic patients

The percentage of circulating CD14⁺ in diabetic and control patients was measured using flow cytometry. A total of 51 subjects were studied, of which 29 were diabetic and 20 were controls (could not collect data for two samples due to experimental errors). Viable leukocytes were gated and the percentage of CD14⁺ cells was plotted. No significant differences were observed between the two groups (Figure 6.2).

Also, total number cells positive if differentiation markers were arrived at by multiplying the percentage of cells positive each marker and total CD14 cells. CD14⁺ cells positive for the markers CD54 ($p < 0.05$), CD36 ($p < 0.05$) and CD11b ($p < 0.05$) were all found to be significantly higher in diabetic patients compared against controls (Figure 6.3).

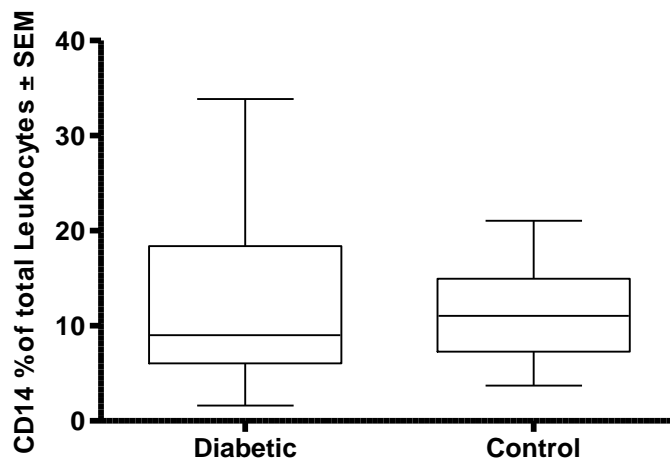


Figure 6.2 Flow Cytometry Studies

Percentage of CD14⁺ cells diabetic (n=29) vs. control (n=20) ($p > 0.05$, unpaired t test).

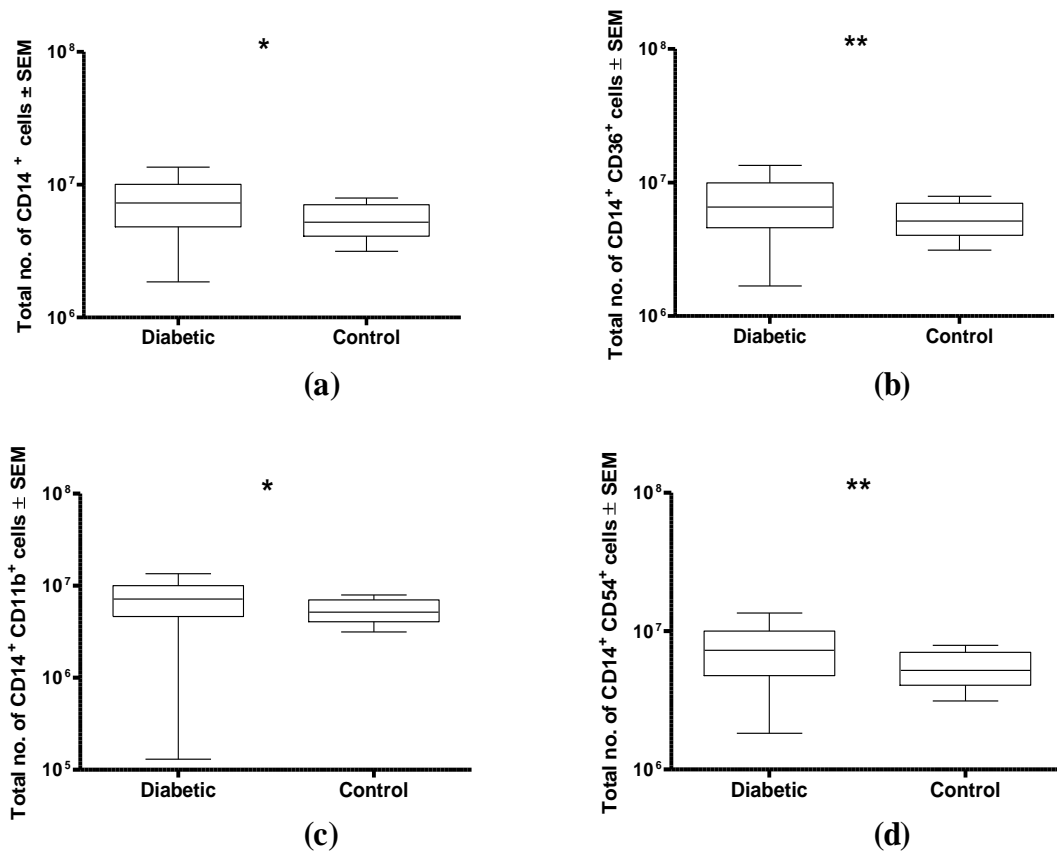


Figure 6.3 Peripheral Monocyte Markers of Differentiation

- (a) Percentage total CD14⁺ cells: $p < 0.05$, diabetic vs. control.
- (b) Total number CD14⁺CD36⁺cells: $p < 0.01$, diabetic vs. control.
- (c) Total number CD14⁺CD11⁺cells: $p < 0.05$, diabetic vs. control
- (d) Total number CD14⁺CD54⁺cells: $p < 0.01$, diabetic vs. control.

Data shown as median in diabetic (n=29) and control (n=20) with whiskers from minimum to maximum (Unpaired t test). * = $p < 0.05$, ** = $p < 0.01$.

Gene expression of GPR132 increased in circulating CD14⁺ monocytes of diabetic patients

Real time RT-PCR was performed to determine the differences between expression of GPR132, GPR120, resistin and FABP4 between control and diabetic subjects (Figure 6.4). GPR132 expression was significantly higher in diabetic CD14⁺ cells compared with controls (Figure 6.5). No change in mRNA expression of resistin was found between the groups (Figure 6.6).

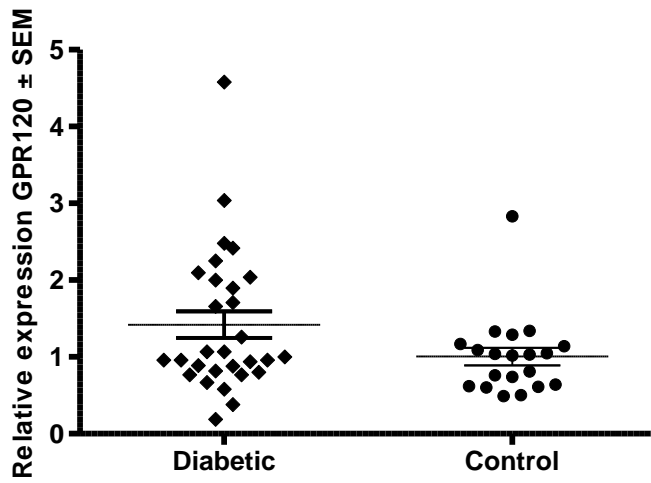


Figure 6.4 GPR120 Gene Expression

GPR120 expression diabetic (n=29) vs. control (n=20). $p > 0.05$.

Data shown are mean \pm SEM (Mann-Whitney test)

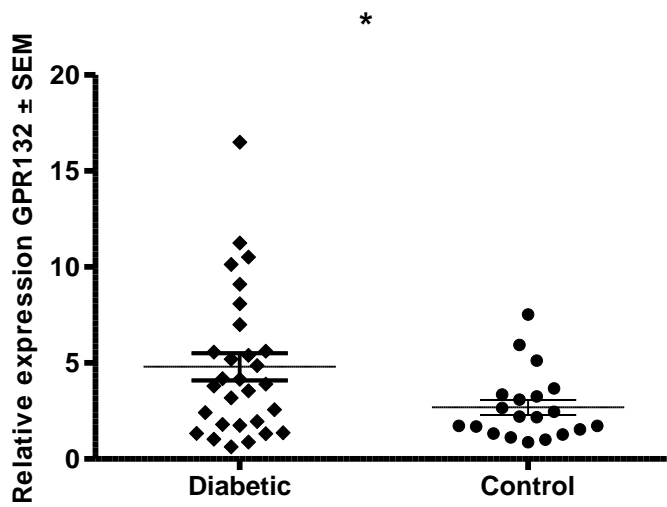


Figure 6.5 GPR132 Gene Expression

GPR132 Gene expression in CD14⁺ cells diabetic (n=29) vs. control (n=20), $p < 0.05$.

Data shown are mean \pm SEM (Mann-Whitney test). * = $p < 0.05$.

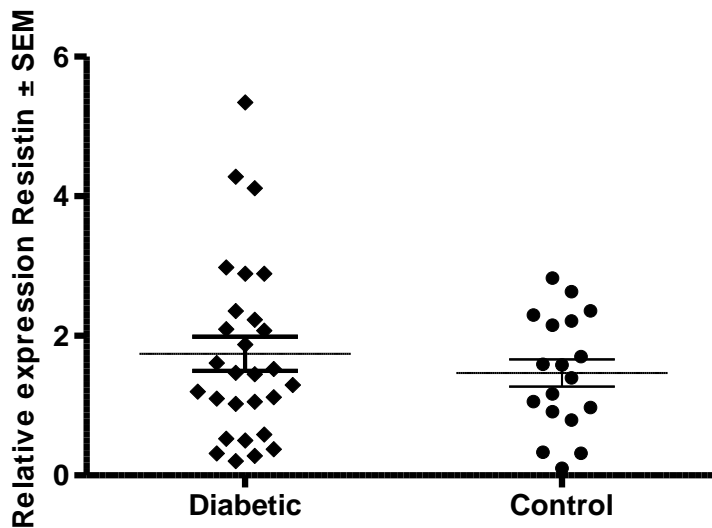


Figure 6.6 Resistin Gene Expression

Resistin Gene expression in CD14⁺ cells diabetic (n=29) vs. control (n=20). $p > 0.05$

Data shown are mean \pm SEM (Mann-Whitney test)

Obesity related cytokines are up-regulated in diabetic patients

Serum from both cohorts of patients was collected. Blood was collected in a serum separation tube that contained serum separator gel. The blood was mixed well and the serum allowed to separate for 30 minutes at room temperature. The tubes were then spun for 10 minutes at 3750g. Serum was then carefully removed and stored at -80°C for protein estimation studies. A 9-plex Human Obesity kit from Bendormed systems was used to detect the presence of different risk markers for obesity and cardiovascular risk. In total, nine cytokines were tested in duplicate for the following markers - sCD40L, Tumour Necrosis Factor Receptor (sTNF-R), osteoprotegerin (OPG), MCP-1, myeloperoxidase (MPO), sICAM, resistin, IL-6 and leptin. IL-6 expression was below the detectable levels. No differences in the expression of markers SCD40L was observed between diabetic and control subjects. Peripheral secretion of TNF-R was higher in diabetic patients ($p < 0.05$) and expression of OPG and MCP-1 was higher in diabetic subjects. (both $p < 0.01$, Figure 6.7). No changes in the expression of MPO and ICAM were observed between the two groups. Resistin and leptin, on the other hand, were markedly increased in diabetic subjects (Figure 6.8).

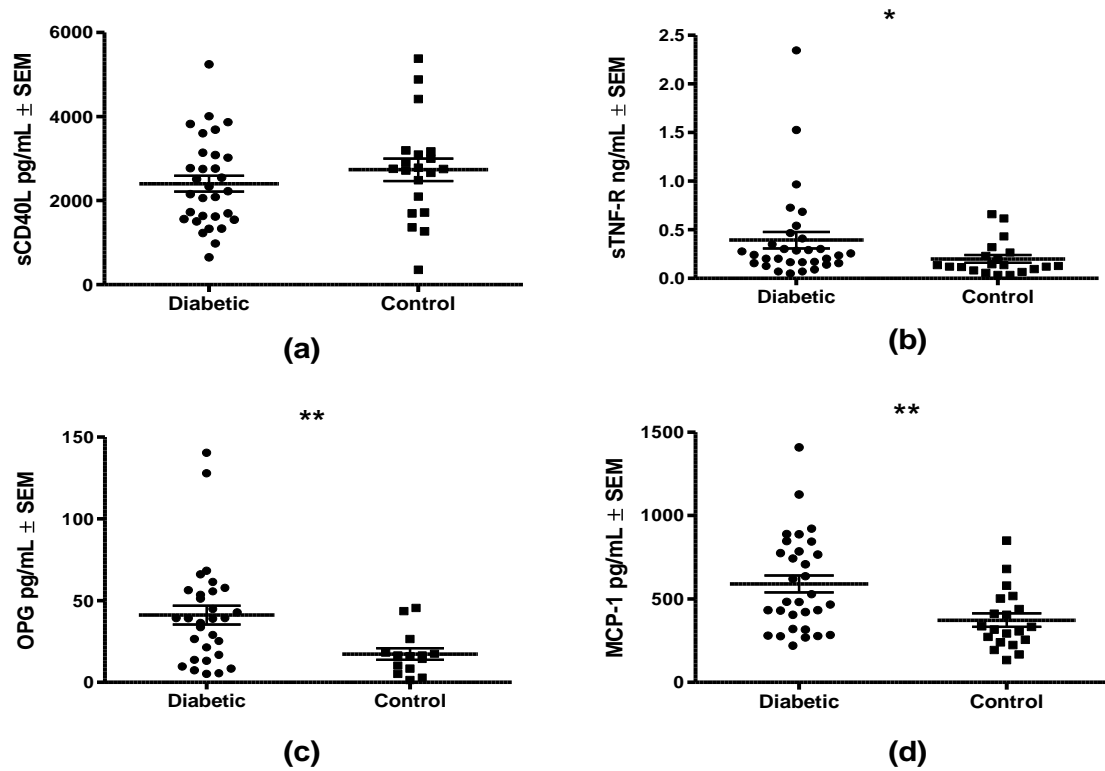


Figure 6.7 Plasma Cytokine Levels

Serum levels of cytokines in diabetic (n=31) and control subjects (n=20)

(a) sCD40L: $p > 0.05$, diabetic vs. control

(b) sTNF-R: $p < 0.05$, diabetic vs. control

(c) OPG: $p < 0.01$, diabetic vs. control

(d) MCP-1: $p < 0.01$, diabetic vs. control

Data shown are mean \pm SEM (Mann-Whitney test). * = $p < 0.05$, ** = $p < 0.01$.

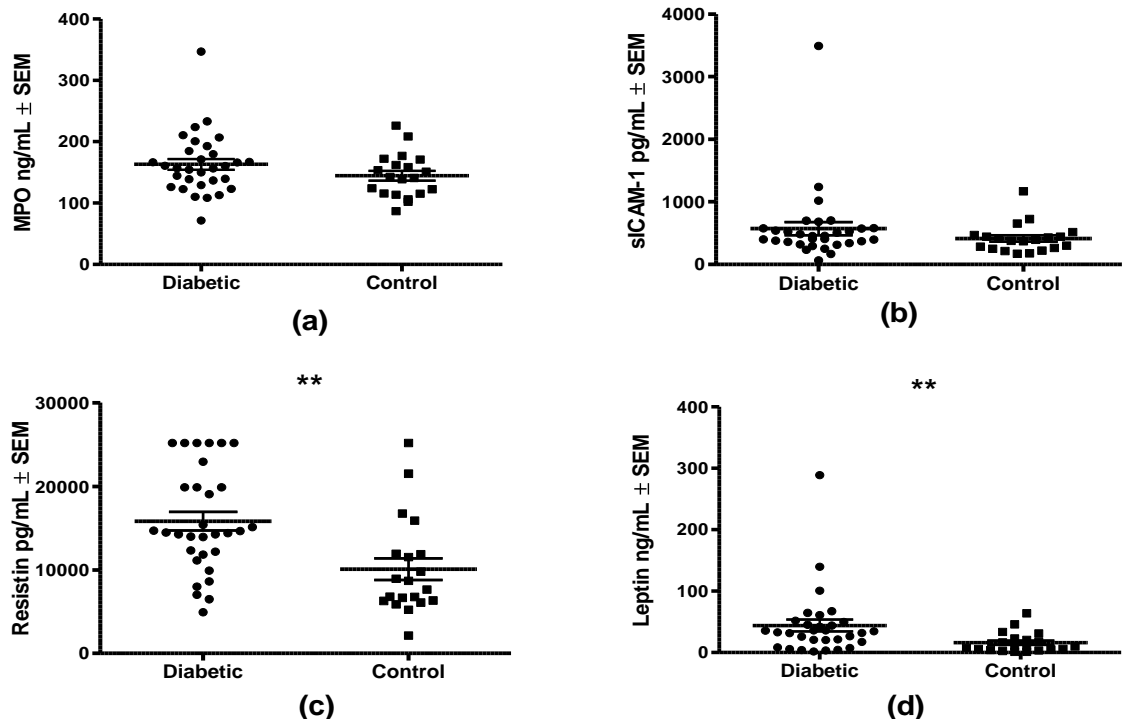


Figure 6.8 Serum Cytokine Levels

Serum cytokine levels in diabetic (n=31) and control (n=20) subjects

- (a) MPO: $p > 0.05$, diabetic vs. control
- (b) sICAM: $p > 0.05$, diabetic vs. control
- (c) Resistin: $p < 0.01$, diabetic vs. control
- (d) Leptin: $p < 0.01$, diabetic vs. control

Data shown are mean \pm SEM (Mann-Whitney test). ** = $p < 0.01$.

Discussion

Patients with diabetes are prone to developing cardiovascular problems.³⁶² They also display increased activation of inflammatory markers that correlate with cardiovascular risk.³⁶³ Monocytes have been shown to be activated in diabetic subjects.³⁴⁹ Though a range of markers are known to be expressed highly in diabetic subjects with high cardiovascular risk, no single marker has proved to be of use in the clinical situation to date. GPR120 and GPR132 have not previously been studied in circulating monocytes of diabetic subjects; the differences in the expression of these receptors could possibly be used in determining the risk of diabetic subjects for developing cardiovascular problems. In this study it was hypothesised that the markers of activation CD54, CD36, CD11b and gene expression of GPRs in CD14⁺ cells would be up-regulated in diabetic subjects displaying increased levels of obesity risk markers.

Flow cytometry studies were employed in this study in order to determine the percentage of CD14⁺ cells expressing different activation markers. All the leukocytes were gated in a dot plot and cells positive for CD14 was selected and the percentage of cells expressing activation markers was analysed. Though no significant differences were found in percentage of CD14⁺ cells among the groups, the total number of CD14⁺ cells and total number of cells expressing CD14⁺CD36⁺ receptors was higher in diabetic subjects. Activation of CD14⁺ monocytes along with an increase in surface receptor CD36^{349, 353} was shown in diabetic subjects. This receptor is involved in uptake of lipids and is known to increase foam cell formation in macrophages.³⁶⁴ Higher levels of CD11b expression in circulating monocytes have been observed in patients with diabetes as a measure for state of activation or priming.^{365, 366} This was also observed in the present study, with the total number of cells positive for CD14⁺CD11b⁺ being higher in diabetic subjects compared with controls. In all, markers associated with monocyte activation were markedly increased in peripheral monocytes of diabetic patients, almost certainly indicative of the general state of inflammation in these subjects.

CD14⁺ cells were separated using magnetic beads by positive selection, and RNA was extracted for gene expression studies. Studies have not previously been performed to investigate the possibility of increased expression of GPR132 in diabetic subjects. Microarray studies have reported an increase in expression of GPR132 in adult

peripheral blood dendritic cells compared with cord blood mature dendritic cells.³⁶⁷ In this study, a marked increase in the mRNA expression of GPR132 was observed in CD14⁺ cells of diabetic subjects compared with controls. It could well be that GPR132 may be a marker for cardiovascular risk as these cells also express other activation markers (as observed in flow cytometry studies). GPR132 expression in macrophages has been implicated in their phagocytic functions which help in clearance of apoptotic debris.³¹⁰ Studies by Bolick et al.^{242, 243} have shown that GPR132 deficient mice exhibit increased atherosclerosis due to lack of GPR132 in endothelium leading to increased monocyte adhesion and pro-inflammatory characteristics exhibited by GPR132 deficient macrophages. It is still unclear what the exact role of GPR132 is in macrophages, and the part it may play in disease progression of atherosclerosis also requires further investigation. GPR132 may be protective in monocytes and its increased expression as a marker of macrophage differentiation may be indicative of activation of protective processes in early atherosclerosis. It is during the later stages of atherosclerosis that increased production of 9-HODE is observed, and at this stage the fatty acid may activate pro-inflammatory processes.⁸⁹ No changes in GPR120 mRNA levels between the groups were observed. This was expected as in the cell line experiments the GPR120 expression was down-regulated with differentiation. The primary cells examined were CD14 positive monocytes that were not stimulated with any fatty acid or PMA. Flow cytometry studies were not performed to determine the expression levels of GPR120 and GPR132 due to lack of availability of antibodies specifically for flow cytometry studies. In future studies, it would be appropriate to also study the changes in the cells when they are treated with HODEs by short-term culture of primary monocytes.

Adipose tissue secretes a number of adipokines including resistin³⁶⁸ (involved in insulin resistance) and leptin contributing to metabolic homeostasis.³⁶⁹ THP-1 cells do not express resistin³⁷⁰ and hence it was not possible to study the changes in expression of this protein during the experiments outlined in previous Chapters. Resistin is thought to be down regulated by the PPAR- γ agonist rosiglitazone.^{371, 372} The fatty acids AA and EPA have also been shown to decrease the expression of resistin in mouse models.³⁷³ In the primary CD14 monocytic cells mRNA studies, we found no changes in their resistin expression. Increased mRNA expression of resistin in diabetic women has been reported contributing to low-grade inflammation and potential atherosclerotic risk.^{350,}

³⁷⁴⁻³⁷⁶ Resistin expression in the serum was expectedly much higher in diabetic subjects in this study. Leptin levels were also significantly higher in diabetic patients. Higher leptin levels in both diabetic subjects, and a correlation with increased body fat has also been reported in non-diabetic subjects. ^{377, 378} In addition, insulin resistant diabetic patients have been reported to have increased serum leptin levels and this study suggested a functional role of insulin in regulating leptin and resistin levels. ³⁷⁹

Serum stored was tested for obesity markers and eight out of nine markers were detected. No changes in the serum levels of sCD40L, MPO were demonstrated between the groups. ICAM-1 is thought to be at least partly regulated by IL-6, ³⁸⁰ with no detectable levels of IL-6 found in the serum in this study, this could explain why no changes in the expression of ICAM was found between the groups (even though the number of CD14 cells expressing CD54 was higher in diabetic subjects). Also, ICAM-1 is mainly secreted by the endothelium, being essential for monocyte adhesion. ³⁸¹ The use of ELISA might well be a more sensitive means of detecting IL-6, though previous studies have also not found any detectible differences in circulating IL-6 using immunometric assays comparing diabetic and control groups. ³⁸²

Two isoforms of the TNF-R receptor are recognised and named according to the protein size - TNFR-I (p60) and TNFR-II9 (p80). ³⁸³ While its exact role is unclear, high levels of serum TNF-R (p60) in the circulation (mainly secreted by adipocytes) has been linked to obesity. ³⁸⁴ Increased secretion of this receptor freely circulating could also neutralise TNF- α thereby controlling the adverse effects caused by excessive TNF- α . ³⁸⁵ Increased levels of TNFR-I was also found to be higher in diabetic subjects in this study. One previous study has suggested that increased levels of this receptor may have an anti-adipogenic effect on adipocytes by decreasing cell proliferation. ³⁸⁶

Elevated levels of OPG have been reported in type 2 diabetic patients, ³⁸⁷ and may be indicative of damage to arterial vasculature by calcification, and therefore a strong predictor of cardiovascular risk in diabetic patients. ^{388, 389} Again, high levels of OPG in the circulation was demonstrated in this study in the diabetic cohort, and indicates their predisposition to cardiovascular disease. MCP-1 expression is increased in macrophage rich areas during atherosclerosis, ^{390, 391} and was also elevated in the circulation of

diabetic subjects.³⁹² We also demonstrated high levels of this chemokine in our diabetic cohort, confirming once more that they are at high cardiovascular risk.

In summary, increased expression of activation markers in diabetic subjects is suggestive of their state of inflammation. The increased levels of GPR132 expression in monocytic cells from diabetic patients could be a novel marker for cardiovascular risk in these patients. Also, the general increased levels of secreted cytokines detected indicate that the diabetic patients are prone to atherosclerosis. It would be interesting to study the effects of HODEs using short-term culture of CD14⁺ cells.

Chapter 7

General Discussion and Future Directions

General Discussion

The overall aim of this work was to examine the role of components of oxidised lipids, HODEs, and the nature of their interaction with monocytes and macrophages. Two characteristic features of atherosclerosis are oxidative stress and accumulation of lipids in the arterial wall. Oxidised lipids are known to regulate the processes of atheroma formation. The role of HODEs, which are abundantly present in atherosclerotic plaque, as well as in the arterial wall is unclear.^{16, 27, 393} This study examined the role of these components of oxidised lipids and how they interact with monocytes, one of the primary cells that are attracted to the inflamed arterial wall, and which differentiate into macrophages.¹⁸ Understanding the interactions between HODEs and monocyte/macrophage cells in the pathogenesis of atherosclerosis could enable us to modulate key signalling molecules, thereby controlling the processes involved in atheroma formation. The salient features of this work are summarised in the following sections with some of the limitations encountered and scope for further studies.

HODEs Induce Specific Effects on Monocytes and Macrophages

In Chapter 3, the effects of HODEs on monocytes and macrophages were investigated, including their effects on cell number, apoptosis, and genes associated with lipid metabolism. A previous study has examined the role of 9-HODE and 13-HODE on U937 monocytic cells but not macrophages. This study reported an increase in apoptosis by 9-HODE but not by 13-HODE.⁴ Another study examined the role of 13-HODE in monocytic apoptosis but did not study 9-HODE.⁵ In the present, study both 9-HODE and 13-HODE decreased cell numbers in monocytes and macrophages. The cell death was due to apoptosis measured using a caspase-based assay. Clinically, during the initial stages of atherosclerosis there is an increased level of lipoxygenase activity resulting in formation of 13-HODE predominantly. In this study 9-HODE was more potent at inducing apoptosis than 13-HODE in monocytes. During the later stages of atherosclerosis, equal amounts of 9-HODE and 13-HODE are derived non-enzymatically.^{91, 95} Both HODEs induced equal levels of apoptosis in macrophages at higher doses.

Here for the first time, expression of receptors for long-chain fatty acid receptor GPR120 and GPR132 was shown to be present on THP-1 monocytes and macrophages using immunocytochemistry. Gene expression studies also revealed an increase in expression of GPR132 in monocytes with HODEs treatment, while in macrophages GPR120 was down-regulated. Natural ligands for GPR120 and GPR132 are not yet known. The latter has been reported to be receptor for 9-HODE.³⁰⁹ While GPR120 has been shown to be a receptor for n-3 fatty acids in mouse models,²³⁷ down-regulation of GPR120 in macrophages could possibly have a protective role. Increased GPR132 expression was also accompanied by a significant increase in transcription factor PPAR- γ and intracellular lipid transporter FABP4 which is involved in foam cell formation.^{141, 142} FABP4 is thought to be regulated by the transcription factor PPAR- γ in monocytes.³¹¹ Both HODEs decreased macrophage release of pro-inflammatory cytokines IL-8, TNF- α , IL-1 β . Overall these experiments suggested that HODEs have effect on macrophages that are protective, and supported the view that these oxidised lipids promote the cells to differentiate into a protective anti-inflammatory M2 phenotype. These data also suggested that some of the effects could be mediated through GPR132, with the transcription factor PPAR- γ also having a major role in controlling these processes (Figure 7.1).

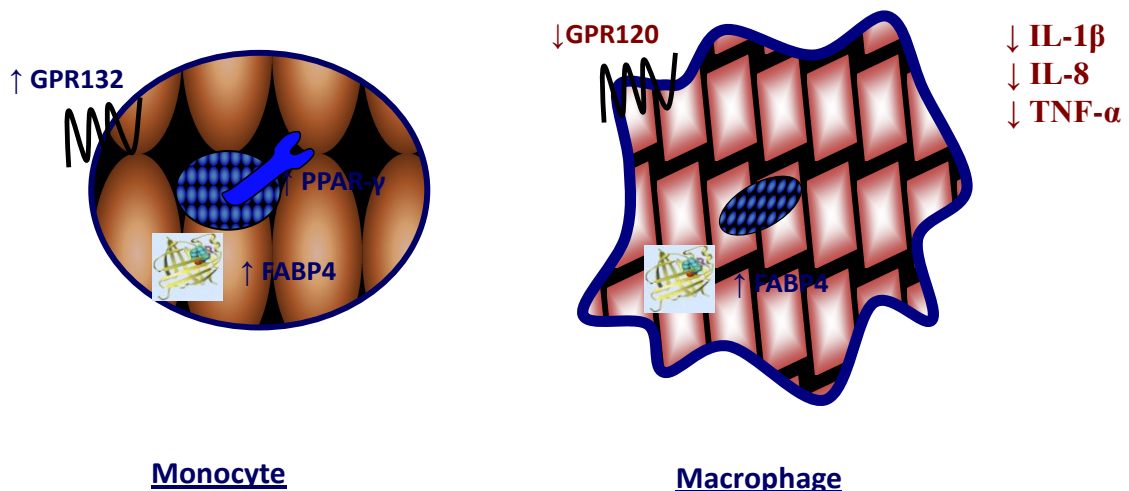


Figure 7.1 Proposed Actions of HODEs on Monocytes and Macrophages

Increased levels of GPR312 observed in monocytes while the expression of GPR120 was decreased in macrophages.

HODEs Promote Differentiation of Monocytes into M2 Macrophage Phenotype

While the previous sets of experiments were carried out to determine the effects of HODEs on monocytes and macrophages individually, further studies investigated the role of HODEs while these cells differentiated. This mimicked what might happen when monocytes are attracted to a site of vascular inflammation during atherosclerosis. Such cells are exposed to various chemoattractants and oxidised lipids. A small concentration of 1nM PMA was added along with HODEs and the effects on cell proliferation, lipid metabolism gene expression, and cytokine release was studied in work presented in Chapter 4.

Monocytes are attracted to sites of inflammation by MCP-1 and, in the arterial wall and in the circulation, these cells are exposed to oxidised lipids.¹⁸ The nature of the latter interaction may determine what type of macrophage they differentiate into. Macrophages are believed to be heterogeneous, but essentially of two phenotypes - M1 and M2. Based on the exposure to TH1 or TH2 cytokines and possibly other ligands, these cells develop into M1 or M2 macrophages respectively. Their functions have also been reported to pro-inflammatory for M1 macrophages and anti-inflammatory for M2 macrophages.²⁷⁶

On exposure to HODEs, there is increased foam cell formation from macrophages, a process that is regulated by PPAR- γ .¹⁴¹ Our study has shown, for the first time, a direct increase in foam cell formation when macrophages are exposed to HODEs (using Oil Red O staining). Markers of differentiation including ITGAM and the principal lipid accumulation receptor SCRA¹⁹³ were up regulated with PMA, but there was no appreciable increase when cells were further stimulated with HODEs. This suggested that these cells had reached their maximum expression levels of the above markers with a low concentration of PMA. One of the other changes observed was increase in expression of SCRB and ABCA1, both involved in reverse cholesterol transport and thus reducing the lipid burden in macrophages.^{67,222}

Lipid accumulation in macrophages is characterised by increased expression of the lipid transporter FABP4 which is thought to be regulated by PPAR- γ .^{141, 142} HODEs could not stimulate FABP4 alone but with a small dose of 1nM PMA, HODEs increased its

expression, possibly driven by PPAR- γ , an increase in the expression of which was also observed in this study. A decrease in activity of SCD is thought to be protective in macrophages.^{323, 331} In the present study, PMA treatment decreased SCD expression, but there was an increase with 9-HODE and 13-HODE treatment. The expression of SCD was further increased when PMA and HODEs were added together. Further study is required to elucidate the effects of HODEs on SCD expression and activity, as well as their relationship with insulin resistance and atherosclerosis.

Increase in the long-chain fatty acid receptor GPR132 was observed when cells were stimulated with a small dose of PMA and HODEs. This study shows that 13-HODE was more potent than 9-HODE. Similar to studies in macrophages, with monocyte differentiation the expression of GPR120 was decreased with HODEs and PMA treatment. GPR120 has been implicated in regulating insulin sensitivity, and may primarily be a n-3 fatty acid receptor.²³⁷ Our study highlights the possible role of HODE in regulating expression of GPR120, the decrease of which is thought to be involved in controlling inflammation. The apoptotic assay suggested that 9-HODE being more potent than 13-HODE in inducing apoptosis when added with PMA. Apoptosis in macrophages is at least partly PPAR- γ regulated, and the process may be protective in early atherosclerosis where 13-HODE is the predominant isoform.³³⁷ The above observations, along with the observed decrease in expression of IL-8 by 13-HODE suggested that HODEs might overall have a protective effect through promoting differentiation of monocytes into macrophages with M2 phenotype (Figure 7.2).

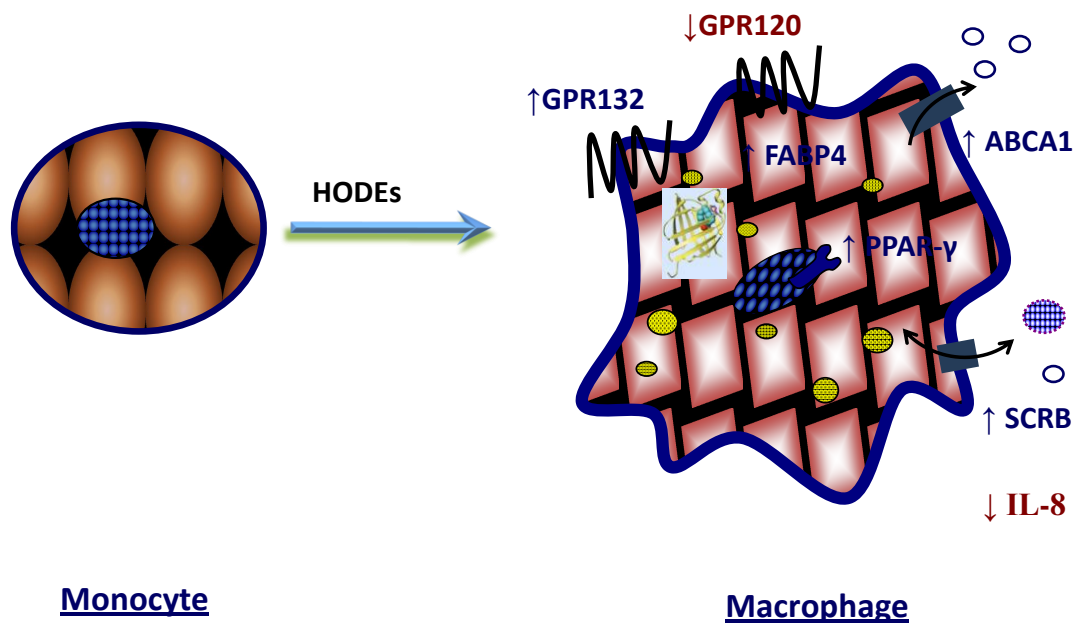


Figure 7.2 Proposed Actions of HODEs on Monocyte Differentiation

HODEs increased levels of GPR132, and decreased GPR120, while increasing expression of molecules associated with reverse cholesterol transport.

Partial Role of PPAR- γ and GPR132 Independent Mechanisms Involved in Regulating Expression of FABP4

Striking changes in expression of GPR132 with HODEs, and literature suggesting a pivotal role for transcription factor PPAR- γ in macrophage differentiation^{276, 337, 394, 395} prompted us to investigate the role of HODEs further. Hence in Chapter 5, the role of PPAR- γ was tested using a specific antagonist T0070907,³³⁹ and gene silencing of GPR132 was employed to determine the specific role of this long-chain fatty acid receptor. The antagonist specific for PPAR- γ decreased expression of FABP4 significantly. When treated with either rosiglitazone or HODEs after antagonist blocking, FABP4 was decreased when compared with the corresponding fatty acid alone, but higher when compared with cells treated with antagonist alone. This suggested a partial role of PPAR- γ in regulating FABP4, since despite it being blocked there was still high expression of FABP4. Cytokine secretion was largely down-regulated when the antagonist was added. Pro-inflammatory cytokines known to be

secreted by macrophages include IL-8, TNF- α , IL-1 β ,²³ and the antagonist blocked their expression, and addition of HODEs or PPAR- γ agonist rosiglitazone³⁴² further reduced secretion of these cytokines. Cytokine secretion in macrophages is known to be controlled by different pathways and regulators including NF- κ B,^{285-287, 396} Jak-Stat pathway,³⁹⁷ activator protein-1,²⁰¹ and TLRs,^{205, 206, 346} thus making it difficult to arrive a definite conclusion on the exact mechanisms of regulating macrophage secretion of cytokines.

The role of GPR132 was tested by successfully transfecting gene silencing oligonucleotides for the first time in this study. Monocytic cells have been documented to be one of the most difficult cell types to transfect.³⁴⁷ Identified as the receptor for 9-HODE,³⁰⁹ GPR132 was postulated to be involved in control of inflammation in the vasculature,^{241-245, 343} and was therefore considered to be an important candidate for HODE signalling in this study. When GPR132 was silenced in macrophages, and its role in regulating expression of FABP4 was investigated, no significant change in expression of FABP4 was observed. This suggests that other, GPR132 independent mechanisms, control the processes of lipid metabolism. Cytokine studies were also indicative of GPR132 independent mechanisms involved in controlling their secretion. While the effects observed with increase in FABP4 and the expression of PPAR- γ was observed with both 9-HODE and 13-HODE and with literature suggesting that 9-HODE is the receptor for GPR132,³⁰⁹ it is likely that other mechanisms are involved in mediating the effects of HODEs on lipid metabolism in THP-1 macrophages (Figure 7.3).

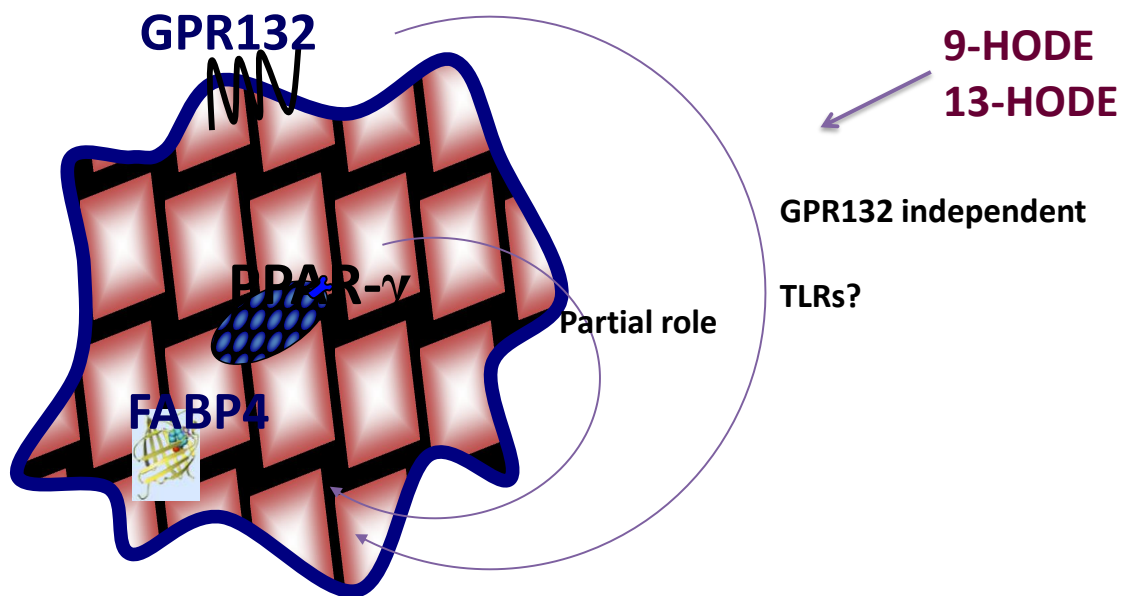


Figure 7.3 Role of PPAR- γ and GPR132 in Macrophage FABP4 Expression

Antagonist and siGPR132 studies are suggestive of a partial role of PPAR- γ , and GRP132 independent mechanisms are involved in regulating FABP4 expression.

Peripheral Monocytes Exhibit Increased Levels of GPR132 in Diabetic Patients

Chapters 3-5 examined the role of HODEs in various processes involved in lipid metabolism and their mechanism on the THP-1 cell line. In Chapter 6 the study was extended to examine monocytes from human diabetic subjects. This might help us understand how relevant the cell line studies are to patients who have high cardiovascular risk. Type 2 diabetic subjects are particularly prone to developing cardiovascular problems.^{362, 363} Monocyte cells in diabetic patients have been shown to be activated,³⁴⁹ with increased levels of pro-inflammatory markers that are associated with increased cardiovascular risk in their circulation.^{350, 374, 377, 387, 391} Blood samples were collected from 52 subjects (including controls) and their serum was also studied for levels of cytokines.

Flow cytometry studies were used to determine the role of activation and inflammation markers in CD11b,³⁶⁵ CD36³⁵⁹ and CD54³⁶⁰ in CD14¹²⁵ monocyte cells. All these

markers of activation were increased in the diabetic cohort suggesting an increased state of inflammation in peripheral monocytic cells. We propose that GPR132 is an indicator for atherosclerotic risk, and is possibly expressed during oxidative stress conditions.³¹⁹ CD14⁺ cells were magnetically separated and real time RT-PCR was performed to determine the expression of GPR120 and GPR132. Expression of GPR132 was much higher in the diabetic cohort compared with the controls. When examined for the inflammatory markers in serum, higher levels of resistin and leptin were detected. These are involved in increasing insulin resistance and obesity respectively.³⁷⁹ Also, increased were levels of markers associated with chemotaxis (MCP-1) and vascular calcification (OPG).

Overall, this study highlights the possible role of GPR132 as a marker for cardiovascular risk and also as a receptor that could be useful on controlling some of the processes involved in atherogenesis. Increased levels of markers of inflammation in the circulation strongly support the observation that diabetic subjects are predisposed to developing cardiovascular problems (Figure 7.4).

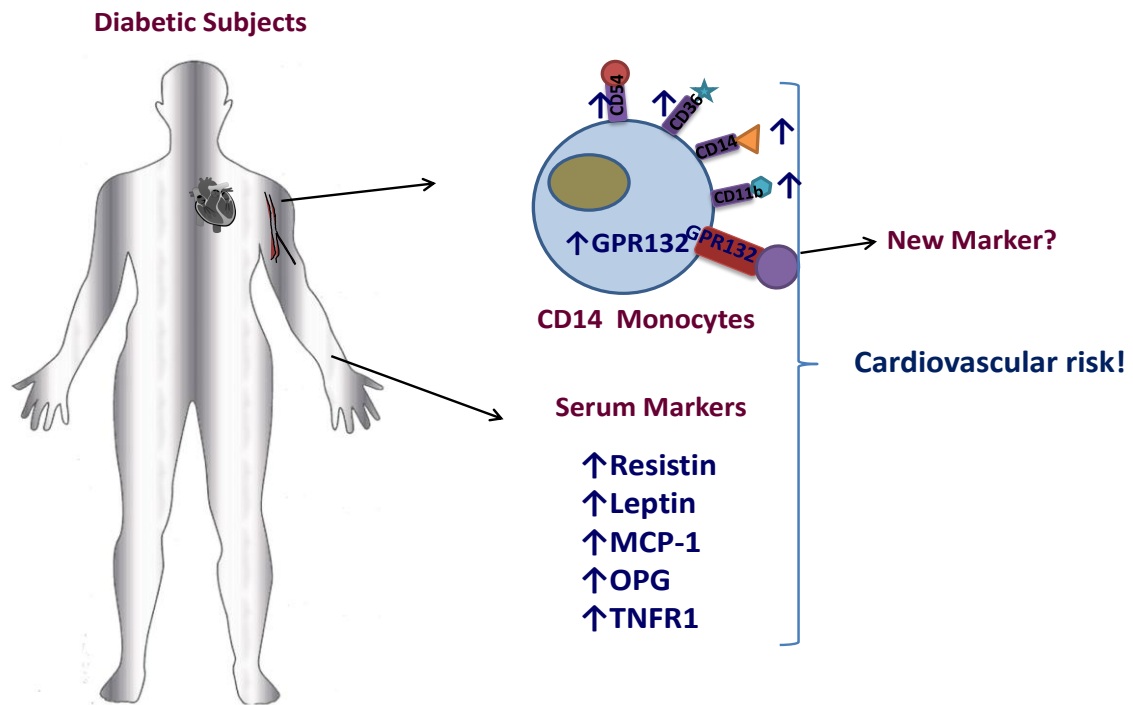


Figure 7.4 Peripheral Monocytes and Serum Markers in Diabetic Subjects

Increased activation markers in monocytes and higher levels of inflammatory markers in serum are suggestive of increased cardiovascular risk. GPR1232 could a potential marker for diagnosing cardiovascular risk.

Limitations and Future Directions

HODEs constitute a class of lipid derivatives that are highly expressed in atherosclerotic plaque. An understanding of their effects on monocytes and other cell types involved in atherosclerosis, obesity and diabetes is essential. This study examined the role of HODEs in macrophage biology using the widely used THP-1 cell line model.¹⁹⁶ There were some limitations in using this line with these cells not expressing resistin,³⁷⁰ and with no flow cytometry antibodies available to date to investigate the cells surface receptors GPR120 and GPR132. Also, transfection of these cells is notoriously difficult.³⁴⁷ GPR132 was one of the major molecules whose expression was increased with HODE treatment and it would be interesting to further study the effects of this receptor which is known to be involved in apoptosis.²⁴³ Studies examining the role of HODEs and their influence on caspase activity could be performed after antagonist blocking of PPAR- γ and siGPR132 in macrophages. Their influence on expression of FABP4 is

significant though no direct effect of GPR132 was demonstrated in this study; it would be useful to determine the levels of FABP4 in circulation of a larger cohort of diabetic patients and to determine whether these correlated with levels of HODEs *in vivo*.

Conjugated linoleic acids (CLAs) are structurally very similar to HODEs, and have been reported to be anti-atherogenic,³⁰⁴ anti-carcinogenic,³⁹⁸ and to have a protective role against anti-obesity.³⁹⁹ CLAs are not substrates for LO.⁴⁰⁰ It would be relevant to determine whether some of the effects of HODEs are replicated by CLAs since the latter have been shown to have a variety of effects relevant to human health. The potential role of a new class of n-7 fatty acids (including 13-HODE which is found in barely, malt and adlay seeds^{99, 100}) needs further examination and may give rise to important nutritional intervention studies. Microarray studies³⁶⁷ could shed light on the different genes that are affected by HODEs in monocytes and macrophages. It would also be appropriate to study the effects of HODEs on short term culture of primary monocyte cells from human subjects and thus to examine their effect on GPR132 and other genes associated with lipid metabolism, insulin resistance and obesity.

Summary

The interaction between oxidised lipids and macrophages constitutes a key aspect of atherogenesis. Understanding the fundamental processes regulated by HODEs and how they influence macrophages (which are involved in all stages of this arterial disease) was one of the primary aims of this study. Studies were directed at understanding the role of HODEs in their interaction with monocytes and macrophages, and during the differentiation and activation of these cells. With recent studies showing that lipids have hormonal functions ('lipokines'), this study explored the possibility that the n-6 fatty acid 9-HODE and n-7 fatty acid 13-HODE may have signalling functions in key aspects of oxLDL-macrophage biology. The important contribution of this study to the body of knowledge includes data showing that both HODEs have immense potential in influencing monocytes to differentiate into a protective M2 macrophage phenotype. The mechanisms involved in these processes were also explored using gene silencing and specific agonists/antagonists to delineate the role of 9-HODE and its receptor GRP132

in macrophages. This area of research is complex, and the study demonstrated a partial role for PPAR- γ in HODEs signalling and that GPR132 independent mechanisms were involved in controlling inflammation. These findings were also explored further in primary monocytes from diabetic subjects who are prone to cardiovascular disease. CD14 monocyte cells had increased levels of activation markers. Increased levels of GPR132 expression in these cells suggests that it has potential as a marker for increased cardiovascular risk. Measurement of serum markers of cardiovascular risk and obesity revealed increased levels of these inflammatory markers in the diabetic cohort again, confirming their predisposition to developing cardiovascular disorders. Further studies in understanding the role of GPR132 in HODEs signalling and apoptosis are required. Nutritional studies, including further investigation of the effects of n-7 fatty acids are required. It would also be pertinent to investigate whether the demonstrated effects of HODEs in this study are relevant to the known biological properties of conjugated linoleic acids which have been shown in numerous animal and human studies to have potentially beneficial effects in relation to risk of insulin resistance, obesity, and atherosclerosis.

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Administrative documentation
has been removed

APPENDIX 2- LIST OF ABBREVIATIONS

AA	Arachidonic Acid
ABCA1	ATP-binding cassette 1
ACS	Acyl-CoA Synthetase
AGEs	Advanced Glycosylation End Products
ALA	α -Linolenic Acid
ALBP/aP2	Adipocyte Lipid Binding Protein
ANOVA	One-way analysis of variance
AP-1	Activator Protein-1
APC	Allophycocyanin
CD36	Fatty acid translocase
CM	Conditioned Medium
COX-2	Cyclooxygenase-2
CSF	Colony Stimulating Factor
DAB	3,3'-Diaminobenzidine
DAPI	4', 6-diamidino-2-phenylindole
DGLA	Dihomo-gamma-linolenic acid
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
EC	Endothelial Cell
EDTA	Ethylene Diamine Tetra-Acetic Acid
EFA	Essential Fatty Acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPA	Eicosapentaenoic acid
ERK $\frac{1}{2}$	Extracellular signal Regulated Kinase 1/2
EtOH	Ethanol
FA	Fatty Acid
FABP	Fatty Acid Binding Protein
FACS	Fluorescence Activated Cell Sorter
FASN	Fatty Acid Synthase
FBS	Fetal Bovine Serum
FFA	Free Fatty Acid

FITC	Fluorescein Isothiocyanate
G2A	G-protein, 2 A-Accumulation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase).
G-CSF	Granulocyte Colony-Stimulating Factor
GLA	Gamma-linolenic acid
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPCR	G protein-coupled receptor
GRKs	GPCR kinase
GW9662	2-chloro-5-nitrobenzanilide
H ₂ O ₂	Hydrogen peroxide
H ₂ S	Hydrogen Sulphide
HDL	High Density Lipoprotein
HETEs	Hydroxyeicosatetraenoic acid
HODEs	Hydroxyoctadecadienoic acid
HPODE	Hydroperoxyoctadecadienoic acid
HRP	Horseradish peroxidase
HUVEC	Human Umbilical Venous Endothelial Cell
ICAM-1	Intercellular Adhesion Molecule-1
IDL	Intermediate Density Lipoprotein
IFN- γ	Interferon- γ
IgG	Immunoglobulin-G
IL-6	Interleukin-6
IL-1 β	Interleukin 1- beta
IL-8	Interleukin-8
ITGAM	Integrin Alpha-M
JNK	Janus kinase
LA	Linoleic Acid
LCFA	Long Chain Fatty Acid
LC-PUFA	Long Chain Polyunsaturated Fatty Acid
LDL	Low Density Lipoprotein
LDLR	Low-Density Lipoprotein Receptor
LO	Lipoxygenase
LOX-1	Lectin like oxidised LDL receptor-1

LpL	Lipoprotein Lipase
LPS	Lipo polysaccharide
LRP	Low Density Receptor Related Protein
LT	Leukotrienes
MAPK	Mitogen-Activated Protein kinase
MCFA	Medium Chain Fatty Acids
MCP-1	Monocyte Chemoattractant Protein-1
M-CSF	Macrophage Colony-Stimulating Factor
mg	Milligrams
MI	Myocardial Infarction
mL	Mililitre
mM	Milimolar
mm-LDL	minimally modified-Low Density Lipoprotein
MNC	Mononuclear cells
MS	Magnetic Separation
MUFA	Monounsaturated Fatty Acid
NBF	Neutral Buffer Formalin
NF- κ B	Nuclear Factor Kappa-B
oxLDL	oxidized LDL
oxoODEs	oxo-octadecaenoic acids
PAI-1	Plasminogen Activator Inhibitor type-1
PE	Phycoerythrin
PECY7	Phycoerythrin-cyanin7
PG	Prostaglandins
PGI	Prostacyclins
PMA	Phorbol Myristate Acetate
PPAR- α	Peroxisome Proliferator-Activated Receptor- α
PPAR- γ	Peroxisome Proliferator-Activated Receptor- γ
PPIA	Peptidylpropyl isomerase A
RANTES	Regulated on Activation, Normal T cell Expresses and Secreted
RES	Reticuloendothelial system
RNAi	RNA interference
ROS	Reactive Oxygen Species

RT-PCR	Reverse Transcriptase -Polymerase Chain Reaction
RXR	9-cis-retinoic acid receptor
SD	Standard Deviation
SCD-1	Stearoyl-CoA Desaturase-1
SCRA	Scavenger Receptors
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide
SMC	Smooth Muscle Cell
T0070907	2-chloro-5-nitro-N-4-pyridinyl-benzamide
TBST buffer	Tris-buffered Saline tween-20; 0.05% Tween-20
TGF- β	Transforming Growth Factor- β
TLR-4	Toll-Like Receptor-4
TNF- α	Tumor Necrosis Factor- α
TR4	Testicular Receptor-4
TX	Thromboxanes
V-CAM-1	Vascular Cell Adhesion Molecule-1
VEGF	Vascular Endothelial Growth Factor
VLDL	Very Low Density Lipoproteins
VSMC	Vascular smooth muscle cells
β -actin	Beta-actin
μ L	Microlitre
μ M	micro Molar
12S-HpETE	12-S-hydroperoxy-5Z, 8Z, 10E, 14Z-eicosatetraenoic acid
12Ss-HETE	12S-hydroxyeicosatetraenoic acid
13-HPODE	13-hydroperoxy-oxydecadienoic acid
13S HODE	13S-hydroxyoctadecadienoic acid
13S-HpODE	13-S-hydroperoxy-9Z, 11E-octadecadienoic acid
15-LOX-1	15-lipoxygenase-1
15S-HETE	15S-hydroxyeicosatetraenoic acid
15S-HpETE	15-S-hydroperoxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic acid
5-LOX	5-lipoxygenase
9S HODE	9S-hydroxyoctadecadienoic acid
9S-HpODE	9-S-hydroperoxy-10E, 12Z-octadecadienoic acid

APPENDIX 3. LIST OF SUPPLIERS

Abcam (Sapphire Biosciences, Redfern, NSW, Australia).

BD biosciences (North Ryde, NSW, Australia).

Beckham Coulter (Gladsville, NSW, Australia).

BIO-RAD (Gladesville, NSW, Australia)

Invitrogen (Mulgrave, VIC, Australia).

Millipore (North Ryde, NSW, Australia).

Miltenyi Biotec (North Ryde, NSW, Australia).

Nunc (Thermo Fischer Scientific, Scoresby, VIC, Australia).

Perkin Elmer (Melbourne, VIC, Australia).

Perkin Elmer (Melbourne, VIC, Australia). Starfrost (Proscitech, Kirwan, QLD, Australia).

Promega (Alexandria, NSW, Australia).

Qiagen Pty Ltd (Doncaster, VIC, Australia).

SAbiosciences and Bender Medsystems Jomar Diagnostics Pty Ltd (SA, Australia).

Sarstedt (Ingle Farm, South Australia, Australia).

Sigma-Aldrich (Castle Hill, NSW, Australia).

Sigma-Aldrich (Castle Hill, NSW, Australia).

Thermo Fischer Scientific (Scoresby, VIC, Australia).

Vector Labs (Abacus ALS, Brisbane, QLD, Australia)

APPENDIX 4. REAGENTS AND SOLUTIONS

1 X PHOSPHATE BUFFERED SALINE (PBS)

For 1000 ml (stock 10X)

8g of Sodium chloride (NaCl)

0.2g of Potassium chloride (KCl)

1.44g of Disodium hydrogen phosphate (Na_2HPO_4)

0.24g of Monopotassium phosphate (KH_2PO_4)

Adjust pH to 7.4. Adjust volume to 1L with additional distilled H_2O .

For 100 ml (working 1 X)

10 mL of stock solution

90 mL of distilled water

FLOW CYTOMETRY BUFFER

100 ml of 1x PBS

2.5 % fetal bovine serum (FBS)

2mL 0.5% EDTA

Mix well. Adjust pH to 7.5. Sterilize by autoclaving. Stored at 4°C

MAGNETIC SEPERATION BUFFER

100 ml of 1X PBS

0.5 % bovine serum albumin (BSA)

2mM of EDTA

Adjust pH to 7.2. Degas the buffer. Sterilize by autoclaving. Store at 2-8°C

0.25% TRYPSIN SOLUTION

2.5g/L trypsin

0.38g/L EDTA

A 0.05% solution was prepared by diluting in RPMI media.

NEUTRAL BUFFER FORMALIN (10%)

Formalin (40% formaldehyde) 100 mL

4g of Sodium phosphate, monobasic, monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)

6.5 g of Disodium hydrogen phosphate (Na_2HPO_4)

Make the volume to 1 L adding distilled water

1X TRIS-BUFFERED SALINE

For 1000 ml (stock 10X)

80g of Sodium chloride (NaCl)

2g of Potassium chloride (KCl)

100mL of 1 M Tris-HCl

Add distilled water making it up to 1 L

Adjust pH to 7.4 and autoclave to sterilize

For 100 ml (working 1 X)

10 mL of stock solution

90 mL of distilled water

For TBS-T, Tween was added as required

1. CONFERENCES AND PRESENTATIONS

Hydroxyoctadecadienoic acids (HODEs) increase apoptosis in human THP1 monocytes and macrophages

V. Vangaveti, U. H. Malabu, V. Shashidhar, B. T. Baune, R. L. Kennedy

Department of Medicine, James Cook University, Townsville, QLD, Australia

Abstract accepted, Endocrine Society of Australia, (to be held) 28-31 August 2011, Perth

Hydroxyoctadecadienoic acids (HODEs) regulate fatty acid binding protein-4 (FABP4) secretion in human monocytes and macrophages

V. Vangaveti, U. H. Malabu, V. Shashidhar, B. T. Baune, R. L. Kennedy

Department of Medicine, James Cook University, Townsville, QLD, Australia

Selected for Oral Presentation Endocrine Society of Australia, (to be held) 28-31 August 2011, Perth

Regulation of fatty acid binding protein (FABP-4) production in human Monocytes and macrophages by Isomers of Hydroxyoctadecadienoic acids (HODES)

R.L Kennedy ¹, V.Vangaveti², V.Shashidhar³, U.H Malabu², BT Baune⁴

¹Medicine, Deakin University, Geelong, VIC, ² Medicine, James Cook University, Townsville ³ Pathology, James Cook University, Townsville, QLD, ⁴Psychhiatry, University of Adelaide, Adelaide, SA, Australia

Isomers of Hydroxyoctadecadienoic acids (HODE) regulate Apoptosis in Human THP-1 Monocytes and Macrophages independent of PPARgamma or GPR132 9G2A)

R.L Kennedy ¹, V.Vangaveti², V.Shashidhar³, U.H Malabu², BT Baune⁴

¹Medicine, Deakin University, Geelong, VIC, ² Medicine, James Cook University, Townsville ³ Pathology, James Cook University, Townsville, QLD, ⁴Psychhiatry, University of Adelaide, Adelaide, SA, Australia

Role of Oxidized lipids in atherosclerosis

V.Vangaveti and R.L. Kennedy

Oral presentation, Celebrating 40 years of research, James Cook University, August 2010

Specific effects of 9- and 13- Hydroxyoctadecadienoic acids on human monocyte activation and macrophage differentiation.

V. Vangaveti and R.L. Kennedy

Australian Society of Medical Research Post graduate conference, Brisbane, June 2010

Poster presentation, Travel grant from School of Medicine and Dentistry.

Omega fatty acids, how do they help us?

V.Vangaveti and R.L. Kennedy

Oral presentation, so you think you can research? Townsville Research Festival, James Cook University, November, 2009

Regulation of Stearoyl-Coa Desaturase by Hydroxyoctadecadienoic Acids

V.Vangaveti and R.L. Kennedy

Australian Society of Medical Research Post graduate conference, Brisbane 2009

Poster presentation, Travel grant from School of Medicine and Dentistry.

Short Chain Fatty Acids influence Stromal Cell-derived factor-1 (SDF-1) regulation in Adipocytes

Thomas L, Vangaveti V, Wood F, Garland S, Kazi M & Kennedy RL

Townsville Research Festival, James Cook University, November 2008

2. PAPERS

Hydroxyoctadecadienoic acids: novel regulators of macrophage differentiation and atherogenesis, Venkat Vangaveti, Bernhard T Baune and R. Lee Kennedy. *Ther Adv Endocrinol Metab. June 2010 1:51-60.*

Free fatty acid receptors – emerging targets for treatment of diabetes and its complications, Venkat Vangaveti, V. Shashidhar, G. Jarrod, Bernhard T. Baune and R. Lee Kennedy. *Ther Adv Endocrinol Metab. October 2010 1:165-175.*

GPR132 is a Novel Marker for Monocyte Activation in Diabetes, But Does Not Mediate Regulation of Fatty Acid Binding Protein-4 Expression by 9-Hydroxyoctadecadienoic Acid

Venkat N. Vangaveti, Venkatesh M. Shashidhar, Bernhard T. Baune & Richard L. Kennedy
(Manuscript in preparation)

Hydroxyoctadecadienoic Acids Regulate Apoptosis in Human THP1 Monocytes and Macrophages

Venkat Vangaveti, Usman H. Malabu, Venkatesh Shashidhar, Bernard T. Baune, R. Lee Kennedy
(Manuscript in preparation)

3. AWARDS

Recipient of School of Medicine and Dentistry faculty fee waiver scholarship, 2007-2010.

Recipient of James Cook University school scholarship, 2007-2010.

Awarded Graduate Research Studies research grant, James Cook University (2008) of value \$2430.

Travel grant from School of Medicine and Dentistry to attend Australian Society of Medical Research (ASMR) for successive years 2009 and 2010.