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The Role of Endocannabinoids in Atherosclerosis

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Cardiovascular disease leads in morbidity and mortality in Western societies with no known cure. NADPH oxidase (Nox) contributes to atherosclerosis through the indirect activation of macrophages leading to the internalization of oxidized low density lipoproteins (oxLDL). Chronic inflammation in activated macrophages contributes to atherosclerosis. Because macrophages are positioned at the cross-roads of lipid metabolism in vessel walls, they are important in the cellular pathology of atherosclerosis. Components of the endocannabinoid (eCB) system are vital to atherosclerotic development, since the eCB system has been found to play an important role in the amelioration of atherosclerosis. The eCB system has several components, including the G-protein-coupled cannabinoid receptors (CB_1 and CB_2); their endogenous ligands, 2-arachidonoylglycerol (2-AG) and anandamide (AEA); and biosynthetic enzymes that produce and degrading these compounds. CB_2 signaling has been shown to upregulate immunoprotective and anti-oxidative pathways, whereas CB_1 signaling has opposite effects. We hypothesized a mechanistic link between scavenger receptor activation and Nox activity, which leads to enhanced 2-AG biosynthesis via a signaling pathway that activates diacylglycerol lipase beta (DAGLB). Activation of CB_2-mediated

signaling by enhanced “eCB tone” can potentially reduce oxidative stress in macrophages. The released 2-AG is subsequently catabolized hydrolytic enzymes, leading to enhanced 2-AGbiosynthesis via activated DAGLB. We first proved that macrophage treated with oxLDL can activate Nox and increase reactive oxygen species production. We used human and mouse macrophages to demonstrate cause and effect. Secondly, we demonstrated that increased levels of superoxide causes enhanced 2-AG biosynthesis within the macrophage, and that upregulation in eCB production is an adaptive response to oxidative stress. Finally, we identified and quantified the serine hydrolases found in smooth muscle cells (SMCs) using an activity-based protein profiling (ABPP)-MudPIT approach that our laboratory has previously done using human macrophages. Additionally, the catabolism of 2-AG by primary SMCs was explored to demonstrate SMCs can hydrolyze 2-AG to its metabolites arachidonic acid and glycerol by the known hydrolytic enzymes. We demonstrated that enhancing endocannabinoid tone within the vessel wall is a valuable strategy to reduce the occurrence of inflammation that leads to atherosclerosis.

DEDICATION

I dedicate this dissertation to my parents, the late Robert and Bessie Suniville; my children, Vashti, Jamie, and Vestori; my grandchildren, Kriestyn, Karson, and Kailei; significant other, Willie Hood; as well as my family. It was with great sacrifice that I was able to accomplish this milestone. I have set the bar, as the first in our family to accomplish this degree, but I will not be the last.

Additionally, to the Soka Gakkai International. It was by not only my faith, but also the faith other SGI members that carried me through the challenges of this degree process. The Daishonin teaches that it is better to conquer oneself than to win a thousand battles. Once one conquer self, the victory is theirs. It cannot be taken. Education and Daimoku are the most powerful weapons which can be used to change the world.

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CHAPTER I

INTRODUCTION TO DISSERTATION

1.1 Introduction

The incidence of cardiovascular disease (CVD) in industrialized countries has remained constant over several years, yet it remains the number one cause of death. Furthermore, though there is a myriad of information and ongoing research pertaining to controlling the underlying factors of CVD, including hypertension, hyperlipidemia, and obesity, among others. CVD is becoming more prevalent in developing countries. Atherosclerosis, which is the major cause of CVD, is a chronic inflammatory disease that involves lipid dysregulation as its major hallmark. This results in the occurrence of plaque formation and the hardening of the vessel wall. There are several homeostatic mechanisms that can act to slow the onset of atherosclerosis, including the upregulation of the endocannabinoid system, enhanced reverse cholesterol transport, and the downregulation of genes that encode pro-inflammatory cytokines. Pharmaceutical therapy has made important advances over the past several decades; however, because CVD remains the number one killer in the United States, it is important to identify targets for new therapeutic agents that will decrease the tendency of cardiovascular disease development.

1.2 Treatment strategies in cardiovascular disease

Each year CVD remains among the top chronic illnesses and the leading health problem in the U.S. According to the American Heart Foundation, cancer deaths pale in comparison to CVD in terms of the number of lives claimed each year (Lori Mosca *et al.* 2011). Although CVD is an equal opportunity disease, more lives of women than men are claimed each year, due to the different warning signs, but both men and women are treated the same in regards to pharmaceutical therapies (Lori Mosca *et al.* 2011; Smith *et al.* 2011; Gouni-Berthold and Berthold 2015).

Currently, treatment strategies for CVD are aimed at relieving the symptoms, reducing risk factors, and increasing blood flow within the heart. Genetic and environmental factors play a significant role in the onset of CVD. The growing trend of a sedentary lifestyle and increased high-fat diet contributes to early onset of CVD. Although genes are obviously hereditary, poor diet and exercise habits can also be passed down from parent to progeny, thus perpetuating a cycle of decreased fitness and declining health. As modern society has become more fast-paced, many people are choosing fast food and processed foods that are high in sodium and saturated fats rather than a heart healthy diet. It is suggested that behavioral changes alone may be a way to decrease the onset of CVD, thus the National Heart Lung and Blood Institute (NHLBI) has proposed dietary approaches to reduce hypertension, which is often the "silent killer" in patients with CVD. However, exogenous toxicants, such as cigarette smoke, can also significantly increase the risk for CVD. Quitting smoking coupled to a healthy diet and exercise are important lifestyle modifications to control heart disease. Cigarette smoke has an arsenal of toxic chemicals that can damage the vascular wall via multiple

pathological pathways (Csordas and Bernhard 2013; Ross *et al.* 2014). Furthermore, several components in cigarette smoke can increase reactive oxygen species levels, such as superoxide, which leads to increased oxidative stress. When combined with other factors, such as an unhealthy diet, limited exercise, and obesity, smoking leads to an enhanced prevalence for peripheral arterial disease. Taken together, limiting behaviors that contribute to oxidative stress can help decrease the risk of myocardial infarctions and sudden death.

Anti-hypertensives and statins are widely used pharmaceuticals to control CVD by decreasing the risk factors of hypertension and hyperlipidemia, respectively. Blood pressure is controlled physiologically by the renin-angiotensin system (RAS). RAS is comprised of angiotensin (Ang) II acting on either type I or type II receptors (AT_1R or AT_2R, respectively), which are members of the G-protein coupled receptor superfamily (Arumugam S 2015). Reactive oxygen species cause a number of dysfunctions within the vascular wall, thus AT_1R blockers are some of the leading therapeutics used to prevent myocardial infarction, stroke, renal disease, and heart failure. For example, sartan drugs, such as Losartan, is a combination of two pro-drugs that are activated in the liver. One of the drugs acts to block the AT_1R, while the other decreases oxidative stress (Arumugam S 2015). Sartan drugs have been shown to play a role in reducing cytokine production, apoptosis, and diminish inflammatory phenotypes in macrophages (Yamamoto *et al.* 2015). Furthermore, in a study using a rat model of vascular calcification, Li et al (2015) revealed that Losartan has the ability to suppress key factors involved in vascular calcification and smooth muscle cell apoptosis. Although there are clear beneficial effects of medicinal therapy to treat hypertension (Chida *et al.* 2015),

more studies are needed to identify the overall biomarkers of cardiac function and lipid parameters. On the other hand, studies have shown that statins have the ability to reduce medial thickness and perivascular fibrosis in the aorta without affecting the inducible and endothelial nitric oxide synthase (Crespo and Quidgley 2015). There are significant clinical benefits with the use of both sartans and statins, however, not without consequences including statin induced myopathy, liver damage, increased blood sugar, and neurological side effects (Fernandez *et al.* 2011; Phan *et al.* 2012; Clinic 2015).

Statins, phospholipase A2 inhibitor, lipoprotein-associated phospholipase A2 inhibitor, RAS inhibitors, and Ang II receptor blockers are anti-inflammatory agents that have been linked to reduced progression of atherosclerosis and decreased vascular inflammatory markers (Husain *et al.* 2015). However, despite advancement in drug therapy the onset of atherosclerosis disappointingly remains the leading cause of mortality, morbidity and decreased quality of life. Being that both endogenous and exogenous chemicals participate in the onset of CVD, current research is directed toward alternative strategies to ameliorate the occurrence of atherosclerosis.

1.3 Research Overview

Oxidative stress contributes to development of atherosclerosis, which is the leading cause of morbidity and mortality in adults. Atherosclerosis is a chronic inflammatory disease that is associated with obesity, diabetes, and an overactive immune response. In addition, the endocannabinoid system (ECS) has also been shown to be involved in atherosclerosis. The ECS is composed of a variety of components, including the G-protein coupled cannabinoid receptors (CB_1 and CB_2); endogenous endocannabinoid ligands, 2-arachidonoylglycerol (2-AG) and anandamide (AEA);

hydrolytic enzymes that degrade these ligands; and cyclooxygenase-2 (COX2), which yields proinflammatory prostaglandin glyceryl esters (PG-Gs) following the oxygenation of 2-AG. CB_2 activation and signaling has been shown to initiate immuno-protective pathways through the modulation of anti-inflammatory mediators; whereas, CB_1 signaling has been linked to pro-inflammatory reactions. Thus, the ECS may contribute to disease pathogenesis and needs to be studied in more depth within the context of atherosclerosis. The links between the ECS, oxidative stress, and atherosclerosis lead us to speculate that modulation of the ECS can reduce the extent of atherosclerosis. Limited knowledge currently exists concerning the scavenger receptors (SR), such as CD36, and the subsequent activation of NADPH oxidase and generation of superoxide in macrophages following engagement with oxidized low density lipoproteins (oxLDL) (Figure 1.1). **We hypothesize that stimulated NADPH oxidase activity and oxidative stress leads to enhanced biosynthesis of 2-AG via a signaling pathway that activates diacylglycerol lipase beta (DAGLβ). Additionally, enhanced "endocannabinoid tone" can potentially reduce the oxidative stress in macrophages.** We will test this hypothesis with the following specific aims:

1.3.1 Specific Aim 1

Determine whether NADPH oxidase activity is increased following activation of SR by oxLDL. Our *working hypothesis* was that ligation of SR by oxLDL will activate NADPH oxidase and increase ROS production. This was explored using HL-60 macrophages-like cells because they express abundant levels of Nox2. Nox2 is the catalytic subunit of NADPH oxidase in macrophages.

1.3.1.1 Overview of Aim 1

There is evidence that apocynin, an NADPH oxidase inhibitor, is a promising therapeutic agent for the treatment of inflammatory diseases. In addition, CD36 is a scavenger receptor expressed on macrophages that interacts with oxidized phospholipids present in oxLDL particles (Park et al 2009). Because NADPH oxidase activation contributes to the upregulation of CD36 (Boyer et al., 2007), it was the *objective* of this aim to determine if a converse response on NADPH oxidase occurs following ligation of SR by oxLDL. We tested the *working hypothesis* that oxLDLs activates SR-evoked signaling and that NADPH oxidase activity could be consequently augmented in VAS2870 and apocynin-treated macrophages, but not control macrophages. The *rationale* for this aim was to determine the change in activity of NADPH oxidase in macrophages during conditions of increased oxLDL exposure, which arises during atherogenesis.

1.3.1.2 Significance of Aim 1

During atherosclerosis, macrophages become foam cells via the uptake of excess fatty acids and cholesterol. The mechanism of reverse cholesterol transport (RCT) in the macrophage is a means of reducing cholesterol accumulation and ROS within the cell. NADPH oxidase plays a vital role in the production of ROS. The mechanism of NADPH oxidase activation (Nox2 isoform in macrophages) involves phosphorylation of a cytosolic $p47^{phox}$ subunit and its subsequent translocation to membranes where Nox2 is found. Inhibition of NADPH oxidase through pharmacological or genetic manipulation has yet to be explored as a means of reducing oxLDL uptake into the macrophage.

1.3.2 Specific Aim 2

Determine whether exposing macrophages *in vitro* to ROS leads to activation of DAGLβ and increased biosynthesis of 2-AG, thereby enhancing macrophage "endocannabinoid tone". *Our working hypothesis* is that a correlation exists between increased levels of superoxide and enhanced 2-AG biosynthesis rate within the macrophage; therefore, elevated 2-AG levels may be an adaptive response to oxidative stress.

1.3.2.1 Overview of Aim 2

Oxidative stress is enhanced during atherogenesis; thus, cells in the vessel wall such as macrophages may become compromised. The *objective of this aim* was to determine whether macrophages exposed to superoxide have an increased rate of 2-AG biosynthesis. The source of superoxide will be either *in situ* xanthine/xanthine oxidase generated extracellular superoxide or intracellularly formed superoxide generated by NADPH oxidase. We tested the working *hypothesis* that enhanced 2-AG levels may be an adaptive response to oxidative stress that results in a positive correlation between increased levels of superoxide and enhanced 2-AG biosynthesis within the macrophage. The *approach* included gene and protein expression analyses of diacylglycerol lipase (DAGL); LC-MS/MS analysis of 2-AG levels in macrophages; and serine hydrolase activity profiling of key enzymes. The *rationale* was to elucidate the role of DAGL in the biosynthesis of 2-AG during oxidative stress. The primary *outcome* of this aim was the characterization of the "endocannabinoid tone" upon exposure of macrophages to oxidative stress (e.g., superoxide). A second *outcome* was an assessment of changes in inflammation in the macrophage in the presence of increased 2-AG concentration. This

aim revealed the role of DAGL in 2-AG production as a consequence of superoxide exposure.

1.3.2.2 Significance of Aim 2

The synthesis and release of 2-AG is stimulus-dependent and relies on the concerted action of phospholipase C-β (PLC-β) and DAGL (Gregg et al., 2012), thus elucidating whether superoxide can affect DAGL activation and alter the ECS is both novel and significant.

1.3.3 Specific Aim 3

Compare the serine hydrolase profiles in human smooth muscle cells (SMCs) and determine the extent of 2-AG metabolism by SMCs. Our *working hypothesis* was that while macrophages are elite producers of bioactive lipid mediators such as 2-AG, other cell types in the vessel wall in addition to macrophages may have roles in the catabolism of this endocannabinoid. Therefore, we identified the 2-AG hydrolytic enzymes and their activities in cultured human SMCs.

1.3.3.1 Overview of Aim 3

Little is understood regarding the metabolism of 2-AG by ECs and SMCs in the vessel wall. Therefore, we profiled the serine hydrolases found in cultured human SMCs using the ABPP-MUDPIT approach that we recently used with human THP1 monocytes/macrophages (Wang et al., 2013). We prepared cell proteomes of cultured primary human SMCs and treated them with the activity-based probe fluorophosphonate (FP)-biotin. We labeled the serine hydrolases with FP-biotin to identify and semi-quantify them following their enrichment on streptavidin beads and on-bead trypsin

digestion followed by LC-MS/MS analysis. Moreover, the extent of metabolism of exogenously added 2-AG to the cultured SMCs was also determined, as our laboratory has done previously using monocytes/macrophages (Xie *et al.* 2010; Wang *et al.* 2013). Both the hydrolytic and oxidative pathways of 2-AG catabolism was examined in these cells.

1.3.3.2 Significance of Aim 3

There is speculation that SMCs produce danger-associated signals that are similar to those of macrophages. Thus they could potentially have the biochemistry to produce the enzymes necessary to lead to the phenotypic changes that lead to SMC foam cell formation. Additionally, serine hydrolase activity has been shown to play a role in the activation of the endocannabinoid receptors due to sustained availability of the ligand 2-AG (i.e., lower activity of 2-AG hydrolytic enzymes leads to higher concentrations of 2-AG). These data could provide evidence for the necessity to explore the SMC further as a means of combating atherosclerosis.

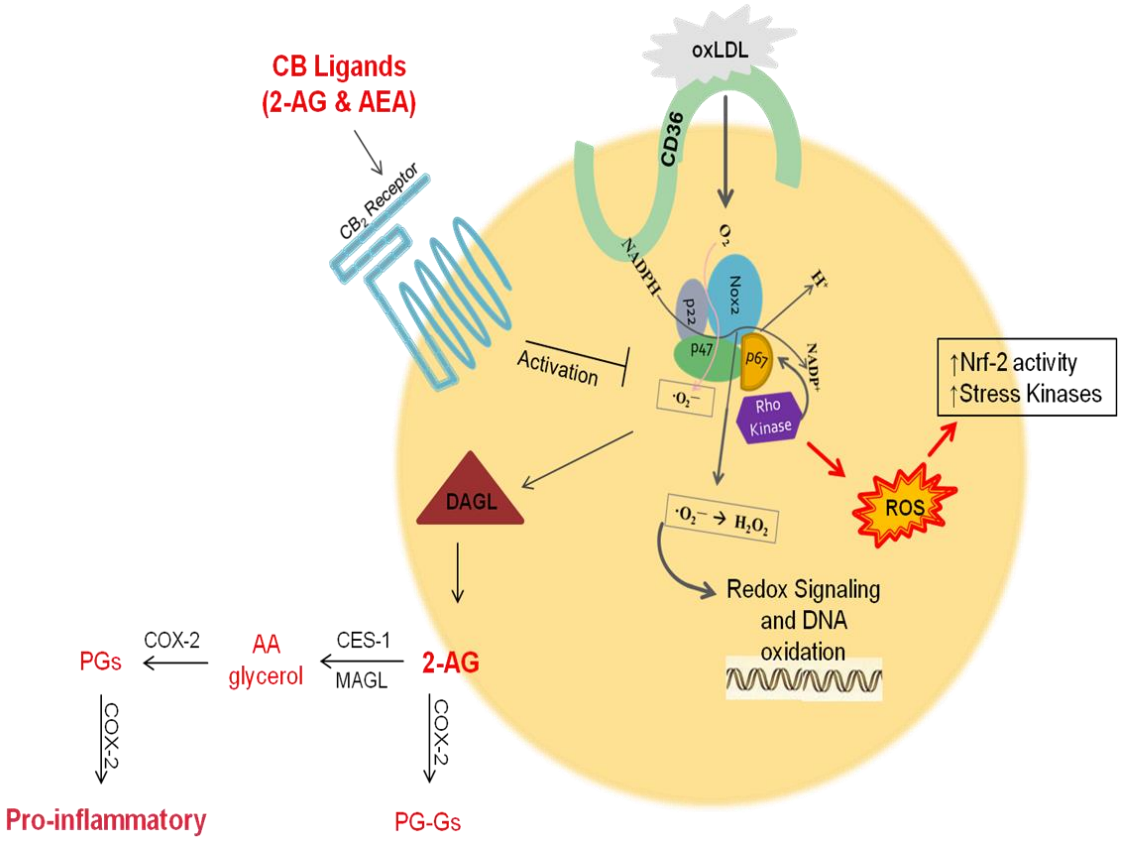

Figure 1.1 Pathways to be investigated in macrophages.

Because DAGLβ catalyzes the biosynthesis of 2-AG, we explored the mechanistic links between scavenger receptor (such as CD36) activation by oxLDL, NADPH oxidase activation, and enhanced 2-AG biosynthesis via DAGLβ activation. Furthermore, we examined the metabolism of the released extracellular 2-AG by cultured primary human smooth muscle cells.
DAGLβ, diacylglycerol lipase beta; oxLDL, oxidized low-density lipoprotein; PPT1, palmitoyl protein thioesterase 1; CES1, carboxylesterase 1.

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CHAPTER II

OXYRADICAL STRESS, ENDOCANNABINOIDS, AND ATHEROSCLEROSIS

2.1 Introduction

Atherosclerosis is the major underlying pathology that causes cardiovascular disease (CVD). Despite considerable investment in the development of new therapies, atherosclerosis remains the leading cause of morbidity and mortality in industrialized societies. At its heart, atherosclerosis is a chronic inflammatory disease caused by inflammatory cascades, dysregulated lipid metabolism, and oxidative stress (Ross et al. 2014). Exogenous and endogenous small molecules – and the enzymes that produce or degrade these molecules – have important roles in these processes. This review will focus on reactive oxygen species (ROS), their important roles in intracellular signaling pathways, and the impact that dysregulated ROS signaling has in pathophysiological processes, such as atherogenesis. In addition, though atherosclerosis is a multifactorial disease involving endothelial cell dysfunction, hypertension, hyperlipidemia, inflammation, and imbalances in ROS formation and degradation, there is also emerging knowledge that the endocannabinoid system has an important role in atherogenesis. Thus, the role of the endocannabinoids will be discussed in the context of inflammation and atherosclerosis.

2.2 Macrophage cholesterol metabolism

Reverse cholesterol transport (RCT) is an endogenous pathway that has the propensity to maintain homeostasis and reduce inflammation in the vessel wall. Cholesterol is an indispensable molecule for proper functioning and organization of the plasma membrane; however, when low density lipoproteins (LDL) substantially outnumber high density lipoproteins (HDL) it becomes necessary for de novo synthesis of HDL via the RCT system. RCT system is induced to restore the balance of LDL and HDL. Ordinarily, free (unesterified) cholesterol is trafficked along endocytic and secretory pathways that involve ATP-binding cassette (ABC) A1 and ABCG1 that efflux lipids and free cholesterol to apolipoprotein A-1 (ApoA-1) for HDL biogenesis (Phillips 2014). It is when LDLs become modified or oxidized that the macrophage has a limited potential to metabolize the lipids into an aqueous phase for facilitated diffusion to translocate the lipids across the cell membrane (Wustner and Solanko 2015). ApoA-1 is an acceptor for free cholesterol thereby forming a mature HDL particle (Brown et al. 1979) followed by the conversion of cholesterol to cholesteryl esters, which can be transferred from HDL to LDL by cholesteryl ester transfer protein (Westerterp et al. 2014). The LDL particles in the circulation are then taken up by LDL receptors on liver cells (hepatocytes). The cholesteryl esters are de-esterified to free cholesterol in the hepatocytes and delivered to the bile for disposal after being converted to bile acids.

Atherosclerosis is caused in part by oxidized (ox)LDL being phagocytosed by macrophages, leading to foam cell formation. Previous reports have indicated that oxLDL interacts with the CD36 receptor (Park et al. 2009) evoking a signaling pathway that activates the NADPH oxidase (Nox) complex (Miller et al. 2011). Park et al (2009)

demonstrated that oxLDL induces actin polymerization in the presence of CD36, resulting in the sustained activation of focal adhesion kinase (FAK) and inactivating the Src homology-2 containing phosphotyrosine phosphatase (SHP-2) due to Nox mediated ROS generation. Because SHP-2 is a phosphatase that diminishes signaling, its inactivation should enhance signaling. With oxLDL being an endogenous Nox activator, it is speculated that oxLDL-derived superoxide contributes to the abundant level of oxLDLs that build up in the arterial wall (Leopold and Loscalzo 2008; Ross et al. 2014). It has been indicated that the genetic deletion (Judkins CP 2010) or pharmacological inhibition (Nguyen-Khoa et al. 1999) of Nox can decrease the lesion formation in $ApoE^{-/-}$ mice.

It has been previously demonstrated that increased risk for atherosclerosis is gender or hormone dependent; for instance, Drummond et al. (2011) indicated that premenopausal females have a lower propensity to develop CVD than their male or ovariectomized female counterparts. These findings are relevant in the present discussion because it was previously shown that elevated levels of estrogens, a sex hormone present in higher amounts in premenopausal compared to postmenopausal women, could modulate NADPH oxidase activity (Miller et al. 2007). Additionally, age contributes to the accumulation of damaged DNA which can activate the transcription factor NF-κB (Cheng et al. 2014). Circulating levels of pro-inflammatory cytokines and the endothelial cell (EC) senescence increase with age (Cheng et al. 2014). Therefore, pharmacological inhibition of NADPH oxidase may be an attractive strategy to reduce atherosclerosis in a way that is not gender or age specific. Although it is generally accepted that dyslipidemia contributes to atherogenesis, modulating metabolic pathways that regulate

the inflammatory response requires further investigation. Therefore, this dissertation is focused on the modulation of the endocannabinoid system as a means of atheroprotection and understanding the mechanisms by which oxidative stress and endocannabinoids are related.

2.3 Oxyradical stress, NADPH oxidase, Nrf2, and atherogenesis

Because of the unique chemistry of oxygen (O_2) and its ability to harvest the rich energy reserves found in nutrients, such as glucose and lipids, life is able to exist on our planet. The ability of living organisms to use oxygen, however, has costs because ROS are significant by-products of aerobic respiration. In some contexts, ROS have beneficial properties that enable the innate immune system to confront microbial invaders. On the other hand, excess ROS can result in macromolecule damage and cell death. Therefore, excess ROS are important mediators of cellular and tissue injury, which in part contributes to disease development. Oxidative stress has been implicated as a causative factor in several diseases and aging (van der Loo B 2000; Hamilton et al. 2001; Hansson et al. 2015). Because of the development of chemical tools that are used as selective probes to detect and quantify specific ROS, such as superoxide and hydrogen peroxide, the biochemical pathways that shape the landscape of oxidative stress in living cells are becoming clearer (Brewer et al. 2015). This has enabled causal inferences instead of mere associations to be made for specific oxyradical species in various physiological processes, which has provided important insights into vascular biology and disease.

NADPH oxidase (Nox), xanthine oxidase, dysfunctional endothelial nitric oxide synthase, cytochrome P450 monooxygenase, and lipoxygenase are found in vascular cells and produce oxyradicals (superoxide) and oxy-nonradicals (hydrogen peroxide) either as

primary products or as by-products of their enzymatic activity. The incomplete reduction of oxygen during mitochondrial respiration also results in superoxide production. In addition, myeloperoxidase catalyzes the reaction between hydrogen peroxide and chloride, which generates the powerful oxidant hypochlorite (HOCl). These reactions are described in Fig. 1. Nox is the only oxidoreductase that produces superoxide as its primary end product, thus it was given the moniker "professional ROS producer" (Jiang et al. 2011). Nox is typically inactive in circulating immune cells, such as neutrophils and monocytes, but in the presence of pro-inflammatory cytokines, such as IL-1β and TNF-α, it becomes activated. A multi-subunit complex is formed, which includes membrane embedded Nox2-$p22^{phox}$ catalytic subunits and soluble regulatory subunits $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and Rho GTPase (Rac1 or Rac2), resulting in the assembly of the Nox holoenzyme (Jiang et al. 2011). Active Nox catalyzes the one-electron reduction of molecular oxygen by NADPH, forming superoxide. Superoxide has a limited diffusion radius in the cell, because it is rapidly converted to hydrogen peroxide and oxygen (dismutation) either spontaneously or via superoxide dismutase (SOD). Furthermore, due to its negative charge, superoxide cannot passively diffuse through lipid membranes as well as anion channels and aquaporins (Al Ghouleh et al. 2013). Although superoxide *per se* is not reactive with cell macromolecules, SOD rapidly reduces its concentration in cells, which is important because this minimizes the Fe^{3+}-catalyzed coupled reaction (Haber-Weiss) between superoxide and hydrogen peroxide that produces hydroxyl radicals, hydroxide ion, and oxygen (Fig. 1). First, superoxide donates an electron to Fe^{3+} to generate Fe^{2+} and O_2, then hydrogen peroxide reacts with Fe^{2+} to yield the hydroxyl radical and hydroxide ion (regenerating $Fe^{3+,}$ thus the net

reaction is catalyzed by ferric ion). As compared with superoxide, the hydroxyl radical is highly reactive and abstracts hydrogen atoms from proteins, DNA, and lipids (mainly unsaturated fatty acids) in the vicinity of its production, thus generating more free radicals and propagating oxidative stress (Ross et al. 2014).

Whereas excess concentrations of superoxide and ROS have obvious toxic properties, these species also have fundamental roles in signaling pathways that enable cells to adapt to stress (Kim and Byzova 2014; Lambeth and Neish 2014). There are several different isoforms of Nox expressed in cells and these enzymes have important roles in cellular homeostasis. For instance, macrophages express an abundant amount of Nox2. Nox2 is activated in response to a variety of physiological stimulants such as insulin, angiotensin II, and sheer stress. The superoxide that is generated can act as a mild reductant because it surrenders an electron to an appropriate acceptor – for example, it can either reduce Fe^{3+} to Fe^{2+} or a second molecule of superoxide to hydrogen peroxide. Signaling pathways activated by superoxide-derived ROS include the stress kinase ERK1/2, which is involved in cell differentiation; JNK-MAPK, which is involved in the regulation of inflammation and cell death; NFκB, a transcription factor for inflammatory and anti-apoptotic genes; Akt, which is involved in regulating metabolic homeostasis; and Ras, Rac, and p38, which regulate several cellular functions such as proliferation, apoptosis, and inflammatory gene expression (Hopkins 2013; Kim and Byzova 2014). Nox2 is implicated in the development of atherogenesis and vascular remodeling. For example, genetic deletion of Nox2 in the high-fat-diet-fed $ApoE^{-/-}$ mouse model ($ApoE^{-/-}Nox2^{-/-}$) caused a reduction in atherosclerotic lesions compared with control $ApoE^{-/-}$ mice (Lambeth and Neish 2014). This finding supports the notion

that pharmacological inhibition of Nox might be an attractive strategy to reduce atherosclerosis (Park *et al.* 2009; Park and Oh 2011; Mangum *et al.* 2015). In addition, vascular endothelial growth factor-induced angiogenesis and neovascularization was impaired in $Nox2^{-/-}$ mice, which implicates Nox2 in wound healing and the generation of vessels (Judkins CP 2010).

A pathological consequence of the excess production of oxidants in the vascular space is the buildup of oxidized low-density lipoproteins (oxLDL) and other oxidized biomolecules in the intima of the vessel wall (Drummond *et al.* 2011). LDLs entrapped in the intimal space are targets of oxyradical fluxes, leading to the chemical modification of lipids and apoproteins in the LDL particle. The resulting oxLDL particles are a hallmark lesion of atherosclerosis. Truncated oxidized phospholipids, such as phosphatidylcholine ($oxPC_{CD36}$), are detected in atherosclerotic plaques and in the circulation of hyperlipidemic subjects (Tojo *et al.* 2005). Circulating monocytes then migrate into the intimal space of the vessel wall and in the presence of macrophage-colony stimulating factor (M-CSF) can differentiate into macrophages. $OxPC_{CD36}$ protrude from the surface of oxLDL like whiskers and their avid and specific interactions with CD36 scavenger receptors promotes the uptake of oxLDL by macrophages and smooth muscle cells, resulting in the uncontrolled uptake of toxic oxLDL particles and the subsequent formation of foam cells (Griendling 2013; Ross *et al.* 2014). OxLDL uptaken by macrophages is an unregulated process due to lack of negative regulation. Consequently, macrophages take up massive quantities of cholesterol and become foam cells. Upon phagocytosis of oxLDL, the store of cholesteryl esters that are abundant in oxLDL are hydrolyzed to free cholesterol (FC) and free fatty acids inside the macrophage

(Silverstein *et al.* 2010; Salomon and Gu 2011). The endoplasmic reticulum (ER) is sensitive to the increased load of FC, which results in significant ER stress. The signaling pathways activated by ER stress cause macrophage foam cells to undergo apoptosis. Apoptosis and secondary necrosis of foam cells lead to development of atherosclerotic plaques, which can result in acute thrombotic complications and death.

Despite abundant evidence to support the oxidative stress hypothesis of atherosclerosis development, antioxidant approaches in human patients have been mostly ineffective at reducing atherosclerosis. Thus, a greater understanding of the temporal and spatial aspects of ROS production in the tunica intima during oxidative stress is needed. Due to the large array of ROS and their limited lifetimes, it is difficult to pinpoint the specific molecular and biochemical pathways that the oxyradicals perturb. However, some key reactions are well known to contribute to atherogenesis. For example, excess levels of superoxide that escape detoxication pathways can react with nitric oxide, resulting in the depletion of this important vasoprotective, anti-inflammatory molecule and the formation of the highly reactive oxidant peroxynitrite (Salomon and Gu 2011) (Fig. 1). Chain reactions within lipid membranes initiated by oxyradicals results in unrestrained lipid peroxidation reactions, which leads to the generation of electrophilic α,β-unsaturated aldehydes (e.g. 4-hydroxynonenal, which is abbreviated 4-HNE), aldehydes, and nitroalkenes (Ross *et al.* 2014). These reactive lipids are electrophiles that can react either reversibly or irreversibly with nucleophiles in proteins and inhibit their normal activities. The resulting "electrophilic stress" caused by lipid peroxidation is implicated in atherogenesis (Feng *et al.* 2003).

Cells have homeostatic mechanisms to control the concentrations of ROS and electrophiles. For instance, glutathione (GSH) is a cysteine-containing tripeptide that quenches intracellular ROS, forming oxidized GSSG in the process, and detoxifies electrophiles (Mukhopadhyay *et al.* 2007; Heumuller *et al.* 2008; Judkins CP1 2010). Furthermore, peroxidases, such as GSH peroxidase and peroxiredoxins, can detoxify both hydrogen peroxide and organic peroxides. In addition, antioxidant concentrations in cells can be increased by consumption of small biomolecules such as N-acetyl cysteine, methionine, or polyphenolic compounds, thereby enhancing intracellular redox homeostasis (Levonen et al. 2014). Although the biochemical pathways for removing ROS and peroxides have been well characterized, chemopreventative strategies to decrease the production of ROS that result from hypertension, hyperlipidemia, diabetes, autoimmune, and inflammatory disease are still under investigation and a matter of debate (Borazjani *et al.* 2011; Levonen *et al.* 2014).

Monocytes adhere to the endothelial cell layer of vessels via their interaction with vascular cell adhesion molecules (VCAM), which are expressed on the endothelial cell surface during inflammation. This causes the development of a positive feedback loop in which NFκB signaling is activated in the endothelial cells, leading to further endothelial cell dysfunction. The subsequent diapedesis of monocytes through the endothelial cell layer and exposure to M-CSF causes the monocytes to differentiate into intimal macrophages. The differentiation process can occur via classical activation (M1 phenotype), which requires pro-inflammatory stimulants such as interferon-γ and lipopolysaccharide (LPS), resulting in the induction of TNF-α (Lambeth and Neish 2014). Alternatively, differentiation can occur via an activation pathway (M2 phenotype)

in which macrophages are exposed to anti-inflammatory cytokines, such as IL-4 or IL-13, which are associated with tissue repair (Levonen *et al.* 2014). Although M1 and M2 are the best studied macrophage phenotypes in atherosclerotic plaques, Kuhn et al. (2011) characterized a Mox phenotype that develops in response to electrophilic oxidized phospholipids and involves the cellular electrophile sensor Nrf2-Keap1. The Mox phenotype has aspects of both M1 and M2. The heterogeneity of macrophage phenotypes found in atherosclerotic plaques has a major role in the inflammatory process, due to the specific phenotype of the macrophage (M1, M2, or Mox) that contributes to the lesion size, composition, and stability of the plaque (Baker *et al.* 2011). Nrf2 is a vital cellular defense transcription factor that protects against the cytotoxic effects of oxidative and electrophile stress. It is a member of the cap' n-collar family and is a basic leucine zipper transcription factor. Nrf2, under basal conditions, is negatively regulated in the cytoplasm by the cysteine-rich Kelch-like ECH associated protein 1 (Keap1). However, when cysteine residues in Keap1 are covalently modified by electrophiles (such as lipid electrophiles) or oxidants, the Keap1-Nrf2 complex dissociates and Nrf2 translocates into the nucleus where it binds to antioxidant response elements (AREs) in the 5'-regulatory region of target genes, thereby initiating transcription (Fig. 3). $OxPC_{CD36}$ and 4-HNE each contain an α,β-unsaturated aldehyde moiety, which is formed during lipid peroxidation reactions. 4-HNE is a freely diffusible molecule, whereas $oxPC_{CD36}$ is associated with oxLDL particles. Both are electrophilic molecules that covalently modify cysteines on Keap1, thereby stimulating Nrf2 transactivation activity (Johnson and Newby 2009). Although several studies have described a role for PPAR-γ in regulating CD36 gene expression (Gordon and Taylor 2005; Kuhn *et al.* 2011), it has also been

shown that CD36 can be induced by $oxPC_{CD36}$ and 4-HNE via Nrf2 activation (Adamson and Leitinger 2011). Thus, Nrf2-mediated regulation of CD36 expression in macrophages is a pathway distinct from that of PPAR-γ. Targeting the Nrf2 pathway could be an attractive strategy for preventing foam cell formation and atherosclerosis.

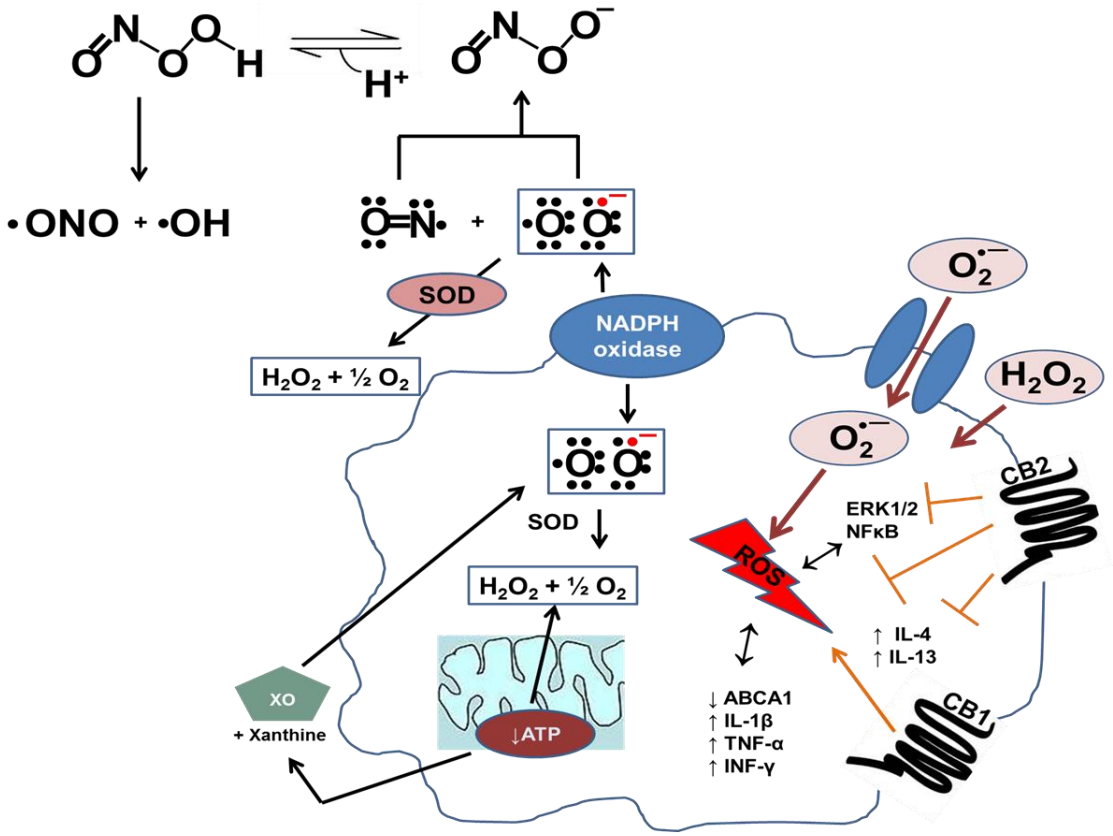

Figure 2.1 Oxidative stress in vascular cells.

Nitric oxide (NO) and superoxide ($O2^{-}$) combine to make peroxynitrite ($ONOO^{-}$) at a rate that is diffusion-limited. Superoxide is rapidly metabolized by superoxide dismutase present in mitochondria, cytoplasm, and extracellular space. Alternatively, xanthine oxidase (XO) can produce superoxide as a byproduct of its activity. Superoxide ($O2^{-}$) and hydrogen peroxide (H_2O_2) cross lipid membranes by either utilizing an ion channel or passive diffusion. Activation of the CB_1 receptor can enhance ROS and pro-inflammatory cytokine production. On the other hand, the activation of the CB_2 receptor is a protective mechanism due to the increased production of anti-inflammatory cytokines.

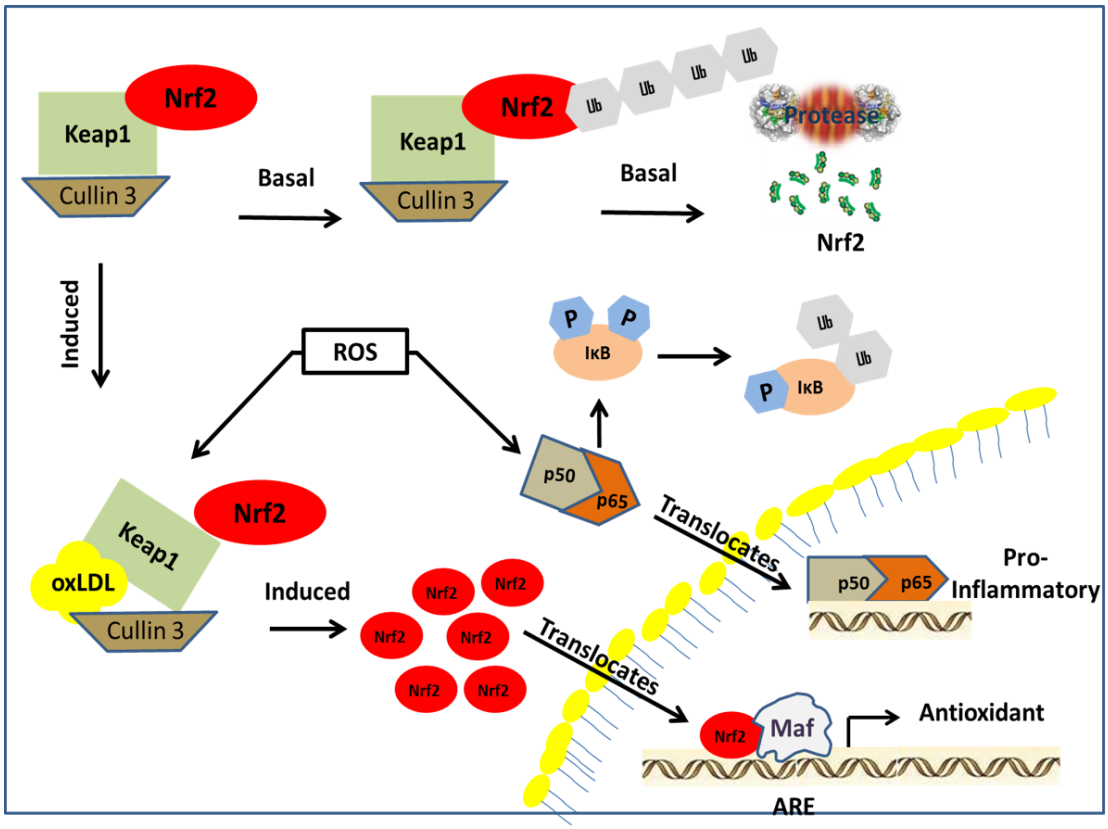

Figure 2.2 Nrf2 activation pathway.

Nrf2 is a member of the bZIP family of transcription factors. It is localized in the cytoplasm under basal conditions by forming a complex with Keap1 and Cullin 3 until activated. Under basal conditions, Nrf2 is ubiquitinated and degraded by proteosomes. During electrophilic stress induced by oxLDL or 4-HNE, the cysteine residues in Keap1 are covalently modified leading to the subsequent ubiquitination of Keap1 and its degradation. As Nrf2 builds up in the cytoplasm, it starts to translocate into the nucleus forming a heterodimer with a Maf protein. The transcription factor complex associates with antioxidant response elements (ARE) in upstream antioxidant genes to initiate transcription. When NFκB forms a complex with IκB in the cytoplasm, it will be ubiquitinated and degraded. However, when IκB is phosphorylated and degraded via the ubiquitin-proteosome pathway, NFκB can translocate into the nucleus, it results in the increased expression of genes that encode pro-inflammatory cytokines.

2.4 Role of endocannabinoids in inflammation

In this section we briefly discuss the role of the endocannabinoids (eCBs) in pathophysiological inflammation; however, for more detailed treatises of this subject, the reader is directed to several excellent publications that describe the eCB system in depth (Levonen *et al.* 2014). ECBs are arachidonoyl-containing lipids that are produced on demand by the metabolism of glycerophospholipid precursors in cell membranes. The best characterized eCBs are 2-arachdonoylglycerol (2-AG) and anandamide (AEA), which are ligands for two G-protein coupled receptors termed cannabinoid (CB) receptors 1 and 2. The eCBs are local-acting lipid hormones that act in an autocrine or paracrine manner. The eCB ligands 2-AG and AEA are rapidly degraded by hydodrolytic enzymes monoacylglycerol (MAGL) and fatty acid amide hydrolase (FAAH) to its metabolites arachidonic acid and ethanolamide, respectively. The role of the CB_1 receptor in neurotransmission has been widely studied in the central nervous system. The CB_1 receptor was identified in the late 1980's by isolating receptors in the brain (Park *et al.* 2009). More recently these receptors have also been established as having a role in the immune response and may contribute to the dysregulation caused by high blood pressure and inflammation (Nagy *et al.* 1998; Adamson and Leitinger 2011). The CB_1 receptor is implicated in pro-oxidative stress/pro-inflammatory responses that are associated with CVD (Ishii *et al.* 2004; Pacher and Steffens 2009; Mackie 2010; Tanasescu and Constantinescu 2010; Booz 2011; Hopkins 2013). The CB_2 receptor, on the other hand, was cloned in the early 1990's from rat spleen and was also detected in the hematopoietic immune system (Devane *et al.* 1988). On the face of it, the CB_1 and CB_2 receptors have similar biochemical functions; they are membrane-bound receptors

coupled to a $G_{i/o}$-protein that when activated can inhibit adenylyl cyclase and modulate calcium channel activity (Pacher and Steffens 2009). However, in contrast to CB_1, the CB_2 receptor activation appears to block and modulate several pro-inflammatory effects, suggesting that downstream signaling pathways evoked by the two receptors are divergent. Several studies have demonstrated that ligand-mediated activation of the CB_2 receptor can counter the inflammation found during oxidative stress (Mukhopadhyay *et al.* 2007). For example, it can attenuate the following physiological and molecular effects: inflammatory cell migration, endotoxin-induced oxidative stress, p38-MAPK activation, and TNF-α secretion (Munro *et al.* 1993; Rajesh M1 2012). Nox activity was also found to be attenuated by a CB_2 agonist in human coronary artery EC (Mackie 2010). CB_2 activation has also been shown to enhance HDL levels, thereby promoting reverse cholesterol transport (RCT) (Pacher and Steffens 2009; Sugamura *et al.* 2010; Turcotte *et al.* 2015). RCT is an important pathway by which non-esterified cholesterol is effluxed from peripheral macrophages onto extracellular ApoA1 and mature HDL acceptors for subsequent transport to the liver. Importantly, the de novo generation of HDL particles via RCT helps to increase circulating HDL levels and reduce atheroma development. ECBs are also vital in modulating immune cell proliferation and apoptosis, cytokine production, macrophage-mediated phagocytosis, and inflammatory cell migration during tissue injury (Booz 2011). Finally, the eCB ligand 2-AG has been specifically implicated in the modulation of inducible nitric oxide synthase (iNOS), nitric oxide (NO), and ROS production in immune cells (Turcotte *et al.* 2015).

Pharmaceutical targeting of the eCB system shows some promise. For instance, the atherosclerotic lesion size decreased in the abdominal aorta of $ApoE^{-/-}$ mice treated

with rimonabant, a CB_1-specific antagonist (Rajesh *et al.* 2010). Pharmacological blockade of the CB_1 receptor or its genetic deletion also enhanced macrophage RCT via ABCA1- and ABCG1-mediated cholesterol transport and reduced atherosclerosis (Sugamura *et al.* 2010; Chouinard *et al.* 2011). Rajesh et al. (2010) demonstrated that chemical blockade of the CB_1 receptor could attenuate Nox expression, MAPK activation, apoptosis, inflammation, ROS, and fibrosis. Whereas CB_1 receptor activation stimulates production of pro-inflammatory mediators, activation of CB_2 receptors in macrophages attenuated TNF-α production and evoked other anti-inflammatory responses. Several studies have indicated that selective activation of CB_2 may render cardioprotective effects (Sugamura *et al.* 2010). For example, JWH-133, a CB_2 agonist, reduced superoxide generation, increased ERK 1/2 and STAT3 phosphorylation, inhibited chemotaxis, and upregulated CD18/CD11b (CR3) expression on inflammatory cells (Sugamura *et al.* 2010).

Therefore, in addition to endothelial dysfunction, hypertension, hyperlipidemia, inflammation, and ROS, the eCB system also appears to be an important factor in atherogenesis (Tsubakio-Yamamoto *et al.* 2008; Pacher and Steffens 2009; Pacher and Kunos 2013). Although the specific mechanistic role of the eCB system in disease development is still enigmatic, links have been postulated between the eCB system, Nox, oxidative stress, and atherosclerosis (Rajesh M1 2012). Because of the large number of oxyradical species that are produced in cells during vascular injury, which significantly complicates the interpretation of experimental data, it is not a trivial task to identify the pathophysiological pathways induced or enhanced by oxidative stress. Nevertheless, several homeostatic mechanisms have evolved to protect against reactive small

molecules, such as ROS and 4-HNE. For example, the enhanced biosynthesis of 2-AG by cells that are "stressed" might be part of a compensatory protective mechanism, via activation of the CB_2 receptor by 2-AG and downstream signaling, that provides anti-atherogenic effects.

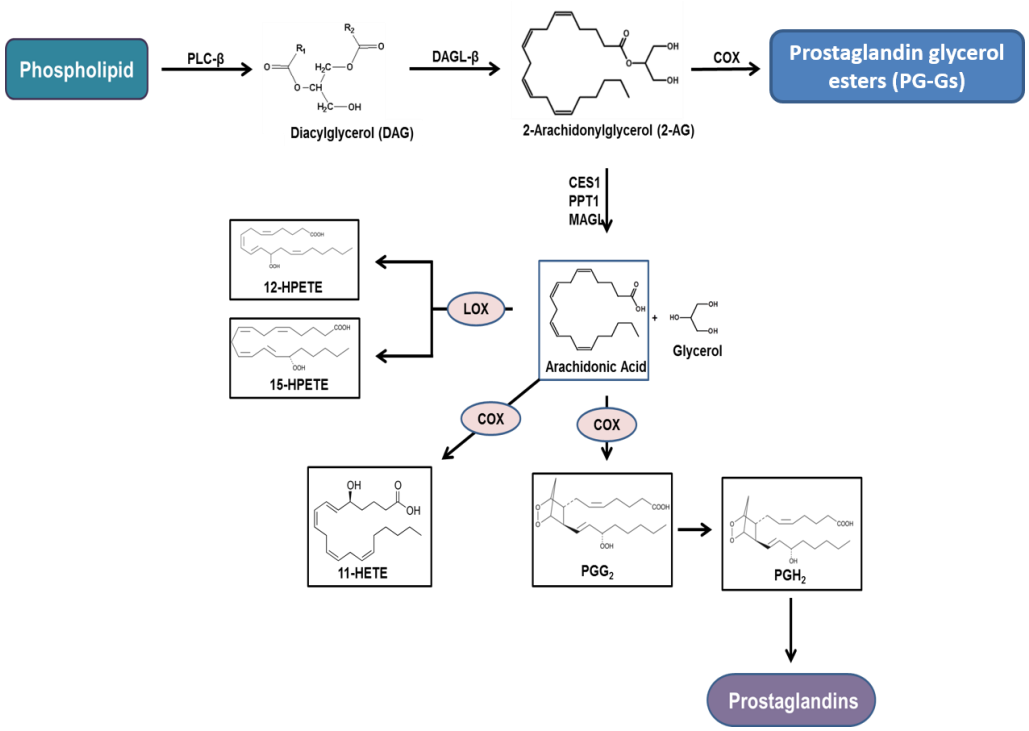

Figure 2.3 2-Arachidonylglycerol biosynthesis and degradation in immune cells.

Phospholipids are metabolized into diacylglycerol (DAG) by phospholipase C-beta (PLC-β). DAG is subsequently metabolized into 2-AG by the enzyme DAGLβ. 2-AG can be degraded by either hydrolytic enzymes [carboxylesterase 1 (CES1), palmitoyl protein thioesterase 1 (PPT1), or monoacylglycerol lipase] or oxidation [cyclooxygenases (COX)]. COX1/2 metabolizes arachidonic acid (AA) into $PG\text{-}G_2$, which are further metabolized into PGH_2 and prostaglandins. COX2, however, can metabolize AA into 11-hydroxyeicosatetraenoic acid (11-HETE). Lipoxygenase (LOX) can metabolize AA into metabolites 12-hydroxyperoxyeicosa-5,8,10,14-tetraenoic acid (12-HPETE) or 12-hydroxyperoxyeicosa-5,8,10,14-tetraenoic acid (15-HPETE).

2.5 Diacylglycerol lipase and Nox

Diacylglycerol (DAG) is a lipid and a second messenger molecule produced by the enzyme phospholipase C-β (PLC-β) (Pacher and Mechoulam 2011; Pacher and Kunos 2013). DAG is hydrolyzed by sn-1-specific diacylglycerol lipases (DAGL) yielding the endocannabinoid 2-AG (Fig. 2) in response to extracellular stimuli, including angiotensin II, thromboxane A2, platelet activating factor, bradykinin, serotonin, glutamate, and acetylcholine (Pacher and Steffens 2009; Pacher and Mechoulam 2011). Two isoforms of DAGL (DAGLα and DAGLβ) were identified in humans, on the basis of the conservation of the intron-exon boundary in human DAGL isoforms with those in Drosophila, fish, and rodents (Anilkumar *et al.* 2008; Mackie 2010). DAGLα is found in high concentrations in brain, whereas DAGLβ is found in high concentrations in immune cells and macrophages (Signorello *et al.* 2011; Shonesy *et al.* 2015). Gao et al. (2010) used DAGLβ$^{-/-}$ knockout mice to show that DAGLβ is a vital enzyme in the biosynthesis of 2-AG. DAGLβ has four transmembrane (4TM) domains, a short cytoplasmic N-terminal sequence, a canonical α/β hydrolase domain that harbors the catalytic residues, and several unconserved loops with potential sites for glycosylation (Astarita and Piomelli 2009). DAGLβ can also be palmitoylated on a cysteine residue in a regulatory loop (Reisenberg et al. 2012). Reversible fatty acyl-posttranslational modifications can modulate protein-protein interactions, cellular trafficking, and substrate access.

The duration of the physiological response to endocannabinoids is regulated by the balance of endocannabinoid biosynthetic and degradative pathways. AEA and 2-AG are both degraded by hydrolysis, yielding arachidonic acid as a common metabolite and ethanolamine and glycerol, respectively. In the mouse brain, ~85% of 2-AG hydrolytic

activity is due to monoacylglycerol lipase (MAGL), with the remaining balance ascribed to two hydrolytic enzymes, α/β-hydrolase domain (ABHD)-6 and ABHD-12 (Hsu *et al.* 2012). In addition, 2-AG and prostaglandin glyceryl esters (PG-Gs), which are cyclooxygenase-derived oxygenated products of 2-AG, are also degraded by carboxylesterase 1 (CES1) (Gao *et al.* 2010; Reisenberg *et al.* 2012). Further adding to the list of hydrolytic enzymes that metabolize endocannabinoids, Wang et al. (2013) identified palmitoyl protein thioesterase 1 (PPT1) as an enzyme that can hydrolyze 2-AG. PPT1 is a monomeric lysosomal hydrolase known to hydrolyze thioester bonds that attach long-chain fatty acids to cysteine residues (Blankman *et al.* 2007; Xie *et al.* 2010; Carr *et al.* 2013).

DAG is also implicated in the regulation of Nox activity (Wang *et al.* 2013), thus DAGLα and DAGLβ are likely to be important hubs in this signaling pathway. Protein kinase C (PKC) is recruited to lipid membranes by the presence of DAG and its kinase activity is activated. This leads to the phosphorylation of the Nox regulatory subunit, $p47^{phox}$. Elevated concentrations of DAG cause a heightened oxidative response in cells and this signal can be terminated by DAG kinase (Wang *et al.* 2013). DAGLβ also appears to indirectly regulate the production of eicosanoids in the brain, liver, and macrophages by increasing the levels of 2-AG in these tissues and cells (Wang *et al.* 2013). When 2-AG hydrolysis was blocked by either chemical inhibition of the principal 2-AG hydrolytic enzyme in brain, MAGL, or its genetic deletion, eicosanoid production was also decreased and neuroinflammation was reduced in a mouse model (Lu and Hofmann 2006). This is because the concentration of the cyclooxygenase substrate, arachidonic acid, had been reduced.

2.6 Conclusion

It now seems apparent that oxidative stress is both a cause and a consequence of atherosclerosis. Superoxide and hydrogen peroxide are implicated in vascular dysfunction through the initiation of diverse signaling and transcriptional pathways (Schlam *et al.* 2013). There are arsenals of enzymes in cells that scavenge oxyradicals to reduce the toxic burden of excess ROS. Emerging evidence indicates that the endocannabinoids have the ability to reduce the production of inflammatory mediators in the vascular wall as a compensatory response to oxidative stress. Pharmacological inhibition or genetic blockade of the key biosynthetic enzyme DAGLβ reduces the production of the eCB ligand 2-AG, indicating that DAGLβ is a key enzyme in establishing the local eCB concentration, or "tone", in a tissue or the vascular space. Furthermore, Nox, the "professional ROS generator", is a promising pharmacological target due to the role it has in superoxide production and CVD pathology. Elucidation of the physiological and pathophysiological processes emanating from Nox activation and the role of eCB ligands in attenuating the inflammation and oxidative stress that result from overactive Nox might be a beneficial treatment modality for CVD. Emerging evidence indicates that the increase in local concentration of 2-AG is an adaptive response to oxidative stress because of its antioxidant and anti-inflammatory activities. Therefore, the genetic and lifestyle factors that lead to atherosclerosis development may be counteracted by either increased biosynthesis (via increased DAGLβ activity) or reduced hydrolysis (via decreased MAGL and/or other hydrolytic enzyme activities) of 2-AG.

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CHAPTER III

OXIDIZED LOW DENSITY LIPOPROTEIN AND OXIDATIVE STRESS

3.1 Introduction

Atheromas in the vasculature are complex lesions comprised of lipids, necrotic cells, cellular debris, and fibrous elements. A large number of studies have focused on the mechanistic pathways of lipid entrapment within the intimal space resulting from dysregulated lipid metabolism, in addition to reversing the narrowing vessel lumen. Under basal conditions, the endothelium is protected against atherogenesis due to laminar blood flow, high levels of superoxide dismutase, and genes pertaining to shear stress that inhibit vascular cell adhesion molecule (VCAM) gene expression (Nomura *et al.* 2011; Hsu *et al.* 2012). Alternatively, areas that are predisposed to lesion formation are sites of branched arteries due to disrupted laminar blood flow. Endothelial cells begin to express increasing amounts of VCAM-1 on the outer membrane when laminar flow is disrupted, thereby recruiting monocytes into the intimal space (Nomura *et al.* 2011). In addition to the upregulated expression of VCAM-1, P- and E-selectin are attracted to the vessel causing the recruitment of monocytes leading to diapedeses and penetration into the tunica intima. Within the intima, the monocytes undergo a morphological change and differentiate into macrophages that express high numbers of scavenger receptors (SRA and CD36) on their surface, which enables the internalization of glycated or oxidized low-density lipoproteins (oxLDLs) (Kim and Byzova 2014).

In this report, we will focus on CD36, a member of the SR-B1 family of scavenger receptors, and the ability of oxLDL to act as a ligand for CD36 thereby increasing NADPH oxidase activity. The general topography of CD36 is evolutionarily conserved. It has 27-amino-acid hydrophobic transmembrane domains near the carboxyl-terminus and an uncleaved signaling peptide at the amino-terminus that includes an uncleaved signal peptide that may potentially be a second membrane spanning domain (Lambeth and Neish 2014). It functions in cholesterol uptake and also facilitates the transfer of long-chain fatty acids into the cell via its fatty-acid translocase (FAT) activity (Libby 2002). A range of diverse and pathologic processes are mediated by CD36 including energy metabolism via phagocytosis of long-chain fatty acids in adipocytes and striated muscle cells, as well as phagocytosis of oxidized lipids in macrophages. It is well established that macrophages engulf oxLDL in an unregulated manner leading to the foam cell morphology and increased pro-atherogenic activities (Silverstein and Febbraio 2000). The localization of CD36 in the cholesterol rich microdomains could potentiate its signaling and internalization functions, as well as the expression capacity increasing with the differentiation of monocytes to macrophages in response to an influx of pro-atherogenic cytokines and increased exposure to LDL and oxLDL (Silverstein and Febbraio 2000).

In macrophages, CD36 plays a major role in pro-inflammatory and pro-atherogenic signaling molecules that induces the intracellular migration of oxLDLs, in addition to generation of ROS, upregulation of focal adhesion kinase (FAK), and inhibition of SHP-2 (Silverstein and Febbraio 2000). Furthermore, platelet activation is suggested to have a direct relationship with the response of CD36 to oxLDLs both in

vitro and in vivo through NADPH oxidase-derived superoxide and reactive oxygen species (Silverstein *et al.* 2010). The binding of oxLDL to CD36 induces a signaling cascade that activates Src kinases, c-Jun N-terminal kinase and intracellular signaling that activates the multi-subunit NADPH oxidase (Park *et al.* 2009; Moore and Tabas 2011; Hopkins 2013; Ross *et al.* 2014; Silverstein 2015). The formation of the NADPH oxidase complex through CD36-oxLDL binding is a significant source of oxidants in the vessel wall leading to chemically reactive molecules migrating across the endothelium. We hypothesize that the activation of NADPH oxidase is linked to enhanced endocannabinoid tone (Chapter 4). It has been well established that the endocannabinoid system is composed of two receptors (CB_1 and CB_2), their ligands and the enzymes that degrade the ligands. Our group has demonstrated that activating the NADPH oxidase complex via intracellular or extracellular-superoxide can increase 2-arachidonylglycerol (2-AG) biosynthesis; which can be abrogated by chemically inhibiting the NADPH oxidase (see Chapter 4). The aim of this study was to determine whether NADPH oxidase activity increases following ligation of CD36 by oxLDL, thus providing the oxidants needed to activate the synthesis of the endocannabinoid 2-AG. The mechanism of NADPH oxidase activation (Nox2 isoform in macrophages) involves phosphorylation of a cytosolic $p47^{phox}$ subunit and its subsequent translocation to membranes where Nox2 is found (Han *et al.* 1997). These studies used the human monocyte/macrophage cell line HL-60 as a model because of its robust expression of Cd36 and Nox2 subunits (Park *et al.* 2009; Silverstein *et al.* 2010).

3.2 Materials and Methods

3.2.1 Chemicals and reagents.

Authentic standards of 2-AG, 2-AG-d_8, and arachidonic acid (AA) were from Cayman Chemicals (Ann Arbor, MI). O,O'-Diethyl p-nitrophenyl phosphate (paraoxon, PO) was a kind gift from Dr. Howard Chambers (Mississippi State University). Avidin-horseradish peroxidase, N-acetyl cysteine (NAC), dimethyl sulfoxide (DMSO), trypan blue solution (0.4% w/v), β-mercaptoethanol, phorbol 12-myristate 13-acetate (PMA), fatty-acid free bovine serum albumin (BSA), penicillin, streptomycin, hydroethidine (HE), and all buffer components were purchased from Sigma (St. Louis, MO). VAS-2870, a selective Nox inhibitor, was purchased from Enzo Life Sciences (Farmingdale, NY). Monoheptadecanoin (MHDG) internal standard, was purchased from NuCheck (Elysain, MN). HyperSep Retain PEP column, BCA reagent and HPLC grade solvents were purchased from Thermo-Fisher. FuGene 6 Transfection Reagent was purchased from Promega Chemicals (Madison, WI). Plasmid Midi Kits were purchased from QIAGEN. Human DAGLβ plasmid was purchased from Origene (Bethesda, MD). Anti-human DAGLβ (ab103100) and anti-V5 tag (ab 182008) antibodies were purchased from Abcam (Cambridge, MA). Goat anti-rabbit IgG and goat anti-mouse IgG antibodies were purchased from Cayman Chemicals. Acetylated oxidized (ox)LDL were from Intracel (Bethesda, MD). Primers for diacylglycerol lipase beta (DAGLβ), CB_1, CB_2, $p47^{phox}$, NOX2, GAPDH, and CD36 were purchased from Invitrogen.

3.2.2 Cells, culture conditions and stimulation.

Human HL-60 cells, THP-1 cells, COS-7 cells, RPMI-1640 medium with and without phenol red (containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium

pyruvate, 4500 mg/L glucose and 1500 mg/L sodium bicarbonate) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Fetal bovine serum (FBS) was purchased from Invitrogen (Grand Island, NY). THP-1 monocytes were passaged in RPMI-1640 containing 10% FBS and 50 µg/mL gentamicin (complete medium) (Magwenzi *et al.* 2015). COS-7 cells were passaged in DMEM medium containing 10% FBS and 10 units/mL penicillin and 10 µg/mL streptomycin (complete medium) (Magwenzi *et al.* 2015; Silverstein 2015). HL-60 cells were passaged in RPMI-1640 containing 10% FBS 10 units/mL penicillin and 10 µg/mL streptomycin (complete medium). Cells were cultured at 37°C in an atmosphere of 95% air/5% CO_2. HL-60 and THP-1 cells were differentiated into macrophage-like cells by adding PMA to the culture medium (final concentration 100 nM) and growing the cells for 72 h.

A human DAGLβ expression plasmid (Origen) was transformed into One-Shot Top10 chemically competent E. coli and the cloned plasmid was purified using QIAGEN Plasmid Midi Kit following the manufacturer's instructions. COS-7 cells were transfected with either human DAGLβ cDNA or empty plasmid (control) using the FuGene 6 Transfection Reagent in Opti-MEM medium and the transfected cells incubated overnight. The transfection medium was removed and replaced with fresh (complete) DMEM medium.

3.2.3 Preparation of cell lysates.

THP-1 monocytes were collected by centrifugation (1000 x g for 5 min), washed with cold phosphate-buffered saline (PBS), resuspended in ice-cold 50 mM Tris-HCl buffer (pH 7.4), and lysed by sonication (three 15 s bursts on ice at 30% maximum power). THP-1 macrophage monolayer (~80-90% confluent) were washed with cold

PBS, scraped into ice-cold 50 mM Tris-HCl (pH 7.4) buffer, and sonicated. COS-7 cells transfected with DAGLβ and control COS-7 cells were harvested with 2 mL Accutase for 5 min. Fresh DMEM containing 10% FBS was added to stop the Accutase reaction. The detached cells were pelleted at 1000 x g (5 min), washed 3 times with sterile PBS, resuspended in ice-cold 50 mM Tris-HCl (pH 7.4) buffer, and sonicated. Protein concentrations of the cell lysates were determined using the BCA reagent according to the manufacturer's instructions (Thermo-Fisher). Cell lysates were used fresh or flash frozen and stored at -80°C prior to use.

3.2.4 Macrophage CD36 expression and NADPH oxidase activity.

The expression of CD36 was determined using RT-PCR. Functional studies examining oxLDL uptake was determined by utilizing oxLDLs and controls were treated in same manner, without oxLDL. NADPH oxidase was first inhibited in the macrophage-like HL-60 cells by pre-incubating the cells in plain RPMI -1640 containing 10 μM VAS-2870 for 30 min. Subsequently, the macrophage were incubated for 24 h with 50 μg/mL oxLDL followed by 10 incubation with 3.2 μM PMA. COS-7 cells were transfected as previously described. Because NOX2 and $p47^{phox}$ are the catalytic subunits of NADPH oxidase, and DAGLβ is a second messenger in the biosynthesis of 2-AG, RT-PCR was done to measurement their mRNA expression levels to determine whether NADPH oxidase is modulated in CD36-oxLDL induced superoxide production.

3.2.5 2-Arachidonoylglycerol biosynthesis by HL-60 cells.

Macrophages were incubated in serum-free medium with 10 μM VAS-2870 for 30 min prior to stimulation, to inhibit NADPH oxidase. Nox was activated via the

addition of PMA (3.2 μM) for 30 min at 37°C. The macrophages were washed in phosphate buffered saline (PBS), scraped in 50 mM Tris-HCl buffer, transferred into microcentrifuge tubes, and sonicated 3 times for 15 sec at 30% power. The oxyradical probe HE (20 μM final concentration) was added to the cells and incubated for an additional 20 mins. The cells were washed, collected in 300 μL of 1:1 methanol: water, centrifuged for 5 min at 16,000 rpm, and supernatant was collected for UPLC analysis to determine the rate of 2-OH-Et^+, the specific oxidative product of HE. The culture medium was collected to measure the production of 2-AG. For 2-AG analysis, chemical reactions were quenched with the addition of 2 volumes ethyl acetate containing 0.1% acetic acid with deuterated 2-AG (internal standard). The samples were vortexed for 1 min and centrifuged at 3,000 x g for 5 min. Alternatively, HL-60 cells were pre-treated with VAS-2870 (10 μM) for 30 mins followed by the addition of 3.2 μM PMA (10 min). The samples were extracted using HyperSep Retain PEP columns. The column was activated on vacuum with 2 mL ethyl acetate followed by 4 mL methanol; 2 mL of 95:5 water: methanol containing 0.1% acetic acid was added to equilibrate. The culture medium was collected and loaded to the column. MHDG was spiked in as internal standard. The column was eluted into a clean glass centrifuge tube with 0.5 mL of methanol followed by 2 mL ethyl acetate. The organic solvent was dried under nitrogen, and resuspended in 1:1 methanol: water for UPLC analysis.

3.2.6 Quantitative real-time PCR.

The SMCs RNA was isolated using RNeasy Plus Mini Kit according to the manufacturer's instructions. NanoDrop ND-1000 spectrophometer (Thermo Scientific, Walham, MA) was used to quantify recovered RNA followed by cDNA synthesis using

iScript Select cDNA Synthesis Kit (BioRad) according to manufacturer's instructions. Real-time PCR of cDNA was performed on a Stratagene Mx3005P thermocycler with Quantifast SYBR Green PCR master mix from Qiagen. Primers are detailed in Table 3.1. The cycle program used for all target genes consisted of 5 min hot start at 95°C prior to 10 s at 95°C for 40 cycles, followed by 30 s at 60°C. The dissociation curve was generated for PCR product quality immediately after analysis. Expression differences were normalized using GAPDH and quantified using the ΔΔCT method. The results generated were in fold differences of gene expression.

Table 3.1 Primer sequence used for quantitative real-time PCR

Gene (Homo sapien)	Forward Sequence	Reverse Sequence
CB_1	CAACAAGTCTCTCTCGTCCT	GATGAAGTGGTAGGAAGGC
CB_2	CACTGATCCCCAATGACTAC	CCACTCCGTAGAGCATAGAT
NOX2	CGAAATGATGGTGACTGGCT	CCCAGCCAGTGAGGTAGATGT
P47phox	ATTCACAAGCTCCTGGACGG	ACACGTCTTGCCCTGACTTT
DAGLB	Hs_DAGLB_1_SG QuantiTect Primer Assay – QT00074319	
GAPDH	Hs_GAPDH_1_SG QuantiTect Primer Assay – QT00079247	

3.2.7 LC-MS/MS analysis.

2-AG analysis was performed on an UPLC-MS/MS system (Waters Acquity UPLC interfaced with a Thermo Quantum Access Max triple quadrupole mass spectrometer) using the method described by Wang et al. (Mangum *et al.* 2015). Extracts were injected (10 µL) onto an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 µm) equipped with a VanGuard pre-column (2.1 mm × 5 mm, 1.7 µm) at 40°C using column oven. For 2-AG, 2-AG-d_8 and MHDG, the mobile phases were a blend of

solvent A (0.1% (v/v) acetic acid in water) and solvent B (0.1% (v/v) acetic acid in methanol). The elution program was described previously (Gaut and Carchman 1987; Ricciarelli *et al.* 2000) and the flow rate was 0.2 mL/min. The column eluate was directed into the mass spectrometer using heated electrospray ionization in the positive ion mode. Single-reaction monitoring (SRM) of analytes were: 2-AG, $[M+H]^+$ m/z 379.2>287.2; 2-AG-d_8, $[M+H]^+$ m/z 387.2>295.2; MHDG, $[M+H]^+$ m/z 344.8>326.8. The internal standard 2-AG-d_8 or MHDG were used for quantification. MS parameters were set as follows: spray voltage = 3500 V, vaporizer temperature = 350°C, sheath gas = 25 units, auxiliary gas = 2 and capillary temperature = 350°C. Scan times were 0.2 s per SRM and the scan width was m/z=0.01. Collision energies and the tube lens voltage were optimized using autotune software for each analyte by post-column infusion of the individual compounds into a 50%A/50%B blend of the mobile phase being pumped at a flow rate of 0.2 mL/min. Xcalibur software was employed for data acquisition and processing.

3.2.8 Statistical analyses.

Data are expressed as means ± SD. Statistical significance between two groups was determined by Student's t-test. Statistical significance between more than two groups of data were compared by one-way ANOVA (Student-Newman-Keuls method). Values of $p < 0.05$ were considered to be significant.

3.3 Results

3.3.1 Macrophage CD36 expression and NADPH oxidase activity.

Our lab previously reported that COS-7 cells overexpressing human DAGLβ treated with arachidonic acid had the ability to increase the production of 2-AG, which could be abrogated upon treating the cells with either a Nox or DAGLβ inhibitor (Chapter 4). These data extended previous reports by demonstrating a vital role for DAGLβ in the synthesis of the anti-inflammatory cannabinoid receptor ligand 2-AG following the activation of NADPH oxidase (Figure 3.1 A).

RT-PCR data revealed an increase in mRNA expression of DAGLβ and the NADPH oxidase regulatory subunit, $p47^{phox}$, but not NOX2, when HL-60 cells were exposed to oxLDLs (Figure 3.1 B). It is well documented that the CB_1 receptor contributes to the inflammatory response seen with the influx of oxidative stress, whereas the CB_2 receptor plays a role in anti-inflammatory responses. Therefore, we used oxLDL-treated HL-60 macrophage-like cells and measured the mRNA levels of the CB receptors to determine whether the treatment would modulate the CB receptor expression (Figure 3.1 C). The data revealed an increased expression of both CB_1 and CB_2 when treated with oxLDL as compared with the control group, although the upregulation of CB_2 was less marked than for CB_1.

3.3.2 Role of carboxylesterases in human DAGLβ overexpressing cells.

Because 2-AG acts locally and is rapidly degraded by hydrolytic enzymes (Wang *et al.* 2013), we determined the JZL184-inhibitable level of *para*-nitrophenyl valerate (pNPV) hydrolysis activity in the HL-60 cell line, which represents the carboxylesterase (CES) activity. We observed that roughly 50% of the pNPV hydrolysis activity in the

HL-60 cells was inhibited by JZL184 (Figure 3.2A), indicating the proportion of pNPV hydrolysis activity that is attributable to CES in the HL-60 cell line. In a subsequent experiment, we treated COS-7 cells overexpressing human DAGLβ with either PO or CPO to inhibit eCB hydrolytic activity (Wang *et al.* 2013), to prevent the degradation of 2-AG. Treatment with both inhibitors significantly increased the level of 2-AG compared to DMSO treated controls (Figure 3.2 B,C).

3.3.3 PMA-induced 2-arachidonylglycerol biosynthesis.

Superoxide is increased following stimulation of HL-60 macrophage-like cells with phorbol ester. Levels of superoxide, as measured using the surrogate compound 2-OH-Et^{+}, which is the specific superoxide-derived oxidized product of the HE probe, were increased after incubations of cells with PMA (Figure 3.3A). Furthermore, the formation of the Nox holoenzyme is a complex pathway that can be influenced by an array of biochemical events, including the liberation of arachidonic acid by phospholipase A2 to promote the conformational changes of $p47^{phox}$ that activate it (Figure 3.3 B). In addition, oxLDL can potentially enhance the biosynthesis of 2-AG, thus increasing the endocannabinoid concentrations in macrophages (Figure 3.6).

To understand the role that NADPH oxidase plays in the production of oxyradicals and 2-AG biosynthesis, we examined whether PMA-induced oxidative stress could be blocked by inhibiting the formation of the Nox subunits. First, we treated macrophage-like HL-60 cells with oxLDL to determine whether DAGLβ protein expression would increase. The western blot analysis revealed an increased band intensity for DAGLβ protein in the oxLDL-treated cells compared to untreated cells (Figure 3.4). We treated cells with PMA in the presence or absence of a Nox inhibitor

and demonstrated that PMA could increase the amount of superoxide produced (as measured by 2-OH-Et^{+}) (Figure 3.5A). Further, 2-AG biosynthesis was also abrogated by addition of the Nox inhibitor (Figure 3.5 B).

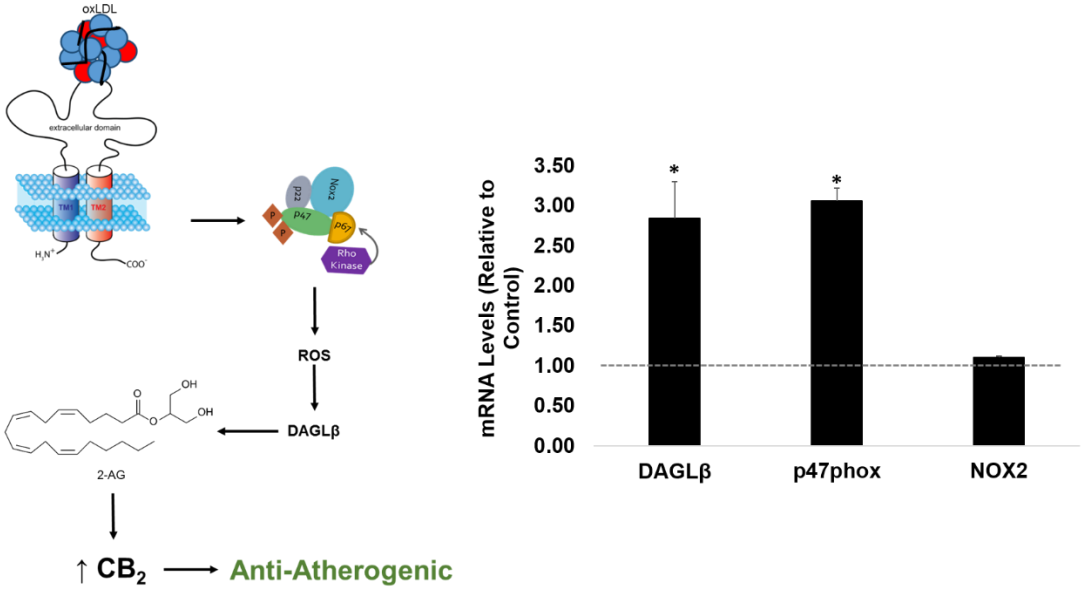

Figure 3.1 oxLDL induced mRNA of DAGLβ, Nox subunits, and cannabinoid receptors.

(A) Scheme of oxLDL binding CD36 to stimulate the NOX2. NOX2 activation leads to the production of ROS enhancing human DAGLβ expression, the second messenger 2-AG production. This mechanistic pathway potentiates anti-atherogenic effects through the upregulation of CB_2 receptor. (B) HL-60 cells were differentiated into macrophages by PMA (100 nM, 72 h) then treated with oxLDL (50 µg/mL for 24 h) or left untreated. The levels of NOX2, p47phox, and DAGLβ mRNA were determined in each group. Data represents mean ± SD n=3. * $p<0.05$, Student's t-test.
OxLDL, Oxidized low density lipoprotein; NOX2, NADPH oxidase; ROS, reactive oxygen species; DAGLβ, diacylglycerol lipase-beta; PMA, phorbol 12-myristate 13-acetate; CB, cannabinoid.

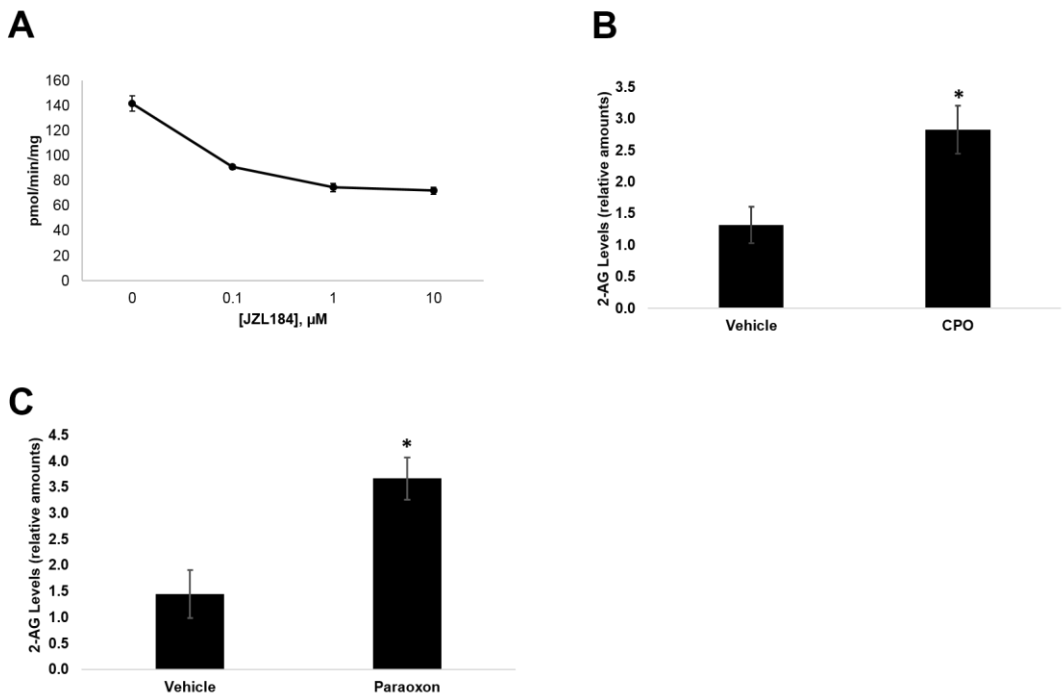

Figure 3.2 HL-60 cells have the biochemical machinery to produce 2-AG which can be hydrolyzed by serine hydrolases.

(A) Activity progress curve for the hydrolysis of pNPVa by CES. HL-60 cells were treated with [0-10 µM] JZL184 for 30 mins followed by pNPVa hydrolysis assay. CES activity was monitored for 5 min at 37°C. The pNPVa was nearly completely hydrolyzed by the CES enzymes. (B) Human DAGLβ overexpressed COS-7 cells lysate (0.25 mg/mL) was incubated with CPO (0.1 µM) for 30 min. The reaction was quenched with two volumes of ethyl acetate containing 0.1% acetic acid doped with 2-AG-d_8, and the organic layer was collected and dried under nitrogen. The residue was re-suspended in 1:1 (v/v) methanol/water to quantify 2-AG levels by LC-MS/MS. (C) Human DAGLβ cells lysate (0.25 mg/mL) was incubated with PO (50 µM) for 30 min and extracted as described above. These data demonstrated a significant increase in 2-AG biosynthesis in the presence of the serine hydrolase enzymes that degrade 2-AG. Data represents mean ± SD n=3. * $p<0.05$, Student's t-test.
Para Nitrophenyl valerate, pNPVa; carboxylesterase, CES; chlorpyrifos oxon, CPO; 2-arachidonylglycerol, 2-AG; paraoxon, PO.

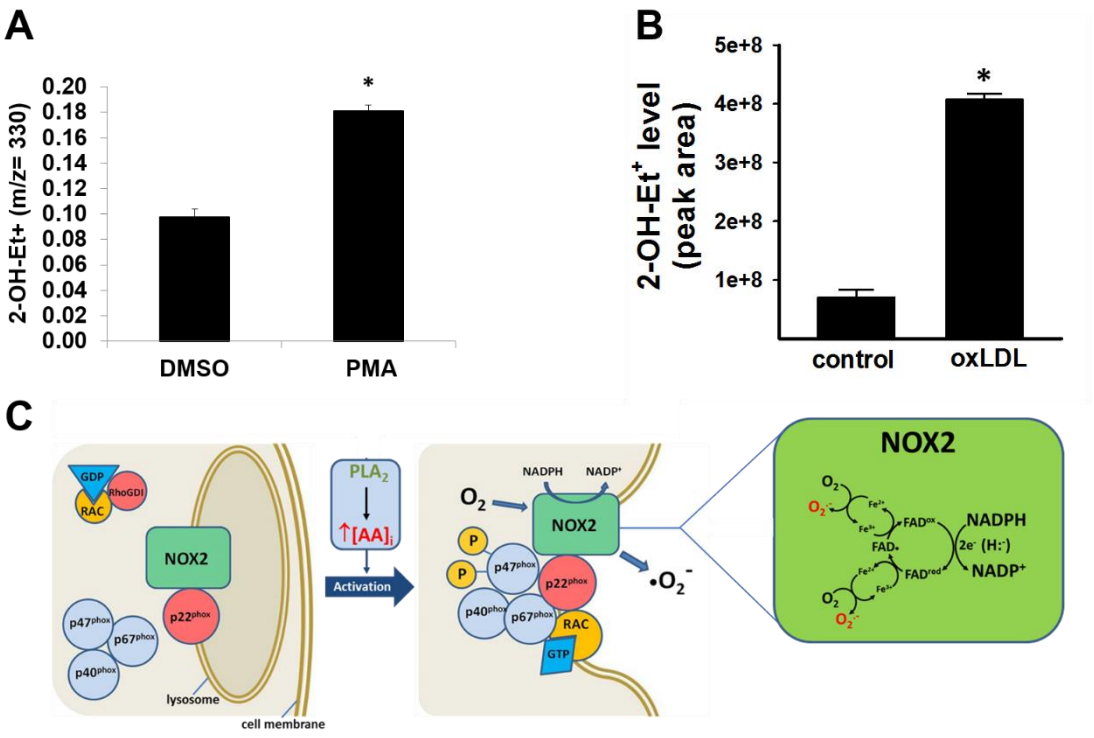

Figure 3.3 Human THP-1 macrophages treated with PMA or oxLDL increased the production of superoxide.

Treatment of THP-1 macrophages PMA (A) and oxLDL (B) heighted the production of 2-OH-Et^+, the specific oxidative product of hydroyethidine (HE) compared to the control. (C) Scheme describing the activation of the Nox multi-subunit complex in cells. Several biochemical events converge to increase Nox activity. These include arachidonic acid (AA_i) being liberated inside the cell by the action of phospholipase A_2 (PLA_2) on glycerophospholipids, which activates protein kinase C (PKC) and promotes conformation changes in p47phox; small G-protein Rac activation; and diacylglycerol (DAG)-mediated PKC activation, which results in the phosphorylation of p47phox (PMA mimics the stimulatory effects of DAG on PKC activity). The output of these various steps results in the functional activation of the catalytic Nox2 subunit. This permits the transfer of 2 electrons from NADPH to the FAD co-factor and the sequential one-electron reduction of two heme moieties, resulting in one-electron reductions of molecular oxygen to yield superoxide anion. Data represents mean ± SD n=3. * $p<0.05$, Student's t-test.

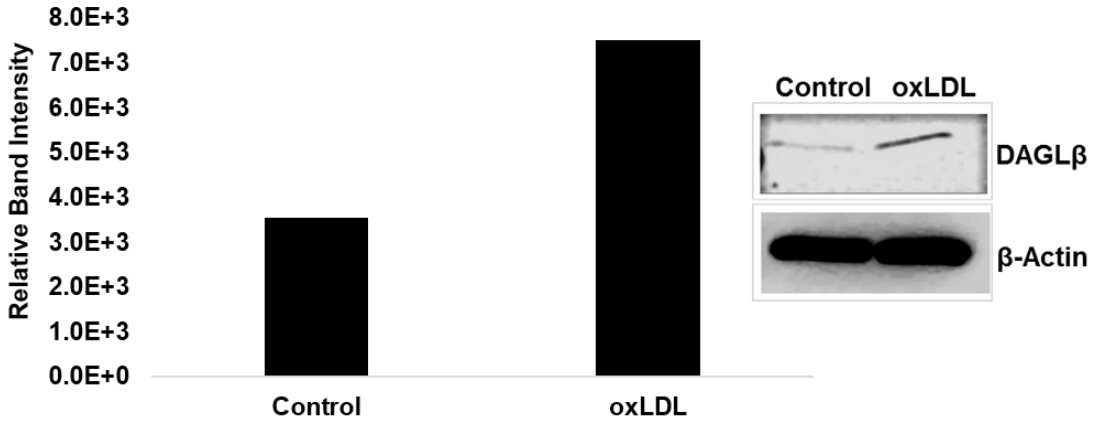

Figure 3.4 OxLDL induced the expression of the key 2-AG biosynthetic enzyme DAGLβ.

HL-60 cells were differentiated to macrophage like cells with PMA (100 nM). The macrophage-like cells were challenged with oxLDL (50 µg/mL) for 24 h exhibited an increase DAGLβ protein expression in oxLDL treated cells compared with non-oxLDL treated controls.
Diacylglycerol lipase beta, DAGLβ; oxidized low density lipoprotein, oxLDL.

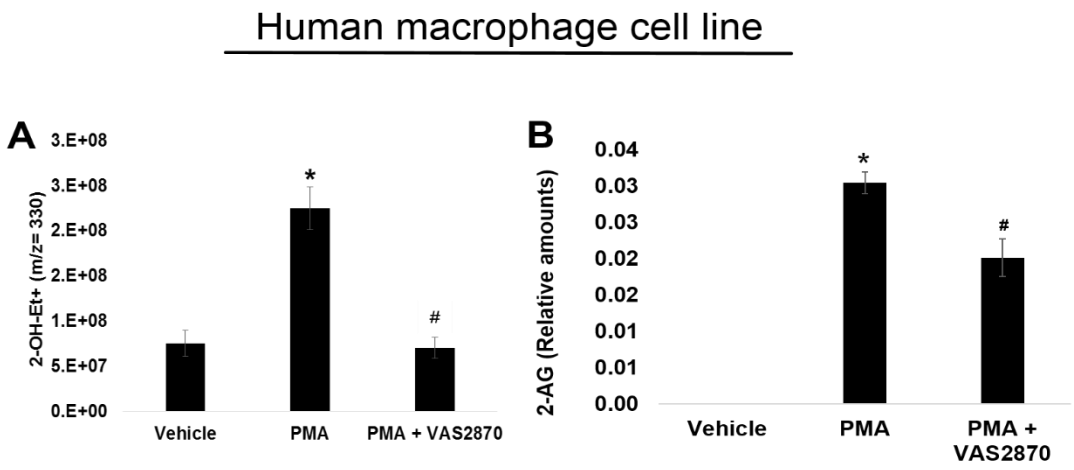

Figure 3.5 PMA-induced superoxide are dependent on Nox activity.

The superoxide-specific product 2-OH-Et (A) and 2-AG (B) levels were significantly higher in HL-60 cells treated with 0.32 µM of PMA, but these data were significantly abrogated with the addition of 10 µM Nox inhibitor, VAS2870. Data represents mean ± SD n=3. * $p<0.05$ relative to vehicle control; # $p<0.05$ relative to PMA–treated cells, one-way ANOVA (Student-Newman-Keuls method).
Oxidized low density lipoprotein, oxLDL; 2-hydroxyehtidium, 2-OH-Et+; NADPH oxidase, Nox.

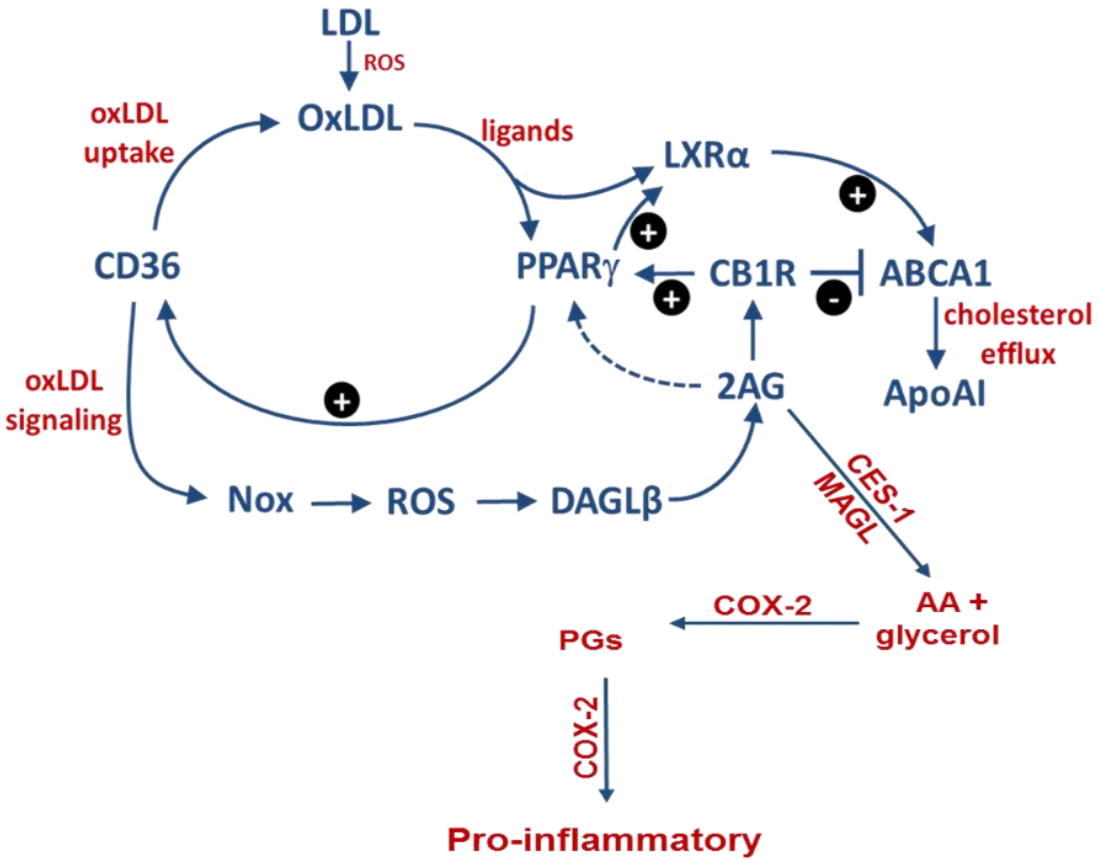

Figure 3.6 Scheme representing pro-inflammatory cascade.

LDL become oxLDL with a high affinity for CD36. The CD36-oxLDL complex activate Nox leading to enhanced ROS. With eCB ligands having equal affinity for both receptors, the pathway can lead to either be pro- or an anti-inflammatory. (Oxidized) Low density lipoprotein, (ox)LDL; NADPH oxidase, Nox; reactive oxygen species, ROS; (endo)cannabinoid, eCB; diacylglycerol lipase beta, DAGLβ, cyclooxygenase, COX; prostaglandin, PG; 2-arachidonylglycerol, 2-AG; arachidonic acid, AA; ATP binding cassette A1, ABCA1; apolipoprotein A1, ApoA1; carboxylesterase, CES; monoacylglycerol lipase, MAGL; peroxisome proliferator-activated receptor gamma, PPARγ; liver X receptor alpha, LXRα.

3.4 Discussion

The formation of lipid-laden foam cells is hallmark in the pathophysiology of atherosclerosis. The recruitment of monocytes to the intima and the subsequent internalization of oxLDL leads to chronic inflammation within the vessel wall. Numerous studies have elegantly described the multiplicity of CD36 functions in response to oxLDL and other mediators due to the inverse relationship between CD36, the principal receptor for cellular cholesterol influx, and ABCA1, a key transporter in cholesterol efflux. An upregulation in PPARγ receptor, which leads to increased CD36 expression, causes a feed-forward action on PPARγ. Macrophage uptake of oxLDL via the upregulation of scavenger receptor CD36 can indeed lead to enhanced oxidative stress in phagocytic cells (Wang *et al.* 2013). Further in a study by Jiang et al (2009) using the non-specific cannabinoid agonist WIN 55,212-2 it was shown that the PPARγ receptor was upregulated, thereby increasing CD36 expression. However, the levels of ABCA1 expression was significantly reduced in the presence of the CB_1 antagonist AM251. Our lab expounded on these data using menadione, a redox cycling agent that produces superoxide, in J774A.1 macrophages. Levels of superoxide following treatment of WIN 55,212-2 were reduced (Chapter 4).

Studies have been conducted as to the role of the cannabinoid system during atherogenesis. There is emerging evidence that chronic low doses of Δ9-tetrahydrocannabinol (THC), the active component of marijuana, can potentially inhibit the progression of atherosclerotic lesions through interactions involving interferon gamma and decreased macrophage infiltration into the intimal space (Steffens *et al.* 2005; Jiang *et al.* 2009). However, there is conflicting data for THC. THC can reduce in

lymphoid cell proliferation, infiltration of macrophages, and interferon gamma secretion, which are characteristics of atheromas (Jiang *et al.* 2009). On the other hand, Chen et al (2015) reported that interferon gamma was enhanced by THC in the C57BL/6 mouse model, but whether this effect was mediated via the CB_1 and/or CB_2 receptors was unclear. The endogenous ligand 2-AG is thought to be an athero-protective by means of downregulating pro-inflammatory cytokines including tumor necrosis factor-alpha (TNF-α), IL-1β, and monocyte chemoattractant protein-1 (MCP-1), while upregulating CD18/CD11b (CR3) (Pacher and Steffens 2009). There is a large body of literature suggesting that 2-AG biosynthesis and the upregulation of CB_2 receptor levels has the ability to reduce atherosclerotic lesions in vessels. Here we demonstrated enhanced levels of 2-AG and increased CB_1 and CB_2 receptor expression, in the presence of oxLDL. Furthermore, when RAW264.7 macrophages were incubated for 24 h with >5 μM of the non-selective CB agonist WIN 55,212-2, the levels of PPARγ were markedly increased (Jiang *et al.* 2009). In agreement with those findings, our data suggest that the oxLDL have the capacity to stimulate the production of oxyradicals leading to the biosynthesis of 2-AG. To support our work on NADPH oxidase leading to enhanced biosynthesis of 2-AG (Chapter 4), we examined whether inhibiting NADPH oxidase would modulate the endocannabinoid system and whether this is a protective response against intracellular oxidative stress. HL-60 cells are widely used in oxyradical studies because they have an abundance of NADPH oxidase (Gaut and Carchman 1987). We demonstrated in this study using this cell line, that the mRNA expression of components of the NADPH oxidase complex was increased when stimulated with PMA, which could be abrogated when NADPH oxidase was inhibited. Similarly, THP-1 and J774A.1 cells pre-treated

with PO or CPO following treatment with PMA expressed similar results (Chapter 4) (Wang *et al.* 2013).

The most effective therapeutic strategies for CVD targets risk factors such as hypertension with the use of angiotensin II receptor antagonist or hypercholesterolemia with statins. Although both these pharmaceutical regimens have their benefits, alternative methods to reduce the prevalence of CVD are needed. Targeting the endocannabinoid system is an emerging strategy to reduce chronic inflammation that leads to atherosclerosis. It has been well documented that increasing the CB_2 receptor (Booz 2011) and decreasing the CB_1 receptor (Pacher and Steffens 2009) on innate immune cells can block the production of pro-inflammatory mediators and decrease the occurrence of reactive oxygen species. Although more research is necessary to detail the specific pathways of activation, we have identified that CD36-oxLDL-evoked signaling results in increased levels of reactive oxygen species activation of the Src kinase family, PKC and the NADPH oxidase (Magwenzi *et al.* 2015). This study provided insight into the role of CD36-oxLDL modulation of the endocannabinoid 2-AG. Since oxLDL can lead to the phosphorylation of p47phox in a Src-CD36-dependent manner (Magwenzi *et al.* 2015), increased CD36 receptor expression can indeed correlate with increased foam cell formation. 2-AG has the ability to modulate intracellular oxidation and inflammation associated with the pathophysiology of atherogenesis to maintaining balance within the cardiovascular wall.

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CHAPTER IV

HEIGHTENED OXYRADICAL STRESS IN MACROPHAGES INCREASES BIOSYNTHESIS OF THE ENDOCANNABINOID 2-ARACHIDONOYLGLYCEROL

4.1 Introduction

The development of cardiovascular disease (CVD) is caused by several environmental, genetic, and lifestyle factors (Libby *et al.* 1997). Atherosclerosis is a primary cause of CVD and is due to altered homeostatic processes that regulate lipid metabolism and inflammation (Ross *et al.* 2014). Initiating events that contribute to the development of atherosclerosis include endothelial dysfunction, hypertension, hyperlipidemia, and inflammation (Ross *et al.* 2014). The resulting pathology leads to the overproduction of reactive oxygen species (ROS) that chemically modify entrapped low-density lipoproteins (LDL), yielding toxic oxidized (ox)LDL. The danger-associated molecular patterns revealed on the oxLDLs can be recognized by scavenger receptors on intimal macrophages, enabling the phagocytosis of these cholesterol-enriched particles and development of macrophage foam cells (Moore and Tabas 2011). Thus, an imbalance in ROS biosynthesis and catabolism in vascular wall cells results in significant oxidative stress and is an important contributor to disease.

ROS, such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), are produced in most cell types in the vessel wall in response to a variety of external stimuli, including

nitric oxide, oxLDL, shear stress, and angiotensin II. These stimuli activate several ROS-generating enzymes including NADPH oxidase (Nox), cyclooxygenase, lipoxygenase, xanthine oxidase, uncoupled nitric oxide synthase, and myeloperoxidase, resulting in significant oxidative stress (Booz 2011; Griendling 2013; Ross *et al.* 2014). The elevated levels of ROS also cause a buildup of oxLDL levels in the vessel wall and the influx of monocytes and neutrophils from the blood stream, resulting in a detrimental inflammatory feedback loop. The increased flux of oxyradicals and the peroxidation of extracellular LDL is a hallmark of atherosclerosis.

OxLDL can stimulate CD36 scavenger receptor-evoked signal transduction pathways in macrophages and platelets, leading to the activation of Nox and the synthesis of superoxide (Miller *et al.* 2010; Rajesh *et al.* 2010; Park *et al.* 2011; Magwenzi *et al.* 2015). Nox is a multisubunit holoenzyme that catalyzes the transfer of electrons from NADPH to molecular oxygen (Lambeth *et al.* 2007). This results in the production of superoxide, which has a limited ability to diffuse through the cell but can cross lipid membranes via anion channels. Superoxide rapidly dismutates to H_2O_2, which has a more tempered reactivity and greater diffusion radius compared to superoxide. Because H_2O_2 is not charged, it is capable of moving through lipid membranes via passive diffusion. The overactivation of Nox can be countered by several compensatory mechanisms, including detoxification of excess superoxide by superoxide dismutase (SOD) (Ross *et al.* 2014), activation of endoplasmic reticulum stress-induced apoptotic pathways (Feng *et al.* 2003), and possibly by the endocannabinoid system (Sugamura *et al.* 2010).

The endocannabinoid (eCB) system is comprised of several components. These include two G protein-coupled receptors, CB_1 and CB_2, which are mainly expressed in the central nervous system and hematopoietic system, respectively. The CB receptors are activated by endogenous arachidonoyl-containing ligands 2-arachidonoylglycerol (2-AG) and anandamide (AEA). Diacylglycerol lipase (DAGL)α and β are the rate-limiting enzymes involved in the biosynthesis of 2-AG in the brain and macrophages, respectively (Hsu *et al.* 2012), while monoacylglycerol lipase (MAGL) is the primary enzyme that degrades 2-AG (Blankman and Cravatt 2013). The CB_1 receptor has important roles in neurotransmission in the central nervous system; whereas, the CB_2 receptor has an immunomodulatory function in peripheral immune responses. Activation of CB_2 can attenuate the following immune processes in macrophages: (i) inflammatory cell migration and endotoxin-induced oxidative stress (Booz 2011), (ii) activation of Nox (Horvath *et al.* 2012), and (iii) p38-MAPK activation and TNF-α production (Pacher and Steffens 2009; Hao *et al.* 2010; Rajesh *et al.* 2010; Hopkins 2013). Furthermore, increased biosynthesis of 2-AG and evoked CB_2 receptor-dependent signal transduction pathways have been associated with the amelioration of atherosclerotic lesions in a mouse model (Sugamura *et al.* 2010). It was also shown that cultured macrophages exposed to oxLDL can produce greater quantities of 2-AG and AEA (Jiang *et al.* 2009). Thus, the endocannabinoid system plays an important role in macrophage function; however, there is a gap in knowledge regarding the specific pathways that lead from exposure to oxLDL to the increases in endocannabinoid levels.

Because it is known that exposure to oxLDL results in increased ROS production by macrophages, the purpose of this study was to explore whether (and how) the

endocannabinoid system becomes activated in macrophages in response to acutely elevated superoxide levels (and heightened Nox activity). Specifically, the levels of 2-AG and AEA were determined following treatments of cultured macrophages with either an extracellular superoxide generating system (xanthine oxidase) or with Nox stimulators (phorbol ester and arachidonic acid). A large body of evidence has suggested that activation of the CB_2 receptor can render cardioprotective effects (Pacher and Steffens 2009; Hao *et al.* 2010; Rajesh *et al.* 2010; Hopkins 2013); therefore, enhanced 2-AG levels may be part of a compensatory mechanism to activate anti-inflammatory and anti-oxidative pathways within vascular cells. We have addressed this issue by inducing high levels of oxidative stress in cells using a redox cycling agent (menadione) and examining the effects of a non-selective cannabinoid receptor agonist (WIN 55,212-2). In this report, we show that oxyradical stress in macrophages increases the biosynthesis of 2-AG in both human and mouse macrophage cell lines and in a cell line engineered to overexpress DAGLβ. Moreover, we showed that WIN 55,212-2 can partly attenuate the oxidative stress induced by menadione.

4.2 Materials and Methods

4.2.1 Chemicals and reagents.

Authentic standards of 2-AG, 2-AG-d_8, arachidonic acid (AA), 1-stearoyl-2-arachidonoylglycerol (SAG), WIN 55,212-2, AM630, and rimonabant were from Cayman Chemicals (Ann Arbor, MI). O,O'-Diethyl p-nitrophenyl phosphate (paraoxon, PO) was a kind gift from Dr. Howard Chambers (Mississippi State University). Avidin-horseradish peroxidase, dimethyl sulfoxide (DMSO), lactate dehydrogenase (LDH), xanthine and xanthine oxidase, trypan blue solution (0.4% w/v), β-mercaptoethanol,

phorbol 12-myristate 13-acetate (PMA), fatty-acid free bovine serum albumin (BSA), penicillin, streptomycin, menadione (MD), hydroethidine (HE), ionomycin, apocynin, U73122, Orlistat, and all buffer components were purchased from Sigma (St. Louis, MO). VAS-2870, a selective Nox inhibitor, was purchased from Enzo Life Sciences (Farmingdale, NY). BCA reagent and HPLC grade solvents were purchased from Thermo-Fisher. FuGene 6 Transfection Reagent was purchased from Promega Chemicals (Madison, WI). Plasmid Midi Kits were purchased from QIAGEN. Human DAGLβ plasmid was purchased from Origene (Bethesda, MD). Anti-human DAGLβ (ab103100) and anti-V5 tag (ab 182008) antibodies were purchased from Abcam (Cambridge, MA). Goat anti-rabbit IgG and goat anti-mouse IgG antibodies were purchased from Cayman Chemicals. Acetylated (ac)LDL and oxLDL were from Intracel (Bethesda, MD).

4.2.2 Cells and culture conditions.

Human THP-1 monocytes, murine J774A.1 macrophages, human HL-60 cells, COS-7 cells, RPMI-1640 medium with and without phenol red (containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4500 mg/L glucose and 1500 mg/L sodium bicarbonate), Dulbecco's modified Eagle's medium (DMEM) with and without phenol red (containing 4 mM L-glutamine, 4500 mg/L glucose, 1 mM sodium pyruvate and 1500 mg/L sodium bicarbonate), Opti-MEM medium, and gentamicin sulfate solution (50 mg/mL) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA). Fetal bovine serum (FBS) and One-Shot TOP10 chemically competent E. coli were purchased from Invitrogen (Grand Island, NY).

THP-1 monocytes were passaged in RPMI-1640 containing 10% FBS and 50 µg/mL gentamicin (complete medium) (Wang *et al.* 2013); J774A.1 macrophages and COS-7 cells were passaged in DMEM medium containing 10% FBS and 10 units/mL penicillin and 10 µg/mL streptomycin (complete medium) (Wang *et al.* 2013); HL-60 cells were passaged in RPMI-1640 containing 10% FBS and penicillin and streptomycin. All cells were cultured at 37°C in an atmosphere of 95% air/5% CO_2. THP-1 cells and HL-60 cells were differentiated into macrophage-like cells by adding PMA to the culture medium (final concentration 100 nM) and growing the cells for 72 h.

4.2.3 Expression of human DAGLβ in COS7 cells.

A human DAGLβ expression plasmid was transformed into One-Shot Top10 chemically competent E. coli and the cloned plasmid was purified using QIAGEN Plasmid Midi Kit following the manufacturer's instructions. COS-7 cells were transfected with either human DAGLβ cDNA or empty plasmid (control) using the FuGene 6 Transfection Reagent in Opti-MEM medium and the transfected cells incubated overnight. The transfection medium was removed and replaced with fresh (complete) DMEM medium. After an additional 48 h incubation, the medium was replaced again with (complete) DMEM medium supplemented with G418 (500 µg/mL) and the cells were maintained in this medium for 3 weeks. The culture medium was replaced every 3 days with fresh medium containing G418 to select for positive clones. After 3 weeks, total RNA was isolated from the DAGLβ-transfected and mock-transfected cells, and the level of DAGLβ mRNA was determined by quantitative-real time-PCR. In addition, the transformed cells were washed with PBS and harvested in 50 mM Tris-HCl (pH 7.4) buffer and sonicated with 3 short bursts on ice. The 1-steroyl-2-

arachidonoylglycerol (SAG) hydrolysis activity of the cell lysates was determined (see below).

4.2.4 Preparation of cell lysates.

THP-1 monocytes were collected by centrifugation (1000 x g for 5 min), washed with cold phosphate-buffered saline (PBS), resuspended in ice-cold 50 mM Tris-HCl (pH 7.4) buffer, and lysed by sonication (three 15 s bursts on ice at 30% maximum power). THP-1 macrophage and J774A.1 macrophage monolayers (~80-90% confluent) were washed with cold PBS, scraped into ice-cold 50 mM Tris-HCl (pH 7.4) buffer, and sonicated. COS-7 cells transfected with DAGLβ and control COS-7 cells were harvested with Accutase (2 mL, 5 min). Fresh DMEM containing 10% FBS was added to stop the Accutase reaction and the detached cells were pelleted at 1000 x g (5 min), washed 3 times with sterile PBS, resuspended in ice-cold 50 mM Tris-HCl (pH 7.4) buffer and sonicated. Protein concentrations of the cell lysates were determined using the BCA reagent according to the manufacturer's instructions (Thermo-Fisher). Cell lysates were used fresh or flash frozen and stored at -80°C prior to use.

4.2.5 2-Arachidonoylglycerol biosynthesis by THP-1 macrophages.

THP-1 macrophage lysates (0.5 mg/mL protein concentration) were treated with $CaCl_2$ (10 μM final concentration) in 200 μL of 50 mM Tris-HCl (pH 7.4) in the absence or presence of 1 mM ethylene glycol tetraacetic acid (EGTA), 10 μM U73122, or 10 μM Orlistat. Incubations went for 5 min at 37°C with shaking (550 rpm). The reactions were quenched by the addition of 300 μL of ethyl acetate (containing 0.1% acetic acid) and fortified with 2-AG-d_8 (internal standard). After centrifugation of samples at 1500 x g (5

min, 4°C), the organic layer was collected in a clean tube and dried under N_2. Residues were reconstituted in 100 µL methanol/water (1:1, v/v) and the levels of 2-AG quantified by LC-MS/MS.

4.2.6 SAG hydrolysis assay.

Cell lysate was diluted to 0.3 mg/mL lysate in DAGL buffer (5 mM $CaCl_2$, 100 mM NaCl, 50 mM HEPES buffer, pH 7.4) (final volume 70 µL). SAG (20 µM final concentration) was added and the reaction duration was 30 min (37°C). Reactions were stopped by adding ethyl acetate (containing 0.1% acetic acid) fortified with 2-AG-d_8. After drying the organic extracts, the residues were reconstituted in methanol:water (1:1, v/v) for LC-MS/MS analysis.

4.2.7 2-AG biosynthesis of human DAGLβ-transfected COS7 cells.

To determine the endogenous level of 2-AG in DAGLβ-transfected COS7 cells as compared to native COS-7 cells, we seeded the cells in two 6 well plates to grow to 80% confluency followed by incubation with 3 µM ionomycin for 30 min at 37°C. The culture medium was extracted with 2-AG-d_8 adulterated ethyl acetate, and the organic extract was dried under nitrogen and reconstituted in methanol:water (1:1, v/v) for LC-MS/MS analysis. In a subsequent experiment, cells were pretreated with 10 µM orlistat, a DAGLβ inhibitor, for 30 min, followed by treatment with 3 µM ionomycin for an additional 30 min. The culture medium was extracted to quantify 2-AG levels and cell lysates were used for the SAG hydrolysis assay.

To determine the role of NADPH oxidase in the DAGLβ-transfected COS-7 cells, the cells were pretreated with a Nox inhibitor (10 µM VAS) for 30 min followed by an

additional 30 min incubation with 10 μM AA to stimulate Nox activity (Mangum *et al.* 2015). The culture medium was extracted to quantify 2-AG levels and cell lysates were used for the SAG hydrolysis assay.

4.2.8 Treatment of macrophages with either extracellular superoxide or Nox activators

4.2.8.1 LDH cytotoxicity assay.

THP-1 monocytes (2 x 10^5 cells/well) were differentiated into macrophages (PMA, 100 nM, 72 h) in a 96-well plate. The macrophages were treated with increasing concentrations of xanthine (0-250 μM) and a fixed amount of xanthine oxidase (0.1 mU/mL) in 200 μL of PBS for 15 min at 37°C in an atmosphere of 95% air/5% CO_2. Cells were monitored for overt cytotoxicity by mixing 100 μL of the culture supernatant with 2 volumes of LDH reagent solution, followed by incubation at 37°C for 30 min to determine LDH activity (at 490 nm in a plate reader). Alternatively, cell lysates were prepared in 50 mM Tris-HCl (pH 7.4) and the intracellular LDH activity was determined (at 690 nm in a plate reader).

4.2.8.2 Quantitation of reactive oxygen species in macrophages.

J774A.1 macrophages or THP-1 macrophages in phenol red-free culture medium (1 x 10^5 cells/well in a 96-well plate) were treated for 30 min with PMA (0.32 μM) or DMSO, followed by the addition of the oxyradical probe HE (20 μM final concentration). Immediately after adding the HE, the fluorescence signal was monitored for 90 min in a Molecular Devices SpectraMax M5 plate reader (ex: 485 nm; em: 590 nm). Fluorescence was normalized against cell protein content to control for variations in cell number in each well.

The Nox activity of a THP-1 macrophage lysate was determined by incubating lysate protein (0.3-0.5 mg/ml) with lucigenin (5 μM final concentration) and NADPH (100 μM final concentration) in 50 mM Tris-HCl (pH 7.4) buffer containing 1 mM EGTA. The resulting luminescence was monitored for 15 min in a Molecular Devices SpectraMax M5 plate reader. Generation of superoxide by a xanthine oxidase system was also determined using the lucigenin probe.

The superoxide-specific product 2-OH-Et^+ (Zielonka *et al.* 2009) was quantified by LC-MS/MS, as previously reported (Mangum *et al.* 2015). Briefly, cells were treated with either PMA (3.2 μM) or oxLDL (50 μg/mL) in phenol red- and FBS-free culture medium for 30 min at 37°C, followed by the addition of the HE probe (20 μM final concentration). After a 20 min incubation with the HE probe, the cells were harvested and washed with PBS. The cells were then sonicated in 300 μL methanol:water (1:1, v/v) and the lysates were centrifuged (16,100 x g for 5 min). The supernatant following centrifugation was loaded into LC vials and analyzed by LC-MS/MS (Mangum *et al.* 2015).

4.2.8.3 Determination of 2-AG levels in macrophages.

THP-1 macrophages (in 60-mm dishes) were pretreated with 1 μM paraoxon (PO) in serum-free medium for 30 min, to prevent 2-AG degradation by hydrolytic enzymes, followed by either a 10-min incubation with 150-μM xanthine–0.1-mU/mL xanthine oxidase or a 24-h incubation with acLDL (20 μg/mL). The culture medium was harvested, fortified with 2-AG-d_8, and extracted with 2 volumes ethyl acetate containing 0.1% acetic acid. The organic layer was collected and dried under N_2, and samples were

reconstituted with 100 µL methanol:water (1:1, v/v). Extracts were analyzed by LC-MS/MS to quantify 2-AG levels.

J774A.1 macrophages were treated with increasing concentrations of PMA (0, 0.32, 3.2, or 32 µM) in 1-mL PBS. After a 10 min incubation, the culture medium was fortified with 2-AG-d_8 and extracted with ethyl acetate to quantify the levels of 2-AG. Due to the short incubations (10 min) and because 2-AG is degraded less rapidly in J774A.1 cells relative to THP-1 cells (Xie *et al.* 2010), the J774A.1 cells were not pretreated with a serine hydrolase inhibitor prior to adding PMA. To determine whether Nox has a role in 2-AG biosynthesis, macrophages were pretreated with a Nox inhibitor (10 µM apocynin) prior to adding stimulants (PMA or AA).

4.2.8.4 Role of cannabinoid receptors in MD-derived superoxide levels in macrophages.

Macrophages (J774A.1 or THP-1) were overlaid with FBS-free and phenol red-free culture medium containing either the CB_1 receptor antagonist rimonabant (1 µM) or the CB_2 receptor antagonist AM630 (1 µM) for 30 min, followed by addition of the non-specific cannabinoid receptor agonist WIN 55,212-2 (10 or 25 µM, 24 h incubation). After removal of the agonists and antagonists, the cells were challenged with the redox cycling agent MD (40 µM) for 30 min. The culture medium was removed and fresh medium containing the oxyradical probe HE (10 µM) was added. After 20 min incubation with the probe, the macrophages were washed and harvested in 300 µL of 1:1 methanol:water, sonicated, and the lysate centrifuged (16,100 x g, 10 min, 4°C). The supernatant was collected for LC-MS/MS analysis of 2-OH-Et^+ (Mangum *et al.* 2015).

In a separate experiment, the macrophages were pretreated with the selective cannabinoid receptor antagonists for 30 min, followed by a 4-h incubation with WIN 55,212-2. MD (40 μM final concentration) was added directly to the culture medium containing the agonists and antagonists (i.e., compounds were not removed before adding MD), and the incubation proceeded for an additional 30 min. The culture medium was removed and fresh medium containing the oxyradical probe HE (10 μM) was added. After 20 min, the macrophages were washed and harvested in 300 μL methanol/water (1:1, v/v), sonicated, centrifuged, and the supernatant collected.

4.2.8.5 LC-MS/MS analysis.

2-AG analysis was performed on a UPLC-MS/MS system (Waters Acquity UPLC interfaced with a Thermo Quantum Access Max triple quadrupole mass spectrometer) using the method described by Wang et al. (2013). Extracts were injected (10 μL) onto an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm) equipped with a VanGuard precolumn (2.1 mm × 5 mm, 1.7 μm). For 2-AG and 2-AG-d_8, the mobile phases were a blend of solvent A (2 mM ammonium acetate and 0.1% acetic acid in water) and solvent B (2 mM ammonium acetate and 0.1% acetic acid in methanol). The elution program was described previously (Wang *et al.* 2013) and the flow rate was 0.4 mL/min. The column eluate was directed into the mass spectrometer using heated electrospray ionization in the positive ion mode. Single-reaction monitoring (SRM) of analytes were: 2-AG, $[M+NH4]^+$ m/z 396 > 287; 2-AG-d_8, $[M+NH4]^+$ m/z 404 > 294. Scan times were 0.2 s per SRM and the scan width was m/z 0.01. The internal standard 2-AG-d_8 was used for quantification.

4.2.8.6 Statistical analyses.

Data are expressed as means ± SD. Statistical significance between two groups was determined by Student's t-test. Statistical significance between more than two groups of data were compared by one-way ANOVA (Student-Newman-Keuls method). Values of $p < 0.05$ were considered to be significant.

4.3 Results

4.3.1 Treatment of human macrophage-like cells with oxLDL enhances superoxide levels

It was previously reported that modified LDLs stimulated Nox activity (and elevated intracellular ROS) in macrophages in a CD36-dependent manner (Shen 2010; Shen 2012; Noda *et al.* 2015). Consistent with this, we found that mRNA levels for the catalytic subunit and cytosolic regulatory protein of Nox (Nox2 and $p47^{phox}$, respectively) were induced in human HL-60 cells by either PMA or all-*trans*-retinoic acid (Figure 4.1A). The rationale for using differentiated HL-60 cells was that these cells express CD36 (Greenberg *et al.* 2006) and Nox2 (Brechard *et al.* 2009). Nox activity in the PMA-primed cells was stimulated by exogenous arachidonic acid, as assessed by the rate of oxidation of the oxyradical probe HE (Figure 4.1B). Moreover, superoxide levels [assessed by measuring the superoxide-specific oxidation product 2-hydroxyethidium (2-OH-Et^+) by LC-MS/MS] in HL-60 macrophages treated with oxLDL were significantly higher than those in untreated controls (Figure 4.1C). These data are consistent with the notion that oxLDL can stimulate Nox activity (and increase superoxide levels) in macrophages (Shen 2010; Shen 2012; Noda *et al.* 2015).

4.3.2 Human THP-1 and murine J774 cells biosynthesize 2-arachidonoylglycerol

Macrophages exposed to oxLDL are also known to make the endocannabinoid 2-AG (Jiang et al. 2009; Xie et al. 2010); however, the mechanistic details for this response have not been clarified. Phospholipase C β (PLCβ) and diacylglycerol lipase β (DAGLβ) are calcium-dependent enzymes that have important roles in signal transduction pathways and are involved in the biosynthesis of 2-AG. PLCβ cleaves phosphatidylinositol 4,5-bisphosphate lipids releasing diacylglycerol (DAG), then DAGLβ specifically hydrolyzes the DAG 1-acyl-2-arachiondoylglycerol at the sn-1 position to provide 2-AG. To understand the connection between oxLDL-derived ROS and 2-AG biosynthesis, we next examined whether 2-AG biosynthesis is increased in human and murine macrophage models under various states of oxidative stress. Human THP-1 cells and murine J774A.1 cells were used because they are widely used models of macrophage biology.

First, we verified that macrophage lysate (THP-1) could be activated by calcium ions to yield 2-AG (Figure 4.2). Addition of PLCβ and DAGLβ inhibitors (U73122 and Orlistat, respectively) blocked the effect of calcium on 2-AG production. Furthermore, addition of the calcium chelator EGTA also prevented 2-AG synthesis. Second, incubation of SAG, a DAGLβ substrate, with macrophage lysate (J774A.1) resulted in a marked increase in 2-AG (~14-fold; Figure 4.3). Together, these data are consistent with literature indicating that elevated calcium levels are vital for the production of 2-AG (Okada et al. 2011; Signorello et al. 2011; Shonesy et al. 2015; Szekeres et al. 2015). It also demonstrates that THP-1 and J774A.1 cells have the biochemical machinery to produce 2-AG.

4.3.3 Macrophages challenged by extracellular superoxide flux

Intact THP-1 macrophages were subjected to oxyradical stress by treatment with an extracellular superoxide generating system (xanthine oxidase; 15 min) (Figure 4.4). Superoxide production by the xanthine oxidase system was verified by the detection of the lucigenin-derived product (Figure 4.4) and the HE-derived 2-OH-Et^+ product (Figure 4.4), a specific product formed by the reaction of the HE probe with superoxide (Zielonka *et al.* 2009). To assess the xanthine oxidase-derived superoxide flux, a cell-free incubation of xanthine oxidase system with cytochrome c for 3 min enabled the flux to be determined (271±2 pmol/min or 1.36±0.01 μM/min). The xanthine oxidase-derived superoxide flux was not overtly cytotoxic to THP-1 cells (Figure 4.5). Moreover, intact THP-1 macrophages treated with the xanthine oxidase system produced increased amounts of 2-AG (2.3-fold) compared with the untreated cells (Figure 4.6). Elevated 2-AG levels were also observed in another cell line (COS-7 cells) treated with the xanthine oxidase system (data not shown).

4.3.4 Activation of macrophage NADPH oxidase is associated with increases in 2-AG levels

We verified that treatment of either J774A.1 macrophages or THP-1 macrophages with PMA (a Nox activator) in the presence of the oxyradical probe HE caused significant increases in the rate of HE oxidation relative to the vehicle-treated cells (Figure 4.7A,B). Furthermore, PMA caused a concentration-dependent increase in 2-AG levels in J774A.1 macrophages (Figure 4.7C). Importantly, the PMA-induced 2-AG production was abrogated by the Nox inhibitor apocynin (Figure 4.7D). Similar findings were observed in PMA-primed HL-60 cells treated acutely with PMA (3.2 μM; Figure

4.8, Figure 4.9). These data taken together suggest that 2-AG biosynthesis is correlated with increased Nox activity and oxyradical stress.

Because modified LDLs can stimulate Nox activity in a CD36-dependent manner (Shen 2010; Shen 2012; Noda et al. 2015), THP-1 macrophages were treated with acLDL (which has high affinity for CD36) in the presence or absence of 1 μM paraoxon, which stabilizes 2-AG by inhibiting carboxylesterase 1-mediated degradation of 2-AG in this cell line (Xie et al. 2010). Exposure of cells to acLDL resulted in increased 2-AG levels compared with control cells (Figure 4.10A). AcLDL alone caused a significant increase in 2-AG levels, while the combination of acLDL and paraoxon led to a slightly greater increase than that of acLDL alone. Furthermore, when murine and human cells (J774A.1 and THP-1 macrophages) were stimulated with the Nox activator arachidonic acid, significantly more 2-AG was produced as compared with DMSO- treated cells (Figure 4.10B). This effect was attenuated in THP-1 cells by a DAGLβ inhibitor (Figure 4.10B). The magnitude of the 2-AG levels was greater in the murine cell line compared with the human cell line, which might reflect the differences in Nox2 expression in mice and human macrophages (Gaines *et al.* 2005).

Superoxide rapidly dismutates to H_2O_2, which is a second messenger in signal transduction pathways (Griendling 2013; Ross *et al.* 2014). The addition of H_2O_2 to either intact living THP-1 macrophages or THP-1 cell lysates increased the levels of 2-AG when compared to vehicle controls (Figure 4.11A,B). This suggested that superoxide/H_2O_2 could stimulate DAGLβ activity. However, when recombinant DAGLβ was pre-treated with H_2O_2 its activity toward SAG was unchanged (Figure 4.12),

indicating that any effects of H_2O_2 on DAGLβ are probably indirect and not due to chemical modification of a cysteine residue that regulates enzyme activity.

4.3.5 Menadione-derived superoxide levels are attenuated by the cannabinoid receptor agonist WIN 55,212-2

Addition of the redox cycling agent MD (40 μM) to J774A.1 macrophages resulted in a significant increase in superoxide levels, as measured by 2-OH-Et^+ (Figure 4.13A,B). A non-selective cannabinoid receptor agonist, WIN 55,212-2, significantly attenuated the levels of MD-derived superoxide (Figure 4.13B). Similar effects of WIN 55,212-2 on superoxide levels were observed in THP-1 macrophages (Figure 4.14A). WIN 55,212-2 did not stimulate superoxide production in cells on its own (data not shown). In addition, the CB_1 and CB_2 receptor antagonists, rimonabant and AM630, respectively, both appeared to abrogate the anti-oxidative effects of WIN 55, 212-2 in J774A.1 macrophages (Figure 4.14B).

4.3.6 DAGLβ-dependent 2-AG biosynthesis following Nox activation by arachidonic acid

Stable overexpression of human DAGLβ expression in COS-7 cells was verified by measuring the DAGLβ mRNA level and SAG hydrolysis activity (Figure 4.15A); both parameters were significantly elevated in the DAGLβ-transfected cells compared to mock-transfected cells. Further, COS-7 cells overexpressing DAGLβ produced more 2-AG than native COS-7 cells when stimulated with ionomycin (Figure 4.15B). Pretreatment of cells with the DAGLβ inhibitor orlistat abrogated the response to ionomycin. Stimulation of DAGLβ-overexpressing cells with exogenous AA resulted in a marked increase in 2-AG levels compared to control (untreated DAGLβ-overexpressing

cells) (Figure 4.15C). This effect was attenuated by either a Nox inhibitor or DAGLβ inhibitor (Figure 4.15C). Similar effects on 2-AG levels were obtained using THP-1 cells treated with AA in the absence or presence of a Nox inhibitor (Figure 4.10B).

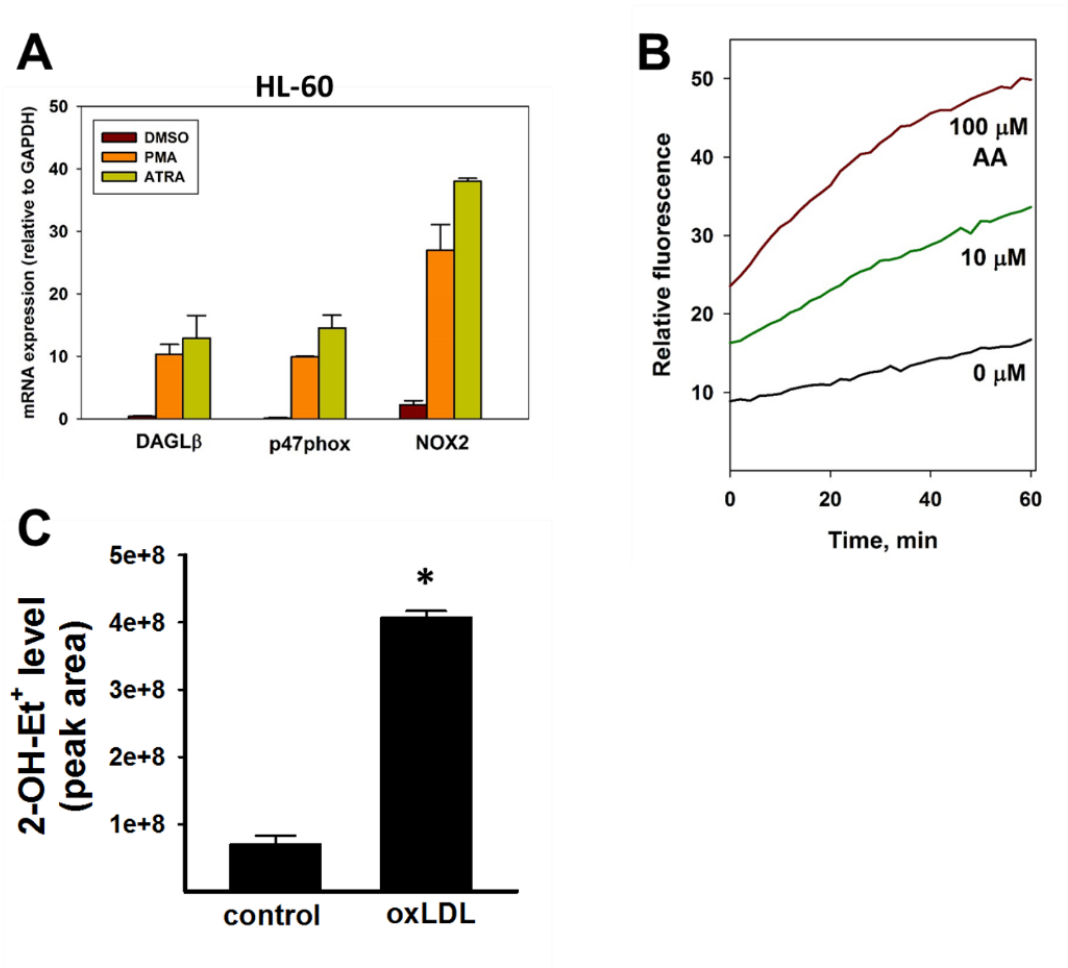

Figure 4.1 mRNA of NADPH oxidase (Nox) components and Nox-derived reactive oxygen species are induced in HL-60 macrophages by various stimulants.

(A) Human HL-60 cells treated with either PMA or all-trans-retinoic acid (ATRA) have increased levels of DAGLβ, $p47^{phox}$, and Nox2 mRNA compared to vehicle (DMSO)-treated cells. (B) PMA-primed HL-60 cells were treated with the indicated concentrations of arachidonic acid (AA), followed by the addition of HE (20 μM) and the rate of HE oxidation was monitored by fluorescence for 60 min. (C) Human HL-60 cells were differentiated into macrophages by PMA (100 nM, 72 h), then treated with oxLDL (50 μg/mL) for 24 h. The levels of superoxide in the cells was determined by adding the oxyradical probe HE for 20 min. After removal of the medium, the cells were extracted in methanol/water (1:1, v/v) (300 μL) and the relative amount of 2-OH-Et^+ (surrogate measure of superoxide) was quantified by LC-MS/MS by measuring the area under the chromatographic peak. Data represents mean ± SD n=3. * $p<0.05$, Student's t-test when two groups are compared, one-way ANOVA (Student-Newman-Keuls method) when more than two groups are compared.

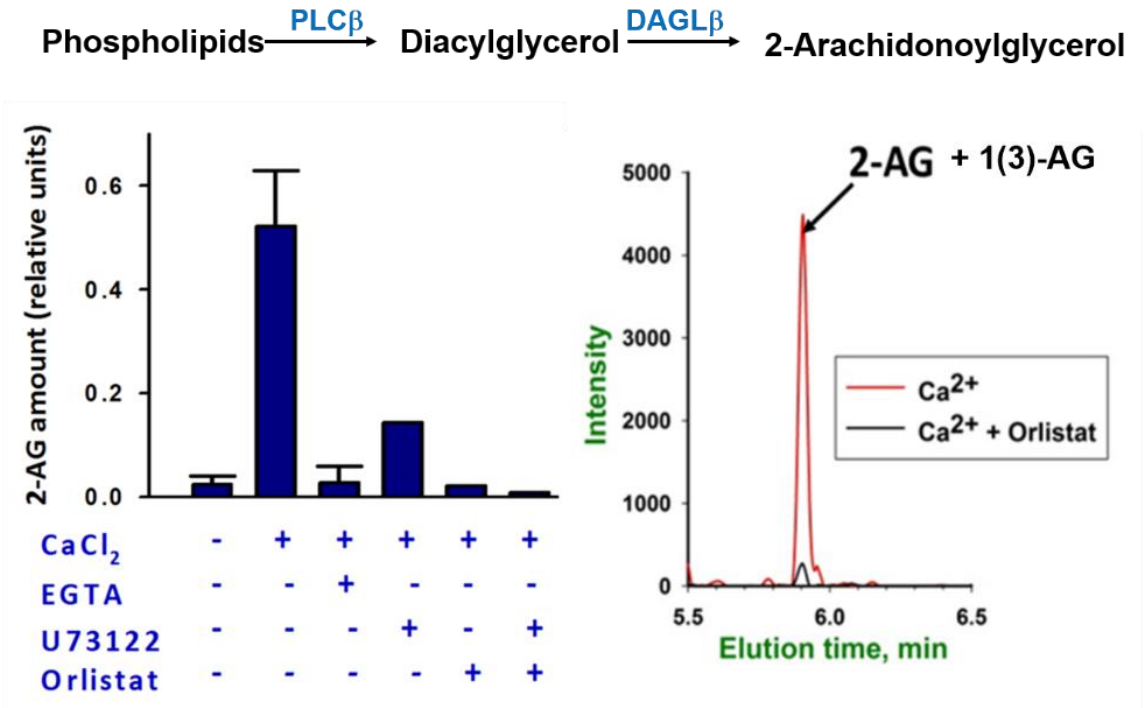

Figure 4.2 THP-1 and J774A.1 macrophages have the biochemical machinery to produce 2-AG.

THP-1 macrophage lysates make 2-AG when stimulated with Ca^{2+}. Calcium activates both PLC-β and DAGLβ activities, which can be blocked with the chelator EGTA (left). U73122 and Orlistat inhibit PLC-β and DAGLβ, respectively. In the presence of one or both of these inhibitors, 2-AG biosynthesis by the THP-1 macrophage lysate was blocked. Mass chromatogram (right) for 2-AG + 1(3)-AG indicated that the isomers of arachidonoylglycerol elute as one peak and that 2-AG biosynthesis was blocked by a DAGLβ inhibitor. Rearrangement of 2-AG to 1(3)-AG due to acyl migration is a well-characterized phenomenon (Shen 2010; Shen 2012; Noda *et al.* 2015). Data represents mean ± SD n=3.* $p<0.05$, Student's t-test.

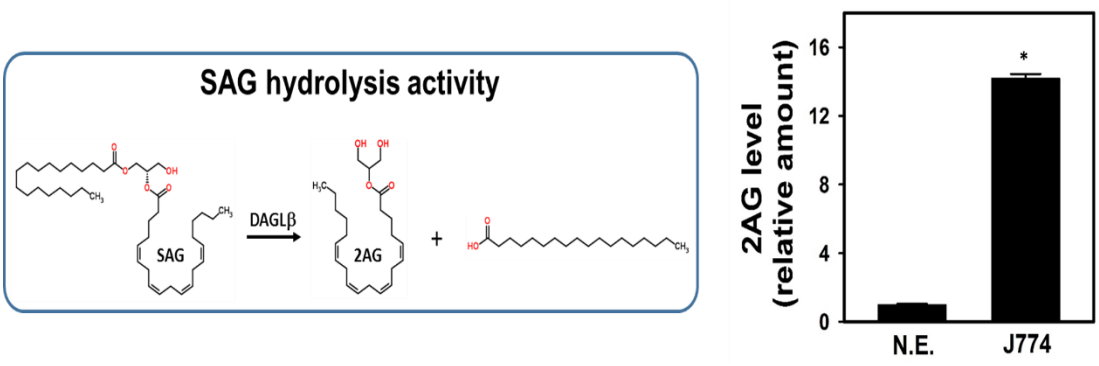

Figure 4.3 J774A.1 macrophages catalyze the formation of 2-AG.

J774A.1 macrophages catalyze the formation of 2-AG from the DAGLβ substrate 1-stearoyl-2-arachidonoylglycerol (SAG). Cell lysate (0.3 mg/ml) was incubated with exogenous SAG (20 μM) for 30 min. The reaction was quenched with one volume of 2:1 (v/v) chloroform/methanol doped with 2-AG-d_8, and the organic layer was collected and dried under nitrogen. The residue was re-suspended in methanol/water (1:1, v/v) to quantify 2-AG levels by LC-MS/MS. N.E., represents the non-enzymatic reaction. Data represents mean ± SD n=3.* $p<0.05$, Student's t-test.

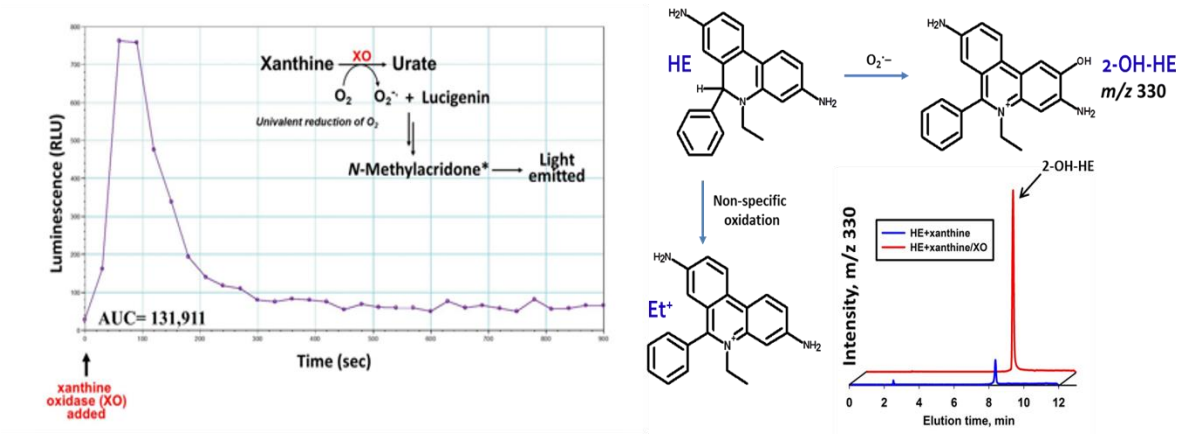

Figure 4.4 THP-1 macrophages treated with extracellular superoxide via a xanthine oxidase system enhanced the amount of 2-AG made by the cells.

The xanthine oxidase system liberates superoxide, which reacts with lucigenin to form the chemiluminescent product N-methylacridone*. The area-under-curve is a measure of the amount of superoxide produced. LC-MS/MS analysis confirmed that the superoxide-specific oxidation product 2-OH-Et^+ was produced when the oxyradical probe HE was added to the xanthine oxidase system (data not shown).

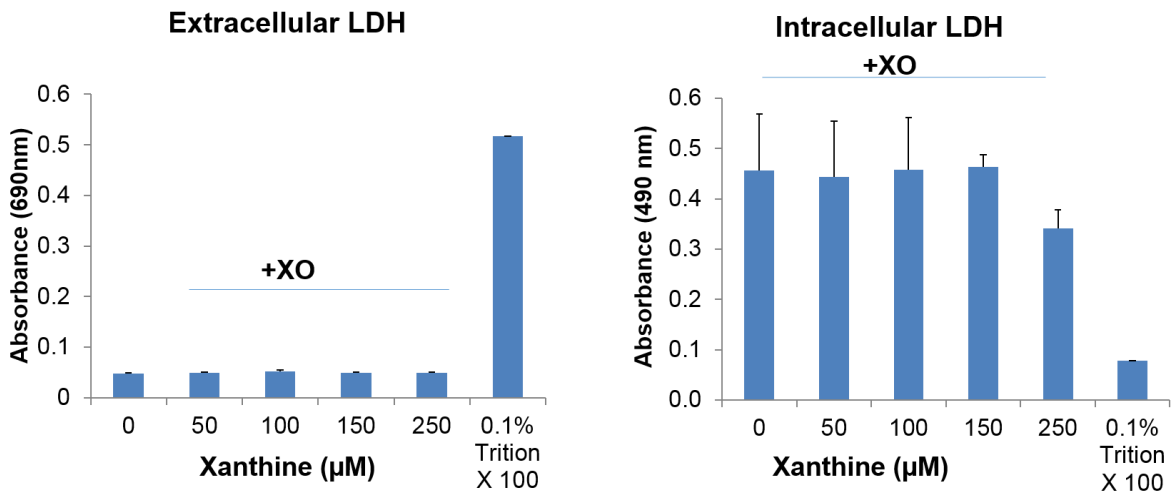

Figure 4.5 Xanthine oxidase system is not cytotoxic to THP-1.

Xanthine oxidase (XO)-derived superoxide was not cytotoxic to THP-1 macrophages following a 15 min incubation and can be used as a model of paracrine superoxide signaling (i.e., superoxide derived from the extracellular space). 0.1% Triton X100 was used as positive control for cell death. Data represents mean ± SD n=3. * $p<0.05$, Student's t-test.

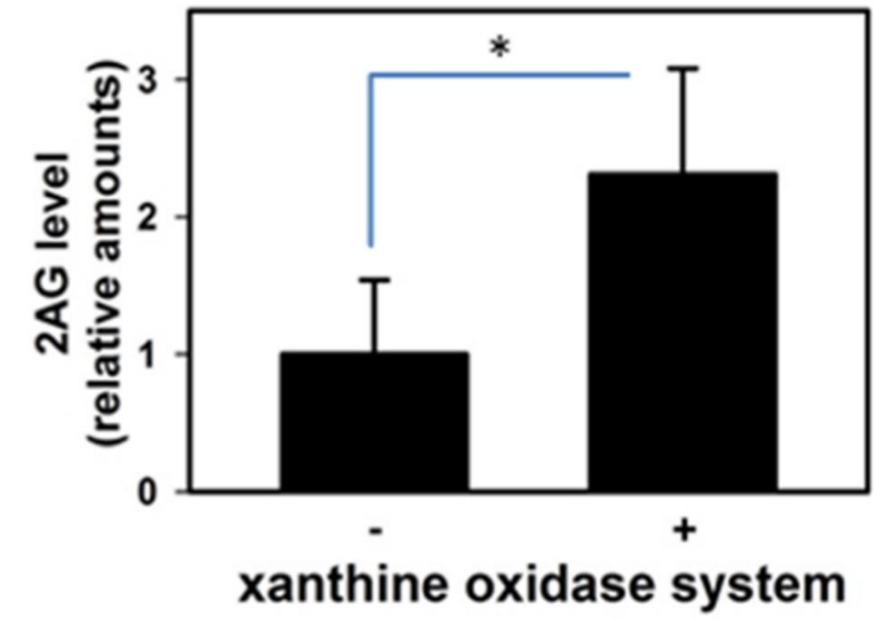

Figure 4.6 THP-1 macrophages treated with extracellular superoxide via a xanthine oxidase system enhanced the amount of 2-AG made by the cells.

Treatment of THP-1 macrophages with the extracellular xanthine oxidase system heighted the production of 2-arachidonoylglycerol compared to the negative control. Data represents mean ± SD. * $p<0.05$, Student's t-test.

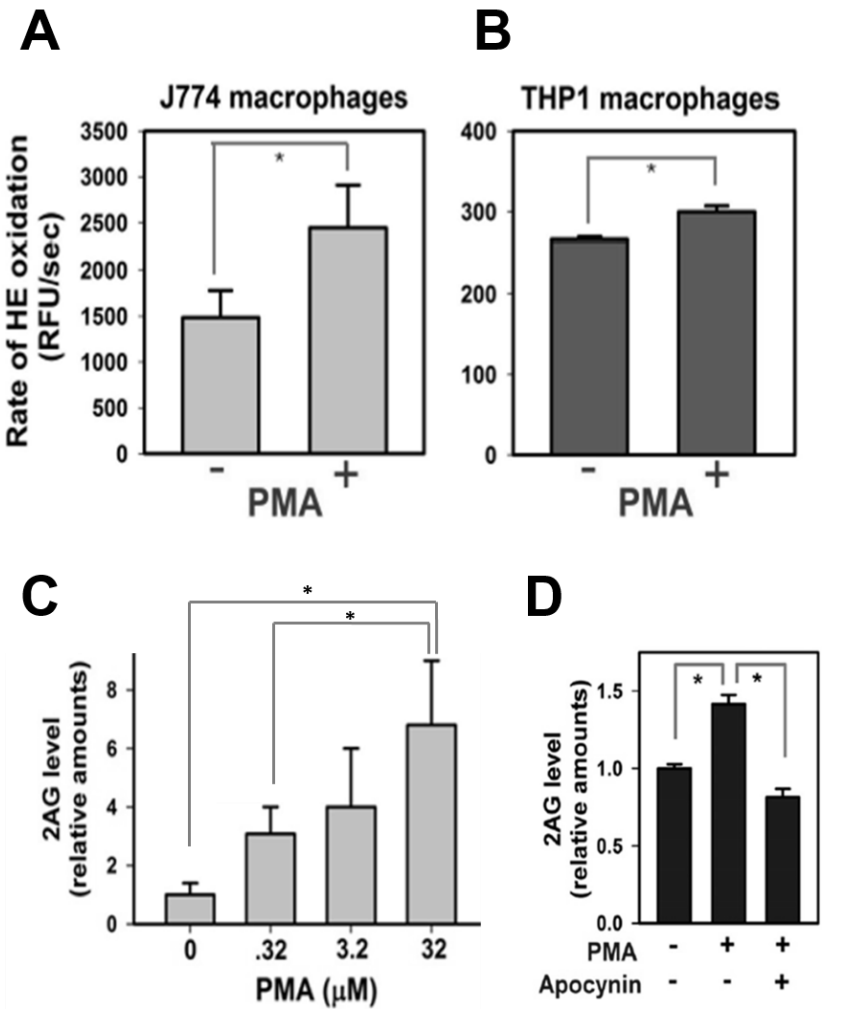

Figure 4.7 PMA-activated macrophages produce elevated amounts of superoxide and 2-AG.

Murine J774A.1 macrophages (A) and human THP-1 macrophages (B) were treated with either DMSO (PMA –) or 0.32 μM PMA (PMA +) for 30 min, then loaded with the oxyradical probe HE (20 μM). The resulting fluorescence was monitored for 90 min at 37°C. PMA-treated murine J774A.1 macrophages and human THP-1 macrophages exhibited a 65% and 13% increase in HE oxidation rates, respectfully, compared to vehicle-treated control. RFU, relative fluorescence units. (C) Murine J774A.1 macrophages treated with the indicated amount of PMA for 10 min exhibited a concentration-dependent increase in 2-AG production, suggesting that 2-AG biosynthesis is correlated with increased oxidative stress. (D) Pre-treatment of J774A.1 macrophages with the Nox inhibitor apocynin abrogated the PMA-stimulated production of 2-AG. Data represents mean ± SD n=3. * $p<0.05$, Student's t-test when two groups are compared, one-way ANOVA (Student-Newman-Keuls method) when more than two groups are compared.

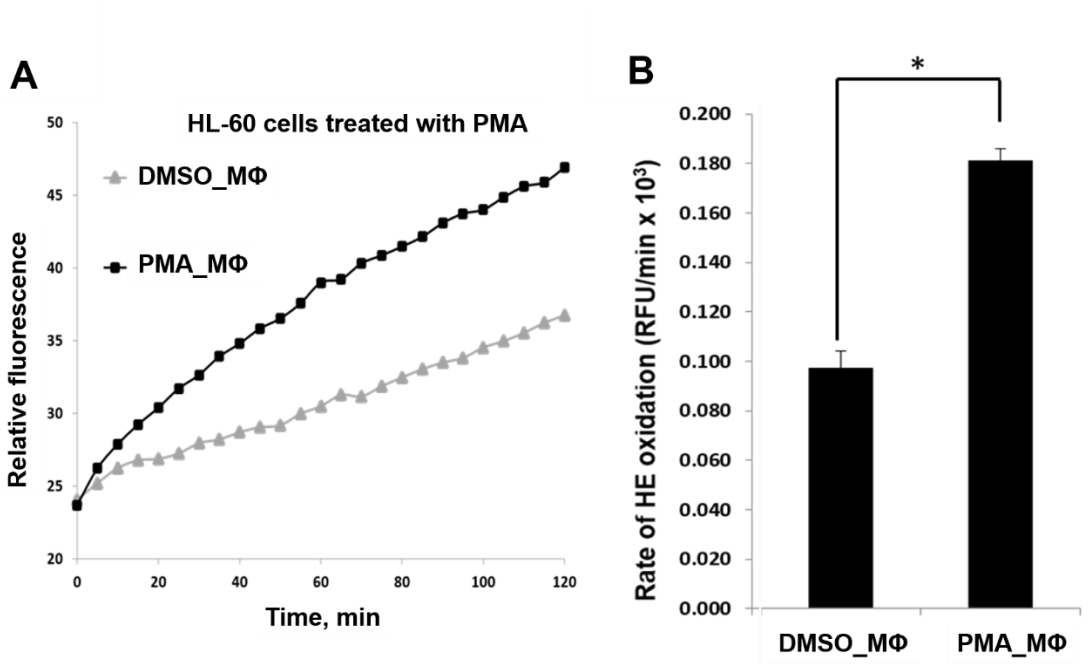

Figure 4.8 PMA-activated macrophages produce elevated amounts of superoxide and 2-AG.

(A) HL-60 cells were differentiated into macrophages (PMA, 100 nM, 72 h), then treated with either PMA (3.2 µM) or DMSO followed by the addition of HE (20 µM final concentration). The rate of HE oxidation was monitored in a fluorescent plate reader for 120 min. (B) Quantitative analysis of the data shown in (A) is provided as a bar graph. Data represents mean ± SD n=3. * $p<0.05$, Student's t-test.

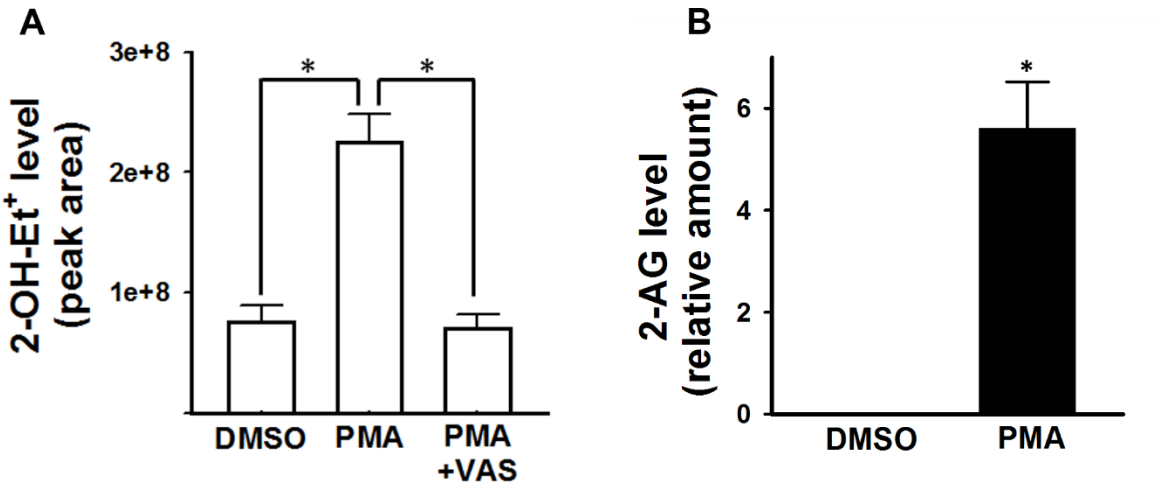

Figure 4.9 Macrophages challenged with acetylated low-density lipoprotein, arachidonic acid, and H2O2 produced elevated amounts of 2-AG.

(A) The superoxide-specific product 2-OH-Et+ was determined in HL-60 macrophages that had been treated with DMSO, PMA (3.2 μM), or PMA (3.2 μM) and VAS (a Nox inhibitor, 10 μM) for 30 min. After 30 min, the culture medium was removed and the cells overlaid with fresh medium containing 20 μM HE, followed by an additional incubation period of 20 min. (B) 2-AG levels were determined in HL-60 macrophages treated with either DMSO or PMA (3.2 μM). Data represents mean ± SD n=3. * $p<0.05$, Student's t-test when two groups are compared, one-way ANOVA (Student-Newman-Keuls method) when more than two groups are compared.

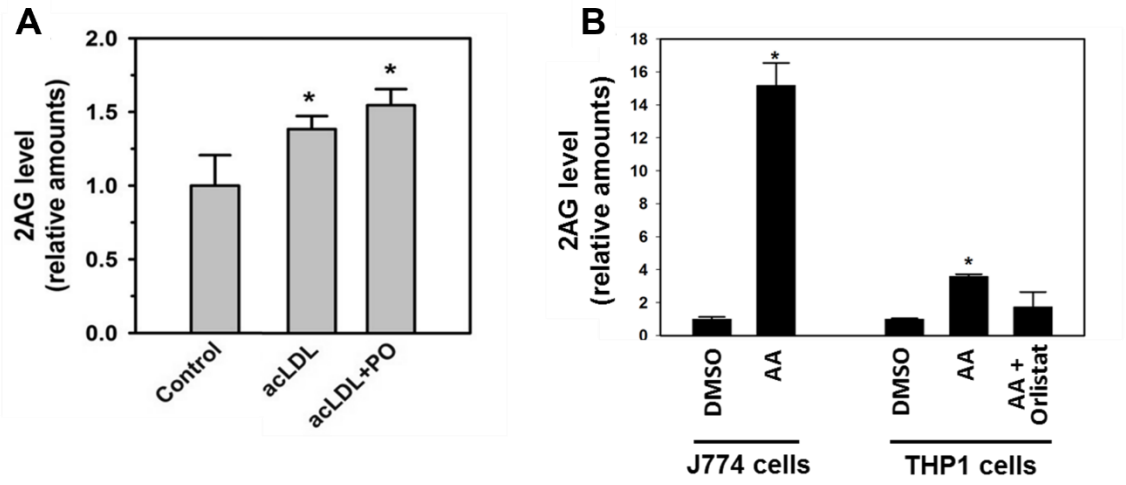

Figure 4.10 Macrophages challenged with acetylated low-density lipoprotein, arachidonic acid, and H_2O_2 produced elevated amounts of 2-AG.

(A) THP-1 macrophages challenged with 20 µg/mL acLDL for 24 h exhibited an increase in 2-AG levels. Challenge with a combination of acLDL and PO also enhanced 2-AG levels, but they were not significantly different from acLDL treatment alone. (B) Exogenous arachidonic acid (AA) stimulated DAGLβ-dependent biosynthesis of 2-AG by macrophages, which was inhibited by orlistat. Data represents mean ± SD n=3. * $p<0.05$, Student's t-test when two groups are compared, one-way ANOVA (Student-Newman-Keuls method) when more than two groups are compared.

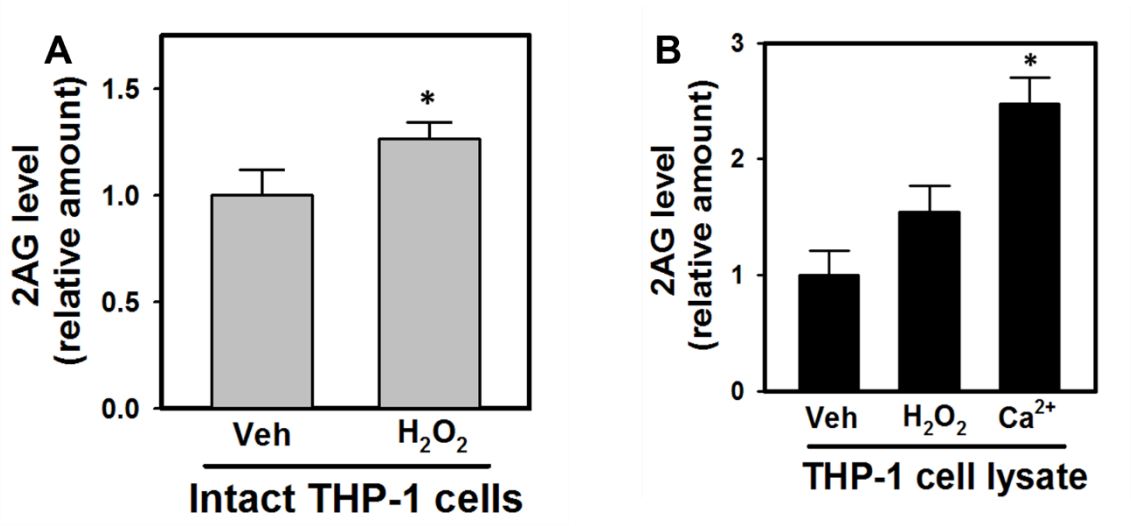

Figure 4.11 Macrophages challenged with acetylated low-density lipoprotein, arachidonic acid, and H_2O_2 produced elevated amounts of 2-AG.

(A) Intact living THP-1 cells were treated with 1-mM H2O2 for 60 min, followed by 2-AG analysis. (B) THP-1 cell lysates were treated with 1-mM H_2O_2 for 60 min, followed by 2-AG analysis. Cell lysate was also treated with Ca^{2+} to stimulate 2-AG synthesis (as a positive control). The vehicle (Veh) for H_2O_2 was water. Data represents mean ± SD n=3. * $p<0.05$, Student's t-test when two groups are compared, one-way ANOVA (Student-Newman-Keuls method) when more than two groups are compared.

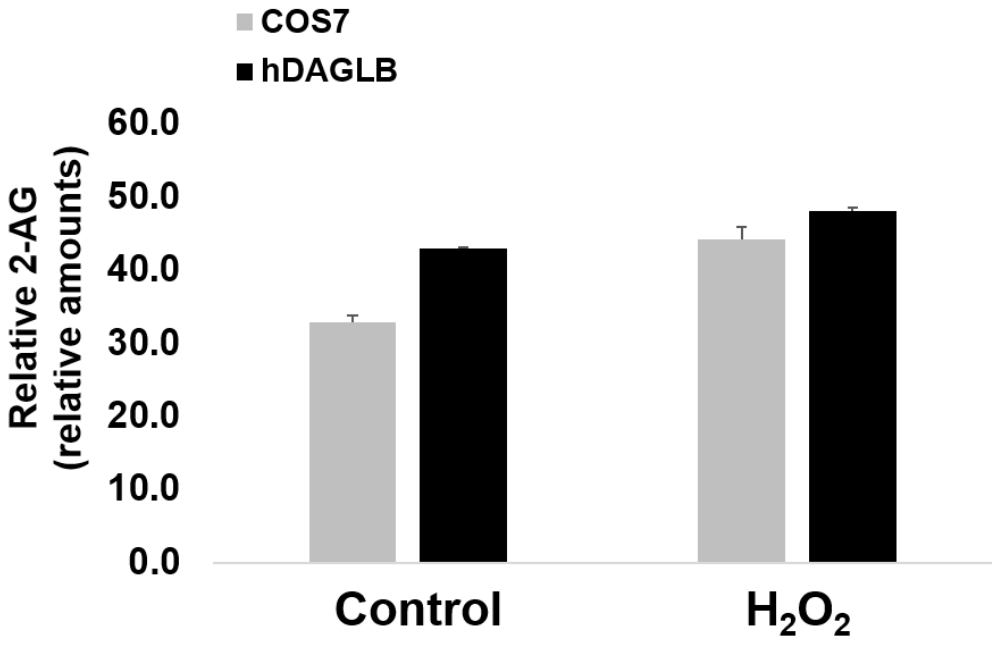

Figure 4.12 Relative 2-AG amounts in macrophages challenged H_2O_2.

Human DAGLβ overexpressed cell lysates were treated with 1-mM H_2O_2 for 90 min, followed by 2-AG analysis. The vehicle (Veh) for H_2O_2 was water . Data represents mean ± SD n=3. * $p<0.05$ (relative to control or vehicle), Student's t-test.

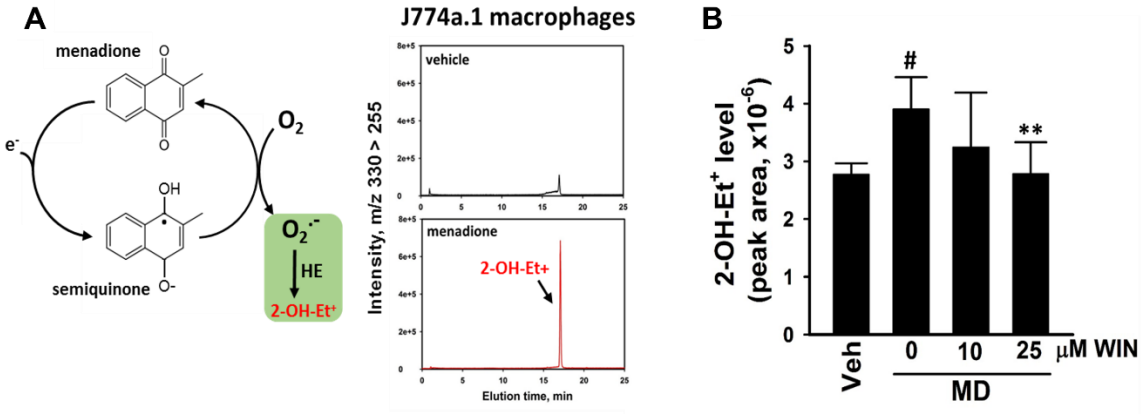

Figure 4.13 Pretreatment of J774A.1 macrophages with the non-selective CB agonist WIN 55,212-2 attenuated 2-OH-Et$^+$ levels following treatment with menadione (MD).

(A) MD is redox cycling agent that produces superoxide in cells. Addition of MD (40 μM) to J774A.1 macrophages for 30 min markedly increased superoxide (2-OH-Et$^+$) levels relative to vehicle. (B) WIN 55,212-2 (WIN) attenuated the levels of the MD-derived superoxide (2-OH-Et$^+$). # $p<0.05$ relative to vehicle (Veh) control; Data represents mean ± SD n=4-9. ** $p<0.01$ relative to MD only-treated cells; * $p<0.05$ relative to MD/WIN–treated cells, one-way ANOVA (Student-Newman-Keuls method).

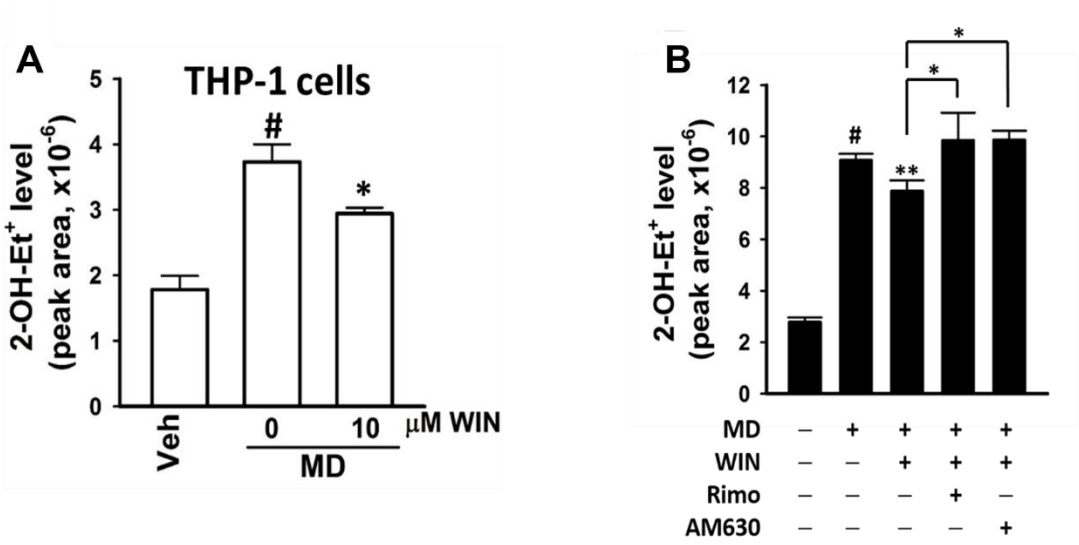

Figure 4.14 Pretreatment of J774A.1 macrophages with the non-selective CB agonist WIN 55,212-2 attenuated 2-OH-Et^+ levels following treatment with menadione (MD).

(A) Addition of MD (40 μM) to THP-1 macrophages for 30 min markedly increased superoxide (2-OH-Et^+) levels relative to vehicle. WIN 55,212-2 (WIN, 10 μM) attenuated the levels of MD-derived superoxide (2-OH-Et^+). (B) The effect of WIN (25μM) on the levels of MD-derived superoxide (2-OH-Et^+) was abrogated by CB_1 and CB_2 receptor antagonists [rimonabant (Rimo) and AM630, respectively]. # $p<0.05$ relative to vehicle (Veh) control; Data represents mean ± SD n=4-9. ** $p<0.01$ relative to MD only-treated cells; * $p<0.05$ relative to MD/WIN–treated cells, one-way ANOVA (Student-Newman-Keuls method).

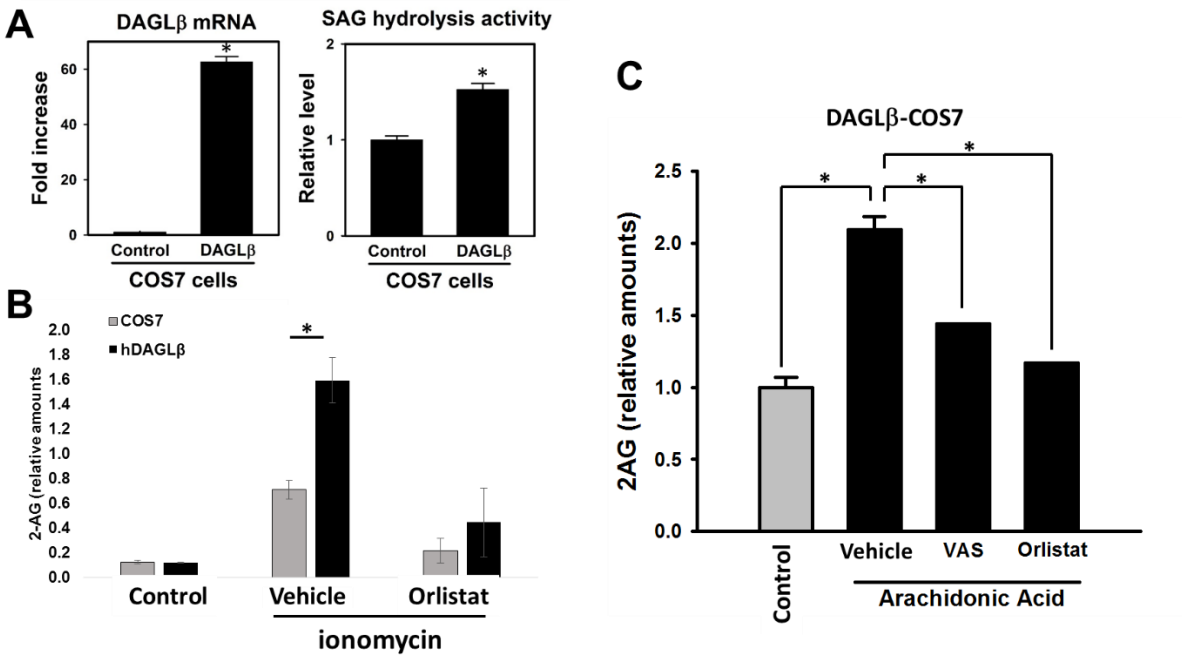

Figure 4.15 COS-7 cells that stably overexpress human DAGLβ produce more 2-AG than control COS-7 cells, and inhibition of Nox activity attenuated 2-AG levels.

(A) Expression of human DAGLβ was verified by measuring its mRNA level in COS-7 cells (left) and the SAG hydrolysis activity of cell lysates (right). (B) 2-AG levels were determined in DAGLβ-COS-7 cells and control COS-7 cells following stimulation with ionomycin in the presence and absence of the DAGLβ inhibitor orlistat. (C) 2-AG levels were determined in DAGLβ-COS-7 cells following stimulation with arachidonic acid in the presence and absence of a Nox inhibitor (VAS) or DAGLβ inhibitor (orlistat). Data represents mean ± SD n=3. * $p<0.05$, Student's t-test when two groups are compared, one-way ANOVA (Student-Newman-Keuls method) when more than two groups are compared.

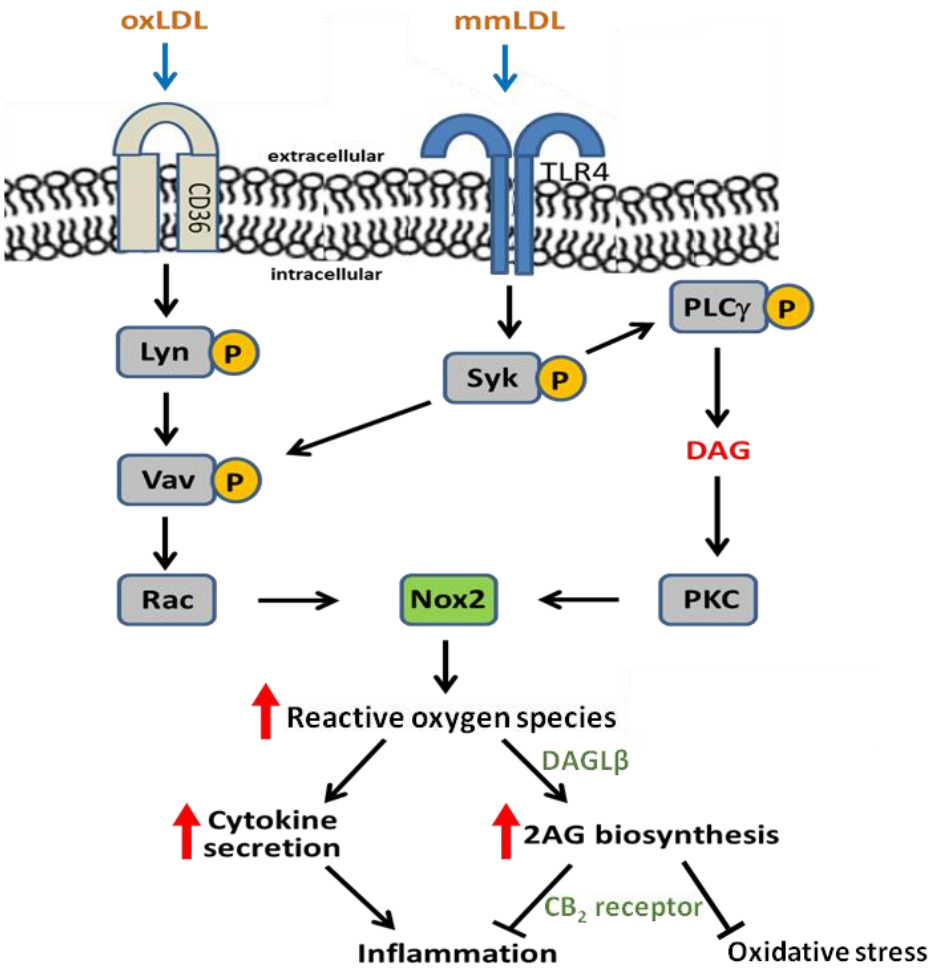

Figure 4.16 Scheme describing how modified LDLs can activate signaling pathways that stimulate 2-AG biosynthesis via Nox activation.

4.4 Discussion

Macrophages have important roles in lipid metabolism and immunity. In this study, we have extended our previous work on Nox in monocytes/macrophages (Mangum et al. 2015) to examine whether stimulation of this enzyme complex modulates the endocannabinoid system by activating the 2-AG biosynthetic enzyme DAGLβ. Nox is the only oxidoreductase that produces superoxide as its primary end product; however, whether Nox (and the reactive oxygen species it produces) interfaces with the bioactive endocannabinoids has not been studied. Thus, we wondered whether overactive redox pathways in immune cells would stimulate downstream compensatory pathways, including increased 2-AG biosynthesis, to reestablish homeostasis. In this study it was demonstrated that 2-AG levels in human THP-1 cells and murine J774A.1 cells, which are two widely used macrophage-like cell lines, were elevated following exposures to either extracellular oxyradicals (via xanthine oxidase system) or intracellular oxyradicals derived from the activated Nox complex. These results suggested that oxyradical stress in the macrophages was causally related to enhanced biosynthesis of 2-AG. In support of this notion, the effects of these stressors on 2-AG biosynthetic activity were blunted by the addition of Nox and DAGLβ inhibitors (Mangum et al. 2015).

Current therapies for cardiovascular disease mainly target well-established risk factors such as high blood pressure and elevated cholesterol levels. However, the endocannabinoid system and oxidoreductases that generate ROS are emerging as attractive targets for reducing inflammation and oxidative stress in the cardiovascular wall. Our data suggest that increased rates of superoxide production, induced by either PMA, arachidonic acid, or a xanthine oxidase system, correlated with increased rates of

2-AG biosynthesis. PMA is a potent activator of protein kinase C (PKC) and the small G-protein Rac; the activated proteins work in concert to stimulate the biosynthesis of Nox-derived superoxide (Schroder et al. 2007; Serrander et al. 2007). Xanthine oxidase, on the other hand, is an oxidoreductase that generates superoxide anion via the oxidation of either hypoxanthine to xanthine or xanthine to uric acid. The increased rates of 2-AG biosynthesis resulting from the elevated superoxide levels might be a compensatory mechanism invoked by oxidative stress. The endocannabinoids have well known homeostatic regulatory functions that help to dampen the effects of noxious stimuli. Consistent with this notion, we showed that the non-selective cannabinoid receptor agonist WIN 55,212-2 attenuated the MD-derived superoxide levels in macrophages. Furthermore, either the selective CB_1 or selective CB_2 receptor antagonists could abrogate the beneficial effect of WIN 55,212-2 (Figure 4.14B). These data indicate that the endocannabinoid system, including the CB_1 and CB_2 receptors and their ligands, might be an important therapeutic target to modulate oxidative stress. The specific signaling pathways evoked by CB_1 and CB_2 receptor activation are undefined in our study; however, CB_1- and CB_2-dependent signaling is known to modulate p38 and ERK1/2 stress kinase activity (Pacher and Steffens 2009), which might account in part for the WIN 55,212-2-dependent decrease in superoxide levels seen here. More work, however, is needed to characterize the integration of signals emanating from the two cannabinoid receptors and how they balance each other to regulate the intracellular redox state.

Our results also showed that increased levels of 2-AG could be produced in macrophages exposed to acLDL (Figure 4.10A). The unregulated phagocytosis of

modified LDLs by macrophages causes foam cell formation; however, blockade of the CB_1 receptor or activation of the CB_2 receptor was shown to reduce foam cell formation (Sugamura et al. 2010) and atherosclerosis development (Booz 2011). In addition, when $ApoE^{-/-}$ mice were treated with WIN 55,212-2, the number of macrophages in plaque lesions was decreased as was the expression of TNF-α, IL-6, and MCP-1 in the plaques (Wang et al. 2010). The beneficial effects of WIN 55,212-2 were blocked with a CB_2 antagonist. It is known that modified LDL can activate Nox activity and increase ROS in macrophages in a CD36-dependent manner (Park et al. 2009). Thus, a compensatory response to Nox activation under these conditions might involve an increase in 2-AG levels to help dampen oxidative stress via CB2 signaling.

The relationship between oxidative stress and endocannabinoids is complex and still poorly understood. However, Rajesh et al. (Rajesh et al. 2010) demonstrated that Nox-derived superoxide levels in cultured human coronary artery endothelial cells were attenuated by a synthetic CB_2-selective agonist. This effect is consistent with the known beneficial properties of CB_2 agonists. For instance, treatment of endothelial cells and smooth muscle cells with a synthetic CB_2 agonist attenuated TNF-α production and evoked other anti-inflammatory responses (Rajesh et al. 2010). On the other hand, CB_1 receptor activation induced the production of pro-inflammatory mediators and substantial oxidative stress (Pacher and Steffens 2009). Because the endocannabinoids bind to CB_1 and CB_2 receptors with roughly equal affinity, an increased endocannabinoid concentration in the vessel wall might elicit both pro- and anti-inflammatory and oxidative effects depending on the relative strength of the signal transduction pathways evoked by CB_1 and CB_2 receptor signaling. Although there is conflicting literature

concerning the role of endocannabinoids in the pathogenesis of atherogenesis (Mackie 2010), it is generally accepted that an imbalance in the endocannabinoid system and the overproduction of Nox-derived superoxide and H_2O_2 disrupts redox circuits and induces inflammatory mediators (Pacher 2007; Drummond *et al.* 2011).

In conclusion, this study provides insight into how Nox signals interface with the activation of the rate-limiting enzyme of 2-AG biosynthesis, DAGLβ. We demonstrated that heightened oxyradical flux via either extracellular (paracrine)- or intracellular (Nox)-derived superoxide leads to increased biosynthesis of 2-AG, thereby enhancing the macrophage "endocannabinoid tone". It is suggested that this is part of a compensatory mechanism to counteract inflammation and oxidative stress, most likely via CB-receptor dependent signaling mechanism and downstream signaling cascades in inflammatory cells (Figure 4.16) (Park et al. 2009).

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CHAPTER V

SERINE HYDROLASE PROFILING OF VASCULAR SMOOTH MUSCLE CELLS

5.1 Introduction

Atherosclerosis is a spatially nonrandom, nonlinear process that occurs at branching points in the arterial tree that are prone to lesion formation due to disrupted blood flow (Tabas *et al.* 2015). The pathophysiology of atherosclerosis as it pertains to phagocytic cells and endothelial cells is well characterized. This chapter focuses on the role that smooth muscle cells (SMCs) play in the cardiovascular disease (CVD) process.

It has been discussed in several reports that macrophages are critical in atherogenesis; however, Tabas et al (2015) suggested that the expression of macrophage derived proteins that are thought to be pro-inflammatory markers of atherosclerosis were actually produced by smooth muscle cells (SMC). The atherosclerotic lesion in its complexity consists of phagocytic cells such as macrophages, endothelial cells, lipids such as oxidized (ox)LDL, and other cellular debris (Hopkins 2013). For example, SMCs produce massive extracellular matrix proteins that contribute to lesion formation and stability; however, the degradation of this "fibrous cap" leads to myocardial infarctions.

The differentiation of SMCs is incompletely understood, but it is clear that they can acquire a broad spectrum of different phenotypes in response to physiological or pathological stimuli. It is speculated that mechanical forces are important in the recruitment and differentiation of SMCs during arterialization. Markers for SMCs

include four actin isoforms nonmuscle β-actin, nonmuscle γ-actin, smooth muscle γ-actin, and most important is the smooth muscle α-actin in addition to nonmuscle-type myosin heavy chain (MHC) type A (NMHC-A) and NMHC-B, which are both expressed in human atherosclerotic lesions (Owens and Thompson 1986; Kuro-o *et al.* 1991; Monical *et al.* 1993). Smooth muscle α-actin comprises 40% of the total cell protein and 70% of total actin in the cells. Smooth muscle α-actin is transiently expressed in various stages of differentiation and in myofibroblasts during wound healing (Owens and Thompson 1986; Owens 1995), and is the first identified marker for SMC differentiation during vasculogenesis. Factors involved in the phenotypic modulation of SMCs include mechanical factors and hemodynamic forces, such as shear stress and tangential wall stress, and involve the reorientation of cells, increased protein and DNA synthesis, and increased production of extracellular matrix (ECM) components such as collagens, elastins, laminin, fibronectin, and glycosaminoglycans (Frink 2002). Decreased smooth muscle α-actin expression is a consequence of increased fibronectin levels, which induces cell migration, proliferation, and differentiation.

The atherosclerotic lesion is an asymmetrical thickening in the vessel wall consisting of two main SMC proteoglycans—chondroitin sulfate proteoglycan (CSPG) and dermatan sulfate proteoglycan (DSPG)—in addition to other ECM proteins and SMCs (Frink 2002). The ECM has profound effects on cell behavior and plays a role in atherogenesis. CSPG, for example, is an active scaffold that is vital in the regulation of transport of plasma constituents and arterial permeability leading to the chemical change in proteoglycans that lead to altered metabolic and biochemical functions (Wight 1989). For example, oxidized low density lipoproteins (oxLDLs) enters the arterial wall from the

circulating blood stimulating increased amounts of proteoglycans synthesis, which enhances lipid accumulation in the intima. The accelerated rate of lipid entrapment leads to changes in SMC phenotype. Previous reports suggest that the phenotypic change in SMCs is not a consequence of the dyslipidemia but a pathological component of the disease (Frink 2002; Tabas *et al.* 2015). The fibrous lesion is a network of ECM and SMCs with increased intracellular pressure within the vessel wall. SMCs can take on a macrophage-like phenotype in which they exhibit scavenger receptors on their surface and have increased Golgi and rough endoplasmic reticulum structures (Owens 1996; Frink 2002). Although SMCs are not as efficient as macrophages, they can phagocytose modified LDL and metabolize cholesterol, although they do not efficiently esterify intracellular free cholesterol. SMCs do not proliferate as macrophage foam cells, but fusion with adjacent cells allows for even greater lipid accumulation. The inability to effectively metabolize oxidized lipids results in apoptosis and the formation of a lipid necrotic core (Frink 2002; Back *et al.* 2015). Additionally, the levels of proteins characteristic of differentiated SMCs, including smooth muscle α-actin, smooth muscle MHC, caldesom, vinculin, and desmin, are reduced. These changes are, at least in part, reversible; however, failure to regulate the differentiated phenotype of vascular SMCs is a contributing factor in the development and progression of atherosclerotic lesions. Tabas et al (2012) suggest a balance of inflammatory and homeostatic responses that regulate the overall atherogenic effects within lesional macrophage-like SMC. Furthermore, plaque rupture is due to matrix metalloproteinases secretion, which contributes to fibrous cap thinning (Libby *et al.* 2014).

Because it has been reported that the endocannabinoids play a role in reducing oxidative stress, and to date the serine hydrolase profile has yet to be determined in SMC, we aim to identify which serine hydrolases are present in the human SMCs and the role these hydrolytic enzymes play in the catabolism of 2-AG. It is well documented that 2-AG and anandamide (AEA) are hydrolyzed by MAGL and FAAH, respectively (Carr *et al.* 2013; Turcotte *et al.* 2015). Because SMCs participate in the inflammation that contributes to atherosclerosis, it is important to inventory the serine hydrolases found within this cell type. Our lab previously utilized the activity-based protein profiling (ABPP)-multidimensional protein identification technology (MudPIT) to identify uncharacterized serine hydrolases found in human macrophages (Wang *et al.* 2013). Therefore, this chapter explores a similar objective to obtain a global view of the hydrolases that are present in SMCs using the same analytical methods. Our approach employs the use of an activity-based protein profiling (ABPP) probe, e.g. fluoro-phosphonate-biotin (FP-biotin), that covalently modifies the catalytic serine residue of serine hydrolases, which can be enriched, proteolytically digested, and identified by LC-MS/MS and MudPIT technology (Xie *et al.* 2010; Wang *et al.* 2013). ABPP enables the activities of enzymes with conserved catalytic mechanisms, such as the serine hydrolase, to be evaluated in their native environments in tissues and cells. ABPP-MudPIT has the ability to distinguish between a wide ranges of target proteins with high specificity. Although macrophages are elite producers of bioactive lipid mediators such as 2-AG, other cell types, such as SMC, may have roles in the catabolism of this endocannabinoid.

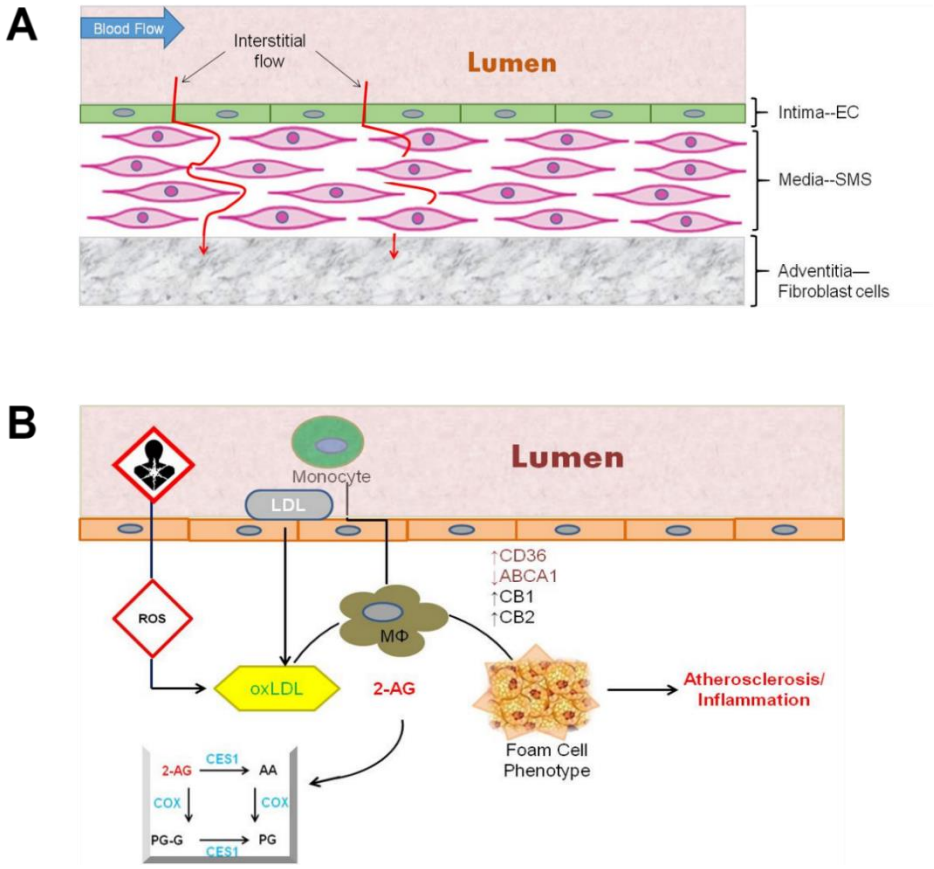

Figure 5.1 Pathophysiology of lipid laden foam cells.

(A) Under basal conditions, the vasculature has the biochemical machinery to metabolize the level of interstitial fluids and other insults that present to disrupt blood flow such as shear stress and LDLs. (B) In the event endothelial dysfunction, toxins or LDL translocate to the interstitial space and become oxLDLs. Monocytes are attracted to the insult. Diapediecisis occurs and monocytes are then differentiated into macrophages, with scavenger receptors on the surface. The unregulated phagocytosis of oxLDL initiate the phenotypic change in macrophages leading to the induction of the inflammatory cascade. The oxLDL bind with high affinity to the macrophage scavenger receptor CD36 which stimulates the production of 2-AG. The ligation of 2-AG the CB_2 receptor initiates the anti-inflammatory response.

5.2 Materials and Methods

5.2.1 Chemicals, cells, and reagents.

Trypan Blue solution (0.4% w/v), β-mercaptoethanol, fatty-acid free bovine serum albumin (BSA), Avidin-horseradish peroxidase, and dimethyl sulfoxide (DMSO), JZL184, Urea, TCEP, IAA, Trypsin, and all buffer components were purchased from Sigma (St. Louis, MO). The activity-based serine hydrolase probe, fluorophosphonate-biotin (FP-biotin), was from Toronto Research Chemicals (North York, Ontario). HPLC grade solvents, trypsin-EDTA, fetal bovine serum (FBS), penicillin, streptomycin, HyperSep Retain PEP column, agarose beads, biospin columns, and were from Thermo-Fisher. Sep-PAK was purchased from Waters. SDS was purchased from Bio-Rad. Human aortic smooth muscle cells, Vascular Basal Medium and Vascular Smooth Muscle cell growth kit were purchased from the American Type Culture Collection (ATCC) (Manassas, VA).

5.2.2 Cell culture conditions.

The human aortic smooth muscle cells were grown to 80% confluency during a 48 h incubation at 37°C, 5% CO_2 in Vascular Cell Basal medium containing 5 ng/mL rh FGF-basic, 10 mM rh Insulin (5 µg/mL EGF:5 ng/mL L-glutamine) 50 µg/mL Ascorbic acid, and 5% FBS.

5.2.3 Preparation of cell lysates.

Adherent cells were incubated with 3 mL trypsin for 5 mins and reactions quenched with 5 mL of culture medium. Subsequently, the cells were washed 2x and collected into ice-cold 50 mM Tris-HCl (pH 7.4) buffer and lysed by sonication (three 15

s bursts on ice at 30% maximum power). Protein concentrations of cell lysates were determined using the BCA reagent according to the manufacturer's instructions (Thermo-Fisher).

5.2.4 Serine hydrolase profile.

Primary human smooth muscle cell proteomes (1 mg/mL, 30 µL reaction volume) was treated with 8 µM fluorophosphonate (FP)-biotin for 1 h at room temperature (vehicle for FP-biotin is DMSO, at a concentration of <1% v/v in the reaction). Reactions with FP-biotin were terminated by addition of 5 µL 6x SDS-PAGE loading buffer (reducing), heated at 90°C for 10 min, and then placed on ice. The labeled proteins were separated by SDS-PAGE, and FP-biotin–labeled serine hydrolases were detected by Avidin peroxidase blotting as previously described by us (Xie *et al.* 2010; Wang *et al.* 2013). This gel-based ABPP approach permits rapid profiling of serine hydrolase activities in the cells.

In addition to SDS-PAGE, cell proteomes were treated as described above using 1 mg/mL in a reaction volume of 1mL. Reactions with FP-biotin were terminated by addition of 25 µL 20% w/v SDS, heated at 90°C for 5 min, and cooled on ice. The labeled proteomes (400 µL) were filtered and centrifuged at 14,000g for 20 mins and washed 3x with 450 µL of 50 mM Tris-HCl. The filter was reversed in a new microcentrifuge tube, the top was washed with 100 µL of 50 mM Tris-HCl and the filter was returned to the original position and washed 3x with 350 µL of 50 mM Tris-HCl by centrifugation at 1,400g for 3 mins. The supernatant was stored overnight at 4°C. The biotinylated proteins were captured with streptavidin beads. The beads were prepared in 1.5 mL EP tubes (200 µL of Agarose beads were centrifuged for 3 min at 1,400g, the

supernate was discarded and beads washed 3x with 1 mL PBS). The samples were allowed to incubate 4 h on rotator to bind to beads. Subsequently, the samples were centrifuged for 3 min, the supernatant was discarded, and 1 mL of 0.2% SDS in PBS was added to the beads and washed 3x with PBS followed by 3x with water for 10 min on rotator. Then, 500 µL of 6 M urea in 50 mM Tris-HCl and 10mM fresh TCEP were added to the beads and incubated for 15 min at 65°C without shaking. The samples were cooled. Fresh 500 mM IAA was added to the samples and incubated 30 min at 37°C in the dark without shaking; 950 µL of 50 mM Tris-HCl was added, centrifuged at 1400g for 2 min and supernatant discarded; 200 µL of 2 M urea in 50 mM Tris-HCl, 2 µL 100mM $CaCl_2$ was added to activate 1 mM trypsin, and 4 µL of Trypsin (20 µg reconstituted into 40 µL of trypsin buffer was added and samples incubated overnight at 37°C on thermo-mixer at 500 rpm.

The bio-spin column was prepared according to manufacturer's instructions, and washed 3x with 400 µL of water. The peptides were collected by adding samples to column and centrifuging at 1400g for 3 mins. The tube was washed with 50 µL of water and transferred to the spin column and centrifuged 3x at 1400g for 3 mins. The eluent was collected and added to 3 µL of 10% formic acid. The peptides were then desalted and activated for evaluation with mass spectrometry. Briefly, Sep-Pak column was activated with 5 mL the addition of 65% ACN plus 35% water containing 0.1% FA (buffer B). The columns were equilibrated with 10 mL of 98% water plus 2% ACN containing 0.1% FA (buffer A). The peptides were loaded to the column and the column was again washed with buffer A. The collection tube was changed to a clean tube and the

protein was eluted 2x with 500 µL of buffer B. The samples were dried under nitrogen for proteomic analysis.

After Proteome Discovery searches, label-free quantization was accomplished by normalizing the spectral counts for each identified serine hydrolase using the commercially available software Scaffold 4.4.5, exactly as we recently published (Wang *et al.* 2013) using ABPP-multidimensional protein identification technology (MudPIT) for the detection of serine hydrolases for a higher resolution and sensitivity than gel-based ABPP.

5.2.5 Database Searching.

All LC-MS/MS samples were analyzed using Sequest (Thermo Fisher Scientific, San Jose, CA, USA; version 1.3.0.339). Sequest was set up to search Gallus_Salmonella_cont_Uniprot.fasta with the digestion trypsin enzyme. Sequest was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 10.0 PPM. Oxidation of methionine and carbamidomethyl of cysteine were specified in Sequest as variable modifications.

5.2.6 Protein Identification.

Scaffold (version Scaffold_4.4.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 65.0% probability to achieve an FDR less than 0.1% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 98.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet

(Nesvizhskii *et al.* 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

5.2.7 Endocannabinoid metabolism of smooth muscle cells.

The cultured SMCs were treated with 10 μM exogenous 2-AG in serum-free culture medium for up to 30 min in order to determine the extent of hydrolytic and oxidative metabolism of this endocannabinoid by the cells. The culture medium was harvested and reactions quenched by the addition of one volume of ACN containing 20 μL of deuterated arachidonic acid. The samples were placed on ice and the supernatant was collected for LC-MS/MS metabolipidomic analysis of 2-AG. To determine if the hydrolytic enzymes that play a role in the degradation of 2-AG, 0.25 mg/mL protein was pre-incubated for 30 min with 1 μL carboxylesterase inhibitor paraoxon or MAGL inhibitor JZL184, followed by a 30 min incubation with exogenous 2-AG. The reactions were quenched by the addition of one volume of ACN and arachidonic acid levels measured by UPLC analysis using well-defined methods published by us (Xie *et al.* 2010; Wang *et al.* 2013). In addition, the extent of cyclooxygenase-mediated oxygenation of 2-AG was determined, as we have already done for human monocytes/macrophages (Xie *et al.* 2010; Wang *et al.* 2013).

5.2.8 2-arachidonylglycerol hydrolysis.

To determine the rate of 2-AG hydrolysis, we seeded a 12 well plate with smooth muscle cells to grow to 80% confluency followed by 30 min incubation with 10 μM 2-arachidonylglycerol as substrate. The culture medium was extracted with 500 nM

MHDG as internal standard using Sep-PAK and dried under nitrogen. The residues were dissolved in 100 µL 1:1 (v/v) methanol:water for LC-MS/MS analysis. In subsequent experiments, the cells were pre-treated with 10 µM VAS2870, an NADPH oxidase inhibitor, followed by an additional 30 min incubation with 10µM 2-AG substrate and the culture medium was extracted.

5.2.9 mRNA expression of Smooth muscle cells

Smooth muscle cells were grown to 80% confluency in a 12 well plate. The cells were treated with pre-treated with 10 µM VAS2870 or DMSO for 30 min followed by 10 µM of AA for an additional 30 mins in an atmosphere of 37°C, 5% CO_2. The culture medium containing 500 nM MHDG was collected and extracted with HyperSep Retain PEP as previously described (Wang *et al.* 2013). The samples were analyzed for 2-AG biosynthesis by LC-MS/MS.

The SMCs RNA was isolated using RNeasy Plus Mini Kit according to the manufacturer's instructions. NanoDrop ND-1000 spectrophometer (Thermo Scientific, Walham, MA) was used to quantify recovered RNA followed by cDNA synthesis using iScript Select cDNA Synthesis Kit (BioRad) according to manufacturer's instructions. Real-time PCR of cDNA was performed on a Stratagene Mx3005P thermocycler with Quantifast SYBR Green PCR master mix from Qiagen. Primers are detailed in Table 5.1. The cycle program used for all target genes consisted of 5 min hot start at 95°C prior to 10 s at 95°C for 40 cycles, followed by 30 s at 60°C. The dissociation curve was generated for PCR product quality immediately after analysis. Expression differences were normalized using GAPDH and quantified using the ΔΔCT method. The results generated were in fold differences of gene expression.

Table 5.1 Primer sequence used for quantitative real-time PCR

Gene (Homo sapien)	Forward Sequence	Reverse Sequence
CB_1	CAACAAGTCTCTCTCGTCCT	GATGAAGTGGTAGGAAGGC
CB_2	CACTGATCCCCAATGACTAC	CCACTCCGTAGAGCATAGAT
CD36	AGGACTTTCCTGCAGAATAACA	ACAAGCTCTGGTTCTTATTCACA
NOX2	CGAAATGATGGTGACTGGCT	CCCAGCCAGTGAGGTAGATGT
$P47^{phox}$	ATTCACAAGCTCCTGGACGG	ACACGTCTTGCCCTGACTTT
DAGLB	Hs_DAGLB_1_SG QuantiTect Primer Assay – QT00074319	
GAPDH	Hs_GAPDH_1_SG QuantiTect Primer Assay – QT00079247	

5.3 Results

5.3.1 Serine hydrolase profile

The proteomic ABPP-MUDPIT analysis identified 24 catalytically active serine hydrolases in human smooth muscle cells (Table 5.2 and Figure 5.2). Surprisingly, we did not identify any carboxylesterase or other secondary enzymes we speculated to be involved in hydrolysis of eCBs. Through use of the FP-biotin activity based protein profiling (ABPP) probe in both gel-based (Figure 5.3) and mass spectrometry-based formats (Figure 5.2), we were able to identify several serine hydrolase enzymes including MAGL, the primary hydrolytic enzyme of 2-AG, and an ABHD11 isoform.

5.3.2 Hydrolysis of 2-AG.

The human smooth muscle cells were shown to metabolically hydrolyze 2-AG to AA when treated with to exogenous 2-AG (Figure 5.4A). However, in the presence of PO and JZL184, CES and MAGL inhibitors, respectively, a decreased rate of 2-AG hydrolysis was evident, which was greater in the cells that were treated with the MAGL inhibitor compared to those treated with the CES inhibitor ($p=0.028$) (Figure 5.4B).

However, S-3030, which is a specific reversible CES1 inhibitor, had no effect on 2-AG hydrolysis activity in SMCs (Figure 5.4B). To determine whether NADPH oxidase has a role in the production of 2-AG, the SMCs were stimulated with arachidonic acid in the presence of VAS-2870 to inhibit NADPH oxidase. Treatment of SMC with ionomycin, a compound known to increase intracellular calcium and 2-AG biosynthesis, followed a similar trend in the presence of VAS-2870 (Figure 5.5A). Furthermore, the data demonstrated that, not only was 2-AG biosynthesis increased with the treatment of AA, it was significantly augmented by the presence of VAS-2870 (Figure 5.5B).

5.3.3 mRNA expression

Our findings indicate that, SMCs have the machinery to produce oxyradical stress as dictated by the expression of mRNA of the NADPH oxidase complex. Furthermore, these data revealed that SMCs have the genes to potentially express the transcripts for the eCB receptors (both CB_1 and CB_2), CD36, NOX2, $p47^{phox}$, and DAGLβ mRNA (Figure 5.6). The presence of the transcripts for the enzyme, DAGLβ, suggest it is feasible to biosynthesize 2-AG in SMCs

Table 5.2 Serine hydrolase profile of human smooth muscle cells

Protein name	Gene	NCBI Gene ID	Molecular Weight	Protease (SP) or Metabolic Hydrolase (mSH)
fatty acid synthase	FASN	2194	**273 kDa**	mSH
dipeptidyl-peptidase 9	DPP9	91039	**102 kDa**	mSH
prolyl endopeptidase	PREP	5550	**81 kDa**	mSH
fibroblast activation protein, alpha	FAP	2191	**88 kDa**	mSH
patatin-like phospholipase domain containing 6	PNPLA6	10908	**146 kDa**	mSH
dipeptidyl-peptidase 4	DPP4	1803	**88 kDa**	mSH
tripeptidyl peptidase II	TPP2	7174	**140 kDa**	SP
neutral cholesterol ester hydrolase 1	AADACL1	57552	**50 kDa**	mSH
N-acylaminoacyl-peptide hydrolase	APEH	327	**81 kDa**	mSH
cathepsin A	CTSA	5476	**54 kDa**	mSH
dipeptidyl-peptidase 8	DPP8	54878	**97 kDa**	mSH
monoglyceride lipase	MAGL	11343	**31 kDa**	mSH
lysophospholipase II	LYPLA2	11313	**25 kDa**	mSH
prolylcarboxypeptidase (angiotensinase C)	PRCP	5547	**56 kDa**	mSH
lysophospholipase-like 1	LYPLAL1	127018	**24 kDa**	mSH
platelet-activating factor acetylhydrolase 1b, catalytic subunit 2	PAFAH1B2	5049	**26 kDa**	mSH
platelet-activating factor acetylhydrolase 2	PAFAH2	5051	**44 kDa**	mSH
sialic acid acetylesterase	SIAE	54414	**58 kDa**	mSH
abhydrolase domain containing 11	ABHD11	83451	**17 kDa**	mSH
lysophospholipase I	LYPLA1	10434	**23 kDa**	mSH
phospholipase A2, group XV	PLA2G15	23659	**47 kDa**	mSH
prolyl endopeptidase-like	PREPL	9581	77 kDa	mSH
retinoblastoma binding protein 9	RBBP9	10741	**21 kDa**	mSH

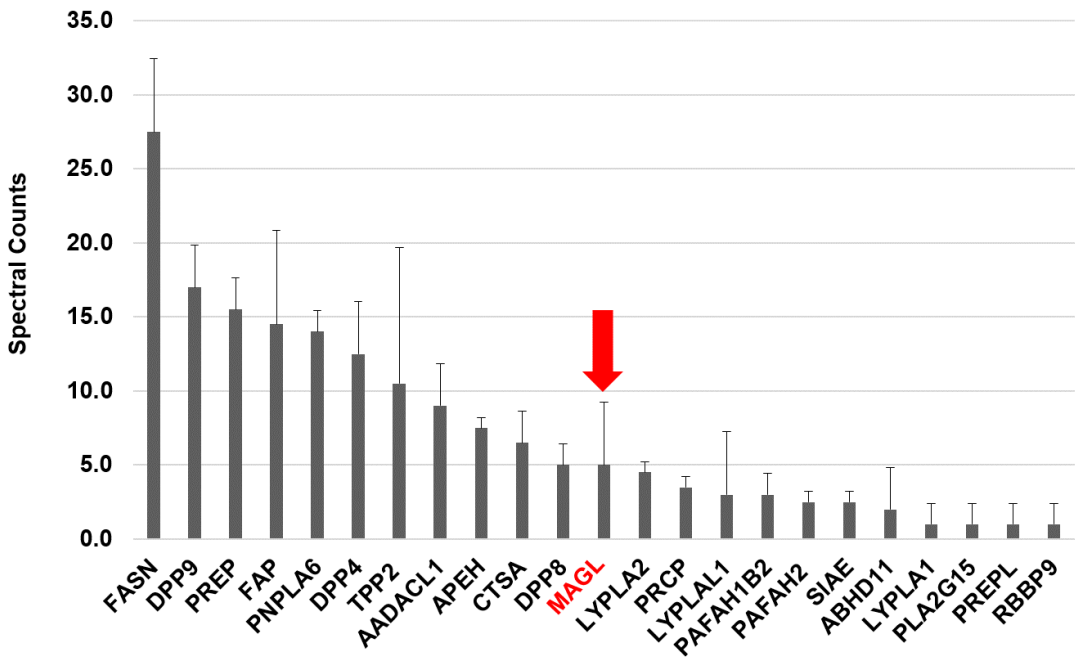

Figure 5.2 Identification and quantification of serine hydrolases in smooth muscle cells.

Serine hydrolase activity profile as determined by FP-Biotin ABPP-MudPIT. Relative spectral counts was used to determined quantification and identification of smooth muscle cells serine hydrolase ABPP-MUDPIT. Importantly, monoacylglycerol lipase (MAGL; highlighted in red), the 2-arachidonylglycerol hydrolytic enzyme, was identified. Data represent means ± SD of triplicate analysis.

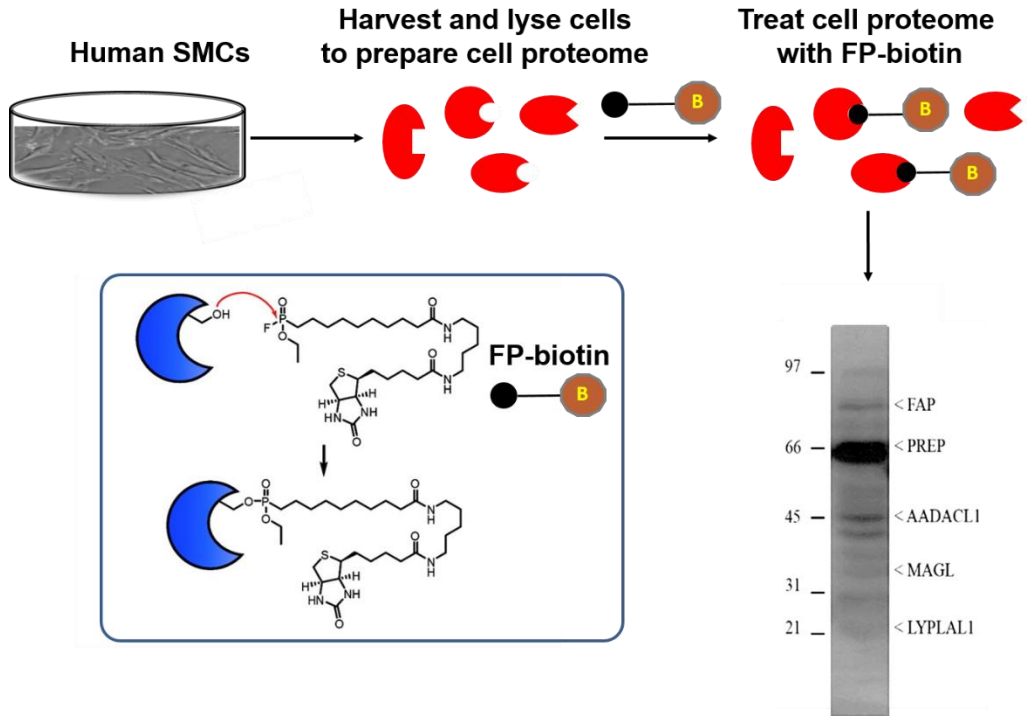

Figure 5.3 Gel based activity based protein profiling of SMCs.

SMCs were grown to 80% confluency. The cells were lysed by sonication and treated with a biotinylated probe. SDS-PAGE revealed five serine hydrolases within the samples of which was monoacylglycerol lipase (MAGL).

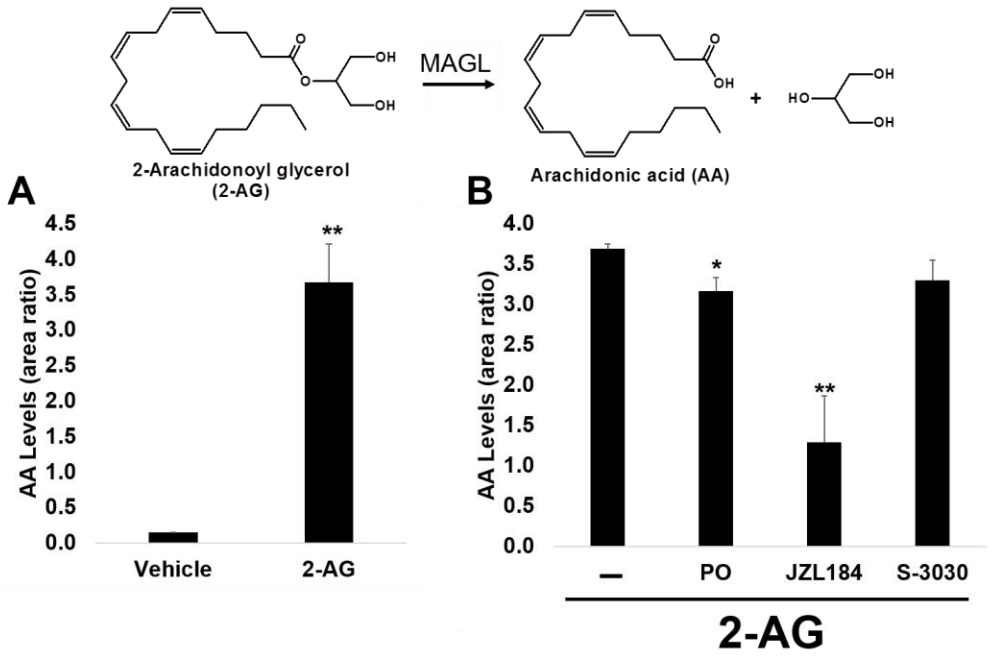

Figure 5.4 2-AG hydrolysis of SMCs.

2-AG can be hydrolyzed by monoacylglycerol (MAGL) to its metabolites arachidonic acid and glycerol. (A) SMCs were treated with 10 μM of exogenous 2-AG for 30 min had an enhanced production of arachidonic acid ($p=0.0004$). (B) These results were abrogated in the presence of PO ($p=0.05$) and JZL184 ($p=0.03$) but not effected by S-3030. Data represents mean ± SD n=3. * $p<0.05$, Student's t-test when two groups are compared, one-way ANOVA (Student-Newman-Keuls method) when more than two groups are compared.

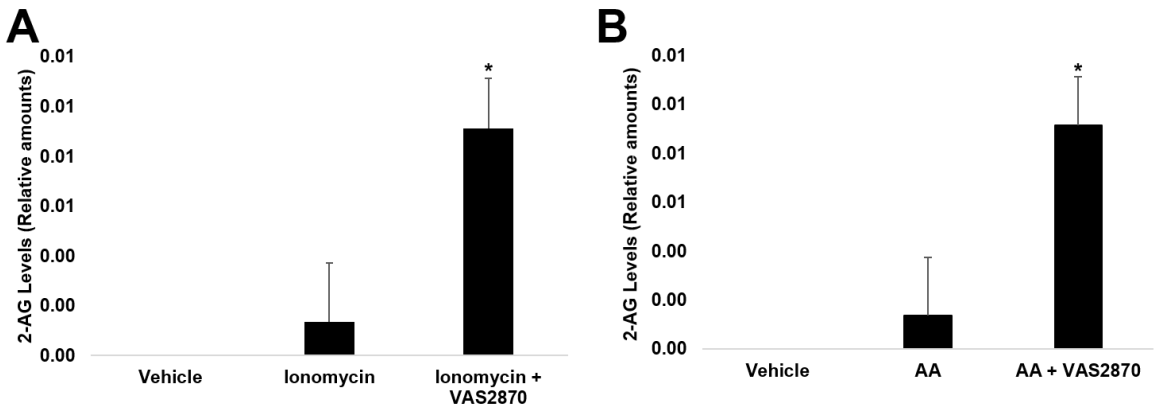

Figure 5.5 2-AG catabolism of SMCs.

(A) SMCs were treated with 1 μM of ionomycin for 30 min had an enhanced production of 2-AG which was further heighted by the inhibition of NADPH oxidase. (B) Using a physiological stimulant, arachidonic acid (10 μM, AA) followed a similar pattern of increased 2-AG biosynthesis which was further enhanced by the inhibition of NADPH oxidase with 10 μM VAS2870. Data represents mean ± SD n=3. * $p<0.05$, Student's t-test when two groups are compared, one-way ANOVA (Student-Newman-Keuls method) when more than two groups are compared.

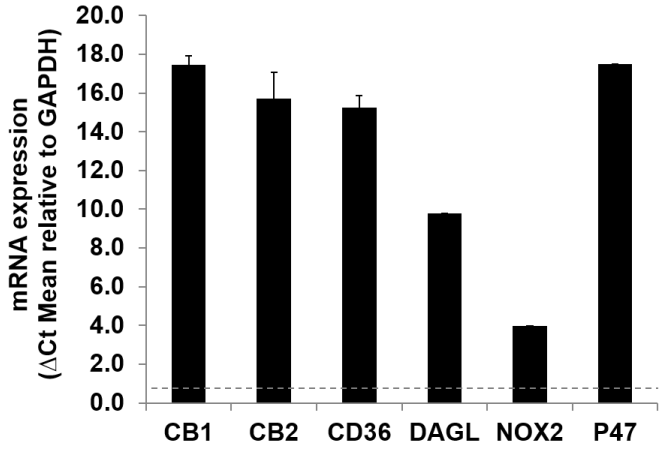

Figure 5.6 mRNA expression of SMC

RT-PCR suggest SMCs have the ability to produce oxidative stress as indicated by the relative gene expression both cannabinoid receptors (CB_1 and CB_2), scavenger receptor CD36, DAGL, and NADPH oxidase subunits, NOX2 and p47phox. Data represents mean ± SD n=3.

5.4 Discussion

Serine hydrolases are the most abundant and diverse classes of enzymes in eukaryotic cells. They include lipases, thio-esterases, amidases, peptidases and proteases (Bachovchin and Cravatt 2012). They represent approximately 1% total of the total proteomes and play a vital role in the physiological processes pertaining to atherogenesis. Serine hydrolases and proteases play roles in cardiovascular disease, such as (a) blood coagulation pathways via activation of zymogens can lead to insoluble fibrin clot formation (Davie and Ratnoff 1964; Macfarlane 1964; Bachovchin and Cravatt 2012), (b) activation of platelets by fibrinogen being cleaved into fibrin via thrombin, the final protease in the clotting cascade (Gustafsson *et al.* 2004), and (c) improve β cell function and promote insulin secretion by dipeptidyl peptidase 4 (Meier *et al.* 2002; Rosenblum and Kozarich 2003).

This is the first report to identify the serine hydrolases present in the human smooth muscle cells. The vast majority of research has been geared toward the use of pharmaceuticals that will inhibit the mechanism of action of these enzymes, but with limited success. As we have demonstrated in previous reports, serine hydrolases CES1, PPT1, MAGL, FAAH, ABHD6 and ABHD12 play a role in the degradation of endocannabinoid ligands, which leads to the production of AA that is metabolized by cyclooxygenases to prostaglandins. These data provide the ground work for endocannabinoid research as a mechanism to modulate inflammation, as seen with atherosclerosis by means of sustaining the presence of 2-AG. Of the hydrolases currently known to degrade 2-AG into its metabolites arachidonic acid and glycerol, MAGL was found to be present at a significant level in the SMCs, whereas CES1, PPT1, and FAAH

were undetectable. The data presented here established that MAGL is the primary hydrolytic enzyme in human SMCs. Because we previously demonstrated in macrophage-like cells that 2-AG biosynthesis could be enhanced when NADPH oxidase was activated, we examined whether a similar mechanisms was in play in SMCs. In the SMCs, however, the response to NADPH oxidase was contrary to findings in the other cell lines. These data indicate the ability of SMCs to produce the ligand 2-AG when intracellular calcium levels rise. It is suggested that the SMCs undergo phenotypic changes that produce markers similar to those of phagocytic cells when stimulated with oxLDL. Products that mimic natural proteins with the ability to convert endogenous substrates into inhibitors of inflammatory markers can be achieved via the serine hydrolases of the SMC.

Small molecule drugs have been clinically approved for the use of targeting serine hydrolases (Bachovchin and Cravatt 2012); however, of the six approved drugs only the DPP4 is found in the SMC. Because modulating the eCB system has become a focus for ameliorating atherosclerosis, the activation of eCB receptors and the enzymes that produce and degrade their ligands to elicit therapeutic benefits of inhibitors with specific targets. The availability of approved marketed drugs that inhibit serine hydrolases has a broad affinity for hydrolases, thus the rate of inhibition could be higher in the face of limited ability of the pharmaceutical industry to create a drug that specifically target a serine hydrolase. Additionally, with the large number of reactive oxygen species available to convolute experimental protocols, further studies are required to validate the increase in 2-AG biosynthesis with the inhibition of the "professional ROS producer" NADPH oxidase and the enzymes that degrade 2-AG once synthesized.

This novel finding of serine hydrolases in SMC will pave the way for research generating genetic or chemical therapies to enhancing the eCB system to decrease the occurrence of atherosclerosis. Studies to include chemical or genetic deletion of these enzymes can potentially reduce (a) the risk of coronary heart disease through PLA2 (Packard *et al.* 2000), (b) preeclampsia and body weight by modulating angiotensin (Ang) II and Ang III with inhibition of PRCP (Mallela *et al.* 2009), and lipogenesis via FASN (Menendez and Lupu 2007). The pursuit of selective inhibitors of SMC serine hydrolases coupled with enhancing 2-AG biosynthesis can lead to the generation of beneficial pharmaceuticals to combat atherogenesis.

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CHAPTER VI

CONCLUSION AND FUTURE DIRECTION

6.1 Conclusion

Despite a sea of research to understand its causes, cardiovascular disease (CVD) still claims the lives of over 17 million people each year in the U.S. Of these deaths, four of five CVD deaths are due to the consequences of either myocardial infarction or strokes. Most individuals at risk for CVD do not display outward signs until the disease has advanced. Moreover, CVD is a secondary complication of hypertension, obesity, and diabetes. The cost of controlling the risk factors and the symptoms of CVD are well above $400 billion dollars per year in the U.S., but surprisingly statin treatment is the most common, least cost effective therapy (Franco *et al.* 2007). It is well known that atherosclerosis is both genetically and environmentally provoked within an individual. Although no one can change their genetics, it is possible to change behavioral patterns, such as increased exercise, healthy diet, and possibly pharmaceutical therapy, to offset the effects of hypertension, obesity, and hypercholesterolemia.

Inflammation is also known to have an important role in CVD. There are several pathways that regulate pro- and anti-inflammatory cascades in cells. Previous research indicated that modulating the endocannabinoid (eCB) system can decrease lesion formation in the aorta. Furthermore, hydrolytic enzymes that degrade eCBs by small molecules might have a role in regulating inflammation.

This dissertation explored the role of the eCB system in monocytes/macrophages that are subjected to oxidative stress. It was demonstrated that both endogenous and exogenous oxyradicals could enhance the biosynthesis of 2-arachidonylglycerol (2-AG), a lipid mediator associated with lowering inflammation. Specifically, examination of how oxLDL-CD36 (scavenger receptor) binding induces oxidative stress and the production of 2-AG was examined in Chapter 3. In chapter 4, the most significant finding concerning oxyradical stress was that an NADPH oxidase stimulant (phorbol ester) or arachidonic acid, extracellular xanthine oxidase, and oxLDL could stimulate oxyradical stress, which resulted in enhanced 2-AG biosynthesis. We were able to identify a role for NADPH oxidase in the increased eCB "tone" (i.e., increased concentration of 2-AG) (Figure 6.1). This was supported by inhibiting the formation of the NADPH oxidase complex and then measuring the levels of 2-AG production, which were consequently reduced. These data support the idea that macrophages have important roles in the metabolism of lipids that have roles in regulating inflammation. There has been recent reports suggesting that upregulating the eCB system can cause Gi/o to inhibit the function of Ras-proximate-1-GTPase activating protein (Rap1-GAP) allowing Rap1-GTP to remain in its "on" state (Figure 6.2). Disallowing Rap1-GTP to shut off leads to downstream signaling that disassemble the Nox complex, thereby suppressing ROS generation (Han *et al.* 2009). Although current therapies for CVD target well-established risk factors such as hypercholesterolemia and blood pressure, the eCB system and the oxidoreductases that generate reactive oxygen species are emerging targets for mitigating inflammation and oxyradical stress in the cardiovascular wall. The research presented in this dissertation confirms previously published data that oxidized

low-density lipoproteins can cause oxidative stress via the ligation of scavenger receptors such as CD36, leading to oxidative and pro-inflammatory responses. However, the connection between increased NADPH oxidase activity and endocannabinoid production is a novel discovery and suggests that oxidative stress can result in the increased biosynthesis of 2-AG.

While ROS appears to induce the biosynthesis of 2-AG, these eCB ligands work locally and are short lived due to their rapid degradation by hydrolytic enzymes. Because macrophages have a vital role in lipid metabolism, they are the most studied peripheral cell types with regard to eCB hydrolytic enzymes. However, this dissertation presented the first report of the nature of the eCB hydrolytic enzymes that are found in human smooth muscle cells (SMCs) in chapter 5. In future studies, phagocytic cells and SMC could be studied together in co-culture to determine the pathways for 2-AG hydrolysis by serine hydrolases, because these cell types are found to neighbor one another in the vessel wall. Additionally, SMCs catabolize 2-AG via monoacylglycerol lipase (MAGL)-dependent hydrolysis and with their close proximity in the vessel wall, SMCs may assist in the degradation of 2-AG released by neighboring macrophages. Several previous reports have suggested that the factors that initiate inflammation in macrophages can also alter the phenotype and function of SMC. Mechanistic pathways that alter the phenotype of SMC resulting in the promotion of a lipid-laden cell phenotype is a viable area for exploration. The data on SMC serine hydrolases will provide additional resources into the investigation of specific inhibitors for serine hydrolases such as MAGL as a therapeutic regimen (Figure 6.3), as opposed to the general hydrolase inhibition that is seen with current drug therapy. To support the notion that CB_2 is a protective response,

Hoyer et al (2011) used ApoE$^{-/-}$CB$_2^{-/-}$ mice that are more prone to atherosclerosis than the ApoE$^{-/-}$ alone to indicate knocking out CB$_2$ increases the superoxide production and increase macrophage infiltration into the vessel wall. The effects of heightened CB$_2$ decreases monocyte migration to the site of vascular inflammation, chemokine receptors, vascular cell adhesion molecule 1 and intracellular adhesion molecule 1 on endothelial cells, and interferon gamma by lymphocytes. This research elucidates the links between oxyradical stress, NADPH oxidase activity, and enhanced 2-AG biosynthesis (Figure 6.3). Because inflammation and oxidative stress are hallmarks of cardiovascular disease, the data in this dissertation coupled with previous reports, provides evidence of compensatory pathways to reduce oxidative stress in macrophages. In addition, it suggest a mechanism by which oxyradical stress can be reduced via enhanced eCB tone.

6.2 Future Directions

Further investigation into the role of the eCB receptors is needed to distinguish between the CB$_1$ and CB$_2$ receptors in oxidative stress. The conundrum of the eCB receptors is that their downstream pathways can counteract each other during intracellular redox state. Therefore, it is important to provide more evidence for the role of each receptor in transducing signals derived from 2-AG, and how this signaling might decrease inflammatory cascades during oxidative stress and shear stress in the vessel wall.

The investigation of CB$_2$ receptor signaling due to oxidative stress and the ability to sustain the presence of the ligand for this receptor could inhibit the inflammation associated with CVD. Although current pharmaceuticals that target serine hydrolases are non-specific, more studies are needed to specifically target SMC serine hydrolases as a

means of abrogating the differentiation of SMC into a lipid-laden phenotype. It is known that inhibition angiotension (Ang) II signaling is associated with decreased CVD symptoms, as seen with the prevalence of sartan prescriptions in the clinical setting. With the SMC serine hydrolases now identified, coupled with the serine hydrolases that have been identified in phagocytic cells, it will be beneficial to explore how stabilizing the levels of 2-AG in these cells can be used as a treatment modality for reducing atherosclerosis.

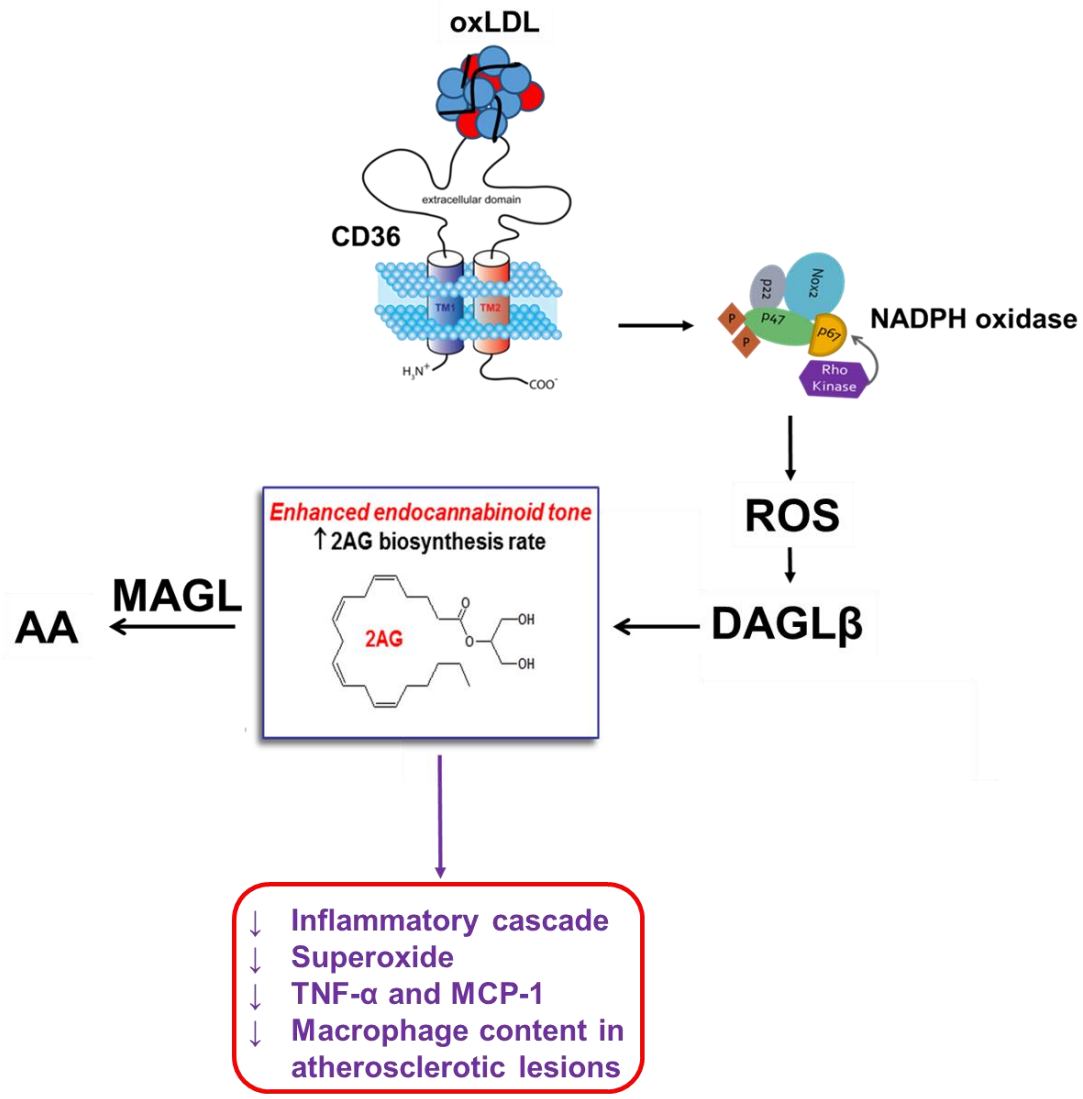

Figure 6.1 Oxyradical stress can activate NADPH oxidase, which results in a compensatory increase in 2-AG synthesis that might have beneficial effects.

The activation of NADPH oxidase by oxLDL binding to scavenger receptors such as CD36 can lead to the upregulation of DAGLβ, the second messenger to the biosynthesis of 2-AG, thereby enhancing "endocannabinoid tone". Additionally, 2-AG can be hydrolyzed by MAGL, which is found in both macrophages and SMCs, to its metabolites AA and glycerol.
Oxidized low density lipoproteins, oxLDL; reactive oxygen species, ROS, diacylglycerol lipase beta, DAGLβ; 2-arachidonylglycerol, 2-AG; monoacylglycerol lipase, MAGL; arachidonic acid, AA; monocyte chemoattractant protein-1, MCP-1.

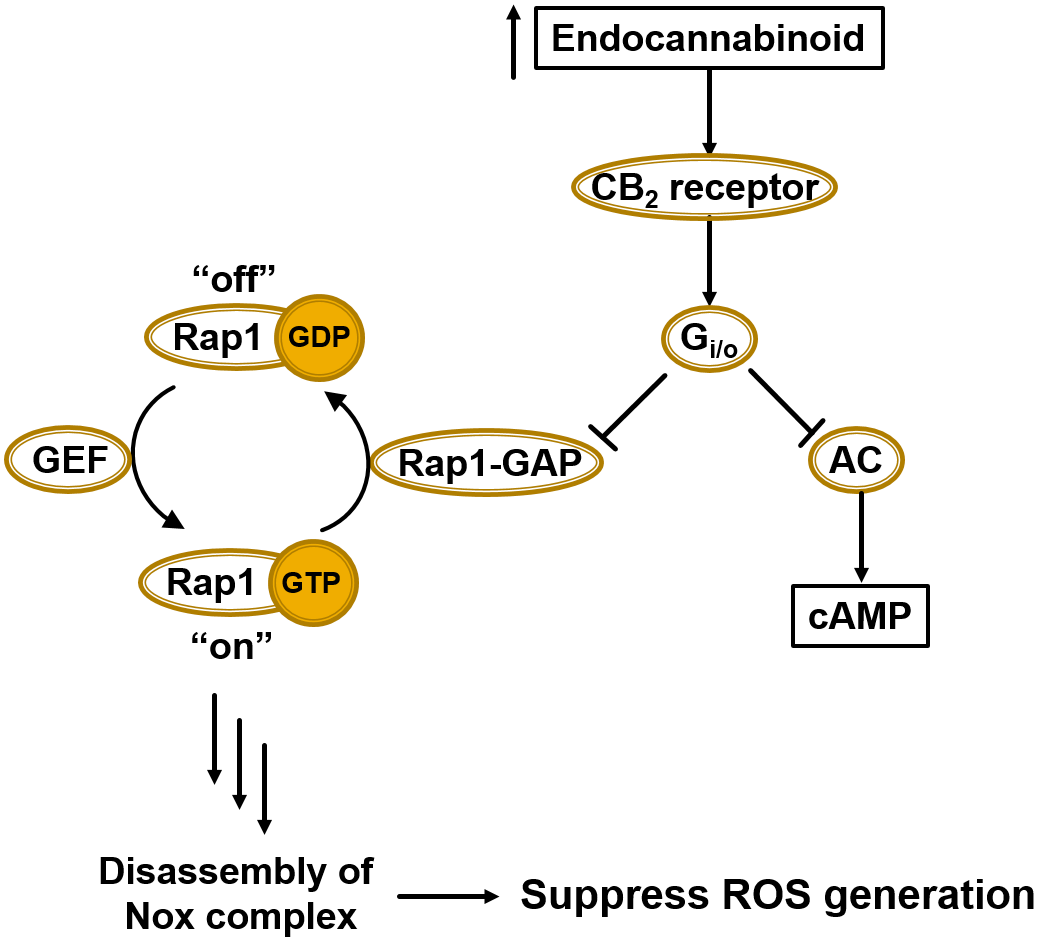

Figure 6.2 Upregulated endocannabinoid system reduce oxidative stress.

The enhanced level of endocannabinoid could be a negative feedback to reduce oxyradical stress. The conical signaling pathway is via $G_{i/o}$, which acts to inhibit adenalate cyclase; however, $G_{i/o}$, could also inhibit the function of Rap1-GAP to maintain Rap-GTP in its "on" state leading to downstream signaling that can potentially disassemble the NADPH oxidase complex, thereby suppressing ROS generation. Cannabinoid receptor 2, CB_2; adenalate cyclase, AC; cyclic adenosine monophosphate, cAMP; Ras-proximate-1, RAP1; guanosine diphosphate/triphosphate, GDP/GTP; GTPase activating protein, GAP; GTP exchange factor, GEF; reactive oxygen species, ROS.

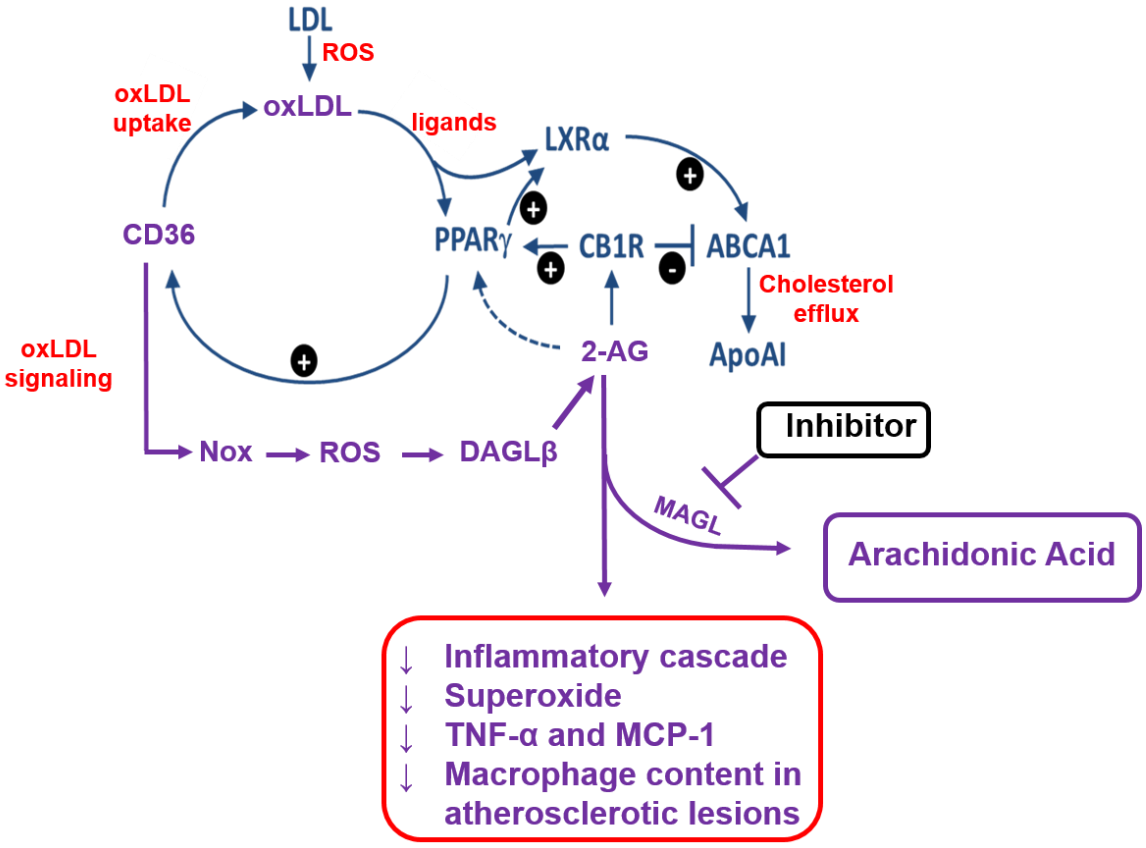

Figure 6.3 Scheme suggesting the significance for therapy.

Oxidative stress can initiate pathways leading to the activation of the Nox complex leading to enhanced ROS. Formation of ROS stimulate the downstream pathway leading to cholesterol efflux to maintain homeostasis. Alternatively, ROS can leads to the upregulation of DAGLβ, the second messenger in the biosynthesis of 2-AG which can potentially lead to anti-inflammatory effects. The compilation of research indicated in these chapters are summarized in purple.

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