OXIDIZED LOW DENSITY LIPOPROTEIN REGULATES APOPTOSIS AND GROWTH FACTOR PRODUCTION IN MACROPHAGES

by

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Abstract

Macrophages and oxidized LDL (oxLDL) both play key roles in the pathogenesis of atherogenesis. This thesis is focused on the effects of oxLDL on macrophage cytokine secretion and macrophage survival. The first project defines some of the mechanisms by which oxLDL increases secretion of vascular endothelial growth factor (VEGF), a pro-inflammatory growth factor known to be involved in atherogenesis. We show that both the protein and lipid components of oxLDL contribute to induction of VEGF secretion. Disruption of the genes for CD36, SR-A, or LOX-1 scavenger receptors had no effect. The atypical protein kinase c (PKC\(\delta\)) was activated by oxLDL, and this activation was essential for the induction of VEGF.

OxLDL could be atherogenic through increasing macrophage number within the plaque. Our group has previously shown that oxLDL induces growth and inhibits apoptosis in macrophages. In the second project, we sought to determine if members of the scavenger receptor family were required for the prosurvival effect of oxLDL in macrophages. We used mouse strains lacking different scavenger receptors and found that oxLDL-mediated survival is not dependent on CD36, SR-A, LOX-1, TLR4, its signaling partner CD14, or Fc\(\gamma\)RIIb. Significant inhibition of oxLDL uptake by a combined inactivation of CD36 and SR-A did not reduce the prosurvival effect of oxLDL.

In the third project, we sought to characterize the oxidative modification of LDL that is responsible for the prosurvival effect. We found that both protein and lipid components of oxLDL can induce growth in macrophages. This seems to be mediated by modification of amino groups in apoB or in phosphatidylethanolamine by lipid peroxidation products.

Further characterization of these oxidation products suggested that unfragmented hydroperoxide or endoperoxide-containing oxidation products of linoleic acid and arachidonic acid derivatize amino groups. When LDL or other proteins are modified in this fashion, they acquire the ability to induce pro-survival signaling pathways in macrophages. HPLC-MSMS studies showed that some of the arachidonic acid-derived lysine adducts are isolevuglandin (isoLG)-derived adducts that contain lactam and hydroxylactam rings. MSMS analysis of linoleic acid autoxidation adducts was consistent with 5 or 6 membered nitrogen-containing heterocycles derived from unfragmented oxidation products.
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<td>ES+</td>
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<td>SR-POX</td>
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<tr>
<td>VCA</td>
<td>vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEC</td>
<td>vascular endothelial cell</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
</tr>
<tr>
<td>VPF</td>
<td>vascular permeability factor</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
</tr>
<tr>
<td>WT</td>
<td>wild-type</td>
</tr>
<tr>
<td>XO</td>
<td>xanthine oxidase</td>
</tr>
<tr>
<td>XTT</td>
<td>2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide</td>
</tr>
<tr>
<td>ε-DNA</td>
<td>etheno-DNA</td>
</tr>
</tbody>
</table>
Acknowledgments

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Dedication

This work is dedicated to my mother, father, and brother. Thank you for your unconditional love and support throughout my life.
Co-authorship statement

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1. Introduction

1.1 Atherosclerosis

1.1.1 Definition and epidemiology

Atherosclerosis is a chronic inflammatory disease of large and medium-sized arteries characterized by the accumulation of lipids, cells, and connective tissue within the arterial wall. Plaques may progressively increase in size so that they eventually compromise blood flow, or the plaque can ulcerate or rupture, leading to clot formation. Either event can cause ischemia in distal regions supplied by that artery. Atherosclerosis is the most common cause of coronary heart disease or CHD (which includes myocardial infarction and angina pectoris), ischemic cerebrovascular accidents (strokes), and peripheral arterial disease (PAD) [1].

The World Health Organization Global Burden of Disease project reported in 2008 that ischemic heart disease followed by cerebrovascular diseases are respectively the top two leading causes of death worldwide. It was also projected in that report that they will remain among the top 4 leading causes of death and that the disease burden for both will continually rise by 2030 [2]. In North America, lifestyle choices have made atherosclerosis a particularly prevalent health issue. Increased attention to smoking cessation and control of risk factors has resulted in a decline in the number of cardiovascular deaths in the past 20 years, but the burden for society remains very high. In Canada, one out of every three deaths is caused by cardiovascular disease with CHD accounting for more than 50% [3]. In the United States of America, one out of every 5 deaths is due to CHD. It is estimated that every minute four Americans will have a coronary episode, of which one will be fatal. In the USA, the estimated 2009 health care cost attributed to CHD and stroke is $165.4 billion [4].

1.1.2 Structure of the atherosclerotic plaque

Even though the clinical presentation varies depending on the site of plaque formation, the pathophysiological mechanisms and the structure of the plaque remains similar. The development of the atherosclerotic plaque has been classified into 6 stages according to the Stary classification [5-7]. Type I lesions or “initial lesions” are generally visible only with a microscope, and show thickening of the
intima and an increased number of macrophages in the intima, some of which contain lipid droplets (macrophage foam cells - Figure 1) [6]. As the lesion further progresses, foam cells increase in number and group together to produce type II lesions. In addition to macrophages, other inflammatory cells such as T lymphocytes have been reported in type II lesions though in small numbers [8]. Quite often, type II lesions become elevated and grossly visible as “fatty streaks”. Type III lesions represent a transitional stage which bridges between early lesions (the first two types) and more advanced types and therefore are considered “intermediate lesions”. Type III lesions are marked by appearance of lipid droplets outside the cells [8]. Expansion and coalition of these free lipid droplets form a distinct “lipid core” which completes the transition from early to a more advanced stage of atherosclerotic plaque (“atheroma” - type IV) [5, 6]. A common theme in all advanced lesions is that they are formed by repeated cycles of inflammation, perhaps driven by modified lipids and the influx of inflammatory cells. The inflammatory response leads to disorganization of normal artery wall architecture [5, 9]. As the atheroma progresses, a distinct fibrous layer (fibrous cap) separates the lipid core from the endothelium, and this is a hallmark of type V lesions. Type VI lesions are those that have undergone complications such as fissuring, ulceration, or dissection - all of which are often associated with thrombosis (Figure 1.1 and [5]).

The mechanisms responsible for plaque initiation and progression are not entirely understood. However, a large amount of information is available and will be reviewed briefly in the following section.

1.1.3 Lipid theory of atherogenesis: cholesterol and low-density lipoprotein

A strikingly early and permanent constituent of all stages of an atherosclerotic plaque is the lipid - mainly cholesterol delivered by apolipoprotein B (apoB) containing lipoproteins. The lipid theory of atherosclerosis proposes a causal role for lipid transudation into the intimal layer and essentially states that increased cholesterol level (mainly low-density lipoprotein (LDL) -associated cholesterol) is atherogenic. Early in the 20th century, Anitschkow showed experimentally that diet-induced hypercholesterolemia by itself is sufficient to induce atherosclerosis [10]. As hydrophobic compounds,
lipids in free form cannot be found in aqueous phases. In plasma, lipids can be found in association with specialized proteins (apolipoproteins) in water-soluble complexes known as lipoproteins (LPs).

Two widely used methods for classification of LPs are based on hydrated density or mobility on agarose gel electrophoresis. Goffman first described different classes of lipoproteins based on analytical ultracentrifugation [11] and the principle of separation of lipoproteins by density was extended to preparative ultracentrifugation by Havel et al [12]. Based on density, lipoproteins are classified as chylomicron (CM) with a density <0.94 g/mL, very low-density lipoprotein (VLDL, 0.94<d<1.006 g/mL), low-density lipoprotein (LDL, 1.020<d<1.063 g/mL), and high density lipoproteins (HDL, 1.063<d<1.21 g/mL). Lees et al. applied agarose gel electrophoresis to separate lipoproteins based on their charge [13]. Based on electrophoretic mobility, mature HDL has α migration, VLDL and nascent HDL have pre-β migration, and LDL has β migration. CM particles are too large to enter conventional agarose gels and are therefore retained at the origin [14].

CM is produced in the intestine with a truncated version of apoB (apoB48) as well as apoA, C, D, and E. The main function of CM is to deliver diet-derived lipids from the intestine to peripheral tissues and the liver. Because of their rapid clearance, fasting blood normally does not contain CM. VLDL is produced in the liver, and has full length apoB (apoB or apoB100) as its major apolipoprotein, but also smaller amounts of apo AI, AII, apoCII, and apoE. Its role is to carry lipids (mainly triglycerides) to peripheral tissue. LDL, a lipoprotein rich in cholesterol with apoB as its only major protein, is formed from VLDL remnants by cholesterol ester transfer protein, which exchanges cholesterol ester in HDL for TG [14]. HDL has apoA-1 as its main protein and is essential for a process called reverse cholesterol transport (RCT) by which excess cholesterol is mobilized from peripheral tissues and plasma to be returned to the liver for biliary excretion. During this process apoA-1 rich lipid-poor HDL, known as nascent HDL, progressively accumulates cholesterol to form more mature HDL particles [15].

Studies of patients with genetic defects leading to abnormal lipoprotein patterns have provided valuable insights regarding the role of individual lipoprotein classes in atherosclerosis. In conditions where concentration of “intact” CMs is elevated (e.g. lipoprotein lipase deficiency), the risk of
atherosclerosis is not increased [16-18]. This may be because the endothelial layer is relatively impermeable to larger lipoproteins (CMs and VLDLs) [19] and also because they have a short half-life. LDL, on the other hand, has a relatively smaller size and a longer half-life of 2 to 3 days [20]. Concentration may also be important as LDL is also the main cholesterol-carrying and atherogenic lipoprotein in humans [9].

The most dramatic example of the importance of LDL comes from patients with naturally occurring mutations in LDL receptor (LDLR) gene, which in patients with heterozygous and homozygous mutations result in two-fold and more than six-fold increases in plasma levels, respectively. The majority of heterozygotes have a cardiac event by the age of 60 and many homozygotes experience heart attacks as teenagers [21].

Epidemiological studies including the Seven Countries study [22, 23] and the Framingham heart study [24, 25] established a strong association between increased total cholesterol levels, and deaths due to CHD. The importance of LDL cholesterol levels has been confirmed in many randomized controlled clinical trials. The interventions employed in these studies involved dietary measures alone (reviewed in [26]) or combined with drugs such as bile acid binding resins [27, 28] or HMGCoA reductase inhibitors (statins) [29]. A meta analysis of 14 trials of statin therapy showed that, over a span of five years, for each mmol/L reduction in LDL cholesterol, there is a ~20% reduction in coronary events, strokes, or coronary artery bypass surgery [30].

However, the lipid theory does not fully explain some features of atherogenesis such as why certain sites are more prone to plaque formation, how foam cells are formed, or how inflammatory features in plaques are initiated and maintained.

1.1.4 Response-to-injury hypothesis: shear stress and endothelial dysfunction

It has been known for many years that atherosclerotic plaques are preferentially located at specific areas of large and medium-sized arteries. This was puzzling especially considering that all risk factors of atherosclerosis, such as hypercholesterolemia, act systemically. For example, a study had revealed that, even though in smaller amounts, plasma components such as albumin, LDL, and HDL
exist in the walls of non-atherosclerotic arteries [31] which suggested a passive diffusion of these elements into the otherwise normal arterial wall. Since the sites of predilection for lesion formation are generally at either curvatures or branch points within the arterial tree, in the mid 20th century, it was believed that disturbances of flow at atherosclerotic-prone sites causes mechanical injury to the endothelial layer [32, 33]. To explain the proliferation of smooth muscle cells in plaque, Ross and Glomset proposed the "response-to-injury" hypothesis. This stated that the initial event in atherogenesis was a direct (albeit unknown) injury to the endothelium causing endothelial desquamation and exposure of the underlying matrix which in turn causes platelets to adhere/aggregate [34, 35]. The activated platelets release platelet-derived growth factor which stimulates smooth muscle cell proliferation. The response-to-injury hypothesis has been refined over several decades. Today, we know that lesions tend to form at sites of disturbed flow, typically associated with either low shear stress or oscillatory shear stress [36, 37].

The cytoskeleton, cell membrane, and intercellular connections between endothelial cells act as a mechanical sensors which respond to changes in shear stress [38]. When flow is disturbed, the net effect of mechanotransduction is to favor activation of pro-thrombotic, pro-oxidative, and pro-inflammatory gene expression in ECs [39, 40] including reduction of endothelial nitric oxide synthase (eNOS) expression [41, 42], increased expression of monocyte chemotactic protein-1 (MCP-1) [43], nuclear factor-κB (NF-κB) activation, intercellular cell adhesion molecule-1 (ICAM-1) expression [44, 45], and induction of oxidant release [46, 47].

It has also been shown that shear stress modulates EC handling of lipids. Firstly, low shear stress causes a sustained activation of sterol regulatory element-binding protein 1 (SREBP1) leading to increased expression of LDLR and LDL binding [48]. Disturbed flow can also promote atherogenesis by increasing endothelial permeability to macromolecules such as LDL [49-51].

### 1.1.5 Response-to-retention hypothesis

This hypothesis is based on evidence suggesting that after apoB containing lipoproteins enter the sub-intimal space, they are retained and achieve high concentrations relative to other plasma
components. Their prolonged retention in this environment (where antioxidants defense is much weaker than plasma) sets the stage for the ensuing events such as modification of native LDL and induction of an inflammatory response [52]. It is also suggested that the characteristic flow dynamics at sites of predilection might lead to changes in matrix composition that favor retention [53]. Among the matrix components of intima, proteoglycans, mostly chondroitin sulfate (CS)-rich biglycan and versican, are believed to bind to apoB and cause LP retention [54-57]. Boren et al. discovered the site on apoB100 that binds to proteoglycans [58] and later a binding site on the surface of apoB48 was also identified [59]. Direct evidence for a role for proteoglycan binding was obtained by Skalen et al. who generated a transgenic mouse that expressed a mutant apoB that did not bind to proteoglycans but had preserved binding to LDLR. Mice expressing the mutant apoB developed significantly less atherosclerosis than controls [60]. However transgenic animals with proteoglycan-binding-defective LDL eventually caught up with their control counterparts in terms of plaque burden. It is now believed that in addition to physical interaction between LDL and proteoglycans, there are additional elements that contribute to retention of LPs. For example, secretory sphingomyelinase (sSMase) can promote retention by inducing aggregation of LDL and by increasing the affinity of LDL for proteoglycans [61, 62]. Other proretentive candidates include lipoprotein lipase (LPL) and secretory phospholipase A2 (sPLA2). Whatever the mechanism(s), lipoprotein retention makes them more susceptible to modifications which advance plaque formation.

1.1.6 Modified low-density lipoprotein and foam cell formation

Macrophage foam cell formation is one of the earliest features of atherosclerosis but is not fully explained by the lipid insudation hypothesis. Cholesterol synthesis and cholesterol uptake are regulated by intracellular sterol content. When the intracellular sterol level is low, SREBPs (the master regulators of intracellular lipid homeostasis) are activated resulting in increased LDLR, fatty acid, and cholesterol synthesis. In the presence of sterols however, lipid synthesis and uptake is significantly inhibited [63, 64]. This feedback mechanism explains why incubation of macrophages with even high concentrations of native LDL does not produce foam cells [65, 66].
In the course of studies to explain how macrophages could accumulate massive amounts of cholesterol, Goldstein and Brown discovered that cultured macrophages could rapidly internalize certain types of modified LDL such as acetylated LDL (Ac-LDL) [67]. They found that the uptake of acetylated LDL was mediated by a saturable high-affinity process, that they termed the scavenger receptor pathway, and that this pathway was not subject to feedback regulation by cellular cholesterol. They hypothesized that native LDL might undergo a similar type of modification in vivo, and that this could lead to its uptake through the scavenger receptor pathway [67, 68]. However Ac-LDL and the other chemically modified forms of LDL that were tested by Brown and Goldstein do not exist in vivo and therefore a search for more physiologically relevant modifications began. Two separate groups independently observed that incubation of LDL with endothelial cells (ECs) could generate a cytotoxic LDL [69, 70]. This form of modified LDL was termed endothelial-cell modified LDL, and it was also capable of inducing foam cell transformation in cultured macrophages. There was partial cross-competition between Ac-LDL and cell-modified LDL for uptake and degradation in macrophages and so initially it was thought they utilized the same pathway [71]. Steinbrecher et al. later showed that this modification by cells involved generation of free radicals and peroxidation of unsaturated fatty acids in LDL [72]. There are other forms of modified LDL that are not oxidized yet can produce foam cells such as: aggregated LDL [73], sphingomyelinase-modified LDL [74], glycated LDL [75], multiple-enzyme modified LDL (E-LDL) [76, 77], and LDL-antibody complexes [78]. However, LDL modified by oxidation is the most widely studied and there is evidence suggesting it may play a role in several stages of atherosclerosis.

1.1.7 Oxidative modification hypothesis of atherosclerosis

The oxidative modification hypothesis [79] holds that LDL becomes trapped in the subendothelial by binding to collagen and other matrix proteins. Its long residence time in a microenvironment where there are several cell types that can promote oxidation [80, 81], and a relative paucity of antioxidant defenses relative to the bloodstream, makes LDL vulnerable to oxidation in this location (Figure 1.1).
There is evidence that oxLDL is present in vivo, both in animal models and humans [82, 83]. OxLDL is immunogenic and autoantibodies against oxLDL are found in higher concentration in serum from animal and humans with atherosclerosis than in controls [83-86]. Immunohistochemical studies have detected oxidation-specific epitopes in atherosclerotic plaques but not in normal arteries [84, 87]. Studying the arterial walls in fetuses of hypercholesterolemic mothers, Napoli et al. showed that oxLDL immunoreactivity can be found even before monocytes are recruited [88].

OxLDL has an impressive repertoire of proatherogenic properties. It upregulates macrophage colony stimulating factor (M-CSF) [89] and monocyte chemotactic protein-1 (MCP-1) expression in ECs and smooth muscle cells (SMCs) [90]. Both of these cytokines play an essential role in lesion development [91-93]. Heavily oxLDL itself is a chemoattractant for monocytes [94] and T cells [95]. It can also activate ECs to express adhesion molecules [96, 97] to promote rolling and tethering of leukocytes, an essential step in atherogenesis [98, 99]. OxLDL also promotes transmigration of monocytes through endothelium by upregulating junctional adhesion molecule C (JAM-C) on the EC surface [100]. The expression of an array of pro-inflammatory cytokines is induced by oxLDL [101, 102]. OxLDL also induces oxidative stress and increases the production of reactive oxygen species (ROS) in macrophages [103]. OxLDL perpetuates endothelial dysfunction by antagonizing nitric oxide (NO) and vasodilation [104]. OxLDL is also involved in events that are important in later stages of atherosclerosis, for example, it induces expression of matrix metalloproteinase 1 (MMP1) [105] as well as tissue factor [106], which could lead to plaque instability and promote thrombosis.

The in vivo mechanism(s) responsible for LDL oxidation remains to be elucidated. Pro-oxidant candidates for which some experimental, genetic, or epidemiological support exist include: lipoxygenases (LPOs) [107-111], myeloperoxidase (MPO) [112-119], nicotinamide adenine dinucleotide (phosphate) or NAD(P)H oxidase [120-124], xanthine oxidase (XO) [47, 125, 126], uncoupled mitochondrial respiratory chain [127, 128], uncoupled endothelial nitric oxide synthase (eNOS) [129-132], and inducible nitric oxide synthase (iNOS) [133]. Endogenous antioxidants (and therefore atheroprotective) candidates include but not limited to: heme oxygenase 1 (HO-1) [134-139], and paraoxonases (PONs) [140-145].
If oxLDL plays an important role in atherogenesis, administering antioxidants that are capable of preventing LDL oxidation in vivo should reduce lesion formation. Several trials of antioxidant administration in animal models of atherosclerosis showed a beneficial effect [146-151]. Epidemiological studies also support a beneficial role for dietary antioxidants [152, 153]. However, several randomized trials of antioxidant supplementation in humans have yielded negative results [154-157]. It has been argued that a clear answer about the role of oxLDL in atherosclerosis requires a detailed understanding and mapping of oxidative events responsible for LDL modification and the development of effective and selective inhibitors of these pathways, as well as the availability of reliable and measurable pathway-specific markers. Even then, an important question would be when one would need to start the antioxidant intervention in humans to reasonably expect an effect. The Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study has found early atherosclerotic lesions in children and adolescents [158, 159]. This means that starting antioxidant treatment in middle age when the disease may already be well established might not show a dramatic benefit.

1.1.8 Immune system and atherosclerosis

The first review paper to highlight the role of immunological processes in atherogenesis was published in 1969 [160]. “Atherosclerosis - an inflammatory disease”, Ross’s definitive review, was published thirty years later [161]. In those three decades we learned that 1) dyslipidemia is not “the only” causal factor (atherosclerosis still remains a challenge in post-statin era [162], 2) inflammation is mechanistically linked to dyslipidemia and lipid oxidation [163], and 3) the role of different aspects of immunity can be tested in animal models of atherosclerosis by targeted manipulation of specific genes.

Many aspects of immunity within the innate and adaptive arms have been shown to modulate atherogenesis. While macrophages, in particular, and T cells are the predominant inflammatory cells within the atherosclerotic plaque, other immune cells such as B cells, mast cells, and dendritic cells also exist in the plaque - all with an activated phenotype [164]. The exact nature of the “harmful” agent(s) that sets off immune effectors is not fully known. However it is thought that modification of LDL (mainly oxidation) renders it immunogenic which is then recognized by the innate and adaptive
immunity [165]. In addition to these “neo-antigens”, certain oxidized lipids such as oxidized phospholipids (oxPL) are inherently pro-inflammatory and will propel the inflammation forward (section 1.4.3 and [166]).

Innate immunity plays an important role in progressions of atherosclerosis. Recruitment of inflammatory cells by chemotactic properties of oxLDL or chemokines it induces was discussed in section 1.1.6. The role of macrophage in atherosclerosis will be further discussed in section 1.2.

Components of the complement system can have either atherogenic and atheroprotective effects [167-169]. There is less direct evidence for the role of other cells of the innate immunity however a mounting body of evidence suggests that mast cells, dendritic cells, and natural killer cells may be important [170]. The humoral arm of innate immunity in the mouse includes a specific type of B cell referred to as B1 cells. They recognize a limited repertoire of antigens that are germ-line encoded and preserved through natural selection (as oppose to massive repertoire of adaptive immunity receptors which are selected through somatic mutations) [171, 172]. These cells are responsible for autoantibodies (IgM class) that are directed against oxidation-specific epitopes and there is strong evidence showing an atheroprotective role for these autoantibodies [173]. The T cell equivalent of B1 cells are natural killer T (NKT) cells which recognize lipid and glycolipid antigens in a CD1 restricted manner. There is evidence showing an atherogenic role for these T cells as well [174].

Adaptive immunity also plays an important role. A combined B and T cell defect reduces atherosclerosis in mouse models [175-178]. However, if the cholesterol level is sufficiently high, atherosclerosis develops even in an immunodeficient background. More selective approaches have shown that adaptive immunity (and innate for that matter) have both atherogenic and atheroprotective effects. CD4+ T cells [178], T cell costimulation [179, 180], and T helper 1 (Th1) polarization with IL-12 and IL-17 [181, 182] promote atherosclerosis while IL-33 (possibly through Th2 polarization) [183], regulatory T cells [184, 185], IL-5 [186], IL-10 [187], and transforming growth factor β (TGF-β) [188] are atheroprotective.

The appreciation for the role of immune system in plaque development and complications has also led to discovery of new potential susceptibility genes [189-191], disease markers, and therapeutic
targets. For example, C-reactive protein (CRP) has been found to be a clinically useful marker for the prediction of atherosclerotic risk in individual patients. Vaccination [192-198] or induction of tolerance [199] with different oxidatively modified LDLs have shown promise as they can reduce atherosclerosis in animal models.
Figure 1.1 Postulated role of oxLDL in the formation of atherosclerotic lesions. LEFT PANEL: In the early stages of atherosclerosis, LDL crosses the endothelial barrier and becomes oxidized within the intimal microenvironment. OxLDL accelerates plaque progression by enhancing expression of adhesion molecules and recruiting inflammatory cells, mainly monocytes. Unregulated uptake of oxLDL by macrophage SRs will generate foam cells. Efflux of cholesterol by HDL (part of reverse cholesterol transport) is a compensatory mechanism which functions to deliver the cholesterol back to the liver for disposal. MIDDLE PANEL: Continuation of process described will lead to increased cellularity within the plaque, luminal bulging of the plaque, fibrous-cap formation by migrating VSMCs and an overall heightened inflammatory response. The inflammatory processes cause thinning of the fibrous cap especially in macrophage-rich shoulder area and formation of physically unstable lipid-rich necrotic core. These processes will render the plaque “unstable” and vulnerable to fissuring or rupture. RIGHT PANEL: Breach of endothelial barrier exposes the underlying thrombogenic tissue leading to platelet aggregation and clot formation. This will further compromise luminal flow causing ischemia of issue supplied by that artery. Reprinted from Cell, Vol(104), Glass CK, Witztum JL, Atherosclerosis: the road ahead, 503-516., Copyright (2001), with permission from Elsevier [9].
1.2 Monocytes, macrophages, and atherosclerosis

1.2.1 Monocyte/macrophage function and heterogeneity

Macrophages phagocytose and remove pathogens, apoptotic cells, cellular debris, and foreign material through scavenger receptors and other phagocytic mechanisms. As an important component of innate immunity, resident macrophages are one of the first lines of defense against pathogens. Through complement and Fc receptors they engulf and internalize opsonized particles by phagocytosis. Their effector function in the innate immune response also involves an array of pattern recognition receptors (PPRs), such as toll-like receptors (TLRs), which recognize epitopes that are specifically associated with pathogens (known as pathogen-associated molecular patterns or PAMPs). In response to PAMP recognition and binding, they become activated and secrete inflammatory mediators that initiate and orchestrate an inflammatory response. Macrophages also are important in acquired immunity as they can process and present antigens - a process by which macrophages communicate with and signal the expansion of alloreactive T and B cell clones. Macrophages can also become activated by lymphocyte-derived cytokines such as IFNγ (classical activation) or IL-4/IL-13 (alternative activation) to combat intracellular pathogens such as listeria, or parasites/allergens, respectively. They are also very important in the resolution phase of an inflammatory response and healing process [200].

Monocytes comprise up to 10% of blood leukocytes in humans. This steady state reflects the constant influx (from the bone marrow) and emigration (from the circulating pool) of cells. Once in tissues, monocytes differentiate into macrophages or dendritic cells. Monocytes all arise from a subgroup of multipotential stem cells in the bone marrow. Monocyte development, proliferation, and differentiation is dependent on colony stimulating factor 1 (csf-1) also known as macrophage colony stimulating factor (M-CSF) and its receptor [201, 202]. Phenotypically and functionally monocytes represent a heterogeneous population. In mice there are two major classes based on size, density, peroxidase activity, cytotoxicity potential and cell surface markers [203]. In the absence of inflammation, a subset of monocytes expresses neither the surface marker Ly6C nor chemokine receptor 2 (CCR2). They, however, express the receptor for fractalkine (CX3CR1+). It is believed that these monocytes are involved in steady state surveillance [204] and also give rise to resident macrophages within tissues. On
the other hand, Ly6C⁺, CCR2⁺, CX₃CR₁low monocytes (the so-called “inflammatory monocytes”) are elicited in response to inflammatory stimuli [205]. In humans, CD14⁺ CD16⁻ monocytes resemble mouse Ly6C⁺ (inflammatory) monocytes whereas CD14low, CD16⁺ monocytes are similar to mouse Ly6c⁻ (resident) monocytes [203]. The relevance of monocyte heterogeneity in atherosclerosis will be discussed in section 1.2.3.

Macrophages do not comprise a homogenous population either and, depending on the microenvironment they reside in and the signals they receive, they exhibit different phenotypes and functions. There is often a tissue-specific phenotype and macrophages in the liver (Kupffer cells), have properties that differ from those of central nervous system macrophages (microglia) or pulmonary alveolar macrophages. In addition to resting macrophages, macrophages are functionally divided into those activated to a “classical”(M1) phenotype or to an “alternative” (M2) phenotype [206]. It is believed that the classification of murine monocytes as Ly6C⁺ vs Ly6C⁻ roughly mirrors the M1 vs M2 classification of human monocytes [204, 207, 208]. However, the precise roles of lineage specificity, microenvironment, and macrophage plasticity in regulating macrophage function are not fully resolved [209].

### 1.2.2 Pattern recognition receptors in macrophages

Compared to B and T cells, macrophages recognize a limited repertoire of epitopes through receptors that are germline-encoded and highly conserved through natural selection. Instead of recognizing specific and unique sequences, they recognize “patterns” associated with exogenous pathogens or altered endogenous components (hence the name pattern recognition receptors) [210].

*Signaling PRRs*: The first signaling PRR to be discovered in humans was a Toll-like receptor (TLR) [211]. Currently 10 human TLRs have been discovered all of which are transmembrane proteins containing leucine-rich repeats (LRRs) outside the membrane. They sense a diverse range of endogenous and exogenous PAMPs [212, 213]. TLRs can further diversify the range of PAMPs they recognize (and even the outcome of their response) by cooperating with other TLRs or other PRRs such as CD14,
CD36, or SR-A scavenger receptors [214-216]. Upon ligand recognition, TLRs profoundly affect macrophage behavior via recruitment of adapter molecules leading to activation of transcription factors such as NF-κB and interferon regulatory factors [217]. In particular, TLR2 and TLR4 have been shown to modulate atherogenesis. Administration of TLR2 [218] and TLR4 [219] ligands exacerbates atherosclerosis. Conversely, different mouse models show significant reduction of atherosclerosis when TLR2 [218, 220, 221] or TLR4 [222, 223] signaling is interrupted. Interestingly, bone-marrow transfer studies have shown that expression of TLR2 by both bone-marrow derived cells and others tissues promotes atherogenesis. It appears that bone marrow-derived TLR2 expression is responsive to exogenous ligands while an unidentified endogenous ligand acts through TLR2 expression in non-bone marrow derived cells [218].

**Endocytic PRRs:** Seminal studies of Goldstein and Brown had suggested the existence of a scavenging receptor that mediated uptake of Ac-LDL [67]. This receptor, which they called the Ac-LDL receptor was purified by Kodama and colleagues, and designated as scavenger receptor A (SR-A) [224]. This group subsequently cloned and expressed various mutants of this receptor to define its functional domains [225, 226]. In the following years the list of SRs has increasingly grown and this superfamily now comprises 8 unrelated classes [227]. Each receptor binds to a variety of closely related ligands derived from pathogens [228] or the host [227]. The main SRs include class A (SR-A types I, II, and III), class B (CD36 and SR-B types I and II), class E (Lectin-like oxidized LDL receptor 1 or LOX-1), class G (scavenger receptor that binds phosphatidylserine and oxidized LDL or SR-PSOX), and class H (fasciclin, epidermal growth factor (EGF)-like, laminin-type EGF-like, and link domain-containing scavenger receptor-1or FEEL-1). An atherogenic role for SR-A [229-232], CD36 [233-235], and LOX-1[236] have been shown in apoE−/− and/or LDLR−/− mice while SR-PSOX [237] and SR-BI mediated lipoprotein uptake [238-240] seem to be atheroprotective. The receptor for advanced glycation products (RAGE) is also another multi-ligand receptor that belongs to the immunoglobulin superfamily and is expressed in macrophages [241]. It was recently shown that RAGE gene inactivation attenuates atherosclerosis in LDLR−/− mice. In this study, part of the effect was attributed to a dampened inflammatory response of macrophages to oxLDL treatment [242].
Macrophages also have an array of cytosolic and secreted PRRs. Nucleotide-binding oligomerization domain (NOD)-like receptors contain leucine-rich repeats, are found in the cytosol, and respond to cytosolic PAMPs [243]. Retinoic acid-inducible gene-I (RIG-I)-like receptor is another example of intracellular PRRs and they respond to viral RNA [244]. The possible role of NOD receptors in atherosclerosis remains to be elucidated [245].

1.2.3 Role of monocytes/macrophages in atherosclerosis

Macrophages along with cholesterol are important constituents of both early and late atherosclerotic plaques (Section 1.1.2 and Figure 1.1). Most foam cells in lesions are derived from macrophages, and the size of the intimal macrophage population correlates well with disease progression and clinical outcome [246, 247]. Monocyte-derived macrophages are dependent on M-CSF for their development, differentiation, and survival [201]. M-CSF deficiency (which results in decreased monocytes and macrophage differentiation/number) in mice lacking either apoE or LDLR caused an almost complete inhibition of plaque formation. Even mutation of a single copy of M-CSF significantly reduced disease progression [91, 248, 249].

Recruitment of monocytes into the intima is a critical event in plaque formation. Broad-spectrum inhibition of CC-chemokines (which mediate monocyte/macrophage recruitment) reduces atherosclerosis [250]. The first step in recruitment is chemotaxis. This is facilitated by monocyte chemoattractant protein (MCP-1) [92, 93, 251, 252]. Both the chemokine receptor CX3CR1 and its ligand fractalkine (CX3CL1) play an important role in atherogenesis [253-255]. Although there is an overlap in ligand binding, these chemokines and their receptors are not redundant as CX3CL1/CCR2/apoE triple knockout exhibited reduced atherosclerosis compared to CX3CL1/apoE or CCR2/apoE double knockouts [256]. Inflammatory (Ly6C+) monocytes are the dominant subtype associated with hypercholesterolemia and most plaque macrophages are derived from this subtype [257, 258]. Monocyte recruitment is dependent on CCR2, CX3CR1, and CCR5 chemokine receptors [257-259]. Rolling of monocytes is another critical step in recruitment which in Ly6C+ monocytes, appears to be dependent on P-selectin glycoprotein ligand-1 (PSGL-1)/P-selectin interaction [260].
Macrophages have a major role in later stages of atherosclerosis as well. Macrophages are an important source of matrix degrading enzymes within plaques [261-263] and prothrombotic tissue factor [264]. Increased macrophage density is associated with plaque instability and rupture [265, 266].

1.2.4 Oxidized LDL and macrophages

Oxidized LDL is thought to be a link between hypercholesterolemia and vascular inflammation [163]. OxLDL is a chemoattractant for monocytes [94]. Once within arterial wall, macrophages interact with oxLDL, mostly through PRRs, which leads to a) foam cell formation through scavenger receptors (this section and also 1.1.6) b) production of inflammatory proteins (this section) c) macrophage survival and increased plaque growth in initial stages of atherosclerosis (Sections 1.2.6) d) macrophage apoptosis resulting in lipid core formation in later stages of the disease (section 1.2.5) e) production of proteases and pro-thrombotic agents (section 1.1.7) which promote plaque instability and clot formation, respectively.

SR-A and CD36 are the main receptors for macrophage uptake of oxLDL and foam cell formation in vitro. Unlike LDLR, SRs are not downregulated by lipoprotein uptake. In fact, CD36 expression is upregulated by oxLDL [267]. Deletion of SR-A [229, 268] leads to a 30-50% reduction in oxLDL uptake and CD36 deletion to a 60% decrease [233]. Deletion of both receptors reduces oxLDL uptake by 80% and prevents in vitro foam cell formation [269].

OxLDL induces expression of inflammatory proteins such as TNF-α [270] and CD40 [271]. CD40/CD40L signaling itself causes plaque growth and instability at least in part by upregulating adhesion molecules and recruiting T cells and macrophages [180]. Oxidized lipids and oxLDL also induce macrophage expression of migration inhibitory factor (MIF) [272, 273], an inflammatory cytokine that promotes recruitment and retention of inflammatory cells. Interestingly, oxLDL inhibits emigration of macrophages out of the plaque. This was shown to be a CD36 dependent phenomenon followed by downstream activation of focal adhesion kinase (FAK). This leads to spreading and anchoring of plaque macrophages to the extracellular matrix.
OxLDL also leads to abnormal intracellular lipid handling. OxLDL is resistant to lysosomal degradation, and leads to the expansion of the number and size of lysosomes in macrophages [274]. It also interferes with reverse cholesterol transport as macrophages loaded with oxLDL show significantly lower sterol efflux compared to Ac-LDL-loaded macrophages [275, 276].

1.2.5 Oxidized LDL and macrophage toxicity and apoptosis

The first property of oxLDL to be reported was its ability to induce cytotoxicity in fibroblasts [69, 70]. It was initially shown that the cytotoxic agent(s) reside(s) within the lipid moiety of oxLDL [277, 278] however later Vicca et al. proposed that, at least with certain methods of LDL oxidation, apoB modification also contributes to oxLDL’s toxic potential [279, 280]. Currently it is believed that the bulk of cytotoxic and pro-apoptotic effects are due to lipid oxidation products such as oxysterols, oxidized phospholipids, and reactive aldehydes (e.g 4-hydroxy nonenal or 4-HNE) produced from decomposition of primary lipid peroxidation products (see section 1.4.1) [281].

OxLDL (or oxysterol)-induced apoptosis seems to be mediated by two major intracellular branches. One involves the mitochondrial pathway [282, 283] with calcium signaling as an upstream event [284]. Calcium release activates two pathways. One involves induction of PKB degradation leading to increased activity of pro-apoptotic Bcl-2 family members BIM and Bad and downregulation of Bcl-XL [285]. The other path is dependent on calcineurin (calcium-dependent phosphatase)-mediated Bad dephosphorylation [286]. The pro-apoptotic profile induced in Bcl-2 family members by these two pathways converges at the mitochondrial membrane to increase its permeability causing cytochrome c release and caspase activation.

Compromising lysosomal integrity/function is another mechanism by which oxLDL might induce apoptosis [287]. This is supported by observations that 1) lysosomal dysfunction precedes apoptosis and 2) inhibition of lysosomal cysteine proteases reduces oxysterol-induced macrophage apoptosis [288]. In other systems, it has been shown that lysosomal dysfunction merges with mitochondrial pathway of apoptosis at the level of Bcl-2 family members and, conversely, caspase
activation can augment lysosomal dysfunction [289, 290]. However, the cross-talk between these two pathways has not been studied in oxLDL-induced macrophage apoptosis.

1.2.6 Oxidized LDL and macrophage growth and survival

Atherosclerotic plaques show active cell proliferation [291, 292] with macrophages comprising the majority of the proliferating cells [293, 294]. More than 10 years after the discovery of its cytotoxic effects, Yui et al. showed that oxLDL can promote growth of mouse peritoneal macrophages [295]. The same group subsequently reported that this effect was mainly due to SR-A-mediated [296, 297] internalization of lysophosphatidylcholine (LPC) [298, 299] followed by intracellular calcium release, protein kinase C (PKC) and Erk1/2 activation, and upregulation of GM-CSF. The autocrine/paracrine action of GM-CSF then caused downstream activation of protein kinase B (PKB) and p38 [300-303]. This growth promoting property of oxLDL was inhibited by addition of anti-inflammatory or statin drugs [304-306]. Studies in our laboratories have confirmed the growth-promoting effect of oxLDL but in our hands this effect was not mediated by LPC. We also showed that a) the mitogenic effect depends on the degree of LDL oxidation and b) oxidative modification of apoB is important for this effect [307, 308].

We and other groups have shown that oxLDL inhibits apoptosis in bone-marrow derived macrophages (BMDMs) [309, 310]. BMDMs are strictly dependent on M-CSF for their growth and survival [311] and more than 50% of cultured BMDM undergo apoptosis after 24 h of M-CSF withdrawal. This makes them a useful model to study apoptosis, because mouse peritoneal macrophages are less dependent on M-CSF and die more slowly, and many dead cells are phagocytosed by their neighbors. As a result, assays for apoptotic cells give falsely low results. Using BMDMs from mice lacking M-CSF or GM-CSF, it was shown that oxLDL-mediated anti-apoptotic effect is not dependent on these growth factors [310, 312].

The inhibition of apoptosis by oxLDL involves multiple signaling pathways. Hamilton et al. reported a role for both Erk1/2 and phosphotidylinositol-3 kinase (PI3K) [309]. The importance of the PI3K/PKB pathway was confirmed by two groups [310, 313]. We showed that activation of PKB by oxLDL results in phosphorylation and proteasomal degradation of IκB-α with release of NF-κB to the
nucleus where it induces the expression of anti-apoptotic Bcl family member, Bcl-X\textsubscript{L} [314]. Activation of PI3K/PKB axis is in part achieved by oxLDL-mediated inhibition of acid sphingomyelinase (aSMase). In fact, aSMase activation upon M-CSF withdrawal leads to ceramide generation - an inducer of apoptosis [314]. Recently our laboratory showed that oxLDL also promotes macrophage survival by activation of eukaryotic elongation factor 2 kinase (eEF2K). One mechanism by which oxLDL activates eEF2K is by increasing sphingosine phosphate levels. This induces intracellular Ca\textsuperscript{++} release, which in turn activates eF2K. OxLDL also prevents the increase in p38 activity which otherwise accompanies growth factor withdrawal, and p38 is a negative regulator of EF2K. eEF2K is a survival mechanism during nutritional deprivation and its activation results in recycling cell components by autophagy and energy conservation by reduced protein translation [315]. A working model of the role of oxLDL in macrophage growth and survival is depicted in Figure 1.2.
Figure 1.2 Working model of oxLDL-mediated inhibition of apoptosis. Upon growth factor withdrawal, acid sphingomyelinase (aSMase) is activated leading to generation of ceramide, which is a strong inducer of the mitochondrial pathway of apoptosis. OxLDL inhibits the sphingomyelinase activity. Additionally, oxLDL may directly activate the PI3K/PKB signaling pathway leading to activation of NF-κB and expression of Bcl-XL. At the same time, oxLDL activates sphingosine kinase which increases sphingosine phosphate levels. OxLDL also inhibits cytokine-withdrawal-induced P38 activation. These processes lead to activation of eEF2K, which in turn promotes autophagic cell survival.

1.2.7 Oxidized LDL-mediated macrophage survival vs apoptosis

OxLDL has been shown to exert both cytotoxic/apoptotic and mitogenic/prosurvival effects on ECs, vascular smooth muscle cells (VSMCs), and macrophages (Table 1). The reason for the discrepant effects on macrophage survival is not clear. However, oxLDL is not a simple well-defined entity. Depending on the mode and degree of oxidation, it may contain a range of bioactive products [316]. For example, minimally modified LDL generated by incubation with ferrous sulfate induced a high level of apoptosis in one study [317], while minimally modified LDL generated by incubation of LDL with LPO-overexpressing cells produced a product that was anti-apoptotic [313]. The degree of oxidation is also important. We found that the potency of oxLDL for inducing cell survival is proportional to the extent of oxidation [307, 310]. The concentration of oxLDL used in in vitro experiments is also crucial. We and others have reported that concentrations of oxLDL less than 70 μg/mL inhibit apoptosis while concentrations higher than 100 μg/mL are toxic [308-310, 318]. Shatrov et al. have shown that pretreatment with low concentrations of oxLDL, protects macrophages from later challenges with toxic concentrations of oxLDL, through induction of manganese superoxide dismutase [319]. Differences in the method used to prepare oxLDL may also explain some of the discrepant results with regard to its effect on apoptosis. For example dialysis or ultrafiltration at the end of LDL oxidation by copper removes toxic water-soluble lipid peroxidation products from the preparation and allows the anti-apoptotic effect to become apparent [320].
Table 1.1  Cell survival and apoptosis with various methods of LDL oxidation

<table>
<thead>
<tr>
<th>Method of LDL modification</th>
<th>Cytotoxic/Apoptotic Effects</th>
<th>Mitogenic/Prosurvival Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>metal ion</td>
<td>[321], [322], [323]<em>, [324]</em>, [325]<em>, [326]</em>, [319]*</td>
<td>[295]<em>, [308]</em>, [309]<em>, [310]</em>, [325]<em>, [319]</em>, [327], [315]*</td>
</tr>
<tr>
<td>heme</td>
<td>[328]</td>
<td></td>
</tr>
<tr>
<td>hypochlorous acid</td>
<td>[279]*</td>
<td></td>
</tr>
<tr>
<td>lipoxygenase (LPO)</td>
<td>[329]</td>
<td></td>
</tr>
<tr>
<td>myeloperoxidase (MPO)</td>
<td>[330]</td>
<td>[315]*</td>
</tr>
<tr>
<td>UV irradiation</td>
<td>[325]*, [331], [332]</td>
<td>[325]*, [333], [334]</td>
</tr>
<tr>
<td>dissolved oxygen</td>
<td>[277], [278]</td>
<td></td>
</tr>
<tr>
<td>multiple-enzyme modified (E-LDL)</td>
<td>[335], [336]*</td>
<td></td>
</tr>
<tr>
<td>cultured cell</td>
<td>[337], [338]</td>
<td></td>
</tr>
<tr>
<td>unsaturated fatty acid auto-oxidation products</td>
<td>[307]*</td>
<td></td>
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<tr>
<td>minimally modified LDL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ferrous ion</td>
<td>[317]*, [339]</td>
<td>[325]*</td>
</tr>
<tr>
<td>LPO-overexpressing fibroblasts</td>
<td></td>
<td>[313]*</td>
</tr>
<tr>
<td>xanthine oxidase</td>
<td>[340]</td>
<td></td>
</tr>
<tr>
<td>dye-sensitized photooxidation</td>
<td>[341]</td>
<td></td>
</tr>
<tr>
<td>oxLDL-immune complexes</td>
<td></td>
<td>[342]*</td>
</tr>
<tr>
<td>LDL modified in vivo</td>
<td>[343], [344]</td>
<td></td>
</tr>
</tbody>
</table>

*Reported effect of modified LDL was observed in monocytes or macrophages
1.2.8 Macrophage survival vs apoptosis in atherosclerosis

Macrophage survival and/or proliferation in atherosclerotic lesions could promote plaque instability if it resulted in an increase in macrophage density and sustained a pro-inflammatory milieu. Recent studies in animal models have in fact suggested that suppression of macrophage apoptosis using p53⁻/⁻ [345] or Bax⁻/⁻ [346] bone-marrow transplantation significantly exacerbates atherosclerosis. On the other hand, enhancing macrophage-specific apoptosis by knockout of apoptosis inhibitory factor-alpha suppresses atherosclerosis in LDL receptor-deficient mice [347]. An atherogenic role for macrophage proliferation was also shown in apoE or LDLR knockout mice lacking the retinoblastoma or p53 gene products [348, 349]. These results support the notion that, at least in early stages of atherosclerosis, macrophage survival is detrimental and therefore induction of apoptosis in these cells could be of therapeutic value. Several recent studies have found that interventions that selectively target macrophages reduce the extent of atherosclerosis and/or promote a more stable plaque morphology [350-353].

Under some circumstances, macrophage apoptosis could be harmful, for example by contributing to the formation of a necrotic-core [354] as this is associated with plaque instability [355]. Therefore, many have proposed a stage-dependent role for macrophage survival and apoptosis, with macrophage survival being atherogenic in early and intermediate stages while macrophage apoptosis favoring instability in advanced lesions [356]. To address this issue, a few studies designed to induce or inhibit macrophage apoptosis in a stage-specific manner were recently conducted. The study by Stoneman et al. used bone-marrow transplantation from transgenic mice expressing CD11b-diphtheria toxin receptor [357]. Their studies confirmed that early induction of macrophage apoptosis reduces atherosclerosis. However, tripling the rate of macrophage apoptosis in established lesions did not promote an inflammatory or unstable plaque phenotype. It was therefore postulated that clearance of apoptotic cells (efferocytosis) might be a protective factor in plaque instability. Efficient efferocytosis promotes clearance of apoptotic cells without release of inflammatory mediators while inefficient efferocytosis leads to secondary necrosis and loss of membrane integrity leading to release of cell contents and a strong inflammatory reaction [356]. Support for this notion came from observations that
efferocytosis is impaired in atherosclerosis [358] and that disrupting apoptotic-cell clearance by macrophages exacerbates atherosclerosis [359]. Apoptotic-cell clearance by macrophages is critically dependent on Mer receptor tyrosine kinase (Mertk). Using stem-cell transfer of Mertk knockout or kinase dead variants, two groups independently showed that inhibition of efferocytosis leads to an increase in apoptotic cells, expansion of the necrotic core, and progression of the disease [360, 361]. In line with a stage-specific role for macrophage apoptosis and clearance of apoptotic bodies, Gautier et al. recently showed that inhibition of apoptosis increases lesion size in early stages while in advanced lesions, the size and complexity of plaques with apoptosis-resistant macrophages exceeded that of controls. Additionally, using an approach similar to Stoneman et al., they showed that sustained induction of apoptosis in advanced lesions resulted in a four-fold increase in lesion size. Short-term induction of apoptosis was also accompanied by accumulation of apoptotic bodies, recruitment of inflammatory cells to the intima, and an overall heightened inflammation [362].
1.3 Vascular endothelial growth factor

1.3.1 Discovery of VEGF and its functions

Vascular endothelial growth Factor (VEGF, also referred to as VEGF-A) belongs to a family of heparin-binding glycoproteins that, in mammals, includes four others members: VEGF-B, C, D, and placental growth factor (PLGF) [363]. The history of VEGF discovery is a testament to its multifunctionality. In 1983, a glycoprotein secreted by tumor cells which strongly increased vascular permeability was partially purified. It was called vascular permeability factor (VPF) [364]. In 1989 Ferrara et al. discovered an endothelial-specific mitogen which they named vascular endothelial growth factor [365]. Sequencing of VEGF and VPF cDNA showed that they encode the same protein [366, 367].

VEGF regulates many aspects of vasculogenesis (de novo generation of capillary beds and vessels from vascular progenitor cells) and angiogenesis (formation of capillary beds through sprouting of existing post-capillary venules). The cardiovascular system is the first organ system to develop during embryogenesis, and vasculogenesis is a critical step in this process. Heterozygote deletion of VEGF gene caused early demise of embryos due to impaired vasculogenesis [368, 369]. One of these studies also revealed a role for VEGF in early hematopoiesis as vegf<sup>+/−</sup> embryos had impaired blood-island formation within the yolk sac [369]. Embryonic development is distinctly dependent on the VEGF gene dose as even modest increases in VEGF expression can be lethal [370].

Early postnatal angiogenesis is also dependent on VEGF [371]. In later postnatal life, the importance of VEGF decreases, but it retains an important role in normal physiology (e.g. wound healing, female reproductive system, and exercise-induced angiogenesis) as well as disease processes such as tumor growth and metastasis, chronic inflammation, and macular degeneration [372]. The first properties of VEGF to be discovered were induction of EC growth and vascular permeability. VEGF induces vasodilatation (enough to cause hypotension upon intravenous administration) and increases vascular permeability. These effects are both mediated by an increase in NO production [373, 374]. It has also been shown that VEGF-induced NO production is one of the upstream events leading to activation of mitogenic pathways such as Ras/Raf/MEK/ERK [375, 376].
Besides inducing endothelial cell growth and permeability, VEGF exerts many others effects at the cellular level. It does this through signaling events initiated by a handful of receptors namely VEGFR1, VEGFR2, and neuropilin coreceptors-1 and -2 [377]. In endothelial cells, not only can VEGF induce proliferation but it also prevents apoptosis - the latter through PI3K/PKB activation initiated by VEGFR2 [378, 379]. VEGF also causes VEGFR2-mediated PI3K/PKB activation which leads to integrin activation [380] and actin reorganization [381] and overall promotes EC migration. It is now established that VEGF induces monocyte migration and chemotaxis [382] which appears to be VEGFR1 dependent [383]. VEGF is also capable of upregulating pro-inflammatory cytokines such as TNF-α and IL-6 in monocytes [384] and cellular adhesion molecules such as VCAM-1 and ICAM-1 in ECs [385].

Angiogenesis and inflammation are interconnected processes. In addition, certain properties of VEGF, especially induction of monocyte chemotaxis and vascular permeability, are inherently pro-inflammatory. Indeed, VEGF has been implicated in pathogenesis of many chronic inflammatory conditions and autoimmune disorders. A few examples include: rheumatoid arthritis [386], inflammatory bowel disease [387] and atherosclerosis (section 1.3.3).

1.3.2 VEGF expression

The main inducer of VEGF expression is hypoxia; however, a variety of other stimuli such as oxidative stress, growth factors, hormones, oncogenes, and tumor suppressor genes also upregulate its expression. These factors mostly work at the level of transcription, however modulation of translational and posttranslational processes also contribute.

_Hypoxia_ is a powerful inducer of VEGF expression acting through hypoxia-inducible factor 1 (HIF-1), a heterodimeric (αβ) transcription factor. Under normoxic conditions, HIF-1α is hydroxylated by oxygen-dependent hydroxylases. When hydroxylated, HIF-1α is a substrate for von Hippel-Lindau E3 ubiquitin ligase and is quickly degraded in proteasomes. Under hypoxic conditions, HIF-1α escapes the oxygen-dependent hydroxylation and degradation, forms a heterodimer with HIF-1β and binds to hypoxia-responsive element (HRE) within the promoter region of VEGF gene, resulting in its activation [388].
Growth factors such as TGF and platelet derived growth factor, gonadotrophic hormones, oncogenes and tumor suppressor genes all can upregulate VEGF transcription. A common route by which these factors exert this effect is activation of receptor tyrosine kinase (RTK) signaling with downstream activation of Raf/Ras/MEK/Erk and PI3K [389]. A major signaling event downstream of PI3K leading to VEGF transcription, is PDK-1 mediated activation of atypical PKC-ζ [390]. Additionally, PKCζ-dependent VEGF induction has been reported downstream of Ras signaling [391]. All these pathways converge at the proximal region of the VEGF promoter and activate transcription through AP-1&2, Egr-1, and the Sp family of transcription factors [389, 392]. Another point of convergence for RTK signaling is signal transducer and activation of transcription 3 (STAT3) [393-395]. There is cross-talk between growth factor or hormone-induced signaling events and HIF-1 induced VEGF expression even in the absence of hypoxia. Unlike hypoxic conditions where accelerated degradation of HIF-1α is the major determinant of HIF-1α levels, under normoxia, growth factor-induced HIF-1α transcription and translation plays the main role. HIF-1α transcription and translation is mediated by diacylglycerol-sensitive PKCs [396] and PI3K [397] pathways, respectively. Downstream of PI3K, HIF-1α translation is induced by activation of p70S6K/mTOR pathway [398]. Another point of cross-talk is increased transcriptional efficiency of HIF-1 resulting from its phosphorylation by P42/p44 MAPKs [399].

Oxidative stress is also capable of inducing VEGF expression. For example, hydrogen peroxide significantly increases VEGF transcription in ECs [400, 401] possibly mediated by PKC and transcription factors NF-κB and AP-1[400]. In gastric cancer cells, H$_2$O$_2$-induced VEGF transcription is mediated by Raf/Ras/MEK/Erk pathway and Sp1 and Sp3 transcription factors [402].

Translational and posttranslational mechanisms also regulate VEGF expression. VEGF translation is modulated by the existence of two independent functional internal ribosome entry sites (IRESs) in the 5’ untranslated region (5’-UTR) of VEGF mRNA. They allow for more efficient cap-independent translation [403, 404]. Additionally, within the 5’-UTR of VEGF mRNA, there exists a binding site for eukaryotic initiation factor 4E. eIF4E binds to mRNA and reorganizes its secondary structure to allow for a more efficient translation [405, 406]. VEGF mRNA stability is also a determinant
of VEGF secretion. It is now known that the binding of RNA-binding protein Hur to the 3’-UTR of VEGF mRNA increases its half-life [407, 408]. This process is dependent on stress activated JNK and p38 kinases [409].

1.3.3 VEGF and atherosclerosis

Oxygenation of tissue by blood in the arterial lumen is restricted by the limits of diffusion (~100-μm). It has been hypothesized that the growth of atherosclerotic plaque beyond this limit is dependent on neovascularization in vessel wall. Highly sensitive micro-computed tomography has been used to reconstruct three-dimensional structure of atherosclerotic aorta in apoB100/apoE double knockout mice, and showed a correlation between the extent of intimal angiogenesis and lesion progression [410]. Experimental data have shown a close association between plaque progression and angiogenesis. For example in the intima of cholesterol-fed apoE deficient mice with plaques of a diameter > 250 μm, the frequency of neovascularization is increased nine-fold relative to smaller plaques. Furthermore, inhibition of angiogenesis can slow plaque growth [411]. Neoangiogenesis also seems to be important in plaque instability and rupture (reviewed in [412]).

VEGF can promote plaque growth by way of its angiogenic properties. In addition, it has pro-inflammatory properties such as recruitment of macrophages and induction of vascular permeability. As mentioned, VEGF can promote monocyte migration through VEGFR1 on the surface of monocytes [383]. A more recent study however, showed VEGFR1 signaling is not essential for monocyte recruitment to the plaques [413]. In this study, it was shown that VEGF-induced MCP-1 production in VSMCs was responsible for monocyte recruitment. Lucerna et al. recently showed that neointimal VEGF upregulation advances plaque growth and promotes plaque instability possibly through recruitment of inflammatory cells (as opposed to promoting angiogenesis) [414]. VEGF is also capable of inducing MCP-1 in ECs [415].

Increased expression of VEGF has been documented in atherosclerotic plaques of humans and animals and this correlated with the plaque stage [416-419]. Increased VEGF delivery in cholesterol-fed apoB100/apoE double knockout mice and in cholesterol-fed rabbits increased the extent of
atherosclerosis [420, 421]. On the other hand, inhibitors of VEGF-dependent angiogenesis inhibited intimal neovascularization and reduced atherosclerosis in animal models of atherosclerosis [422, 423]. It was recently shown that oral vaccination against VEGFR2 (flk-1) reduced its expression in endothelial cells of LDLR−/− mouse and also reduced neoangiogenesis with an overall atheroprotective effect [424].

The clinical significance of VEGF in atherosclerosis is still under debate [425]. VEGF could also be beneficial as it can promote arteriogenesis and establish collateral perfusion. Clinical trials such as VIVA [426] and others (reviewed in [427]) have suggested a potential therapeutic role for this cytokine in management of coronary artery disease. A gene polymorphism study suggested a protective role for VEGF [428]. Asahara et al. have shown that VEGF promotes endothelialization after interventions such as stenting and reduces the risk of stent re-stenosis [429]. However, other groups have shown that increased VEGF correlates with increased hyperplasia and re-stenosis [430, 431]. The Kuipo Angiogenesis Trial (KAT) showed no effect of VEGF in preventing re-stenosis [432]. Overall, VEGF may be a double-edged sword. Within the plaque environment, it is implicated as an important factor in lesion neovascularization, which may lead to plaque growth, instability, and rupture. On the other hand, VEGF can act to reestablish perfusion within an area by promoting angiogenesis mediated collateral formation.
1.4 Lipid oxidation

Lipid peroxidation can be initiated by exposure to strong oxidants such as singlet O$_2$ or ozone (O$_3$), or through reactions catalyzed by light (photoxidation), radical-producing agents, or enzymes (enzymatic oxidation). In the following sections, oxidation of different lipids will be discussed with a focus on oxidation of polyunsaturated fatty acids (PUFA).

1.4.1 Oxidation of polyunsaturated fatty acids

The main polyunsaturated fatty acids (PUFAs) in LDL are linoleic acid (LA; 18:2, n-6) and arachidonic acid (AA; 24:4, n-6). Each molecule of LDL contains 1100 molecules of LA and 153 molecules of AA, mostly esterified to phospholipids or cholesterol [316]. LA and AA are also released during lipolysis of triglyceride-rich lipoproteins. Arachidonic acid can also be released from cell membrane phospholipids by PLA$_2$ as the initial step in the arachidonic acid cascade, which involves three major branches: cyclooxygenase, lipoxygenase, and epoxidase [433-435].

Non-enzymatic oxidation of PUFAs generates products some of which structurally differ from their enzymatically-generated cousins. At first, as a simpler model, I will explain the general mechanism for autoxidation of LA (with two unsaturated bonds) and at each relevant section expand that to explain more complicated products of AA autoxidation.

LA has one active bis-allylic methylene group (carbon 11, in the middle of: –C=C–C–C=C– (Figure 1.3)). The resonance structure makes carbon 11 electron-rich and prone to hydrogen abstraction, which yields an unstable radical intermediate with a hybrid pentadienyl configuration (Figure 1.3). This structure can react with oxygen either at carbon 9 or 13 to produce the corresponding 9- or 13-hydroperoxy radical. This also results in rearrangement of the double bond to form a conjugated diene (conjugated denotes the two double bonds are separated by one saturated bond). These hydroperoxy radicals can now propagate the oxidation process as they are avid electron acceptors. After hydrogen abstraction, they will form more stable hydroperoxides (OOH). In non-enzymatic peroxidation systems the ratio of 9- to 13-hydroperoxide is almost one. Considering cis and trans stereoisomers, there will be 4 different combinations possible as there are two double bonds. The relative concentrations of final
products depend on many factors such as the temperature or presence of antioxidants. The generation of 11-hydroperoxy-LA (a non-conjugated diene) has been under debate for years, however, Brash et al. have shown that in the presence of 5% α-tocopherol, this isomer can account for up to 10% of initial products [436]. A schematic presentation of these steps is depicted in Figure 1.3. A hydroperoxide can attack a double bond of another fatty acid to generate an epoxide. Additionally, hydroperoxides are relatively labile. This can lead to formation of alkoxyl (RO·) and hydroxyl (-OH) radicals which can propagate the oxidation and also produce ketones, epoxide, or alcohols. Example of ketone and epoxide formation from hydroperoxide can be found in Figure 1.3. Hydroperoxides can also undergo fragmentation to produce aldehydes [437] or oligomerize with other OPs.

AA autoxidation follows a similar pattern, however, since AA has three bis-allylic methylene groups, three different pentadienyl radicals can be formed. Therefore the number of products greatly increases. The primary products of AA peroxidation are the 5-, 8-, 9-, 11-, 12-, and 15-hydroperoxide isomers. LA and AA hydroperoxides can undergo a second hydrogen abstraction to form dihydroperoxides. When there are more than two unsaturated bonds such as in linolenic acid (18:3) or AA, the hydroperoxide isomers are not produced in equal amounts. For example, the “outer” hydroperoxides of arachidonic (5- and 12-) are far more abundant than “inner” (8-, 9-, 11-, and 12-) hydroperoxides. This is due to rapid 1,3 cyclization of the “inner” hydroperoxides [438, 439] to produce epidioxides. A 1,5 cyclization will produce a bicyclic endoperoxide which, in AA autoxidation, is the pathway for production of prostaglandin-like OPs [440], the precursor structures for isoprostanes (isoPs) and isolevuglandins [441, 442]. Generation of isoPs is accomplished by rearrangement of the bicyclic endoperoxide precursor with an intramolecular attack by a carbon-centered radical on a double bond resulting in formation of a cyclopentane ring (Figure 1.4). They can be biologically active and some of them are useful markers of non-enzymatic lipid peroxidation in vivo [443]. The cyclization is inhibited by antioxidants, which result in equal concentrations of outer and inner hydroperoxides [439].

Levuglandins (LGs) are reactive dicarbanoyl ketoaldehydes that are generated during enzymatic oxidation of arachidonic acid by cleavage of C-C and O-O bond of the bicyclic peroxide nucleus of a bicyclic endoperoxides (e.g. prostaglandin H) [444]. Isolevuglandins (isoLGs) are generated via similar
rearrangements during non-enzymatic oxidation of AA (Figure 1.4 and [442]). As aldehydes, LGs and isoLGs can form adducts with amino groups such as lysine residues and covalently modify proteins [445] which is discussed in more detail in section 1.4.5. Recently Fessel et al. discovered a new class of AA OPs - called isofuranes (isoFs) [446]. They showed that, with increased oxygen tension, the carbon-centered radical is attacked by oxygen to form a hydroperoxide radical (instead of intramolecular attack by a double-bond to form isoPs). Subsequent rearrangements produce a tetrahydrofuran ring (hence the name isofurane). Yin et al. recently described another new class of arachidonic acid oxidation products termed dioxolane-isoprostanes [447]. These oxidation products have both a bicyclic endoperoxide and a cyclic peroxide functionality on the same molecule.

LA and AA oxidation products can also undergo oligomerization. The simplest such reaction would be the reaction of two lipid oxyradical (e.g. LOO-) with each other, to yield a dimer radical. In biological systems lipid peroxidation often occurs in bilayer membranes or lipoproteins, and in these systems dimers are usually composed of fatty esters (containing hydroperoxy, hydroxyl, or keto functional groups) linked through their peroxide groups [448]. These dimers may undergo thermal cleavage (fragmentation) to produce volatile compounds (next paragraph).

Fragmentation (or decomposition) is another mechanism for formation of secondary products from primary products of PUFA oxidation. Two major mechanisms explaining fragmentation products are: homolytic β-scission [449] and Hock cleavage [437]. In homolytic scission, both products are radicals (each retaining an unpaired electron) instead of ions (one fragment losing and the other gaining an electron). The results will be aldehyde-, alkyl- (if saturated), and/or alkene- (if unsaturated) radicals. Of course, these radicals seek stabilization by reacting with electron donors to produce alkanes or alkenes, or react with hydroxyl groups to make alcohols and aldehydes. Hock cleavage involves breaking of C-C bond with an insertion of oxygen in between which will in turn rearrange to form oxo or keto groups. Based on these mechanisms, 9 and 13 hydroperoxy LA fragmentation products include (but are not limited to) 2,4 decadienal, 3-nonenal, hexenal, 4-hydroxy nonenal (4-HNE), and pentane. It is important to remember that unsaturated secondary oxidation products can themselves undergo the same processes (dimerization or decomposition) to give more products. This leads to accumulation of
unsaturated aldehydes and dialdehydes (such as malondialdehyde or MDA) in advanced stages of oxidation. Figure 1.3 on next page illustrates some of the primary and secondary OPs of LA autoxidation. Figure 1.4 illustrates products of phospholipid oxidation (1-palmitoyl 2-arachidonyl phosphatidylcholine or PAPC in particular).
Figure 1.3 Autoxidation of linoleic acid (LA). In the initiation step, a hydrogen atom is abstracted from bis-allylic methylene group (carbon 11) which leads to formation of pentadienyl radical. Addition of oxygen will produce 9- or 13-hydroxy conjugated diene radicals which themselves are capable of propagating the oxidation chain reaction. In the presence of α-tocopherol, another product, 11 hydroperoxy LA, comprises about 10% of primary oxidation products. Boxed areas present two examples of monomeric unfragmented oxidation products of LA. Further oxidation and rearrangements (RA) can produce epoxy alcohol (right) or epoxy keto compounds (left).
1.4.2 Oxidation of cholesterol and cholesterol esters

Cholesterol oxidation products (oxysterols) can be generated in vivo by enzymatic or non-enzymatic reactions. Different members of the cytochrome P450 family of oxidases can enzymatically oxidize (hydroxylate) carbons in the cholesterol ring or in its side-chain. Mitochondrial CYP27A1 hydroxylases attack carbon 27 to produce 27-hydroxy-cholesterol (27OH-chol - formerly known as 26OH-chol). Other side-chain hydroxylases produce 24 and 25OH-chol [450] which carry important functions in cholesterol and bile acid homeostasis. For example, 27OH-chol formation is the first step in the alternative pathway of bile acid synthesis [451]. The classical bile acid pathway is regulated by CYP7A1 hydroxylase which inserts a hydroxyl in the 7α-position of the sterol ring, the first product in the classical pathway. CYP3A4 oxidizes cholesterol to form 4βOH-chol [452]. 24, 25-epoxy cholesterol (24,25 epox-chol), an activator of liver X receptor (LXR), is another important oxysterol formed enzymatically by a shunt pathway during cholesterol biosynthesis [453]. This oxysterol has recently received some attention as it seems, in macrophages, increasing this oxysterol can promote cholesterol efflux through LXRα-activated transcription [454, 455].

Cholesterol makes up to half of the lipids within cell membranes and under some conditions it may be more susceptible to oxidation than PUFAs [456]. Non-enzymatic cholesterol oxidation gives rise to four major products: hydroperoxy-, hydroxy-, keto, or epoxy- cholesterol. The bond dissociation energy between hydrogen and carbon 7 (in the B ring) is relatively low (88Kcal/M) [457] which explains why this site is frequently attacked during non-enzymatic cholesterol oxidation. After the initial hydrogen abstraction, addition of another oxygen atom produces 7α or 7β hydroperoxy radical (7α/7βOO·-chol). Similar to PUFA oxidation, this hydroperoxy radical by itself can propagate the oxidation process. By abstraction of a hydrogen atom, the radical is reduced to the corresponding hydroperoxide (7α/7βOOH-chol). Further hydrogen abstraction and loss of water will produce the stable product hydroxyl or keto cholesterol (7α/7βOH-chol and 7α/βK-chol). Another pathway for formation of keto-cholesterol is through Russell mechanism where two cholesterol hydroperoxy radicals form keto- and hydroxy-cholesterol and release one singlet oxygen (O2) [458]. The third pathway to keto-cholesterol involves enzymatic (dehydrogenase) or non-enzymatic loss of hydrogen from the hydroxyl group [459].
The non-enzymatic reaction occurs via hydrogen abstraction by a hydroperoxy radical – a reaction that promotes termination of the chain reaction [460]. Another major product of cholesterol oxidation is 5,6 epoxy cholesterol - either α or β epimers - (5α,6α or 5β,6β epox-chol, respectively). A mechanism for cholesterol epoxide formation is hydroperoxy radical attack at the 5 and 6 carbon (the double bond of the B ring) with formation of a hydroperoxy intermediate. Dissociation of an alkoxy radical thereafter leaves an epoxide behind [461]. Another pathway of epoxy cholesterol formation is unique to cholesterol esters such as cholesterol linoleate. Fatty acyl peroxidation (R-OOH formation) and intramolecular rearrangement leads to formation of epox chol esterified to an alkoxy (R-OH) group [462]. Exposure to non-radical ROS (such as O2, O3, and HOCl) can also oxidize cholesterol. The main products are 5OOH-, 6OOH-, or 7OOH-chol for O2 and products such as 5β,6β epox-chol for ozone (reviewed in [458]). HOCl oxidation of cholesterol can commonly happen during phagocytosis where local concentration of this ROS increases. Signature products (like 5,6 dichlorinated sterols) of such oxidation were described by Hazen et al. [463]. Of course, HOCl can produce other cholesterol oxidation products such as (α or β) 5,6epox-chol and 4OH-chol [464].

In plasma the top four oxysterols, in decreasing order of abundance, are: 27OH-, 24OH-, 7αOH-, and 5,6β epox-chol [465]. Side chain oxidation is not generally observed with non-enzymatic pathways indicating that most plasma oxysterols are enzymatically generated. Oxysterols found in copper oxidized LDL, in decreasing order of abundance, are: 7K-, 7OOH-, 7OH-, 5,6 epox-chol [466] which reflects the non-enzymatic (free-radical induced) nature of mechanisms involved. Other forms of oxidatively modified LDL more or less exhibit the same pattern [467, 468] but it seems that 7K-chol is a more abundant in the LDL that has been extensively oxidized by copper while 7OOH-chol is found more in enzymatic or metal-free oxidation systems [468]. Studies of animal and human atherosclerotic lesions have shown that the concentration of oxysterols is significantly higher within plaques compared to normal intima. For example one study showed increased levels of 7OH-, 7OOH-, 5,6 epox-, and 7K-chol [469]. In this study, 27OH-chol was also relatively abundant which hints both enzymatic and non-enzymatic processes could be relevant within atherosclerotic plaques. Other studies have shown that
27OH-chol is the main oxysterol in the plaques followed by 7K- and 7OH-chol [468]. The abundance of 27OH-chol in plaques could be due to the activity of mitochondrial CYP27A1 within macrophages.

1.4.3 Oxidation of phospholipids

Glycerophospholipids (PLs) contain two fatty acyl groups esterified to a glycerol backbone with a polar head group (such as phosphocholine) at the sn-3 position. Oxidized PLs (oxPLs) are biologically active and can even serve as markers for cardiovascular diseases [470]. Commonly the fatty acyl esterified to the sn-2 position of glycerol backbone is AA or (even more frequently) LA. PLPC (1-palmitoyl, 2-linoloyl, phosphatidylcholine) and PAPC are in fact among the most abundant PLs (and sources of AA and LA) in vivo. As such, PL oxidation is more-or-less governed by the same mechanisms as in PUFA oxidation (described in section 1.4.1). PLs can undergo enzymatic (e.g. lipoxygenases) or non-enzymatic oxidation. PAPC, for example can generate primary or secondary OPs, some of which are shown in Figure 4. OxPLs might play a role in atherogenesis mainly due to their pro-inflammatory properties. They have shown to promote macrophage recruitment, induce binding of monocytes to ECs [471, 472], increase expression of inflammatory cytokines [473-475], increase thrombogenicity by increasing TF production [476] or by mimicking platelet activating factor (PAF) activity [477]. In addition, oxPLs act as PAMPs and these can be recognized by CD36 [112, 478].
**Figure 1.4 Autoxidation of phospholipids.** Conjugated dienes are formed through oxidation of arachidonic acid on sn-2 position of 1-palmitoyl,2-arachidonyl, glycerophosphocholine (PAPC) (1). Further oxidation will generate a hydroperoxy (OO·) radicals. Through rearrangements (RA) it can generate prostaglandin-like compounds (3, 4). Further rearrangements can generate isoprostanes, isolevuglandins, or isothromboxanes (5). PEIPC can undergo dehydration to produce PEIPC. On the other hand, hydroperoxy radical can propagate oxidation by hydrogen abstraction from another PUFA and convert into hydroperoxide (2). Arachidonic acid-derived hydroperoxides in PAPC can decompose to produce proinflammatory truncated oxPLs such as POVPC and PGPC and ligands of CD36 (HODiPC for example) (7) or free reactive aldehydes such as HNE. (HOOA-PC: 5-hydroxy-8-oxo-6-octenoic acid esters of LPC, HODiA-PC: 5-hydroxy-8-oxo-6-octenedioic acid esters of LPC. KOOA-PC: 5-keto-8-oxo-6-octenoic acid esters of LPC, PEC-PC 1-hexadecanoyl-2-(5,6-epoxyisoprostane A2 oyl)-sn-glycero-3-phosphocholine, PEI-PC 1-hexadecanoyl-2-(5,6-epoxyisoprostane E2 oyl)-sn-glycero-3-phosphocholine,
POVPC 1-palmitoyl, 2- (9-oxononanoyl)-sn-glycero-3-phosphocholine, PGPC: 1-palmitoyl, 2-glutaroyl-

sn-glycero-3-phosphocholine, HNE 4-hydroxy-trans-2-nonenal). Reprinted from Biochimica et
Biophysica Acta, Vol(1772), Fruhwirth GO, Loidl A, Hermetter A, Oxidized phospholipids: from
molecular properties to disease, 718-736, Copyright (2007), with permission from Elsevier [479].
1.4.4 Studying lipid oxidation

Simple methods of studying lipid peroxidation include colorimetric and spectrophotometric techniques. Conjugated dienes are produced during peroxidation of PUFA, and can conveniently be monitored by their UV absorption at 234 nm. 2-Thiobarbituric acid (TBA) can react with a few secondary products of PUFA oxidation such as malondialdehyde (MDA). These TBA-reactive substances (TBARS) bind to two TBA molecules and generate a pigment with red/pink color and maximum absorbance at 532-535 nm [480]. Lysine derivatization by PUFA oxidation products generates fluorescent products with a maximum excitation/emission at 350/430 nm. The fluorescence spectrum for MDA-modified proteins is slightly different (excitation/emission of 390/475) [481]. DNA modification by OPs of AA produces fluorophors with maximum excitation/emission of 315/420 nm [482]. The above methods are simple and convenient, but are semiquantitative at best and provide no structural information.

A variety of chromatographic methods can be used to resolve OPs. Thin-layer chromatography (TLC) has been used to separate secondary products of LA oxidation. However the resolution of this method is usually inadequate to separate all products even in simple systems [483]. For that reason, TLC is mainly limited to qualitative studies of OPs. More powerful chromatographic methods include gas chromatography (GC) [484, 485] and high performance liquid chromatography (HPLC) which with chiral columns allows separation of cis and trans stereoisomers [486, 487]. GC is mostly used for volatile oxidation products such as aldehydes, ketones, and hydrocarbons. HPLC is useful in studying thermally unstable OPs such as hydroperoxides. High performance size exclusion columns have also been successfully used to resolve monomeric, dimeric, and oligomeric secondary products [488].

Mass spectrometry (MS) is a technique that has revolutionized the research in this field. MS can be coupled with HPLC or GC and used in tandem with another mass spectrometer (MSMS). In MSMS, the “parent” ion is fragmented and the daughter ions separated and further monitored to give more information regarding the structure of the parent ion. In the setting of GC-MS, since higher temperatures and volatile compounds are studied, the compounds in vapor state are bombarded with electron impact ionization (EI) to charge and fragment the compound. With HPLC-MS, however, there is the problem of
compounds being in the solvent phase and labile with harsher methods of GC-MS. Thermospray, electrospray ionization (ESI) [489], matrix-assisted laser desorption ionization (MALDI) [490], and atmospheric-pressure chemical ionization (APCI) [491] are examples of softer ionization techniques. With ESI and MALDI, there will be practically no fragmentation of compounds which allows for studying the non-volatile and thermally unstable OPs. A special variety of ESI, uses injection of a central complexing ion (such as silver ion) to generate coordinated charged ions. This technique, called coordinated ion-spray mass spectrometry (CIS-MS), greatly increases the sensitivity [492]. Harvilla et al. applied CIS-MS to the study of OPs and showed that Ag+ adducts of (hydro)peroxides, with a mass of M+107 or M+109, behave exactly the same way (in terms of fragmentation) as free OPs - with an added advantage of superior sensitivity [493].

Lipidomics has lagged behind proteomics at least in part because of the difficulties associated with analysis of this group of compounds. Complexities of reactions during oxidation, lack of standards, and instability of products have further complicated research in the field of lipid oxidation. The major technological breakthroughs have occurred mostly within the past 20 years and this field is still in its formative years.

1.4.5 Modification of proteins by fatty acid oxidation products

A major mechanism by which oxidative stress exerts its deleterious effects is modification of macromolecules. For example, bifunctional aldehydes generated through decomposition of OPs such as 4-HNE and MDA are capable of modifying DNA or proteins. DNA modification by these OPs produces etheno-DNA (ε-DNA) which is potentially genotoxic. Excision repair will release modified bases that can be traced in the urine [494].

Modification of proteins by oxidized lipids is believed to create a fluorescent lipofuscin-like product [495]. Lipofuscin is a pigment granule that accumulates intracellularly. Its abundance increases with age and represents repeated cycles of oxidative damage. When accumulated in macrophages [496] or atherosclerotic lesions [497], some authorities refer to this pigment as “ceroid” which is principally made up of OPs covalently linked to proteins. The best studied OPs responsible for modification of
lysine residues are reactive aldehydes such as MDA. The proposed reaction mechanism for MDA involves Schiff-base formation by reaction of an aldehyde group with primary amino groups of proteins (Figure 1.5A). Decomposition of OPs can also create γ-hydroxy aldehydes (such as 4-HNE) which are more reactive than MDA (Figure 1.5B). After the initial reaction through their aldehyde group as described for MDA, the intra-molecular cyclization and dehydration generates a pyrrole ring (Figure 1.5B). Another mechanism for addition of such reactive aldehydes involves Michael-adduct formation. The aldehyde group can then form a Schiff-base with another lysine residue to cross-link the protein or interact with the hydroxyl group on the same adduct to make a hemiacetal group (Figure 1.5B) [479, 498, 499]. IsoLGs, as γ-ketoaldehydes, are extremely reactive. In fact assaying them in biological systems them has proved to be challenging as they form Schiff-base adducts within seconds [500]. Subsequent reactions will generate pyrroles, hydroxyl lactams, and lactams as described above for γ-hydroxy aldehydes (Figure 1.6 and [442]).

Except for isoLGs, all the OP-induced modifications described above require fragmentation of oxidation products to allow reactivity. Fruebis et al. have suggested that reactive aldehydes derived from fragmentation cannot account for the majority of fluorescence generated by modification of protein by OP [501]. They proposed a mechanism by which hydroperoxides modify proteins without the need for decomposition (Figure 1.7). To support this hypothesis, the same group developed a polyclonal antibody that recognized hydroperoxide-modified proteins but not native or aldehyde-modified proteins. Using this polyclonal antibody, they showed that such epitopes exist in vivo [502].

Modification of proteins by oxidized lipids is of interest as it can alter their function. As well, OP modification can be used as a marker of oxidative damage. For example, oxidatively modified LDL has been detected in atherosclerotic lesions, and oxidized LDL has been found to have numerous biologic properties that could accelerate atherogenesis. Haberland et al. showed that modification of 15% of lysine residues within apoB by MDA renders it a ligand for scavenger receptors. Interestingly, to create SR ligands, non-oxidative modifications (acetylation or succinylation) had to modify at least 60% of lysines [503, 504]. Steinbrecher showed that extensive oxidation of LDL with copper ion modifies nearly 30% of apoB lysine residues and generates the typical fluorescence pattern discussed for OP-
modified proteins [505]. This type of oxidation is not limited to protein modification but also results in extensive oxidation of LDL lipids and hydrolysis of PC to LPC [505]. To determine if a given effect of oxLDL was due to protein modification by OPs, our lab developed a simple model for LDL modification in which OPs of AA or LA are used to modify LDL under conditions where oxidation of LDL lipids itself is inhibited [506]. This oxidation product-modified LDL (OP-LDL) was shown to share many biological properties of copper-oxidized LDL. For example OP-LDL is taken up avidly by macrophages [274] and is able to compete with oxLDL for uptake even in SR-A knockout mice [268]. OP-LDL also induces macrophage growth [307]. It has been shown that autoantibodies against epitopes created by OP-protein modification exists in apoE knockout mice which supports the notion that such modifications occur in vivo [87]. Immunohistochemical studies have also shown the existence of OP-modified epitopes in hypercholesterolemic rats [507]. However the structure of these epitopes in OP modified LDL has not been elucidated. A different group later developed another metal-ion independent, peroxidation-dependent method to modify LDL [508]. One importance of such models is that they provide a more physiological alternative to copper-oxidized LDL.
**Figure 1.5 Protein modification by reactive products of PUFA oxidation.** A) Aldehydes can form Schiff bases by reacting with epsilon amine groups of lysine residues on proteins. Schiff bases are potentially reversible, but bifunctional aldehydes such as malondialdehyde (MDA) can form stable cross-link proteins by this mechanism. B) γ-hydroxy alkenals are significantly more reactive than MDA. Similar to A, they can form a Schiff base with primary amines of lysine residues. However, they can then form a pyrrole ring through reaction of amide with hydroxyl group. Alternatively, they can react, through Michael addition, with amino acids containing primary amines (R’-NH2) or thiol (R’S) groups. Dehydration of the adduct can then form a hemiacetal ring. Reprinted from Biochimica et Biophysica Acta, Vol(1772), Fruhwirth GO, Loidl A, Hermetter A, Oxidized phospholipids: from molecular properties to disease, 718-736, Copyright (2007), with permission from Elsevier [479].
Figure 1.6 Protein modification by levuglandins and isolevuglandins Levuglandins (LG) and their non-enzymatically-generated counterparts, isolevuglandins (isoLGs), are extremely reactive. Within seconds of generation they form Schiff base adducts with lysine residues of proteins which later form nitrogen-containing heterocycles such as pyrrole by cyclization and loss of water. Continued oxidative processes will lead to generation of hydroxylactams and lactams. Reprinted from Molecular Nutrition & Food Research, Nov 2005; Volume (49), Pages:1050-62, Zhang W, Salomon RG, Oxidized phospholipids, isolevuglandins, and atherosclerosis, Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission [509].
Figure 1.7 Concerted reaction between linoleic acid hydroperoxides and lysine. As proposed by Fruebis et al., unfragmented hydroperoxides may modify the primary amine of lysine residues. This involves the nucleophilic displacement of hydroperoxy group onto an unsaturated bond and consequent formation of an epoxy group. The secondary amine (imino group) can then react with either carbon of the epoxy ring to generate nitrogen containing 5- or 6-membered heterocyclic adducts ((1) and (2), respectively). Adapted from Figure 3B in Fruebis J, Parthasarathy S, Steinberg D, Evidence for a concerted reaction between lipid hydroperoxides and polypeptides Proceedings of the National Academy of Sciences of the United States of America, 1992; Volume (89), Pages: 10588-10592, [501]. Used with permission.
1.5 Objectives

Previous studies reported that oxLDL can induce VEGF secretion in macrophages. However, there is neither agreement nor complete understanding of the signaling mechanisms underlying this effect. As well, there is very little information about the possible requirement for plasma membrane receptors for oxLDL to induce VEGF. The objectives of studies in chapter 3 were to shed light on the signaling pathways and determine the possible contribution of major scavenger receptors in oxLDL-mediated VEGF secretion.

Several groups have also reported that oxLDL promotes survival and growth in macrophages. However, very little information has been published regarding the involvement of scavenger receptors in mediating growth and survival. The studies in chapter 4 were designed to test if an endocytic or signaling pattern recognition receptor(s) might be required for oxLDL-mediated macrophage survival.

The 5th chapter of this dissertation is a study to try to define the molecular structure that was required for growth and survival signaling in macrophages. To make this analytically feasible, we chose to characterize our model of modified LDL generated by oxidation products of polyunsaturated fatty acids.
2. Materials and methods

2.1 Materials

Phenazine methosulfate (PMS), arachidonic acid, linoleic acid, L-lysine-methyl ester dihydrochloride, phosphatidyl-L-serine, 1-earoyl lysophosphatidylcholine, acetic anhydride, butylated hydroxytoluene (BHT), cholesterol, pyrrole, sphingomyelinase (SMase), myeloperoxidase (MPO), catalase, octyl glucoside, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT), N-methyldibenzopyrazine methyl sulfate, and trinitrobenzenesulphonic acid were obtained from Sigma-Aldrich (Mississaug, ON, Canada). Egg phosphatidylcholine (PC) and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) were obtained from Northern Lipids (Vancouver, BC, Canada). Cayman Chemical (Ann Arbor, MI) supplied 4-hydroxyhexenal and 4-hydroxy nonenal. Ceramide-1-phosphate was from Avaniti Polar lipids (Alabaster, AL). Fetal bovine serum (FBS), DMEM and RPMI 1640 medium, sodium pyruvate and L-glutamine were from Gibco Invitrogen. Fisher Scientific (Vancouver, British Columbia, Canada) supplied 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS), glucose, L-tyrosine, sodium borohydride and sodium hypochlorite. Glucose oxidase was from Roche Diagnostics (Laval, PQ). Centriplus 20 ultrafilters were obtained from Amicon (Beverly, MA). Antibodies to PKB, Erk2, phospho-PKB (Ser-473), and phospho-Erk1/2 (Thr-202/Tyr-204) were purchased from Cell Signaling Technologies. Protein G Sepharose beads and anti-PKCζ (C-20) and isotope control antibodies were obtained from Santa Cruz (Santa Cruz, CA). Nitrocellulose membranes, protein standards, and Bradford protein assay kits were purchased from Bio-Rad (Hercules, CA). Ficoll-paque and reagents for enhanced chemiluminescence were from Amersham Biosciences. BCA protein assay reagents were from Pierce (Milwaukee, WI). Recombinant human M-CSF and mouse and human VEGF ELISA kits were purchased from R&D Systems (Minneapolis, MN). The fluorescent dye 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was from Molecular Probes. [γ-32P] labeled ATP was purchased from PerkinElmer (Waltham, MA). Propidium iodide, RNase A, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) were obtained from Invitrogen (Burlington, ON, Canada). Titan gel lipoprotein kit was from Helena Laboratories (distributed by Inter Medico, Markham, Ontario).
Defined fetal bovine serum (FBS) was from HyClone (Logan, UT). All other reagents including inhibitors were Calbiochem products (San Diego, CA).

2.2 Animals

CD-1 mice were from the UBC Animal Care Unit. Wild-type C57/bl6, TLR4, CD14, and FCγRIIb knockout mice were all purchased from The Jackson Laboratory (Bar Harbor, MA). CD36/SR-A double knockout mice were generously provided by Dr. M. W. Freeman [269]. LOX-1⁻/⁻ mice were generated in the laboratory of Dr. T. Sawamura, and were kindly sent to us by Dr. Osamu Cynshi, Chugai Pharmaceutical Corp, Tokyo. Genotypes of offspring of knockout mice were verified by PCR. All animal procedures were in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of British Columbia.

2.3 Cell Culture

RAW 264.7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM supplemented with FBS 10%, L-glutamine and sodium pyruvate (1 mM final concentration). Cells were harvested using a Teflon cell lifter. For experiments, FBS concentration was 1% (v/v).

Resident mouse peritoneal macrophages (MPMs) were isolated by peritoneal lavage with PBS [510]. 500,000 cells were plated in each well of a 12-well plate in 1 mL RPMI, 10% FBS, L-glutamine, sodium pyruvate. For human monocyte-derived macrophages (hMDMs), peripheral blood mononuclear cells were isolated by Ficoll-paque centrifugation. Monocytes were isolated from mononuclear cells by adherence and were differentiated to macrophages in the presence of M-CSF for 7 days, changing the media every 48 h. All experiments using human macrophages started at the 7th day post isolation.

Bone marrow derived macrophages (BMDMs) were isolated from femurs of 6-8 weeks old wild-type or knockout animals as described [310]. Cells were plated in 10-cm diameter tissue culture plates and incubated overnight in DMEM containing L-glutamine, sodium pyruvate, 10% FBS, and 10% L-cell conditioned media as a source of macrophage colony stimulating factor (M-CSF). Non-adherent cells
(monocytes) were then collected, and differentiated to macrophages by a further 5-day incubation in the same medium. Macrophages were scraped off the plate using a Teflon cell lifter, counted and re-plated at specified densities and incubated overnight in the same medium. In all experiments using BMDMs, cell treatment started at the 7th day post isolation.

2.4 Lipoprotein isolation, modification, and labeling

LDL (d = 1.019 to 1.063 g/mL) was isolated by sequential ultracentrifugation of EDTA-anticoagulated fasting plasma obtained from healthy normolipidemic volunteers as described [310]. Prior to oxidation, LDL was dialyzed to reduce EDTA concentration to 10 μM. Different degrees of oxidation were achieved by incubating 200 μg/mL LDL for 2, 5, or 24 h at 37 °C with 5 μM copper sulfate. Oxidation was stopped by addition of 40 μM butylated hydroxytoluene (BHT) and 300 μM EDTA [310]. The modified LDL was then washed and concentrated to about 1 mg/mL using Amicon Centriplus 20 ultrafilters.

Ferrous-ion-modified LDL (FeLDL) was prepared by dialyzing 200 μg/mL LDL against PBS containing 1 μM FeSO₄ until LDL-associated carotenoids were consumed as judged by color change [511]. Similar to copper-oxidized LDL, FeLDL was washed and concentrated using ultrafilters.

Acetylation of LDL was performed by sequential addition of aliquots of acetic anhydride as described by Goldstein, Brown, and colleagues [512]. Hydroxyl alkenal-modified LDL was prepared by addition of 4-hydroxyhexenal (0.5 mg) or 4-hydroxynonenal (1.5 mg) to 1 mg of LDL dissolved in PBS containing 10 μM EDTA. These modified LDL preparations were extensively dialyzed to remove unbound reactive material.

For modification of LDL by fatty acid oxidation-products, 10 mg neat arachidonic acid or linoleic acid was autoxidized in a glass test tube by exposure to air at 37°C for 72 h and the water-soluble component was incubated overnight at room temperature with LDL in the presence of 300 μM EDTA and 50 μM BHT [506].

p-hydroxyphenylacetaldehyde (p-HA)- modified LDL was generated by addition of 1 mL of 2 mM pHA to 1 mg of LDL and incubated for 24 h at 37°C. As a source of pHA, NaOCl (0.9 M) was
sequentially added to L-tyrosine (2 mM) in ice-cold phosphate (20 mM) buffer containing 100 µM DTPA (pH 7.0) until a final 1:1 molar ratio was achieved. The solution was then warmed for 1h at 37°C and filter sterilized. Efficient conversion of L-tyrosine to pHA occurs under these conditions [513].

Fluorescence labeling of LDL and oxLDL was done by addition of 75 µL of 3 mg/mL DiI solution to 4 mg (protein) of native or oxidized LDL in 8 mL of lipoprotein-deficient serum (d>1.21 g/mL plasma fraction). After incubation for 8 h at 37ºC, labeled lipoproteins were reisolated by ultracentrifugation. DiI was extracted from lipoproteins with isopropanol and fluorescence was measured using a microplate fluorometer (Fluoroskan Ascent FL, Thermo LabSystems) with excitation/emission wavelengths set at 530/590 nm. Within the range of 0 to 1000 ng, a linear correlation existed between DiI concentration and fluorescence intensity with a lower detection limit of 5 ng diI. In preparations used in these experiments, 15-18 and 9-12 ng of DiI was incorporated into each µg (protein) of nLDL and oxLDL, respectively.

2.5 Lipoprotein characterization and analytical procedures

Purity of LDL isolation and extent of modification was confirmed by agarose gel electrophoresis using a Titan gel lipoprotein kit. Lipoprotein bands were visualized by Fat Red staining. Electrophoretic migration was determined by measuring the distance between the point of origin and the leading edge of lipoprotein. apoB modification was quantified and expressed as the migration of the modified LDL relative to that of native LDL.

Lipoprotein concentration was calculated based on the amount of protein measured by bicinchoninic acid (BCA) assay and expressed as milligrams per milliliter. Due to reactivity of OP products with BCA reagent, the Lowry protein assay [514] was used for protein measurements in OP-, 4-HHE-, 4-HNE-, and pHA-modified LDLs.

2.6 Modification of lysine-methyl-ester by PUFA oxidation products

3 mg of oxidation products of AA or LA (as described in section 2.4) dissolved in 300 µL of PBS were added to 200 µL of lysine-methyl-ester or LME (from a freshly prepared aqueous solution of
10 mg/mL, pH 8.0) and total volume was adjusted to 1 mL with PBS. The mixture was incubated at room temperature overnight in airtight glass tubes.

2.7 Lipid extraction, liposome preparation and modification

Lipid extraction from lipoproteins was carried out according to the Bligh and Dyer method [515] with Hajar’s modification [516] to increase the yield. One mg of native or oxidized LDL in 0.5 ml PBS containing 10 mM HCl were mixed with ice-cold methanol:chloroform (2:1) and vortexed. Then a solution containing 2 M KCl, 0.2 M H₃PO₄ (in water) mixed 1:1 with chloroform was added and vortexed again. Centrifugation at 2000 rpm for 10 minutes yields an upper aqueous layer and a lower organic (lipid) layer with denatured protein at the interface.

For liposome preparation, the organic phase from the lipid extraction was dried under nitrogen and the lipids were resuspended by vortexing in 1.5 mL of liposome buffer (150 mM NaCl containing 0.1 mM EDTA and 10 mM HEPES, pH 7.5). The mixture was passed 10 times through a 0.1 µM polycarbonate membrane at 37°C under nitrogen flow using a Lipex mini-extruder. Concentrations of liposomes are expressed according to the initial amount of LDL protein used for extraction. Phospholipid liposomes were prepared by mixing 10µM of DPPE (for PE liposomes) or egg PC (for PC liposomes) with equimolar cholesterol and vortexing in 2 mL of liposome buffer and extruded as described above. Liposome concentrations are expressed in terms of initial concentrations of phospholipid assuming 95% recovery after extrusion. The size of liposomes was determined by a submicron particle sizer (Nicomp Model 270). The mean diameter of PC was 136±20 nm, and that of PE liposomes was 126±53 nm.

Liposomes were modified by PUFA oxidation products as follows: OPs of AA or LA (described in section 2.4) were resuspended in 1 mL of PBS and incubated with liposomes (2 mg total lipid) for 8 h at room temperature.

2.8 apoB solubilization

After the extraction as described in previous section, the middle (protein) layer was washed with 2 mL of ice-cold H₂O and 2 ml of acetone. After a final wash with water the apoB was solubilized in 0.5
mL of a 15 mg/mL aqueous solution of octyl glucoside containing 10 mM NaOH for 30 minutes at room
temperature. Excess detergent was then removed by dialysis against PBS.

2.9 Cell viability assay

Macrophage cell number in MPMs was estimated by the XTT formazan method, which
measures the rate of reduction of the XTT dye by mitochondria. We have previously documented a close
correlation between macrophage number and formazan dye reduction [308]. Peritoneal macrophages
were isolated as described in 2.3, and plated at 1*10^4 cells/well with 100 µL RPMI 1640 containing 10%
FBS. After overnight incubation, each well was supplemented with 50 µL of RPMI medium containing
25µM PMS and 1 mg/mL XTT. After 4 h of incubation in at 37°C, absorbance was measured at 450 nm.

For later experiments involving survival in BMDMs, MTS was substituted for XTT as it permits
a 1-step assay procedure [310]. BMDMs from wild-type or knockout mice were harvested and expanded
in culture for 5 days as described in 2.3. Cells were harvested with a Teflon scraper, counted, and each
well was seeded with 3*10^4 cells in 100 µL RPMI 1640 containing 10% FBS and 10% L-cell
conditioned media. Cells were incubated overnight to allow them to adhere. At the start of each
experiment, wells were washed twice with PBS and cells incubated in fresh media with or without M-
CSF and indicated concentrations of native or modified lipoproteins. At the end of the experiment, 50 µL
RPMI 1640 containing 333 μg/mL MTS and 25 ng/mL PMS was added to each well. After 2 h
incubation at 37°C, absorbance at 490 nm was measured. Where applicable, inhibitors were added in
fresh media, 45 minutes prior to addition of native of modified LDL. All cell viability experiments were
done at least in triplicate.

2.10 Apoptosis assay

Propidium iodide (PI) staining and FACS analysis was used to quantitate the sub-diploid
population. At the end of each time point, BMDMs from wild-type or knockout mice were permeabilized
using ethanol 70% (v/v) for 1 h at 4°C and washed twice in PBS containing 0.01% glucose. Cells were
then resuspended in the same buffer plus RNase A (final concentration 0.1 mg/mL) and PI (0.12
mg/mL). Fluorescence was measured with a BD FACS Canto. The data were analyzed with FCS Express Pro Software Version 3 (De Novo Software, Thornhill, Canada).

2.11 ELISA

Conditioned medium was collected and centrifuged at 14,000 rpm for 10 min. The VEGF concentration in the supernatant was determined using the Quantikine human and mouse VEGF ELISA kits (R&D Systems) according to the manufacturer’s instructions. The result was normalized to total cell protein content. Where applicable, inhibitors were added 45 min before adding the native or modified LDL.

2.12 Immunoblotting

Cells were lysed by ice-cold solubilization buffer (containing 50 mM Tris-HCl PH 7.7, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 2.5 mM EDTA, 10 mM Na plus freshly added sodium orthovanadate (0.2 mM), sodium molybdate (1 mM), and protease inhibitor cocktail containing aprotinin (10 µg/mL) and leupeptin (10 µg/mL). Thereafter, lysates were centrifuged at 14,000 rpm for 10 min and protein content of the supernatant was determined by BCA assay. Sample concentrations were adjusted to 1 mg/mL and sample buffer was then added to each lysate and heated for 5 minutes at 90 ºC. Equal amounts of protein were loaded onto an SDS-PAGE separating gel (10%) with low molecular weight protein ladder (Biorad) for calibration. Protein was then transferred to nitrocellulose paper, blocked for 1 h in Tris-buffered saline (TBS) containing 5% skim milk with 0.1% Tween 20, and then incubated overnight with the primary antibody anti-phospho PKB or Erk2 in TBS and 0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody at 1:1000 dilution for 1 h. Bands were visualized using enhanced chemiluminescence. As a loading control, phospho-antibodies were stripped with Tris buffer containing SDS (2%) and β-2-mercaptoethanol (100 mM) at 50ºC for 20 minutes, washed, and reprobed with antibody to unphosphorylated PKB or Erk2.
2.13 PKCζ immunoprecipitation and in vitro kinase assay

Cell lysates were prepared as for western blots. For each condition, equal amounts (500 µg/mL) of protein were incubated overnight with 2 µg of anti-PKCζ (C-20) or isotope control antibodies at 4°C. Antigen/antibody complexes were pulled down using protein G Sepharose beads and washed twice in lysis buffer and then twice in kinase buffer (described below).

Kinase buffer composition was β-glycerophosphate (25 mM), MOPS (20 mM), EGTA (5 mM), EDTA (2 mM), and MgCl₂ (20 mM), sodium orthovanadate, and sodium molybdate. Each kinase reaction also contained bead/protein complex, myelin basic protein (5 µg), phosphatidylserine (4 µg) and [γ-³²P] ATP (0.166µCi). The reaction was carried out at 37°C for 20 minutes, and was stopped by addition of hydrochloric acid. The mixture was then spotted on P81 filter papers and washed with o-phosphoric acid. Radioactivity on filters was measured using a liquid scintillation analyzer. The specificity of the reaction for PKCζ was verified using an in vitro kinase inhibitor (PKCζ pseudosubstrate) according to the manufacturer’s instructions. This inhibitor completely blocked the oxLDL-induced increase in kinase activity.

2.14 Lipoprotein uptake assay

Uptake of DiI-labeled LDL was compared in BMDMs from wild type, LOX-1, and CD36/SR-A double knockout (DKO). BMDMs were seeded at 10⁶ cells/well of 6-well plates and incubated with 25 µg/mL of either unlabeled lipoprotein (autofluorescence control) or DiI labeled native LDL or oxLDL (DiI-LDL and DiI-oxLDL, respectively). After 24 hours, cells were washed with PBS and harvested using a Teflon cell scraper. Equal aliquots were taken for protein measurements. The remaining cells were centrifuged and the pellet was dissolved in isopropanol. DiI fluorescence of each condition was measured (excitation: 530 nm, emission: 590 nm) in triplicates with unstained control as background. A serial dilution of DiI was used to create a standard curve. Results were normalized to protein content and expressed as percentage of DiI-oxLDL uptake in wild-type macrophages.

In MPMs, uptake of fluorescently labeled lipoproteins was compared in macrophages from wild-type and CD36/SR-A double knockout (DKO) mice using flow cytometry. MPMs from WT or DKO
mice were incubated with 25 µg/mL of DiI-LDL, DiI-oxLDL, or unlabeled lipoprotein (unstained control). After 24 hours cells were washed twice with PBS and harvested using Teflon cell-scraper and then resuspended in PBS containing 1% BSA. Up to 20,000 events were acquired on a BD FACS Canto. The data were analyzed with FCS Express Pro Software Version 3 (De Novo Software, Thornhill, Canada). DiI uptake for each condition was calculated by determining the geometric mean of the corresponding fluorescence profile after correction for fluorescence in the unstained control (expressed as Δ mean fluorescent intensity or ΔMFI).

2.15 Quantitative reverse transcription polymerase chain reaction

RNA was isolated from macrophages using an RNasy mini kit (Qiagen, Mississauga, ON, Canada). Nucleic acid concentration and purity were tested by measuring absorbance at 260 and 280 nm, respectively with an Ultrospec 3000 spectrophotometer (LKB Pharmacia Biotech). Total RNA was utilized as a template for first strand cDNA synthesis using Supertranscriptase II and OligoT primers (Invitrogen, Burlington, ON, Canada) according to the manufacturer’s instructions.

The resulting cDNA was amplified using the VEGF forward primer 5’-AGTCCCATGAATGATCAAGTTCA-3’ and the reverse primer 5’-ATCCGCATGATCTGCATGG-3’. β-actin gene expression was determined as a reference with forward primer 5’-AGGCCCAGAGCAAGAGAGG-3’ and reverse primer 5’-TACATGGCTGGGTGTTGAA-3’. These sequences were obtained from previously published work [517]. qPCR was performed with an ABI Prism 7300 Sequence Detector (Applied Biosystems) using a QuantiTect SYBR Green PCR Kit (Qiagen, Mississauga, ON). Amplification was performed according to the ABI 7300 standard protocol with data collection at stage 3, step 2 (60°C 1 min). All assays were performed in triplicate and data were expressed as fold increase relative to no-treatment control.

2.16 Hydroperoxide measurement

Hydroperoxide content was measured by a lipid hydroperoxide assay kit (Cayman Chemical Co., Ann Arbor, MI) according to manufacturer’s protocol. This assay measures the hydroperoxide content
directly by redox reactions relying on hydroperoxide-dependent ferric ion generation which then reacts with a chromogen giving color with maximum absorbance at 500 nm. Background was determined by measuring the absorbance after treatment with triphenylphosphine multiplied by 1.28 to correct for the effect of triphenylphosphine on the chromogen.

### 2.17 Mass spectrometry and tandem mass spectrometry

In early experiments, unit mass and MSMS data were obtained on an Esquire Ion Trap instrument (Bruker Daltonics, Billerica, USA). A Micromass LCT time of flight (TOF) system (Micromass, Manchester, UK) was used to perform accurate mass measurements. Samples were dissolved in methanol at a concentration of 1 mg/mL and injected into the electrospray ionization source at a flow rate of 10 μL/min. Mass spectrometers used for HPLC-MS and HPLC-MSMS are described below.

### 2.18 High performance liquid chromatography coupled mass spectrometry and tandem mass spectrometry

The chromatographic system consisted of a Waters ACQUITY UPLC coupled with a photodiode array (PDA) detector and a ZQ single quadrupole or a Waters/Micromass Quattro Micro triple quadrupole mass spectrometer (Waters Corp., Milford, MA). Oxidation products or adducts were resolved on a BEH C18 column (1.7 μm, 50 mm x 2.1 mm; Waters, Milford, MA) equilibrated with 20:80 acetonitrile/water. In early experiments, the acetonitrile gradient was ramped to 100% from 0.2 – 10 min at 0.8 ml/min with ~ 1/3 split to the MS and column temperature was 35 °C. 50 mM ammonium acetate was used as the aqueous modifier. MS data were acquired in both ES+ and ES- mode. In ES+ capillary was at 3 kV, cone voltage 30 V, source and desolvation temp were 100 & 250 °C, respectively and desolvation gas flow 800-1000 L/h. In ES- capillary was 2.5 kV and remaining parameters the same as for ES+. OP products and especially OP-LME adducts were more efficiently ionized in ES+ mode.
High resolution mass measurements and MSMS data were also acquired using a quadrupole time-of-flight mass spectrometer (SYNAPT™ High Definition Mass Spectrometry™ system; Waters, Milford, MA, USA). This system was coupled to an ACQUITY ultra-performance liquid chromatography (UPLC) system similar to the chromatographic set up mentioned above. The following acetonitrile gradient was used: 0-0.2 min, 10-10%; 0.2-2.0 min, 10-15%; 2-25 min, 15-40%; 25-31 min, 40-90% at 0.3 mL/min. 20 mM ammonium acetate was used as the buffer in the aqueous phase. Inject volume was 10 µL and total run time was 35 min, including the 100% acetonitrile flush and re-equilibration. ES+ data were collected using ionization conditions similar those noted for quadrupole analyses with the instrument operating in V mode, tuned to approximately 10000 resolution and using leucine enkephalin for lock mass correction. m/z 50-1000 calibration was carried out by infusion of a sodium formate solution with root mean square deviations < 1 parts per million (ppm) for the ion clusters. Scan data were collected using 0.5 sec scans from m/z 100-600 using collision energies of 6 and 4 V, respectively in the instruments 2 collision cells. MSMS data for selected ions were collected using 1 sec scans from m/z 50-600, with the quadrupole at unit resolution and using ramps of 10-30V or 15-30 V in collision cell 1 and either 6, 12 or 16 V in collision cell 2 to yield varying degrees of fragmentation.

For fluorescence detection of adducts, a Waters Alliance LC coupled with a PDA and 2475 multi wavelength Fluorescence Detector System was used (with excitation/emission set at 350/430 nm). For these measurements, oxidation products were resolved on a 2.1x150 Xterra MS C18 5 µm column with a 20 mM ammonium acetate/acetonitrile gradient as follows: 0.2-2.0, 10-15%; 2-25, 15-40%; 25-31, 40-90%; 34.25-34.5, 90-100%, 36.25-36.5 100-10%. Total runtime was 40 minutes. Column temperature was 35ºC and flow rate was 0.3 mL/min.

2.19 Statistical analysis

Unless stated otherwise, all results are expressed as means ± SD of pooled data from 3 to 5 experiments as specified in figures. Comparison of two means was done using a 2-tailed Student’s t test. For comparing more than two means one-way ANOVA was used followed by Tukey’s post hoc analysis to
measure the level of statistical significance between groups. In Figure 3.14, where the mean of control is compared to other conditions, Dunnett’s post hoc analysis was carried out after ANOVA.

The level of significance of difference is indicated in the graphs as follows: * \( p \leq 0.05 \), ** \( p \leq 0.01 \), and *** \( p \leq 0.001 \). ANOVA and post hoc analyses were carried out with GraphPad Prism 4 software (La Jolla, CA).
3. OxLDL-mediated VEGF secretion in macrophages

3.1 Introduction/rationale

As discussed in section 1.3.3, VEGF is thought to play an important role in atherosclerosis. Increased expression of VEGF has been documented in atherosclerotic plaques of humans and animals and correlates with the extent of disease progression [416-419]. Macrophages seem to be an important source of VEGF in atherosclerotic plaques [416]. OxLDL has been shown to increase VEGF expression in macrophages [418, 518, 519] however, the cellular mechanisms by which oxLDL induces VEGF secretion are not well understood. In particular, it is unclear how the type and extent of oxidation of LDL influences its induction of VEGF, or if macrophage scavenger receptors are required.

Recently, LOX-1 has been implicated as a receptor for oxLDL in endothelial cells [520] and in bovine articular chondrocytes [521], and it was suggested in the latter paper that oxLDL acts through LOX-1 to induce VEGF expression in chondrocytes. However, no reports to date have addressed whether either of the two main receptors involved in oxLDL uptake in macrophages (SR-A and CD36) are required for VEGF upregulation by oxLDL in these cells. As well, only limited information has been published about the mechanism by which oxLDL stimulates VEGF secretion. Inoue et al. showed a potential role for peroxisome proliferator-activated receptor γ (PPARγ) [518], and Salomonsson et al. reported that oxLDL increased VEGF mRNA stability, partly via p38 stress activated MAPK [519]. However, many factors that increase VEGF act through receptor tyrosine kinases (RTKs) which then signal via two major downstream signal transduction pathways, PI3K and Ras-dependent MAPK pathways (section 1.3.2) but these pathways have not been examined with regard to VEGF induction by oxLDL.

The objectives of the studies in this chapter were to determine how different types and degrees of LDL modification affect VEGF induction, to determine if SR-A, CD36 or LOX-1 were required, and to define the signal transduction pathways involved in enhancement of VEGF expression by oxLDL.
3.2 Results

3.2.1 OxLDL induces VEGF expression and secretion in macrophages

To confirm the previous data, we measured the expression and secretion of VEGF in RAW264.7 with qPCR and ELISA, respectively - as described in the methods section. Figure 3.1A shows that extensively oxidized LDL, but not native LDL, increased VEGF secretion in RAW 264.7 cells in a time-dependent manner. This increase reached statistical significance as early as 12 hours. Figure 3.1B shows that the increase in VEGF secretion by oxLDL in RAW 264.7 cells was concentration-dependent. Figures 3.1C shows that the increased VEGF secretion is at least in part achieved by increasing mRNA levels. The increase in VEGF mRNA became statistically significant after 6 hours of oxLDL treatment.

To verify that oxLDL can also increase VEGF expression in primary macrophages, we measured VEGF secretion in response to oxLDL in mouse peritoneal macrophages and human monocyte-derived macrophages. We found that oxLDL caused a dose-dependent increase in VEGF secretion in both hMDMs (Figure 3.2A) and in MPMs (Figure 3.2B). Compared to RAW264.7 and MPM, hMDMs showed slower kinetics with regards to oxLDL-mediated VEGF secretion.

3.2.2 Type and degree of modification influences oxLDL-mediated VEGF secretion

To determine the effect of oxidative modification of LDL on VEGF secretion, cells were treated with LDL oxidized to various degrees. The mildest degree of oxidation, which resulted mainly in lipid peroxidation, was with ferrous ion (FeLDL). More extensive oxidation was obtained by exposing LDL to Cu^{++} for 2, 5, and 24 h to produce a range from mildly to heavily oxidized LDL (oxLDL2, oxLDL5, oxLDL24, respectively). Degree of oxidation was monitored by agarose gel electrophoresis (Figure 3.3B). FeLDL had the lowest mobility of the modified LDLs, and there was a progressive increase in migration with time of exposure to copper. For oxLDL, the electrophoretic mobility correlated with the amount of VEGF secretion (Figure 3.3A). FeLDL used in our experiments had less apoB100 modification than oxLDL2 as judged by electrophoretic mobility (Figure 3.3B), but the difference in VEGF secretion between cells incubated with FeLDL and oxLDL2 did not reach statistical significance.
Figure 3.3A also shows that FeLDL was less potent than oxLDL5 ($p = 0.004$) or oxLDL24 ($p < 0.001$) at increasing VEGF secretion.

To determine if selective modification of apoB100 by lipid peroxidation products without oxidation of LDL lipids has an effect, we tested the effect of LDL modified by fatty acid oxidation products (OP-LDL). Figure 3.4A shows that OP-LDL preparations with comparable electrophoretic mobilities to that of oxLDL24 significantly increased VEGF secretion compared to nLDL, but they appeared less potent than oxLDL24 (Figure 3.3A $p = 0.003$). Similar results were observed in MPMs and hMDMs using different preparations of OP-LDL (Fig. 3.4C &D). As a control we also used Ac-LDL which is a non-oxidative chemical modification of apoB100 that renders it a ligand for scavenger receptor A. Although Ac-LDL had an electrophoretic mobility similar to that of oxLDL24, suggesting that there was a similar degree of modification of lysine residues, it had no significant effect on VEGF secretion in any of the 3 cell types tested (Fig. 3.4A, C and D). This indicates that an oxidative modification, and not merely a charge change of apoB sufficient to confer scavenger receptor recognition, is required to induce VEGF.

### 3.2.3 OxLDL and ceramide phosphate both increase macrophage secretion through different mechanisms

Ceramides are important lipid messengers which carry out important functions such as inhibition of cell proliferation and angiogenesis, and induction of apoptosis [522]. OxLDL can modulate intracellular ceramide homeostasis. For example, our lab has shown that oxLDL inhibits macrophage apoptosis at least in part through inhibiting acid sphingomyelinase activity, thereby blocking ceramide generation [314]. An anti-angiogenic effect has also been shown for ceramide [523]. Interestingly, many of the effects of oxLDL can be mimicked by Cer-P. For example, Cer-P is mitogenic for macrophages, and blocks macrophage apoptosis [522]. In addition, Cer 1-P has been involved in inflammatory responses through stimulation of the cytosolic form of PLA$_2$ and the subsequent generation of prostaglandins [522]. We therefore decided to examine VEGF secretion in response to Cer-P. Figure 3.5 shows that Cer-P caused a dose-dependent increase of VEGF secretion in RAW267.4 cells. To inhibit
Cer-P activity we added sphingomyelinase (SMase) to the medium to increase ceramide levels in the cell membrane. Figure 3.6 shows that pre-treating RAW267.4 cells with SMase at 0.6 U/mL or higher led to a decrease in basal as well as in Cer-P stimulated VEGF secretion. However, it did not inhibit oxLDL-mediated VEGF secretion. In fact, a trend toward increased VEGF secretion was observed (Figure 3.6).

### 3.2.4 Scavenger receptors and oxLDL-mediated VEGF secretion

Figure 3.7 shows that there was no difference in VEGF secretion in MPMs from wild-type and SRA<sup>+</sup>/CD36<sup>+</sup> DKO mice. It also shows that MPMs from LOX-1 knockout mice exhibited the same induction of VEGF secretion by incubation with extensively oxidized LDL as MPMs from wild-type mice. These results indicate that signal transduction mediated by SR-A, CD36, or LOX-1 does not play a significant role in VEGF secretion by macrophages.

### 3.2.5 VEGF secretion is independent of oxLDL uptake

Our lab has already shown that in unstimulated peritoneal macrophages, LOX-1 does not significantly contribute to oxLDL uptake [524]. SR-A and CD36 however are both important for macrophage uptake of oxLDL (Section 1.2.4). To determine if uptake of oxLDL was important for VEGF secretion by oxLDL, in parallel with experiments described above, oxLDL uptake was measured in CD36/SR-A double knockout (DKO) MPMs. Figure 3.8A shows the fluorescence plot of one experiment demonstrating a significant reduction of DiI-oxLDL uptake in DKO compared with wild-type cells. The DKO cells formed a bimodal population, with most cells showing a very low median fluorescence similar to macrophages exposed to native LDL, but also an unexplained minor population with an intermediate level of fluorescence that formed a shoulder on the low-fluorescence population. Figure 3.8B shows pooled results for mean fluorescence intensity from 4 independent experiments. DiI-oxLDL uptake was similar between MPMs from wild-type and DKO mice (p = 0.65) and as expected, in macrophages from DKO mice DiI-oxLDL uptake was reduced to almost 20% that of cells from wild-type mice (p < 0.001). Taken together, these uptake studies and VEGF measurements show that oxLDL uptake is not essential for its effect on VEGF secretion.
3.2.6 OxLDL-mediated VEGF secretion is independent of MAPKK

Ras and PI3K can activate two important downstream kinases, MAPK and PKB/Akt, both of which may be involved in regulating VEGF expression (section 1.3.2). To assess the role of MAPK, RAW 264.7 cells were treated with PD 98059, a specific MAPKK inhibitor. At the incubation times and concentrations of inhibitors used, no toxicity from the inhibitor was evident by MTS assay. Figure 3.9A shows that PD 98059 reduced the basal levels of VEGF secretion but oxLDL retained the ability to increase VEGF secretion in the presence of PD 98059 at concentrations as high as 50 µM. The activity of MAPKK inhibitor on its target was verified by immunoblotting for phosphorylated Erk1/2, the downstream target of MAPKK (Figure 3.9B).

3.2.7 PI3K is important in oxLDL-induced VEGF secretion

To assess the role of PI3K, RAW 264.7 cells were treated with the PI3K inhibitor LY 294002. Incubation times longer than 18 h or concentrations higher than 30 µM reduced cell viability to 80% (data not shown). Therefore a shorter time-point of 14 hours was used to test the effect of this inhibitor. Figure 3.10A shows that LY 294002 significantly inhibited VEGF secretion. Wortmannin, another inhibitor of PI3K, also significantly reduced VEGF secretion ($p < 0.05$ data not shown). However, the effect of LY 294002 was greater than that of wortmannin. It is possible that this difference is due to the shorter biological half-life of wortmannin. The activity of LY 294002 on their targets was verified by immunoblotting (Figure 3.10B). VEGF secretion by hMDMs was similarly inhibited by LY 294002 (Figure 3.11).

3.2.8 OxLDL-induced VEGF secretion is independent of classical and novel PKC isoforms

PDK-1 acts as a central hub in phosphoinositide signaling. It phosphorylates and activates many members of AGC (cAMP-dependent, cGMP-dependent and protein kinase C) family of kinases including all isoforms of PKC [525]. PKCα and PKCζ have been implicated as key regulators of VEGF expression [391, 526, 527]. To evaluate roles of conventional and novel PKC isoforms we took two approaches. One was to test the effect of RÖ-31-7549 and RÖ-32-0432, which are both inhibitors of
conventional and novel, but not the atypical, PKC isoforms. Neither of these compounds inhibited oxLDL-mediated VEGF secretion (Figures 3.12A and B). Second, we exposed RAW 264.7 cells to phorbol 12-myristate 13-acetate (PMA) for 24 h to downregulate expression and activity of conventional and novel PKC isoforms [528]. PMA treatment by itself increased basal levels of VEGF secretion. OxLDL was capable of further increasing VEGF even after PKC depletion by prolonged PMA treatment (Figure 3.12C) which suggests neither conventional nor novel PKC isoenzymes are involved in VEGF upregulation by oxLDL.

3.2.9 PKCζ mediates oxLDL-induced VEGF secretion

Next, to examine the role of PKCζ, we used a cell-permeable pseudosubstrate inhibitor of this atypical PKC isoform. This inhibitor slightly but significantly ($p < 0.05$) reduced VEGF upregulation at 1 μM, and at 5 μM it caused more than 50% inhibition of oxLDL-mediated VEGF secretion (Fig. 3.13). To verify that oxLDL in fact activates PKCζ, we carried out kinase assays on immunoprecipitated PKCζ. It is known that LPS increases PKCζ activity in macrophages [529]. OxLDL’s ability to increase PKCζ activity was comparable to this positive control (more than 3 fold increase with LPS (1μg/mL, 10 minutes) and oxLDL24 (25μg/mL, 120 minutes). Results in Figure 3.14 show a time-dependent augmentation in PKCζ activity following oxLDL treatment with increasing to more than 500% of control at 50 minutes. This increase in PKCζ activity was reduced to baseline in the presence of a PKCζ pseudosubstrate inhibitor (not shown).

3.3 Summary/discussion

In the studies presented in this chapter, we used three different types of macrophages namely RAW 264.7 cells, mouse peritoneal macrophages, and human monocyte derived macrophages. RAW 264.7 macrophages, originally generated through transformation by Abelson Leukemia virus, exhibit many properties and functions of primary macrophages without a requirement for activation (for example by PMA)[530]. Normal intima is devoid of resident macrophages and therefore the major source of intimal macrophages is blood monocytes that are recruited to the site of LDL deposition.
Therefore, hMDM is a frequently used model, even though hMDMs show some differences compared to macrophages isolated from atherosclerotic lesions. Mouse peritoneal macrophage is one of most commonly used primary cells in atherosclerosis research. In addition to closely resembling macrophage behavior in artery wall [531], it is an attractive model as functions of specific gene products can be assessed via their deletion in transgenic mice.

Our results demonstrate a dose-dependent increase in secretion of VEGF by these macrophages in response to extensively oxidized LDL and are in agreement with previous reports in different cell types [418, 518, 519]. We also found that LDL mildly oxidized with iron, which has mostly peroxidation of lipids and little modification of apoB [472] and LDL modified by exposure to fatty acid oxidation products, which has mostly derivatization of apoB with fatty acid oxidation products but little change to LDL lipids [506] both induced VEGF secretion. This seems to suggest that oxidation of either the lipid or the protein components of LDL may lead to VEGF induction. Our findings with mildly oxidized LDL are in line with recent results that show oxidized phospholipids can induce VEGF secretion in monocyte-derived macrophages [532]. On the other hand, our findings contrast somewhat with the report of Ramos et al., who proposed that LPC in oxLDL may increase VEGF expression and secretion in macrophages [418]. In that study however, the maximal effect of LPC on VEGF was significantly less than that of oxLDL and we suspect that other components of oxLDL were probably also contributing to VEGF upregulation, as we have shown for the growth-inducing effect of LPC in MPM [418]. We found that Ac-LDL had no significant activity on VEGF secretion, indicating that an oxidative rather than a chemical lysine modification is required for VEGF induction.

Other groups have suggested that ligands of PPARγ can upregulate VEGF in macrophages [533]. Of interest, 9- and 13-hydroxy-(S)-10,12-octadecadienoic acid (9- and 13-HODE, respectively) were suggested by Inoue et al to account for part of the increase in VEGF secretion mediated by oxLDL [518]. However; the concentrations of ligands used in that study were very high. The specificity of some PPARγ ligands such as ciglitazone and prostaglandin J2 (used in the above-mentioned study) is also in doubt, as many effects attributed to these ligands have been reproduced in PPARγ-null cells [534]. In addition, potent and specific PPARγ agonists were not able to induce VEGF expression in primary
macrophages [535]. Synthetic LXR agonists acting through LXR can induce VEGF in macrophages [535], and oxysterols (which are LXR agonists) have been shown to increase VEGF expression in rat vascular smooth muscle cells [536]. Further studies are required to define the quantitative importance of oxysterols in VEGF induction by oxLDL. In our studies, the ability of ceramide to reduce basal levels of VEGF secretion is in line with other studies showing anti-angiogenic properties for ceramide [523]. Additionally, our results show for the first time that Cer-P can increase VEGF secretion. However, it seems that oxLDL and Cer-P are using different mechanisms as ceramide antagonizes Cer-P-induced (as expected) but not oxLDL-induced VEGF secretion.

OxLDL is not a simple well-defined entity. Depending on the method used to oxidize LDL, there can be wide variation in the nature and extent of protein or lipid modification. [316]. The initial stage of metal-catalyzed oxidation is accompanied by consumption of LDL antioxidants followed by a propagation phase during which lipid peroxidation of mainly polyunsaturated fatty acids (PUFAs) and cholesterol occurs. Further oxidation includes a decomposition step where oxidized PUFAs are fragmented and covalently bind to lysine residues on apoB100. This lysine derivatization leads to recognition of extensively oxidized LDL by SR-A and loss of recognition by LDL receptors [510]. Due to complexities inherent in these steps, any two preparations may not be identical. In our results with OP-LDL, oxLDL24, Ac-LDL, and FeLDL, different preparations showed very reproducible effects. However one caveat in our studies is that the experiments with oxLDL2 and oxLDL5 were done with same preparations.

CD36 and SR-A are two scavenger receptors that together are responsible for more than 80% of binding and uptake of oxLDL by macrophages [269]. We found that macrophages lacking both these receptors had the same induction of VEGF secretion by oxLDL as wild-type mice, which rules out any essential role for either SR-A or CD36 signaling in VEGF induction. Secondly, it also suggests that induction of VEGF secretion is not dependent on receptor-mediated uptake of oxLDL. One could hypothesize that 20% of uptake might be still enough for increased VEGF secretion; however, considering the dose-dependent nature of response to oxLDL, one would expect to see a significant difference between wild-type and DKO cells. Our results also rule out an essential role for LOX-1
scavenger receptor. The observed discrepancy between our results and previous reports could be explained by the fact that previous reports used endothelial cells [520] or articular chondrocytes [521]. In addition to cell-type differences, in both studies, pre-incubation with anti-LOX-1 blocking antibody was used to demonstrate a role for this receptor whereas we used macrophages from LOX-1 knockout mice. One would expect that the knockout model would represent a more specific and complete inactivation of LOX-1 than the antibody.

As discussed in section 1.3.3, VEGF has a complex role in atherosclerosis and may act as a double-edged sword in that it tends to accelerate lesion progression but can also improve revascularization in ischemic areas of myocardium. OxLDL increases VEGF secretion but can also antagonize potentially beneficial effects of VEGF. For example, oxLDL can inhibit VEGF-induced endothelial progenitor cell differentiation [537] and also can inhibit VEGF-induced NO-dependent migration of endothelial cells [538]. This suggests that if oxLDL induces VEGF in plaque, the angiogenic effect will predominate and potential beneficial effects of VEGF on endothelium would be inhibited by oxLDL. Thus, finding signaling pathways that link atherogenic stimuli (such as oxLDL) to VEGF but do not block beneficial effects of this cytokine could be therapeutically important. The regulatory mechanisms involved in increased VEGF secretion in response to oxLDL have not been clearly dissected. Previous studies indicated a role for PPARγ and P38 MAPK but in those studies each pathway was shown to have only a partial effect [518, 519]. Many stimuli act through different receptor tyrosine kinases (RTKs) followed by activation of PI3K/PKB and Ras dependent MAPK pathways and both of these pathways are activated in macrophages in response to oxLDL [310]. We found that MAPKK activity was involved in the control of baseline VEGF secretion but MAPKK inhibition did not block oxLDL-induced VEGF secretion. On the other hand, PI3K inhibitors significantly inhibited oxLDL mediated VEGF secretion. Another caveat in our studies is that pharmacological inhibitors are not 100% selective and specific for their targets. For example wortmannin may inhibit MAPK at higher doses and LY 294002 can inhibit casein kinase 2[539]. However, the fact that both these inhibitors were able to inhibit oxLDL-mediated VEGF secretion suggests that the effect is mediated by their common (and intended) target. One of the potential targets of PI3K is PKC. We found no evidence that
conventional and novel PKC isoforms are involved. Our finding that prolonged exposure to PMA increases VEGF expression corroborates the findings of Shih et al. In those studies PKCζ was suggested to play a key role in PMA-mediated VEGF upregulation [526]. In our hands, oxLDL was still able to further upregulate VEGF with extended PMA treatment. This also fits well with findings of Ijichi et al. who showed that brief and prolonged PMA treatment both induce VEGF expression, and that angiogenic stimuli such as hypoxia are still able to increase VEGF in the presence of PMA, consistent with a role for atypical PKC isoforms [540]. We showed that PKCζ is activated by oxLDL and also demonstrated that a cell permeable pseudosubstrate inhibitor of PKCζ significantly blocked oxLDL-mediated VEGF secretion. This suggests that activation of this atypical PKC isoform is important for VEGF induction by oxLDL.
**Figure 3.1 OxLDL increases VEGF expression and secretion in RAW264.7.** Macrophage VEGF secretion in response to native LDL (nLDL) or LDL that had been extensively oxidized with Cu^{++} for 24 h (oxLDL24) was measured by ELISA as described in the methods. Panels represent mean values ± SD from at least 3 independent experiments with duplicates for each condition. 

A) Time course of VEGF secretion by RAW 264.7 cells. Medium from untreated cells (CTL) or cells treated with 25 µg/mL of nLDL or oxLDL24 was assayed at the indicated time points. 

B) Effect of oxLDL concentration in RAW 264.7 cells. VEGF was assayed in medium after 24 h incubation with increasing concentrations of nLDL or oxLDL24. Level of significance of difference between oxLDL and corresponding nLDL control is indicated as follows: * p ≤ 0.05, ** p ≤ 0.01, and *** p ≤ 0.001. No difference was observed between cells treated with nLDL or control (CTL) cells exposed to medium alone. 

C) VEGF mRNA was measured in RAW264.7 by qPCR at baseline, 3, and 6 h after treatment with oxLDL24 or nLDL. Results
were normalized to β-actin and expressed as fold increase of oxLDL24 treatment relative to nLDL. *** denotes level of significance of \( p \leq 0.001 \) between baseline (0 h) and 6 h conditions.
Figure 3.2 OxLDL increases VEGF secretion in primary macrophages. Macrophage VEGF secretion in response to nLDL or oxLDL24 was measured as described in the methods. Panels represent mean values ± SD from 3 independent experiments with duplicates for each condition. A) VEGF secretion by hMDM. Medium was assayed for VEGF after 48 h of treatment with increasing concentrations of nLDL or oxLDL24. B) VEGF secretion by MPM. Medium was assayed for VEGF after 24 h with no treatment (CTL) or with 25 μg/mL of nLDL or oxLDL24. Level of significance of difference between oxLDL and corresponding nLDL control is indicated as follows: * p ≤ 0.05, ** p ≤ 0.01, and *** p ≤ 0.001. No difference was observed between cells treated with nLDL or control (CTL) cells exposed to medium alone (Not shown in panel A).
Figure 3.3 Effect of degree of LDL oxidation on VEGF secretion. A) VEGF secretion by RAW264.7 cells was measured after 24 h of treatment with 25 µg/mL of nLDL or the same concentration of LDL that has been modified with ferrous ion (FeLDL) or with copper ion for 2, 5, or 24 h (oxLDL2, 5, or 24, respectively). The panel represents mean values ± SD from 3 independent experiments with duplicates for each condition. Level of significance of difference between nLDL control and each condition is indicated as: * p ≤ 0.05 and *** p ≤ 0.001. B) With point of origin at the minus sign, electrophoretic mobility of nLDL and modified LDLs was assessed on agarose gel (Titan gel lipoprotein kit). Lipoprotein bands were visualized by Fat Red staining and the mobility was determined by measuring the distance between the point of origin (minus sign) and the leading edge of the lipoprotein and was expressed relative to the mobility of nLDL (relative mobilities as follows: FeLDL: 1.5, oxLDL2: 2.8, oxLDL5: 4.9, and oxLDL24: 6.2).
Figure 3.4 Effect of type of apoB modification on VEGF secretion. LDL was extensively modified by acetylation (Ac-LDL), by exposure to copper ion for 24 h (oxLDL24) or by exposure to oxidation products of fatty acids (OP-LDL) as described in methods. Panels represent mean values ± SD from at least 3 independent experiments with duplicates for each condition. A) VEGF secretion by RAW264.7 after 24 h treatment with 25 µg/mL of nLDL, oxLDL24 or Ac-LDL and varying concentrations of OP-LDL. B) Bars represent electrophoretic mobility of modified LDLs used in these experiments relative to nLDL C) VEGF secretion in MPMs from CD-1 mouse after 24 h incubation with 25 µg/mL of oxLDL24, nLDL, Ac-LDL or OP-LDL. D) VEGF secretion in hMDM after incubation for 48 h with 25 µg/mL of nLDL or oxLDL24, or 40 µg/mL of Ac-LDL or OP-LDL. For panels C) and D), the electrophoretic mobilities of different LDL modifications relative to nLDL were 6.8, 6.2, and 4 for Ac-LDL, oxLDL24 and OP-LDL respectively. (* p ≤ 0.05 and *** p ≤ 0.001 between nLDL control and the specified conditions).
Figure 3.5 Ceramide-1-phosphate increases VEGF secretion in RAW267.4 cells. Macrophage VEGF secretion was measured in medium after 24 h incubation with oxLDL, nLDL, or increasing concentrations of ceramide1-1-phosphate (Cer-P) and expressed as mean values ± SD from at least 3 independent experiments with duplicates for each condition. Level of significance of difference between each condition and nLDL control is indicated as ** $p \leq 0.01$ and *** $p \leq 0.001$. 
Figure 3.6 Ceramide inhibits Cer-P-mediated, but not oxLDL-mediated VEGF secretion.

Following 24 h incubation with or without indicated concentrations of sphingomyelinase (SMase), media was changed and RAW267.4 cells were treated with oxLDL (25 μg/mL), nLDL (25 μg/mL) or Cer-P (50 μM) and VEGF secretion was measured in medium after 24 h incubation. Bars represent the difference between the mean and the upper range from 2 independent experiments with triplicates for each condition.
Figure 3.7 LOX-1, SR-A, and CD36 are not essential for oxLDL-mediated VEGF secretion. VEGF secretion in MPMs from wild-type (WT), LOX-1, and SR-A/CD36 (DKO) knockout mice was measured after 24 h incubation with 25 μg/mL of oxLDL24 or nLDL. Each bar represents pooled data ± SD from three independent experiments.
Figure 3.8 OxLDL uptake is significantly reduced in SR-A/CD36 double knockout MPMs. A) The histogram represents lipoprotein uptake from one experiment in wild-type (WT) and SR-A/CD36 double knockout (DKO) MPMs. Unlabeled oxLDL is used as unstained control. DiI-LDL and DiI-OxLDL represent fluorescently labeled nLDL and oxLDL, respectively. B) DiI uptake was measured as described in the methods. Data represent the means and SD of pooled data from four independent experiments.
Figure 3.9 MAPKK is not essential for oxLDL-mediated VEGF secretion. A) Role of mitogen activated protein kinase was examined using the selective MAPKK inhibitor PD 98059. RAW 264.7 cells were treated with oxLDL24 or 5 µL of DMSO as vehicle control with or without pre-incubation for 45 min with the indicated concentration of PD 98059. The graph represents data from one experiment done in duplicate and was confirmed in two independent experiments. B) The activity of PD 98059 on MAPKK in RAW 264.7 cells was verified by evaluation of phosphorylation status of the downstream target MAPK1 (Erk2) by immunoblot.
Figure 3.10 Role of PI3K in oxLDL-mediated VEGF secretion in RAW 264.7 cells. A) RAW 264.7 cells were incubated for 14 h with oxLDL24 or nLDL, with or without 45 min pretreatment with indicated concentrations of LY 294002, a PI3K inhibitor. Bars represent mean values ± SD from three independent experiments with duplicates for each condition. (***: p ≤ 0.001 between oxLDL24 and the specified treatments). B) The activity of 15 µM LY 294002 in RAW 264.7 cells was verified by evaluation of PKB phosphorylation by immunoblot.
Figure 3.11 Role of PI3K in oxLDL-mediated VEGF secretion in hMDMs. Human MDMs were incubated for 48 h with oxLDL24 or nLDL, with or without 45 min pretreatment with indicated concentrations of LY 294002. Bars represent means ± SD from two independent experiments with triplicates for each condition. (**: p ≤ 0.05 between oxLDL24 and the specified conditions).
**Figure 3.12 OxLDL-induced VEGF secretion is independent of classical and novel PKC isoforms.**

RAW 264.7 cells were pretreated with increasing concentrations of A) RÖ 32-0432 or B) RÖ 31-7549, inhibitors of both conventional and novel PKC isoforms. C) RAW 264.7 cells were preincubated with or without 1 μM phorbol 12-myristate 13-acetate (PMA). After 24 h, medium was replaced with fresh medium containing 25 μg/mL oxLDL, or no oxLDL. After an additional 24 h incubation, medium was harvested for VEGF immunoassay. All graphs represent data from one of two or three similar experiments all carried out in duplicate. Error bars represent mean ± range.
Figure 3.13 PKCζ mediates induction of VEGF secretion by oxLDL. RAW 264.7 cells were pretreated with increasing concentrations of a myristoylated PKCζ pseudo-substrate peptide (PKCζ-PS) for 1 h prior to addition of oxLDL24. After 24 h of incubation, VEGF was measured. Bars represent means ± SD from three independent experiments with duplicates for each condition. Level of significance of difference between each condition and oxLDL24 is indicated as ** p ≤ 0.01 and *** p ≤ 0.001. No significant effect of PKCζ-PS by itself was observed on basal levels of VEGF.
**Figure 3.14 OxLDL induces PKCζ activation.** RAW 264.7 cells were treated with 25 μg/mL of oxLDL24 and PKCζ kinase activity was measured as described in Methods. Results were normalized and expressed as fold increase relative to no treatment control. The graph represents pooled data from four independent experiments (*: p ≤ 0.05 between 50 minutes of oxLDL24 treatment and control).
4. Receptors for oxLDL and its prosurvival effect in macrophages

4.1 Introduction/rationale

Macrophage survival and proliferation play an important role in pathogenesis of atherosclerosis (section 1.2.8). The signaling pathways mediating prosurvival effects of oxLDL have been partially defined (section 1.2.6). However, no studies have been reported that characterize the signaling events upstream of PI3K activation. The fact that signaling events are triggered within seconds to minutes after addition of oxLDL suggests a receptor-mediated mechanism. The studies presented in this section were carried out to determine if one of the pattern recognition receptors (PRRs) expressed by macrophages is required for anti-apoptotic signaling by oxLDL in macrophages. Important macrophage PRRs and their significance in atherogenesis and oxLDL uptake were discussed in sections 1.2.2 and 1.2.4, respectively. The major scavenger receptors I studied were SR-A, CD36, and LOX-1. As well, I tested a role for signaling PRRs including TLR4 and its signaling partner CD14. FcγRIIb, a member of immunoglobulin receptor superfamily, was also studied. At least in mice, FcγRIIb has been shown to bind to oxLDL with high affinity [541].

4.2 Results

4.2.1 LOX-1 signaling is not required for oxLDL prosurvival activity

LOX-1 is a major receptor for oxLDL in endothelial cells [542], but is also expressed in monocyte-derived macrophages [542, 543] and macrophages in atherosclerotic plaques [544]. There is evidence showing that LOX-1 can trigger cell signaling in response to oxLDL [545-548]. To address the role of LOX-1 in oxLDL-mediated macrophage survival, we compared the ability of oxLDL to promote survival in cytokine-deprived BMDMs from wild-type and LOX-1 deficient mice. Figure 4.1 shows that after 24 h there was no significant difference in the viability of wild-type and LOX-1 deficient cells treated with oxLDL. Additionally, there was no significant difference between wild-type and LOX-1 BMDMs in the percentage of sub-diploid cells after incubation with oxLDL (Figure 4.2). To measure the contribution of LOX-1 to oxLDL uptake in BMDMs, uptake of fluorescently labeled oxLDL was
compared between LOX-1 and WT BMDMs, and as show in Figure 4.5B, there is no significant difference.

4.2.2 OxLDL-mediated macrophage survival is independent of CD36 or SR-A receptors

CD36 [233-235] and SR-A [229-232] have both been shown to contribute to atherogenesis in animal models. It has also been reported that these endocytic receptors are capable of initiating cell signaling upon ligand binding [549, 550]. To test the effect of CD36 and SR-A, the ability of oxLDL to promote survival or inhibit apoptosis were compared between BMDMs from wild-type mice and from mice recombinant for deletion mutations in both these receptor genes. Figure 4.4 shows that oxLDL but not native LDL increased BMDM viability measured with the MTS assay in cells from both wild-type and DKO mice. There was no significant difference in viability after 24 h of M-CSF deprivation between wild type and DKO BMDMs at any concentration of oxLDL (Figure 4.4A). Longer time points (48 h) also did not show any difference with oxLDL in WT or DKO BMDMs (Figure 4.4B). Figure 4.5 shows the number of sub-diploid cells relative to the control condition (incubated without M-CSF or oxLDL (oxLDL 0 µg/mL)). Again, oxLDL was equally effective in reducing apoptosis in WT and DKO BMDMs. After 24 h of treatment, 50 µg/mL of oxLDL appeared slightly more effective at reducing sub-diploid population in DKO BMDMs (P = 0.03) but there was no difference after 48 h incubation and so the statistical significance at 24 h is probably spurious.

4.2.3 OxLDL-mediated macrophage survival is independent of oxLDL uptake

SR-A and CD36 together are responsible for most of the uptake of oxLDL in mouse peritoneal macrophages [269]. To confirm the functional inactivation of these receptors for oxLDL, in parallel with the above-mentioned experiments, uptake of fluorescently labeled oxLDL was measured in BMDMs as described in the methods section. Figure 4.5A shows that uptake of oxLDL in DKO BMDMs was reduced by 60% compared to wild-type.
4.2.4 Toll-like receptor 4 (TLR4) is not essential for oxLDL-mediated survival

Signaling through TLR4 is believed to play an important role in atherosclerosis [219, 222, 223]. Interestingly, minimally oxidized LDL can initiate TLR4-dependent signaling [101]. At least certain oxidation specific epitopes on oxLDL have been reported to act as PAMPs to initiate TLR4-mediated signaling [551]. To determine if TLR4 is responsible for the anti-apoptotic effect of oxLDL, we measured viability and apoptosis in TLR4 deficient macrophages. As shown in Figure 4.6A, there was no difference between viability of wild type and TLR4 deficient BMDMs in response to different concentrations of oxLDL. As well, oxLDL was equally effective in reducing the sub-diploid population in TLR4−/− as in wild type BMDM (Figure 4.7).

4.2.5 CD14 is not required for oxLDL prosurvival activity

In addition to the fact that CD14 acts as a signaling partner of TLR4 [552], some types of oxidatively modified LDL are reportedly recognized by (and bind to) CD14 [553]. Moreover, studies have shown that SR-A ligands can activate PI3K pathway via a CD14 dependent, SR-A independent pathway [554]. To determine whether CD14 plays a role in oxLDL-mediated survival, we compared oxLDL viability in BMDMs from wild type and CD14 knockout mice. OxLDL was able to increase viability (Figure 4.8) and inhibit apoptosis (Figure 4.9) in CD14-null BMDMs as effectively as in wild-type cells.

4.2.6 Fc gamma receptor IIb (FcγRIIb) and oxLDL-mediated survival

FcγRIIb, a macrophage receptor for Fc region of IgG, also binds to oxLDL with high affinity [541]. However, oxLDL increased viability in FcγRIIb-deficient macrophages to the same extent as in wild type cells (Figure 4.10). With FcγRIIb deficiency, we observed a slight increase in the sub-diploid population in all conditions (with or without M-CSF) which reached statistical significance for oxLDL 25 µg/mL treatment (Figure 4.11). This difference represented only ~10% of oxLDL ability to inhibit apoptosis and in FcγRIIb−/− BMDMs, oxLDL was still able to significantly decrease sub-diploid
population compared to no M-CSF control or LDL treatment ($p<0.0005$ for oxLDL at 25 and 50 µg/mL, Figure 4.11).

### 4.3 Summary/discussion

In these studies we evaluated macrophages from mice lacking a variety of receptors for oxLDL and found that none of these receptors were required for the prosurvival effect of oxLDL. LOX-1 is an endocytic scavenger receptor that has been shown to trigger cell signaling in response to oxLDL [545-548]. Our findings however show that in BMDMs LOX-1 is not required for oxLDL-mediated prosurvival signaling events. These observations are supported by a recent publication showing that in unstimulated MPMs, LOX-1 inactivation does not reduce oxLDL internalization [524].

CD36 and SR-A together account for the majority of oxLDL uptake and are thought to play a role in foam cell formation. Kunjathoor et al. showed in their studies that ~85% of oxLDL uptake in mouse peritoneal macrophages is mediated by these two receptors [269]. In our hands, 60% of oxLDL uptake in BMDMs was dependent on the combined effect of these receptors. This difference is probably due to proportionately greater receptor-independent uptake of oxLDL in BMDM compared to peritoneal macrophages. In fact in studies presented in chapter 3, similar to [269], we found that up to 80% of oxLDL uptake in MPMs is mediated by these two receptors. Another possible explanation for this cell-type specific difference could be that the pattern of SR expression (or compensatory mechanisms) between BMDMs and MPMs is different (with CD36 and SR-A being quantitatively more important in MPMs). In any case, considering the dose-dependent nature of oxLDL-induced prosurvival effect, the fact that oxLDL sustains its ability to promote survival in spite of 60% reduction in uptake suggests that uptake of oxLDL is not required for its anti-apoptotic effect. Although endocytic scavenger receptors are not generally considered as signaling receptors, there are reports that CD36 [550, 555] and SR-A [549] can initiate signaling upon ligand/receptor binding. Our results show that neither SR-A nor CD36 is responsible for oxLDL-induced signal transduction.

Toll-like receptor signaling is thought to be involved in the inflammatory processes that are important in the pathogenesis of atherosclerosis. Our results show that the TLR4 do not play a role in
anti-apoptotic effect of oxLDL in BMDM. This is in line with other experiments carried out in our laboratories showing that BMDMs from C3H/HeJ (LPS-unresponsive TLR4 mutant [556]) or TLR2 knockout mice also exhibited the same viability pattern in response to oxLDL [557].

In our hands, FcγRIIb-deficient BMDMs exhibited a general increase in sub-diploid population compared to wild-type which became statistically significant for only one of oxLDL treatments. One point worth noting is the genetic background of FcγRIIb knockout animals used in our experiments. FcγRIIb knockout mice come from a mixed Sv129/C57BL6 genetic background. The controls used in our studies have pure C57BL6 background and the marginal difference between the two cells could have been caused by a residual Sv129 DNA carryover. Strain-specific differences in the effect of FcγRIIb deficiency has been observed previously [558]. However this is not a likely explanation for the increased sub-diploid population as the observed trend was discordant with parallel MTS results where an increase in viability of FcγRIIb-deficient BMDMs was seen. While this marginal trend might be spurious, it is plausible in the sense that a decrease in apoptotic cell clearance in FcγRIIb-null macrophages could lead to a larger sub-diploid pool. Although FCγRs have been shown to be involved in clearance of apoptotic cells in vivo, no reports exist in literature regarding direct (immunoglobulin-independent) clearance. It is however known that that FcγRIIb deficiency leads to an autoimmune phenotype and an exaggerated IL-12 response in antigen-presenting cells incubated with apoptotic thymocytes [559]. In any case, this difference observed in our experiments at best can account for only ~10% of oxLDL ability to inhibit apoptosis which rules out an essential role for FcγRIIb.

Other receptors of interest for future studies include SR-PSOX (also known as CXCL16) receptor for advanced glycation products (RAGE), and SR-BI. SR-PSOX is a high affinity receptor for oxLDL and phosphatidylserine [560]. Aslanian et al have shown that murine SR-PSOX accounts for ~34% of oxLDL uptake. Unexpectedly however, they showed an atheroprotective role for this scavenger receptor [237]. An increased number of apoptotic cells in the plaques of SR-PSOX-null mice were observed which was attributed to ineffective clearance of apoptotic cells. This putative role in apoptotic cell clearance was suggested as a mechanism for atheroprotection by SR-PSOX. To our knowledge there are no reports on SR-PSOX signaling in response to oxLDL. It would be interesting to see if SR-PSOX
is required, at least partially, for oxLDL-mediated anti-apoptotic signaling in macrophages. Although not classified as a scavenger receptor, RAGE, is also another multi-ligand receptor that belongs to the immunoglobulin superfamily and is expressed in macrophages [241]. It was recently shown that RAGE deactivation attenuates atherosclerosis in LDLR$^{-}$ mice. In that study, it was shown that RAGE triggers cell signaling in response to oxLDL and in fact, part of the protective effect of RAGE deletion was attributed to a dampened inflammatory response of macrophages following oxLDL treatment. In their studies, Sun et al. show that RAGE is required for oxLDL-mediated MAPK signaling however they did not study the possible role of RAGE in other signaling pathways induced by oxLDL [242]. We have shown before that MAPK signaling by oxLDL is not essential for its prosurvival effect [310] but it would be interesting to test the effect of oxLDL-induced prosurvival pathways such as PI3K and calcium signaling in RAGE-deficient BMDMs. Interestingly, Kinslinger et al. have shown that N$^{\varepsilon}$-carboxymethyllysine adducts are ligands of RAGE [561]. In addition to glycoxidation, lipid peroxidation followed by protein modification is capable of generating N$^{\varepsilon}$-carboxymethyllysine adducts (section 1.3.5 and [562]) , therefore it would be interesting to study and compare the contribution of this receptor to the prosurvival effects of oxLDL and OP-LDL. As discussed in section 1.2.2, SR-BI is an atheroprotective scavenger receptor through its roles in reverse cholesterol transport [238-240]. Different groups have shown that SR-BI contributes to cellular bindings and degradation of oxLDL [563, 564]. Additionally, it has been shown that SR-BI is capable of triggering signaling events and activating kinases (such MAPK and p38) upon ligand binding [565, 566]. These properties makes SR-BI another interesting candidate for mediating the anti-apoptotic effects of oxLDL in macrophages.
**Figure 4.1 LOX-1 is not essential for oxLDL-mediated survival.** BMDMs from wild-type (WT) or LOX-1 knockout mice were seeded at $3 \times 10^4$ cells/well in 96 well plates. After overnight incubation in media containing M-CSF, they were washed and incubated for 24 h without M-CSF, but with indicated concentrations of oxLDL. Viability was then measured by the bioreduction of MTS as described in the methods. The results represent means ± SD of pooled data from four independent experiments. Viability is expressed as a percentage relative to that of cells treated with M-CSF. None of the differences between corresponding WT and LOX-1 conditions reached statistical significance.
Figure 4.2 LOX-1 is not essential for the anti-apoptotic effect of oxLDL. BMDMs from WT or LOX-1 mice were plated at $10^6$ cells/well in 6-well plates. Following overnight incubation in M-CSF-containing media cells were washed and incubated for 24 h in medium containing M-CSF or no M-CSF and indicated concentration of oxLDL. The sub-diploid population was measured by flow cytometry after propidium iodide staining. In the upper panel, histograms of each condition from one representative experiment are shown. The sub-diploid population was measured by counting the cells with fluorescence intensity under the region depicted by the red line. Results in the lower panel represent the means ± SD of pooled data from three independent experiments which are expressed relative to control cells incubated with neither M-CSF nor oxLDL. None of the differences between corresponding WT and LOX-1 conditions reached statistical significance.
Figure 4.3 SR-A and CD36 are not required for oxLDL-mediated survival. BMDMs from wild-type (WT) or SR-A/CD36 double knockout (DKO) mice were seeded at $3 \times 10^4$ cells/well in 96 well plates. After overnight incubation in media containing M-CSF, they were washed and incubated without M-CSF, but with indicated concentrations of native LDL (nLDL) or oxLDL for A) 24 h or B) 48 h. Viability was then measured by the bioreduction of MTS as described in the methods. Results represent the means ± SD of pooled data from four independent experiments. Viability is expressed as a percentage relative to that of cells treated with M-CSF. None of the differences between corresponding WT and DKO conditions reached statistical significance.
Figure 4.4 OxLDL-mediated inhibition of apoptosis is independent of SR-A and CD36. BMDMs from WT or CD36/SR-A double knockout (DKO) mice were plated at 10^6 cells/well in 6-well plates. Following overnight incubation in media containing M-CSF, cells were washed and then replenished with medium containing M-CSF or no M-CSF together with the indicated concentration of nLDL or oxLDL. A) The sub-diploid population was measured by flow cytometry after propidium iodide staining. Histograms from one representative experiment are shown. The sub-diploid population was measured by counting the cells with fluorescence intensity under the region depicted by the red line. Results in B) and C) are means ± SD of pooled data from four independent experiments at 24 h incubation (B) or 48 h (C) expressed relative to control cells incubated with neither M-CSF nor oxLDL. Unless specified, the difference between WT and the corresponding DKO is not statistically significant.
Figure 4.5 OxLDL uptake in BMDMs depends on SR-A/CD36 but not LOX-1. BMDMs from wild-type (WT), CD36/SR-A double knockout (DKO) (A), or LOX-1 knockout mice (B) were seeded at $10^6$ cells/well in 6-well plates. They were then washed and incubated for 24 hr with fluorescently labeled oxLDL or nLDL (DiI-oxLDL and DiI-LDL, respectively). Cell content of DiI was then measured by fluorimetry after isopropanol extraction and expressed as percentage of DiI-oxLDL uptake in corresponding WT BMDMs. (NS: not significant).
Figure 4.6 TLR4 does not contribute to the prosurvival effect of oxLDL. BMDMs from wild-type (WT) or Toll-like receptor 4 (TLR4) knockout mice were seeded at $3 \times 10^4$ cells/well in 96 well plates. After overnight incubation in media containing M-CSF, they were washed and incubated for 24 h without M-CSF, but with indicated concentrations of nLDL or oxLDL. Viability was then measured by the bioreduction of MTS. Results represent the means ± SD of pooled data from three independent experiments, each in triplicate. Viability is expressed as a percentage relative to that of cells treated with M-CSF. None of the differences between corresponding WT and TLR4 conditions reached statistical significance.
Figure 4.7 TLR4 is not essential for the anti-apoptotic effect of oxLDL. BMDMs from WT or CD14 mice were incubated for 24 h in medium containing M-CSF or no M-CSF and indicated concentration of nLDL or oxLDL. The sub-diploid population was measured by flow cytometry after propidium iodide staining. In the upper panel, histograms of each condition from one representative experiment are shown. The sub-diploid population was measured under the region depicted by red line. The lower panel shows means ± SD of pooled data from three independent experiments expressed relative to control cells incubated with neither M-CSF nor oxLDL. None of the differences between the WT and the corresponding TLR4 reached statistical significance.
Figure 4.8 CD14 is not essential for the prosurvival effect of oxLDL. BMDMs from wild-type (WT) or CD14 knockout mice were seeded at 3 \times 10^4 cells/well in 96 well plates. After overnight incubation in media containing M-CSF, they were washed and incubated for 24 h without M-CSF, but with indicated concentrations of nLDL or oxLDL. Viability was then measured by the bioreduction of MTS. Results represent the means ± SD of pooled data from three independent experiments, each done in triplicate. Viability is expressed as a percentage relative to that of cells treated with M-CSF. None of the differences between the WT and corresponding TLR4 reached statistical significance.
Figure 4.9 CD14 does not contribute to the anti-apoptotic effect of oxLDL. BMDMs from WT or CD14 mice were incubated for 24 h in medium containing M-CSF or no M-CSF and indicated concentration of nLDL or oxLDL. The sub-diploid population was measured by flow cytometry after propidium iodide staining. In the upper panel, histograms of each condition from one representative experiment are shown. The sub-diploid population was measured under the region depicted by the red line. The lower panel shows the means ± SD of pooled data from three independent experiments which are expressed relative to control cells incubated with neither M-CSF nor oxLDL. Unless specified, the difference between WT and the corresponding CD14 is not statistically significant.
Figure 4.10 Fc gamma receptor IIb (FcγRIIb) is not required for the prosurvival effect of oxLDL.

BMDMs from wild-type (WT) or FcγRIIb knockout mice were seeded at 3 x 10⁴ cells/well in 96-well plates. After overnight incubation in media containing M-CSF, they were washed and incubated for 24 h without M-CSF, but with indicated concentrations of nLDL or oxLDL. Viability was then measured by the bioreduction of MTS. Results represent the means ± SD of pooled data from three independent experiments, each done in triplicate. Viability is expressed as a percentage relative to that of cells treated with M-CSF. With oxLDL treatment, there was no statistically significant difference between the WT and FcγRIIb knockout macrophages.
Figure 4.11 Anti-apoptotic effect of oxLDL and Fc gamma receptor IIb (FcγRIIb). BMDMs from WT or FcγRIIb⁻/⁻ mice were incubated for 24 h in medium containing M-CSF or no M-CSF and indicated concentration of nLDL or oxLDL. The sub-diploid population was measured by flow cytometry after propidium iodide staining. In the upper panel, histograms of each condition from one representative experiment are shown. The sub-diploid population was measured in the area depicted by the red marker. The lower panel shows the means ± SD of pooled data from three independent experiments which are expressed relative to control cells incubated with neither M-CSF nor oxLDL. Unless specified, the difference between the WT and FcγRIIb knockout macrophages is not statistically significant.
5. Fluorescent adducts formed by reaction of oxidized unsaturated fatty acids with amines induce macrophage growth

5.1 Introduction/rationale

Macrophages play a key role in pathogenesis of atherosclerosis from its conception to complications (section 1.2). In particular, macrophage burden correlates well with the disease progression and clinical outcome [246, 247]. As discussed in section 1.2.6, several groups including our own have shown that oxLDL promotes macrophage growth and survival. OP-LDL, or LDL modified by oxidation products of fatty acids (OPs), was used in our laboratory as a model for metal-independent oxidatively modified LDL (section 1.4.5). In OP-LDL, unlike extensive modification of LDL with copper ion, endogenous lipids are not oxidized and LPC content is not increased. Even though lysine residues of apoB become derivatized, the apoB itself is not fragmented [268, 506, 510]. Epitopes recognized by antibodies specific to this type of modification are shown to exist both in vivo and in LDL oxidized by other methods [87, 507]. Martens et al. showed that OP-induced modification of not only LDL but also proteins such as albumin or immunoglobulins confer mitogenic properties towards macrophages [307]. OPs and protein modification by them were discussed in sections 1.4.2 and 1.4.5, respectively. The studies presented in this chapter were carried out to further define OPs and adducts they generate through reacting with lysine residues with relation to macrophage growth and survival.

5.2 Results

5.2.1 Both lipid and protein moiety of oxLDL promote macrophage growth

Sakai et al. had suggested that LPC is important in oxLDL-mediated survival [298, 299]. Martens et al. subsequently challenged this report with data showing that in MPMs, the effect of macrophage growth is not due to LPC [307]. In contrast, they found that lysine modification of apoB is essential, as prior methylation of apoB lysine residues abolished growth-promoting properties of oxidatively modified LDL. To determine more rigorously whether the lipid or protein moiety is contributing to the induction of growth by oxLDL, we extracted the lipid and separately reconstructed the
lipid and protein moieties of oxLDL. The ability of these fractions to promote macrophage growth was compared. Figure 5.1 shows that both lipid and protein fractions can promote growth of MPMs.

5.2.2 OPs react with lysine residues

To confirm that the lysine residue is the target of reactive OPs, polylysine was preincubated with OPs and the mixture was then added to LDL. Figure 5.2 show that this pre-treatment with polylysine completely abolishes induction of growth. Preincubation with polylysine also significantly prevents lysine modification on apoB as judged by relative electrophoretic mobility measurements (1.2 vs. 6.6-fold that of native LDL with or without polylysine pre-treatment, respectively).

5.2.3 Amine modification by OPs is sufficient for growth induction

We were somewhat surprised by the observation that lipid extracts of oxLDL can promote macrophage growth (Figure 5.1). One possibility for this finding could be the modification of amine-containing phospholipids by OPs. To explore this possibility, phosphatidylethanolamine (PE)-containing liposomes were modified by OPs from arachidonic and linoleic acid (AOP-PE and LOP-PE, respectively). Figure 5.3A shows that OP-PE liposomes are capable of promoting growth in MPMs - although not quite to the same extent of OP-LDL. On the other hand, the product of incubation of phosphatidylcholine (PC) liposomes, which lack the free amino group, with OP did not induce growth (Figure 5.3B). Additionally, Figure 5.3B shows that prior treatment (and reduction) of OPs with sodium borohydride abolished the growth-promoting effect of OP-PE liposomes.

5.2.4 Borohydride-reducible functional groups in OPs are essential for the growth-promoting effect

Similar to OP-PE (Figure 5.3B), we observed that pre-treatment of OPs with borohydride will abolish the growth-promoting effect of OP-LDL (Figure 5.4). Figure 5.4 additionally shows that borohydride treatment after LDL modification does not diminish the ability to promote growth. This suggests that borohydride-reducible functional groups are important for growth-promoting adduct
formation while such functional groups are not required after modification. The electrophoretic mobility of LDL was reduced by 50% with borohydride pretreatment (not shown) which suggests some reactive OPs are not amenable to reduction by borohydride.

5.2.5 Alkenals do not account for the growth-inducing activity of OPs

Borohydride-reducible functional groups include reactive aldehydes. Aldehydes are commonly generated during lipid peroxidation of unsaturated fatty acids (section 1.4.1) and are capable of modifying lysine residues and creating fluorescent adducts (section 1.4.6). In fact, aldehydic reactive products are reportedly the main components of oxidized fatty acids responsible for lysine modification of apoB [316, 505, 567]. To test if these reactive aldehydes could be responsible for the growth-inducing derivatization, we modified LDL with 4-hydroxynonenal (HNE) or 4-hydroxyhexenal (HHE), the major fragmented aldehydic products of arachidonic acid oxidation [316], to generate HNE-LDL and HHE-LDL, respectively. Figure 5.5 shows that neither HNE-LDL nor HHE-LDL induced macrophage growth.

5.2.6 Hydroperoxides and/or endoperoxides contribute to the induction of growth by OPs

Other borohydride-reducible functional groups in OP would include hydroperoxides and endoperoxides. Triphenylphosphine (TPP), a reducing agent selective for hydroperoxides and endoperoxides, was preincubated with OPs and the mixture was then used to modify LDL. Table 5.1 shows that TPP substantially, although not completely, reduced the ability of OP to modify lysine residues (based on electrophoretic mobility) and diminished the ability of modified LDL to induce growth. This suggests that at least part of OP reactivity responsible for lysine modification and growth induction is due to hydro (and/or endo) peroxides.
TABLE 5.1 Hydroperoxides and/or endoperoxides contribute to the induction of growth by OPs.

Arachidonic acid or linoleic acid (100 μmoles) was autoxidized for 48 h, and dissolved in 1 ml CHCl₃. Aliquots were then incubated with or without a 2-fold molar excess of triphenylphosphine for 12 h at 4°C. Lipid hydroperoxide content of each sample was assayed in triplicate. An aliquot equivalent to 6 μmoles of fatty acid was mixed with 1 mg LDL in PBS for 8 h, and agarose gel electrophoresis of each LDL sample was performed. Each LDL sample was also tested for effects on macrophage viability using the MTS assay. Viability results are normalized to the value with LOP-LDL. Similar results were obtained in two replicate experiments.

<table>
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<tr>
<th></th>
<th>Relative electrophoretic mobility</th>
<th>macrophage viability</th>
<th>LOOH assay mol/mol LOP or AOP</th>
</tr>
</thead>
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<tr>
<td>LDL</td>
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<tr>
<td>LOP-LDL</td>
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<td>100%</td>
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<tr>
<td>AOP-LDL</td>
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<tr>
<td>(AOP+TPP)-LDL</td>
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<td>68%</td>
<td>1.1 ± 0.3</td>
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</tbody>
</table>
5.2.7 Autoxidation of arachidonic and linoleic acids produces mainly unfragmented oxygenated species

The OPs of LA and AA were studied using electron ion spray-mass spectrometry (ESI-MS). Figure 5.6 shows the results of ESI-MS in positive ion mode. Unoxidized AA (M_{AA} = 304) under our experimental conditions existed as protonated (m/z = 305) and sodated (m/z = 327) forms. Unoxidized LA (M_{LA} = 280) mainly existed as sodated form (m/z = 303). ESI-MS of AOP shows emergence of new masses in the range m/z 325 to 420, and with LOP in the range m/z 315 to 400. Table 5.2 shows the results of MS studies of a time-course of AA and LA oxidation for 24, 48, and 72 hours. With increased degree of oxidation, more than 90% of AA or LA is consumed while the relative peak intensities for OPs increase. An aliquot of every time-point was also used to modify LDL and the degree of lysine modification of apoB was estimated by agarose electrophoresis. Table 5.2 shows that the increase in relative abundance of OPs correlates with the ability to modify LDL. The m/z values of the most abundant products are consistent with addition of up to 6 and 4 oxygens to AA and LA respectively, without fragmentation. Table 5.2 also includes predicted structural assignments based on each m/z. These studies suggest that by 24 h, most of the arachidonic acid has been oxidized by addition of two molecules of oxygen (i.e. addition of four oxygen atoms at two of the four double bonds) consistent with the formation of two hydroperoxides or epidioxides. This was further supported by olefinic proton number in NMR studies carried out by our collaborator Dr. Guenther Eigendorf. Subsequently there is an increase in the proportion of fatty acid molecules with lower degrees of oxygenation, presumably reflecting secondary rearrangement or decomposition of hydroperoxides, perhaps to epoxides, ketones, and hydroxides. The proportionate amount of the ion representing the 2-oxygen atom addition product increased in parallel with those of the 3- and 4-oxygen atom addition products, so no inferences could be drawn from the time course study as to which species was responsible for modifying LDL.

We further studied autoxidized linoleic acid with MSMS. Isolation of the ion at m/z 335 and collision with helium yielded a main fragment in positive ion mode with m/z 225, consistent with 9-hydroxy-10,11-epoxy linoleic acid fragmenting at the methyl end of the epoxide (+Na). A minor ion at m/z 221 was found, consistent with fragmentation at the methyl end of the 13-hydroxy-10,11-epoxy-
octadecadienoic acid. No ions were detected that would have corresponded to fragmentation at either side of a 9-hydroperoxide. While 9-hydroxy-10,11-epoxy linoleic is capable of reacting with amino groups, it is also possible that the 13-hydroperoxide was responsible for reacting with amino groups but was not detected in the mass spectrometer due to decomposition. To determine if the reactive 2-oxygen addition product was a hydroperoxide or an epoxy-hydroxy compound, we subjected linoleic acid that had been oxidized to reduction with sodium borohydride. Before reduction, the 3 most abundant ions were m/z 335, m/z 357, and m/z 317 corresponding to M+32+Na\(^+\), M+32+2Na\(^+\)-H\(^+\), and M+32+Na\(^+\)-H\(_2\)O respectively. These ions accounted for 70\% of the total. After reduction with 100 mM NaBH\(_4\) about half of the total di-oxygenated product was reduced to a mono-oxygenated species. We conclude from this that the oxidation product contains an approximately equal mixture of hydroperoxy-octadecadienoic acid and epoxy-hydroxy-octadecenoic acid.

Neither 9,10-epoxyoctadec-12Z-enoic acid nor 12,13-epoxyoctadec-9Z-enoic acid reacted with LDL (not shown). However, the keto, epoxy-hydroxy, or epoxy-keto forms would be more reactive and might react with amino groups under some conditions.
**TABLE 5.2 Time course analysis of polyunsaturated fatty acid autoxidation products.** 9 mg of neat arachidonic acid or linoleic acid were incubated for 24-72 h in an air atmosphere at 40°C. At each time point, a sample of oxidized fatty acid was analyzed by mass spectrometry. A parallel sample was dissolved in PBS, and incubated for 24 h with LDL. Agarose gel electrophoresis was then performed to assess the extent of LDL modification. Results are representative of three independent experiments.

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<tr>
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<tr>
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5.2.8 OP modification of lysine and apoB generate fluorescent adducts

As mentioned, we and others have reported that copper-oxidized LDL has characteristic fluorescence properties, with maximum excitation at 350-360 nm and maximum emission at 430 nm (section 1.4.4 and [505, 568]). To see if OP modification of LDL generates the same fluorescence pattern, we compared the fluorescence of AOP-LDL and LOP-LDL with that of oxLDL. In addition to nLDL control, Ac-LDL was used as a non-oxidative lysine modification control. Similar to oxLDL, AOP-LDL and LOP-LDL exhibited a more than 20-fold increase in fluorescent intensities (excitation/emission of 350/430 nm) compared to nLDL. Acetylation of LDL on the other hand, produced no increase in fluorescence intensity (Figure 5.7A). We next measured the fluorescence of adducts formed by modification of lysine methyl ester (LME) with OPs. Figure 5.7B represents the fluorescence intensities of serial dilutions of OP-LME reaction mixture depicting an increase in the fluorescence with OP-LME while there was no fluorescence associated with OP alone. Lysine methyl ester contains two potentially reactive amino groups. However, the alpha amine of lysine is 6 time less reactive compared to the epsilon amine [569] and it is believed that esterification of the carboxyl group further reduces reactivity of the alpha amine. Hence, although the alpha amine was not protected, at least 90% of adducts are anticipated to be on the epsilon amino group. Figure 5.7B shows that the increased fluorescent intensity of different concentrations of OP-LMEs while there was no increase in fluorescence of OP alone. Fluorescent scanning of LOP-LME revealed a fluorescence spectrum (Figure 5.7C) almost identical to what has been reported for oxLDL [505].

5.2.9 Unfragmented linoleic acid autoxidation products can modify lysine methyl ester

Modification of LME by LOP was chosen as the initial adduct to study because there are fewer potential products than with AOP modification LOP were used to modify lysine methyl ester (LME). The LOP-LME reaction mixture (equivalent to 10 μg of LOP) was injected into the ionization source of an Esquire Ion Trap instrument. Figure 5.8A shows a representative mass spectrum analysis of LOP-LME in positive ion mode. In addition to LME (m/z = 161) and masses known to exist in LOP, new products were found that only existed in LOP-LME reaction moisture. The m/z for these products (such
as 455, 457, 477) were all within the 450-510 range suggesting that adducts are formed by reaction of LME with unfragmented linoleic acid containing one to four atoms of oxygen. Figure 5.9B lists the predicted m/z of possible LOP-LME adducts with masses found in our experiments highlighted in red.

5.2.10 Putative structures of LOP-LME adducts

Fruebis et al. had suggested that OPs of unsaturated fatty acids could modify proteins through a concerted reaction involving the amine group of lysine residues and reactive functions (such as hydroperoxy groups) on OPs ([501] and Section 1.4.5). According to this model, adducts with nitrogen-containing heterocycles (such as pyrrole, pyrroline, or pyridine) are produced (Figure 1.7). Fukuzawa et al. have shown that keto-oleic acid can modify amine containing amino acids without fragmentation leading to generation of hydroxylactam-containing adducts with the same fluorescent properties we observed in OP-LME [570]. To further characterize the adducts in LOP-LME, major ions unique to LOP-LME were analyzed by electrospray ionization tandem mass spectrometry (ESI-MSMS). Table 5.3 represents the putative parent ion composition for the major daughter ions of each mass. Figures 5.9A shows the MSMS plot of m/z = 455. The empiric formula for M = 454 is C_{25}H_{46}N_{2}O_{5}, and based on the reaction mechanisms proposed by Fruebis et al. and Fukuwaza et al., one can propose several putative structures for the parent adduct that could produce the observed daughter ions upon fragmentation (Figure 5.9B & C– schemes a) through e)).

Loss of H_{2}O is a common phenomenon in MSMS analysis of fatty acids and their derivatives and this explains the peak at m/z = 437 (Figure 5.9A). Fragmentation of the carboxylic acid-containing side chain adjacent to the heterocycle accounts for the 144, 312, and 295 daughter ions. The relative abundance of m/z = 142 compared to 144 can be explained by existence of an unsaturated bond within the aliphatic chain between the heterocyclic structure and the carboxyl (Figure 5.9C scheme e)) or by desaturation of the aliphatic chain upon fragmentation. Scheme c) depicts how such dehydrogenation might occur with transfer of the hydrogens to an oxo functional group (or the neighboring unsaturated bond of a pyrroline ring - not shown in the scheme) to produce a hydroxyl group (or to saturate the
double bond). Additionally, a charge-remote fragmentation, which is commonly seen with fatty acids and their derivatives [571], can explain how a daughter fragment can undergo dehydrogenation (Scheme d)).

Lou et al. have reported conditions under which tertiary amines can undergo spontaneous dehydrogenation during fragmentation [572]. Scheme e) in Figure 5.9C is an application of this observation that provides an alternative explanation for the daughter ions of m/z = 455. Upon dehydrogenation, the daughter ion containing the cyclic moiety is already positively charged (N+) and does not require further protonation in positive ion mode resulting in m/z of 311 and 295 in positive ion mode. The fact that hydroxypyrrolidine has 4-fold higher fluorescence than pyrrole (see below) makes it an attractive candidate for the core structure of the adduct. An example of a structure with pyrrolidine core is given in scheme a). Table 5.3 shows the major daughter ions for other adducts including m/z = 457. The ion with m/z of 457 can be considered a hydrogenated form of 455. The detection of core fragments 312 and 296 and as well as the 144 fragment representing the carboxyl side chain (see figure 5.9A) is consistent with this assumption. Emergence of fragments with m/z of 391 and 341 however, could be a sign for the existence of other isobaric (m/z = 457) compounds within LOP-LME mixture. Another less likely explanation could be that saturation of the double bond is accompanied by intramolecular rearrangements leading to a different pattern of fragmentation.

Species with higher m/z followed the same fragmentation pattern with generation of dehydrated pseudoparent ions. In addition they commonly generated fragments with m/z = 335 and 319 which is possibly the sodated form of core structures (m/z = 312 and 296, respectively) that were observed with m/z of 455 and 457.

These results showed that adducts may contain 5 or 6 membered nitrogen heterocycles, most likely a pyrrole, pyrroline, or a derivative of. To determine if pyrrole derivatives might explain the fluorescence at 350/430 nm wavelengths, we compared fluorescence of pyrrole with that of its derivatives 3-pyrroline and 3-hydroxypyrrolidine. No fluorescence was seen with 3-pyrroline but that of 3-hydroxypyrrolidine was about 4-fold higher than that of pyrrole (data not shown).
TABLE 5.3 ESI-MSMS analysis and possible assignments of adducts of lysine methyl ester with autoxidized linoleic acid

<table>
<thead>
<tr>
<th>parent ion composition assignment</th>
<th>daughter ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>455 LME+LA+OOH-H₂O+ H⁺</td>
<td>437, 423, 378, 312, 296, 144, 142</td>
</tr>
<tr>
<td>457 LME+LA+O+H⁺</td>
<td>438, 391, 379, 341, 312, 296, 144</td>
</tr>
<tr>
<td>473 LME+LA+OOH+H⁺</td>
<td>455, 419, 413, 357, 335, 319, 144</td>
</tr>
<tr>
<td>477 LME+LA+OOH-H₂O+Na⁺</td>
<td>459, 335, 317</td>
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<tr>
<td>489 LME+LA+2OOH-OH⁻</td>
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<td>473, 335, 319, 211, 195</td>
</tr>
<tr>
<td>505 LME+LA+OOH+OOH+H⁺</td>
<td>487, 405, 351, 333, 317, 211, 195</td>
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</table>
5.2.11 Unfragmented arachidonic acid autoxidation products can modify lysine methyl ester

As discussed in section 1.4.1, AA autoxidation yields far more products compared to LA. For example, isolevuglandins alone, which are found in AOP but not LOP, comprise 64 isomeric structures. To study adducts formed by AOP, we used HPLC coupled to a ZQ single quadrupole spectrometer. Initially, an acetonitrile gradient (20 to 100%) from 0.2 – 10 min was used. Figure 5.10 depicts the results of a representative run. The total ion current (TIC) of the eluent in positive ion mode (Figure 5.13 A) shows an increase in some peak intensities but no peak could be uniquely assigned to AOP-LME. We therefore obtained mass spectra of 30-second fractions of the AOP-LME chromatogram and compared these with spectra from AOP and AA controls. Figure 5.10B shows the MS analysis of the eluent (from 120 to 180 seconds) for AOP-LME, AOP, and AA. New masses with m/z of 475, 491, 493, and 509 were found only in AOP-LME. Each of these masses seemed to elute in a relatively large number of fractions. For better chromatographic resolution, longer runs were used and Figure 5.11 shows the results of single ion recordings (SIRs) of the above-mentioned masses with optimized running conditions (total run time of 35 minutes) as described in methods (Sections 2.18). Each plot depicts an overlay of SIR of one m/z for AOP and AOP-LME mixture which clearly demonstrates these masses exist only in the reaction mixture. Each mass produced at least a dozen chromatographic peaks. Retention times suggest that with addition of oxygen, adducts become more hydrophilic. Un-oxidized arachidonic acid eluted at 30.5 minutes (not shown) while adducts eluted between 3 and 23 minutes.

The charge in positive ion mode can be due to protonation or addition of sodium or potassium. To determine if adducts found in positive ion mode are protonated we carried out SIRs of 473, 489, 491, and 507 in negative ion mode. Figure 5.125A shows the SIRs of m/z = 507 in negative ion mode and m/z of 509 in positive ion mode. The overall pattern of the negative ion SIR of m/z = 507 is almost identical to that of 509 in positive ion mode suggesting that m/z = 509 is protonated. In general, AOP-LME adducts where less efficiently ionized in negative mode. Comparing the ion intensities for example, negative ion mode underestimates the intensity of m = 507 by 34 fold compared to m/z = 509 in positive mode (Figure 5.125A). The efficiency of ionization in negative mode further decreased for adducts with lower m/z to a point that m/z = 473 was quite poorly ionized in ES-. To see if these adducts in positive
ion mode are singly charged, we looked at isotopic distribution of each m/z using a time-of-flight (TOF) analyzer. Inserts a, b, and c of Figure 5.12 show one-unit mass increases for different isotopic distributions which suggest that adducts were singly charged. We therefore concluded that in positive ion mode, these adducts accept a single proton.

We then studied the elemental composition of adducts by a SYNAPT™ High Definition Mass Spectrometry™ (HDMSTM) System operating in TOF mode for exact mass measurements. MassLynx software (4.1) was used for the analysis. The parameters used in analyses included concentration (parts per million (PPM)), estimated/calculated number of double bonds (double bond equivalent (DBE)), and i-FIT™ value. i-FIT™ measures the probability that the isotope distribution of a theoretical elemental composition will fit the experimental data (the lower the i-FIT value, the higher the probability of a match). Table 5.4 shows elemental composition analysis results of one representative peak for each m/z = 475, 491, 493, and 509. Considering all the criteria that are used in the analysis (such as i-FIT values) and also that adducts are most likely protonated (Figure 5.12), the most likely empirical formulae for adducts are as follows: C_{27}H_{43}N_{2}O_{5} (for m/z = 475), C_{27}H_{43}N_{2}O_{6} (for m/z = 491), C_{27}H_{45}N_{2}O_{6} (for m/z = 493), and C_{27}H_{45}N_{2}O_{7} (for m/z = 509). Analyses of other chromatographic peaks for each m/z produced similar compositional predictions.
Table 5.4 Elemental composition analysis of AOP-LME adducts. Elemental composition of m/z = 475, 491, 493, 509 was predicted based on exact mass measurements using a TOF analyzer. MassLynx software (4.1) was used with the following parameters: Tolerance = 10.0, parts per million (PPM), Double bond equivalent (DBE): min = -1.5, max = 50, Number of isotope peaks used for i-FIT = 3, Elements: C:10-40, H:0-100, N:0-5, O:0-10, 23Na:0-1. The most probable empirical formulae are highlighted. (i-FIT™: lower value increases the probability that the isotopic pattern of the elemental composition matches a cluster of peaks in the spectrum).

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<th>Mass</th>
<th>Calc. Mass</th>
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<th>PPM</th>
<th>DBE</th>
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5.2.12 AOP-LME adducts include isolevuglandin-modified LME

We next carried out MSMS studies on AOP-LME adducts with a SYNAPT™ HDMS™ system operated in TOF. Figure 5.13A depicts the MSMS plot for m/z = 509 and 491. The MSMS plots represent the pooled data from all chromatographic peaks from each mass. The ion at m/z = 491 seems to be the dehydrated form of 509 as its fragmentation pattern was identical to that of 509. The fragmentation products included the pseudoparent ions (with loss of one or more molecules of water) and additional fragmentation products. An identical fragmentation pattern has been reported before by Brame et al. for hydroxylactam adducts of isolevuglandin-modified proteins [442]. Inserts (c) to (f) of Figure 5.16A, schematically represent the structure of these daughter ions. Exact m/z of 84.1 is consistent with it being generated from fragmentation followed by cyclization of the lysyl portion of molecule as has been observed before [442]. Similarly, m/z of 475 likely represents the dehydrated form of m/z = 493 (Figure 5.14). The fragmentation pattern of these adducts was identical to lactam-containing adducts formed by modification of lysine by isolevuglandins (Figure 5.14B & [442] ). HPLC-MSMS studies of AOP-LME showed that the daughter ions existed in all peaks of a single species (m/z = 493 for example). Figure 5.15 represents the HPLC-MSMS study of m/z = 493.3 after AOP-LME (equivalent to 10 μg of AA) was resolved by a ACQUITY UPLC system and the eluent was studied by SYNAPT™ as described in methods. Daughter ions 84.1, 233, and 144 are seen in all peaks reflecting that these fragments are present regardless of which isolevuglandin the adduct is derived from. Similar results were obtained with m/z = 509.3. We therefore conclude that the adducts are derived from different isolevuglandins (i.e. isoLG, iso[4]LG, iso[7]LG, and iso[10]LG).

5.2.13 Fluorescent peaks of AOP-LME correspond to isolevuglandin-derived adducts.

To see if these adduct could have contributed to fluorescent signature observed in AOP-LME, AOP and AOP-LME were resolved with a Waters Alliance LC (on a 2.1x150 Xterra MS C18 5 μm column) coupled with 2475 multi wavelength fluorescence detector system. Fluorescent peaks of the eluent (excitation/emission wavelengths: 350/430) were found to exist between 10 to 25 minutes which clearly corresponds to the region were adducts are eluted. As an example, Figure 5.18 shows that many
peaks of the m/z = 491 correspond to fluorescent peaks of the eluent. Given the number of peaks for each mass and also the fact that there is a great overlap between the elution times of these masses, it is not possible to conclude that one mass contributes to the fluorescent peaks more than other masses. In fact, it is more likely that all adducts - containing a lactam or hydroxylactam as a core structure - may have more or less the same fluorescent properties. Therefore the fluorescent peaks in the eluent reflect the sum of contribution of different masses.

5.3 Summary/discussion

Protein modification by lipid peroxidation products is a common event in many age-related and pathological conditions. Lipid peroxidation products are potentially genotoxic through modification of nucleic acids [573]. Additionally, it has been shown that fragmented aldehydic products can drastically change gene expression pattern especially genes regulating antioxidant, heat shock, ER stress, and nutrient deprivation responses [574].

The experiments described in this chapter yielded significant new observations regarding a particular form of amino group modification by unfragmented autoxidation products of linoleic or arachidonic acid. First, our NMR and MS studies indicate that this metal-free method for autooxidizing polyunsaturated fatty acids causes minimal fragmentation of the carbon backbone despite the fact that nearly all of the fatty acid molecules are oxidized. Second, these oxidized fatty acids are capable of forming covalent adducts with free amino groups on proteins or phosphatidylethanolamine. Third, fluorescence and MSMS studies strongly suggest the formation of a pyrrole-type adduct of the oxidation products with amino groups. Fourth, these adducts mimic the ability of copper-oxidized LDL to inhibit macrophage apoptosis whereas adducts with various other reactive oxidation products such as 4-HNE and 4-HHE had no biologic activity with regard to macrophage growth and survival.

The biologic relevance of modified aminophospholipids is supported by reports showing that oxidized phospholipids (not covalently associated with apoB) are recognized by the scavenger receptor CD36 [478, 575] and are also biologically active [576]. Our results with OP-modified PE liposome
confirms that prosurvival activity requires a relatively small structure, namely a small molecular domain containing an amino group coupled to an oxidized fatty acid by a pyrrole-type adduct.

Our studies do not define if there is a unique structural adduct that promotes growth upon modifying apoB. Autoxidized fatty acid preparations are expected to contain a large number of reactive products including lipid peroxides, epoxides, 2-alkenals, 4-hydroxy alkenals, and (in the case of arachidonic acid) other biologically active compounds such as isoprostanes and isolevuglandins [442, 448, 493, 577, 578]. Fragmented aldehydes such as HNE and HHE are widely recognized for their ability to modify proteins and DNA. However in our hands, HNE or HHE-modified LDL could not promote survival in macrophages. Furthermore, Fruebis et al. showed that these hydroxy-aldehydes cannot account for the fluorescence observed with adducts of lysine and linoleic acid oxidation products [501]. Brame et al. have shown that autoxidation of arachidonic acid generates oxidation products that are several order of magnitude more reactive than HNE [442]. This is especially significant since HNE is stated by some experts to be one of the most reactive fatty acid peroxidation products [567].

Similarly, MS analysis showed no evidence that aldehyde fragments were being generated. Additional evidence that the reactive products in our OP have not undergone fragmentation of the carbon backbone is derived from NMR studies carried out by our collaborator Dr. Guenter Eigendorf (Department of Chemistry, University of British Columbia) which indicate no detectable formation of aldehydes despite oxidation of more than 90% of the starting polyunsaturated fatty acid. Hence, it seems reasonable to conclude that hydroperoxides, endoperoxides, and/or unfragmented aldehydic products seem to be the main peroxidation products responsible for conferring prosurvival effect to modified LDL.

While studying AOP-LME with HPLC-MS, we were able to find newly generated masses with mass to charge ratios and fragmentation patterns that very well fitted with isolevuglandin derived lactam and hydroxylactam-containing adducts. A pyrrole ring-containing adduct of lysine methyl ester derived from any of the isolevuglandins will have an m/z of 477 in positive ion mode (which was not found in our system) but further oxygenation of pyrrole ring produces lactam (+16, m/z = 493) and hydroxylactam (+32, m/z = 509) containing adducts which were found in our reaction mixture. This fits
very well with previous reports showing that, unless air is strictly excluded, all of lysyl-pyrrole adducts of levuglandins and isolevuglandins will be oxidized to lactam and hydroxylactam [442]. We also found two masses corresponding to dehydrated lactam and hydroxylactam adducts (m/z = 475 and 491, respectively). The MSMS studies and fragmentation patterns did corroborate the assumption that these are dehydrated forms. Similarly, Salomon et al. had reported that lysyl-lactam and lysyl-hydroxylactam adducts do exist as sodated [M+Na], protonated [M+H], and dehydrated [M-H2O+H] species [579]. The relative intensities of our major ions were similar to studies by Brame et al. A difference we observed was the existence of m/z = 144 daughter ion. Since this fragment existed in all peaks of both lactam and hydroxylactam adducts, it is most likely due to a core fragment as opposed to fatty acid side chains where different fragments are expected based on structural isomerism. This difference might be due to use of lysine methyl ester in our system (while Brame et al. used lysine). In the absence of the esterified methyl, fragmented lysine will easily cyclize to generate the m/z = 84.1 fragment. To positively determine the origins of the 144 fragment, parallel MSMS experiments with lysine, LME, and lysine ethyl ester (LEE) should be carried out. If still present in all three conditions, the fragment (m/z = 144) is more likely to have originated from an aliphatic side chain. Otherwise, it is expected to observe m/z of 144 and 168 for LME and LEE, respectively.

Although we are not aware of published fluorescence spectra for isolevuglandin-modified LDL, our results suggest that lysyl-isolevuglandin adducts or related structures may be the source of fluorescent peaks (excitation/emission:350/430 nm) observed in our chromatographic studies. On the other hand, since linoleic acid autoxidation cannot generate isolevuglandins, it is clear that the isoLG-lysine structure is not essential for generation of fluorescence or (more importantly) for induction of growth in macrophages. Salomon et al. have shown that oxidized LDL contains isoLG-lysyl and LG-lysyl adducts [579]. It is also known that LG-modified LDL behaves similar to oxLDL in that it is recognized and taken up avidly but degraded poorly by macrophages [580]. It would be interesting to determine if LDL modified by iso[4]LG (E) [581] has prosurvival activity in macrophages.
It is also known that oxLDL competes for the same receptor(s) with isoLG (or LG)-modified LDL while Ac-LDL is not a competitive inhibitor [580]. This suggests that isoLG-LDL is recognized by a receptor other than SR-A. It would be interesting to study the contribution of other scavenger receptors such as CD36, LOX-1, or RAGE to isoLG-LDL uptake. One caveat to consider in interpreting such experiments however, is the possibility that isoLG is cooperating with other reactive OPs for modification of lysine and induction of macrophage growth. It has been reported that, even when in excess molar ratio, only 16 molecules of LG bind to a molecule of BSA [445]. Even though it appears that in oxLDL the isoLG-lysyl epitopes are four times more abundant than LG-lysyl epitopes [579], if similar limitations exist for apoB modification with isoLG, then it would difficult to measure/interpret partial contributions of isoLG.

The finding that the prosurvival effect of OPs was almost completely abolished by pretreatment with NaBH₄ suggests that this biologic activity involves formation of adducts with ketones, hydroperoxides, or aldehydes. The partial inhibition of formation of bioactive adducts by triphenylphosphine supports the conclusion that at least part of the prosurvival effect was mediated by adducts with hydroperoxides (and/or endoperoxides).

The mass spectrum for LOP-LME showed only very low-intensity new ions in the region between m/z 250 and 343 where one might expect to find adducts of lysine methyl ester with fragmented aldehydes derived from linoleic acid hydroperoxide. However, there were several prominent new ions in the range m/z 434-505 that could represent lysine adducts with linoleate-derived oxidation products having an intact 18-carbon backbone. It has been proposed that linoleic acid-derived hydroperoxides [501] and keto oleic acid [570] can modify lysine residues to produce 5 membered pyrrole, lactam, or hydroxylactam (and possibly 6 membered pyridine) heterocycles with characteristic fluorescent spectra very close to what we found. The fragmentation pattern of m/z = 455 is consistent with products suggested by both mechanisms. We postulated that the existence of 142/144 doublet, and 312 daughter ions is consistent with fragmentation of hydrocarbon chain adjacent to the heterocycle (Figure 5.9B). However, a fragmentation of LME, just below N of the heterocycle is also consistent with these daughter ions. This type of fragmentation is similar to what is believed to happen in isoLG-LME adducts. To
positively trace the origin of these daughter ions, it is possible to compare the MSMS profile of adducts of LME with lysine and [U-13C] lysine. If m/z = 144 still present in MSMS analysis of LOP-lysine and LOP-[U-13C] lysine, it would be an indication that m/z = 144 originated from the fatty acid and contains the carboxy terminus.

Although it is quite possible for autoxidation products of arachidonic acid to proceed with the same chemical reactions proposed above (and by Fruebis et al. and Fukuzawa et al.) for linoleic acid, the only major masses identified in our HPLC-MS studies of AOP-LME were shown to be isoLG-LME adducts. Considering the high reactivity of isoLGs, it is not surprising that, when present, they would be modifying amine groups long before any other reactive components. It is still likely however that other adducts are present in the mix but have not been discovered due to lower intensities. On the other hand, it is also known that higher number of double bonds (trienes systems and beyond) increases the likelihood of producing bicyclic endoperoxides that are prostaglandin-like precursors [582] and can generate γ-ketoaldehydes (such as isoLGs) by a spontaneous cleavage of the endoperoxide moiety. Pryor et al. have proposed a mechanism by which hydroperoxides of a diene system (e.g. linoleic acid) may produce such bicyclic endoperoxides [582]. Zhang et al. ([583]) have also shown the production of hydroperoxide and dihydroperoxide unconjugated dienes which are capable of using mechanisms proposed by Pryor et al. to generate such bicyclic endoperoxides. This suggests that LOP may contain γ-ketoaldehydes with similar reactivity and behavior to isoLGs. However to our knowledge, there are no reports of unfragmented γ-ketoaldehydes being produced from linoleic acid autoxidation. Additionally, adducts produced from γ-linolenic acid (18:3) but not linoleic acid are recognized by anti-iso[4]LG-lysine antibody [579] which suggests that prostaglandin-like precursors do not exist in LOP. The structures we proposed here for LOP-LME adducts may contain pyrrole, lactam, or hydroxylactam core, a feature that is shared by isoLG-lysine. However, the hydrocarbon side chains in the LOP-LME adducts are on positions 2 and 5 of the heterocycle while in the isoLG-adducts, they are on positions 3 and 4. Salomon et al. have shown that the length of residues on positions 3 and 4 is the major determinant for recognition by anti-LG-lysine and anti-iso[4]LG-lysine antibodies while differences on positions 2 and 5 are not recognized [579].
In BMDMs, oxLDL inhibits apoptosis by activation of PI3K/PKB axis [310], blocking ceramide generation [314], and inducing calcium oscillations and activation of calcium-dependent kinases such as eIF2K [315]. Similarly, in peritoneal macrophages, oxLDL induces growth by activation of PI3K/PKB axis [308]. It would be interesting if oxLDL, AOP-LDL, LOP-LDL use the same intracellular signaling pathways for inducing macrophage survival. Our results in chapter 4 suggest that the survival signaling by oxLDL may not require internalization of the modified LDL, and it would be of interest to identify what proteins oxLDL or OP-LDL interact with on the cell surface.

Since linoleic and arachidonic acids are present in significant amounts in vivo, our findings have potential importance not only for atherosclerosis but also for any other condition with increased oxidative stress. For example, advanced lipid peroxidation modified proteins have been reported to correlate significantly with periodontal disease (inflammatory disease of the gingiva and adjacent soft tissue) [584] and lipid-induced glomerular disease [585]. Numerous articles in the literature implicate fragmented aldehydes as the key injurious lipid peroxidation products in atherosclerosis [316, 586], Alzheimer’s disease [587], as well as liver damage induced by ethanol [588], iron overload [589], prooxidants [590]. In addition, 4-HNE is reported to increase AP-1 binding to DNA and thereby to induce expression of the pro-fibrotic cytokine TGF-1beta [591]. In contrast, our results with adducts of unfragmented oxidation products show that they can activate prosurvival pathways in macrophages. Reagents that can detect these unfragmented adducts in vivo might lead to novel screening tools for diseases related to lipid peroxidation. Furthermore, strategies to modulate their generation might be of therapeutic value.
Figure 5.1 Lipid and protein fractions of oxLDL promote macrophage growth. Lipid and protein of native or oxLDL were extracted by the Bligh-Dyer method. The fractions were subsequently reconstituted as described in the methods. MPMs from CD-1 mice were isolated and seeded in 96 well plates at $10^4$ cells/well density and incubated for 24 hours with indicated concentrations of oxLDL or lipoprotein fraction. Viability was then assessed with the XTT method. The graph represents the means ± SD of pooled data from three independent experiments, each done in quadruplicate. The difference between nLDL and all other conditions (at corresponding concentration) was statistically significant ($P < 0.05$).
**Figure 5.2 OPs react with lysine residues.** Arachidonic acid was thermally autoxidized (AOP) as described in the methods. A 300 µL aliquot was preincubated for 2 hr with 400 µL PBS containing 1 mg polylsine and then used to modify LDL (AOP/Lys-LDL). The control (AOP-LDL) was not treated with polylsine. Mouse peritoneal macrophages (MPMs) from CD-1 mice were isolated and plated in 96 well plates at $10^4$ cells/well density and incubated with indicated concentrations of AOP-LDL or AOP/Lys-LDL for 24 hours. Viability was then assessed by the XTT method.
Figure 5.3  Amine modification by OPs is sufficient for growth induction. A) LDL and phosphatidylethanolamine (PE) liposomes were modified by thermally oxidized arachidonic acid and linoleic acid (AOP-LDL, AOP-PE, and LOP-PE, respectively). MPMs were isolated and seeded in 96-well plates at $10^4$ cells/well density and incubated with indicated concentrations of LDL or the above-mentioned modifications and viability was assessed after 24 h by the XTT method. B) Phosphatidylcholine (PC) or phosphatidylethanolamine (PE) containing liposomes were modified by AOP or AOP that had been pretreated with 75mM sodium borohydride for 2 h ((AOP+ BH$_4$)-PE). Viability was assessed as in A). Similar results were observed in two other experiments.
Figure 5.4 Borohydride-reducible functional groups in OPs are essential for the growth-promoting effect. Sodium borohydride (100mM) was used to reduce arachidonic acid oxidation products (AOP) before or after LDL modification ((AOP+BH₄) LDL and (AOP-LDL)+BH₄, respectively). Viability of MPMs was assessed by XTT method after 24 h incubation with indicated concentrations of these modifications. The graph represents the means ± SD of pooled data from three independent experiments, each done in triplicate. P < 0.05 between (AOP+BH₄)-LDL and AOP-LDL or (AOP-LDL)+BH₄.
Figure 5.5 Alkenals do not account for the growth-inducing activity of OPs. LDL was modified with 4-hydroxyhexenal or 4-hydroxynonenal (HHE-LDL and HNE-LDL, respectively) as described in methods. MPMs were isolated and plated in 96 well plates at 10^4 cells/well density and incubated with indicated concentrations of modified lipoproteins for 24 hours. Viability was then assessed by XTT method. Similar results were observed in 2 independent experiments.
Figure 5.6 ESI-MS analysis of neat and thermally oxidized linoleic and arachidonic acids. 10μg arachidonic (AA) or linoleic acid (LA) or their thermally oxidized (72 h) products (AOP and LOP, respectively) were dissolved in methanol and injected into the electrospray ionization source of an Esquire Ion Trap instrument. In positive ion mode, AA ($M_{AA} = 304$) existed as protonated $[M_{AA}+H]^+$ or sodated $[M_{AA}+Na]^+$ ions. In this set of experiments, major masses found in AOP, in descending order of abundance, are as follows: 375 [$M_{AA}+Na+3O]^+$, 359 [$M_{AA}+Na+2O]^+$, 391 [$M_{AA}+Na+4O]^+$, and 343 [$M_{AA}+Na+1O]^+$. LA ($M_{LA} = 280$) mainly existed as sodated forms (303: $[M_{LA}+Na]^+$ or 325: $[M_{LA}+2Na-H]^+$). The most abundant masses in LOP correspond to addition of up to three oxygens in sodated state (335: $[M_{LA}+Na+2O]^+$, 319: $[M_{LA}+Na+1O]^+$, and 351: $[M_{LA}+Na+3O]^+$).
**Figure 5.7 OP modification of lysine and apoB generate fluorescent adducts.** Fluorescent intensities of 25 μg/mL of nLDL and LDL that had been modified by copper sulfate, AOP, LOP, or acetylation (oxLDL, AOP-LDL, LOP-LDL, and Ac-LDL, respectively) (A) or increasing concentrations of OP-LME and OP (B) was measured with excitation/emission wavelengths set at 350/430. The results in A) and B) represent pooled data from at least 3 preparations and expressed as fold increase relative to nLDL. C) Fluorescent spectra of LOP and LOP-LME.
**Figure 5.8 ESI-MS analysis of adducts formed by modification of lysine methyl ester with oxidized linoleic acid.** A) Mass spectrum (100-600 range) of LOP-LME acquired on an Esquire Ion Trap instrument. m/z of 161 = LME and 300 < m/z < 370 corresponds to OPs. New masses (420 < m/z < 510) were found exclusively in LOP-LME reaction mixture. B) Predicted masses of given assignments assuming no fragmentation of acyl chain. Masses highlighted in red represent the most abundant masses found in our analyses.
A) LOP-LME MS/MS 455 (ES+)

B) Molecular Formula = C_{25}H_{46}N_{2}O_{5}  
Molecular weight = 454.3

a) 

b) m/z=469.3  \rightarrow  m/z=455.3
Figure 5.9 MSMS analysis and putative structures of m/z = 455 in LOP-LME. A) LOP-LME was analyzed by ESI-MSMS in ES+ scan mode which reveals two pseudoparent ions ([M-H2O]+ and [M-2H2O]+) plus major daughter ions. B) Shows the empirical formula and molecular weight of m/z = 455.

Scheme a) depicts several putative structures based on the concerted reaction suggested by [501] while scheme b) represents a pathway proposed by Fukuzawa et al. ([570]) where a hydroxylactam (m/z = 469) can be produced by modification of lysine with keto-oleic acid. A derivative of such compound with a m/z = 455 with daughter ions is also depicted. C) Explains how a daughter ion with an unsaturated c-c bond can account for the 142 fragment. The upper section of scheme d) exhibits a charge-remote fragmentation commonly seen in MSMS analysis of fatty acids and their derivatives leading to creation of unsaturated bonds with a loss of hydrogen [571]. Lower section of scheme d) exhibits predicted fragmentation products and their m/z based on this model. Scheme e) exhibits the possibility of dehydrogenation and generation of C=N double bond.
**Figure 5.10 HPLC-MS study of AOP-LME adducts.** A) 10μg (or equivalent of) arachidonic acid (AA), AA oxidation products (AOP), and AOP-LME reaction mixtures were resolved by HPLC on a BEH C18 column (1.7 μm, 50 mm x 2.1 mm) and eluted with a 20-100% acetonitrile gradient. The effluent was analyzed with a ZQ single quadrupole mass spectrometer. Total ion current in positive ionization scan mode is illustrated in (A). B) The mass spectrum (200-700 range) of each sample for the 2.00 to 2.50 minutes (red marker) of chromatogram is shown. New masses with m/z of 475, 491, 493, and 509 were found only in AOP-LME reaction mixture.
Figure 5.11 m/z of 475, 491, 493, and 509 are unique to AOP-LME reaction mixture. AOP and AOP-LME (equivalent to 10μg AA) were resolved on a BEH C18 column (1.7 μm, 50 mm x 2.1 mm). An acetonitrile gradient (10- 90%) was used with a total run time of 35min, including the 100% acetonitrile flush and re-equilibration. The eluents were directed to a ZQ single quadrupole mass spectrometer. Single ion recordings in ES+ mode for m/z of 475, 491, 493, and 509 were compared between AOP and AOP-LME.
Figure 5.12 AOP-LME adducts are singly charged and protonated species. AOP-LME (equivalent of 10μg of AA) was resolved on a BEH column and directed to a ZQ single quadrupole mass spectrometer. Panels represent the single ion recordings of A) 507 (ES-) and 509 (ES+), B) 491 (ES-) and 493 (ES+), and C) 489 (ES-) and 491 (ES+). High resolution isotopic distribution for m/z of 509, 493, and 491 are shown in a), b), and c), respectively.
A) TOF MSMS 491.30ES+
AOP-LME 226

B) m/z = 509.3
Formula Weight = 508.3
Molecular Formula = C_{27}H_{44}N_{2}O_{7}

(a) m/z = 509.3
(b) -H_2O m/z = 491.3

(c) -2H_2O m/z = 473.3
(d) m/z = 84.1

(e) m/z = 413

(f) m/z = 144
m/z = 330
**Figure 5.13 m/z = 509 is an isolevuglandin-derived hydroxylactam-containing adduct.** A) MSMS analysis of AOP-LME (equivalent of 10μg of AA) acquired on a SYNAPT system operating in TOF mode for m/z = 509 and 491. Plots represent the pooled data from all the chromatographic peaks of each mass. Intensities are normalized to the highest peak (226 and 1.42e3 for m/z = 491 and 509, respectively). B) Molecular weight, empirical formula and structures of parent ions (491 and 509) and their fragmentation products. Iso[4]LG derived adduct is used as an example.
Figure 5.14 m/z = 493 is an isolevuglandin-derived lactam-containing adduct. A) MSMS analysis of AOP-LME (equivalent of 10μg of AA) acquired on a SYNAPT system operating in TOF mode for m/z = 493 and 475. Plots represent the pooled data from all the chromatographic peaks of each mass. Intensities are normalized to the highest peak (884 and 119 for m/z = 493 and 475, respectively). B) Molecular weight, empirical formula and structures of parent ions (495 and 493) and their fragmentation products. Iso[4]LG derived adduct is used as an example.
**Figure 5.15 HPLC-MSMS analysis of m/z = 493.** AOP-LME (equivalent of 10μg of AA) was resolved on a BEH column with acetonitrile gradient (10-90%) on an ACQUITY UPLC system coupled to SYNAPT™ High Definition Mass Spectrometry™ (HDMS™) System operating in TOF mode. Panels represent the total ion current for MSMS study of m/z = 493.3 (lowest panel) and chromatograms for m/z of 84.1, 332, and 144 daughter ions. In the lowest panel, the numbers below retention times (in red) represent the most abundant fragment within that peak.
Figure 5.16 Fluorescent peaks of AOP-LME correspond to m/z of 491. AOP or AOP-LME (equivalent of 10µg of AA) was resolved by a Waters Alliance LC with an Xterra 5µm C18 column coupled with a PDA and 2475 multi wavelength Fluorescence Detector System. Acetonitrile gradient (10-100%) was used as described in methods. The fluorescence of the eluent was measured with excitation/emission set at 350/430 nm (upper panel). In parallel, similar chromatographic set up was coupled to a PDA and the ZQ single quadrupole mass spectrometer. Single ion recordings for adducts was carried out with the lower panel representing that of m/z = 491 in ES+. PDA readings at 205 nm (for AA) and 234 nm (for conjugated dienes) were used to align the chromatograms. In both panels, the numbers associated with the peaks represent retention times.
6. Summary

This thesis focuses on oxidized LDL and its effects on macrophages. The first project dealt with the mechanisms by which oxLDL induces production and secretion of VEGF. This project focused on oxLDL-mediated VEGF secretion at three different levels: 1) the ligand: what moiety of oxLDL triggers this event? 2) the receptor: are scavenger receptors involved? and 3) the intracellular events: what signaling pathways are required?

Our results with nLDL, acetylated LDL, and LDL that had been modified by oxidation with copper or ferrous ions or by exposure to autoxidation products of arachidonic acid showed that oxidative modification of either its lipid or protein component can induce VEGF expression. Higher degrees of oxidation of LDL conferred higher potency to induce VEGF. Studies from knockout animals revealed that LOX-1, SR-A, and CD36, three of major scavenger receptors, are not required for this effect. Additionally, we found that uptake of oxLDL may not be essential for VEGF induction as scavenger receptor-deficient macrophages still secreted VEGF efficiently in spite of an 80% decrease in oxLDL uptake.

Signaling studies revealed that mitogen-activated protein kinase (MAPK) pathway is not important for oxLDL-mediated VEGF secretion in macrophages even though it is important for baseline levels of VEGF secretion. PI3K pathway, on the other hand, was shown to be essential for VEGF induction by oxLDL. We also show that an atypical protein kinase C isoform (PKCζ) is required for VEGF secretion. Although we did not directly demonstrate this, we believe that PKCζ activation is downstream of PI3K, possibly through phosphoinositide-dependent kinase-1 (PDK-1) activation. We also, for the first time, show that oxLDL increases the activity of PKCζ in macrophages.

The importance of finding a pathway essential for oxLDL-mediated VEGF secretion lies in the Janus-like effects of this growth factor in atherogenesis. VEGF on one hand is potentially atherogenic because it can increase vascular permeability, recruit inflammatory cells, and promote angiogenesis within the plaque, which could drive plaque growth. On the other hand, VEGF has therapeutic potential by promoting the establishment of collateral circulation to compensate for an occluded segment in an artery. Interestingly, not only does oxLDL increase the intra-plaque VEGF, but it also antagonizes some
of potential effects of this growth factor, tipping the balance towards an atherogenic profile. Therefore, targeting molecular mechanisms that link oxLDL to VEGF expression in lesions (as opposed to a non-selective suppression of VEGF) can restore the more favorable balance.

Our laboratory has been studying the prosurvival and mitogenic properties of oxLDL towards macrophages, as this may be a mechanism by which macrophage populations in the intima are expanded. We have shown that the PI3K-PKB pathway as well as EF2K activation are required, but to date we have not defined upstream signaling events or if a cell surface receptor is involved. I took advantage of the availability of transgenic mice lacking one or more scavenger receptors to see which ones, if any, are required for the anti-apoptotic effects of oxLDL in bone marrow derived macrophages. My results indicated that none of these receptors (SR-A and CD36, LOX-1, TLR4, CD14, or FcγRIIb) are essential for activating the oxLDL prosurvival pathway. Furthermore, the anti-apoptotic effect was not affected by the rate of oxLDL uptake. Three major receptors that remain to be explored in this regard are SR-PSOX, SR-BI, and RAGE.

LDL modified by oxidation products of unsaturated fatty acids was developed in our laboratory to facilitate the study of oxidative protein modification of LDL. The final project I undertook was centered on characterization of this type of modification especially with respect to its prosurvival potential.

These studies showed that both protein and lipid moieties of oxLDL are capable of promoting survival and growth in macrophages. It appears that oxidation products of unsaturated fatty acids can derivatize amine groups (of whether the lysine residues in apoB or phosphatidylethanolamine) resulting in promotion of growth and survival. Studies with borohydride and triphenylphosphine show that the reactive functions required for these modifications include aldehydes, ketons, hydroperoxides, or endoperoxides. LDL modified with alkenals (eg. 4-HNE) derived from fragmentation of fatty acid hydroperoxides showed no prosurvival activity.

MS analysis showed that the OPs we generated to modify LDL were largely unfragmented even though more than 80% of the fatty acid molecules were peroxidized. To study the structure of the adducts formed when these OP react with amines, we mixed oxidation products with lysine methyl ester
Mass spectrometry showed that LOP-LME and AOP-LME both contain masses that correspond to formation of a covalent adduct with an unfragmented oxygenated fatty acid. In the case of AOP-LME, some fragments generated by MSMS were consistent with isolevuglandin adducts.

Another aspect of this project studied the fluorescent properties of these adducts. Similar to oxLDL (and modification of any protein by lipid peroxidation product) a specific fluorescent pattern with maximum emission and excitation of 350/430 nm was observed with LDL and LME modification by AOP and LOP. It also appears that isolevuglandin-lysyl adducts could have contributed to this fluorescent pattern within AOP-LME mix.

This project has many potential applications in the field of atherosclerosis and beyond. Firstly, it characterizes a metal-free method for oxidative modification of LDL. Secondly, since arachidonic acid and linoleic acid are the two most abundant unsaturated fatty acids in vivo, any condition with increased oxidative stress could lead to the generation of similar adducts. These adducts have therefore the potential to become markers of oxidative damage. Unlike many current markers of oxidative damage, which are relatively unstable and have a short half-life, these adducts are quite stable. Analogous to diabetes and glycated hemoglobin (HbA1C), a long-term (months) marker of glucose control in diabetic patients, these adducts may be used to give information on the cumulative effect of oxidative damage over a longer period of time. This by itself can have many other applications such as defining a biological age (as opposed to a chronological age) or monitoring cases with chronic oxidative injury, such as smoking, and create more incentive for behavior modification. Thirdly, our results highlight the fact that such adducts may not merely be markers of oxidative damage, but they may in fact contribute to disease pathogenesis by virtue of their biological activities. Therefore developing strategies that isolate or neutralize these adducts or block their recognition by PRRs that mediate detrimental effects have therapeutic applications.
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