THE EFFECT OF OXIDIZED LDL ON MACROPHAGE CELL GROWTH:

IMPLICATIONS FOR THE PROGRESSION OF EARLY
STAGE ATHEROSCLEROSIS

by

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ABSTRACT

Early atherosclerotic lesions are characterized by the presence of cholesterol rich, macrophage-derived foam cells. It has recently been shown that macrophage proliferation occurs during the progression of early lesions and that oxidized low density lipoproteins (oxidized LDL) stimulates macrophage growth. Possible mechanisms by which oxidized LDL causes macrophage growth include potentiation of mitogenic signal transduction by a component of oxidized LDL following internalization, by interacting with integral plasma membrane proteins coupled to signalling pathways, or by direct or indirect activation of growth factor receptors on the cell surface.

The present study was undertaken to further elucidate the factor(s) and cell surface receptor(s) that are involved in the growth-stimulating effect of oxidized LDL in macrophages, and to characterize some of the early intracellular signaling events by which oxidized LDL mediates macrophage cell growth. In this study, the growth stimulating effect of LDL was specific, in that neither native LDL nor acetyl LDL produced such an effect. Growth stimulation by oxidized LDL was shown to dependent on degree of LDL oxidation, requiring at least 15 hours of copper incubation to achieve significant levels of growth stimulation. Incubation of oxidized LDL with fatty acid-free BSA resulted in a 97% decrease in lysophosphatidylcholine (lysoPC) content, but only a 20% decrease in mitogenic activity. Similarly, treatment of native and acetyl LDL with phospholipase A_2 (PLA_2) led to a 90% increase in lysoPC formation, but only enhanced the ability of acetyl LDL to stimulate growth by 30% compared to that of oxidized LDL. When LDL was pretreated with PMSF to inhibit platelet activating factor-acetylhydrolase (PAF-AH) prior to oxidation, the hydrolysis of oxidized phospholipids to lysoPC was reduced by 70-80%, resulting in a 30% increase in growth stimulation over untreated oxidized LDL. Furthermore, oxidized LDL induced macrophage growth was reduced by approximately 60-70% with the PAF receptor antagonist L659,989, while growth of scavenger receptor type A class I/II (SR-AI/II) deficient cells was reduced by only 30% compared to control macrophages. In

THP-1 macrophages, oxidized LDL was also found to increase protein-tyrosine phosphorylation and cause a 2-fold increase in phosphatidylinositol 3-kinase (PI3-kinase) activity compared to similar concentrations of native LDL. Finally, oxidized LDL induced macrophage growth was shown, in part, to require the activation of PI 3-kinase, as growth stimulating activity was reduced by 40-50% if cells were pretreated with PI 3-kinase inhibitors (100 nM wortmannin or 20 uM LY294002). The main findings of this study suggest that oxidized phospholipids, not lysoPC, account for the growth effect of oxidized LDL in macrophages, that PAF receptor activation may be required for the observed growth stimulating effect, and that at least part of the growth stimulating effect of oxidized LDL in macrophages is a result of PI 3-kinase activation.

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G protein	Guanine nucleotide protein
kDa	Kilodaltons
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
lysoPC	Lysophosphatidylcholine
LY294002	2-(4-morpholinyl)-8-phenylchromone
MAPK	Mitogen-activated protein kinase
PA	Phosphatidic acid
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PI	Phosphatidylinositol
PI 3-kinase	Phosphatidylinositol 3-kinase
PI 3-P	Phosphatidylinositol 3-phosphate
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol-12-myristate-13- acetate
PMS	Phenazine methosulfate

PMSF	Phenylmethylsulfonyl fluoride
PTK	Protein tyrosine kinase
RPMI	Roswell Park Memorial Institute
SE	Standard error of the mean
SR-AI/II	Scavenger receptor type-A class I/II
TLC	Thin layer chromatography
XTT	2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide

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DEDICATION

This work is dedicated to my father, Hendrik Martens, and father-in-law, Wilfred Molnar, in appreciation of the many insights into life that they have shared with me, and in the hope that they will continue to enjoy thier lives to the fullest for many years to come.

Chapter 1. Introduction and Background

1.1 The Pathogenesis of Atherosclerosis

Atherosclerosis is a chronic progressive disorder that affects medium-sized and large arteries, ultimately producing clinical manifestations through impairment of blood flow in target organs. In coronary atherosclerosis, impaired blood flow to the heart leads to the most serious manifestations of the disease, resulting in angina or myocardial infarction. Atherosclerotic lesions are characterized by the deposition of lipid (principally cholesterol), fibrous matrix components (collagen), and by smooth muscle cell proliferation in the arterial intima. Numerous risk factors that increase the likelihood of atherosclerosis have been identified, including abnormal plasma lipoprotein levels, high blood pressure, smoking, diabetes, obesity, family history of premature coronary disease, diet, psychosocial stress, and a sedentary lifestyle. Some of these risk factors can be modified resulting in a reduction in the risk of coronary disease. However, the mechanisms by which risk factors operate are not yet fully understood. The pathogenesis of atherosclerosis is extremely complex, involving an array of circulating blood proteins, lipoproteins, growth factors, cytokines and vasoregulatory molecules, and their interactions with cellular components of the arterial wall (1).

Perhaps the most important and best studied of the major risk factors for atherosclerosis is hypercholesterolemia. Low density lipoprotein (LDL) is the major carrier of cholesterol in blood, and is the source of most of the cholesterol found in atherosclerotic lesions (2-7). In early lesions, most cholesterol is found in the form of cytoplasmic lipid droplets within macrophages, and it is believed that cholesterol accumulation within these cells is an important initial step in the formation of more advanced lesions (8-14). The mechanism by which macrophages in the arterial intima accumulate massive amounts of cholesterol is not obvious, as the activity of the LDL receptor is regulated by intracellular cholesterol content, and incubation of cultured macrophages with high concentrations of normal plasma LDL does not cause lipid

accumulation (15-16). However, subsequent studies showed that certain chemical modifications of LDL lysine residues, such as acetylation, converted the LDL molecule to a form that was recognized by a specific receptor on macrophages termed the acetyl LDL or scavenger receptor (SR-AI/II). This modification of LDL was significant because internalization of this receptor and its bound "modified" LDL particle was not regulated by cellular cholesterol. This unregulated uptake of modified LDL allows massive cholesterol deposits to accumulate in macrophages in vitro (15, 17-18) leading to their transformation into foam cells, the main components of early atherosclerotic "fatty streaks".

The search for a potential "physiologic" ligand for the scavenger receptor led to the discovery that some cultured cells, notably arterial endothelial cells and smooth muscle cells, were capable of inducing oxidative modification of LDL, and that oxidatively modified LDL could bind with high affinity to this receptor which is expressed in macrophages (19-26). However, more recent studies have now shown that the uptake of oxidized LDL in macrophages is only in part attributable to the scavenger receptor. These studies have shown that as much as 70% of the uptake of oxidized LDL cannot be accounted for by the SR-AI/II alone (27). Attempts to identify additional oxidized LDL receptors has led to the identification of several other membrane proteins that bind oxidized LDL in both transfected cells and ligand blots, including the $F_c\gamma RII$-B2 receptor (28), the human monocyte antigen CD36 and its mouse homologue (29), the closely related SR-BI scavenger receptor (30), the collagenous based macrophage receptor (MARCO) (31), and a 94 - 97 kDa protein occurring on mouse peritoneal macrophages identified as macrosialin, the mouse homologue of human CD68 (32). Although several of these proteins have been shown to internalize significant amounts of oxidized LDL, because of overlapping ligand specificity with the SR-AI/II receptor, it has been difficult to evaluate their relative importance in the uptake of oxidized LDL by macrophages.

1.2 Mechanisms of Oxidation and Structural Changes Associated with LDL

The physiochemical changes that occur in the LDL particle that impart both scavenger and oxidized LDL receptor recognition are complex and not fully understood. Oxidation of LDL involves a transition metal-catalyzed, free radical-mediated lipid peroxidation process in which the polyunsaturated fatty acyl residues in LDL lipids are degraded to a variety of peroxidation products (33). These products include hydroperoxy and hydroxy fatty acids (34-36) aldehydes and hydroxyaldehydes (37-38) and more complex aldehydes (38). Aldehydic lipid peroxidation products are highly reactive, and can cause derivatization of free amino groups of apolipoprotein B (21,39). Such derivatization of lysine epsilon-amino groups of apolipoprotein B results in an overall negative charge on the LDL molecule. It is believed that the resulting negative charge of the oxidized LDL particle is responsible for its interaction with the scavenger receptor, as derivitization of these amino groups has been shown to prevent interaction with the LDL receptor, but conversely permit binding to and unregulated uptake by scavenger receptors on phagocytic cells (21,38,40-41). During the oxidation process, apolipoprotein B is also modified through radical-mediated scission of peptide bonds, and probably also by direct reaction with fatty acyl hydroperoxides (42). Oxidative modification of LDL is also accompanied by oxidation of cholesterol esters and free cholesterol (43), and by hydrolysis of oxidized phosphatidylcholine to lysophosphatidylcholine through the action of a lipoprotein-associated phospholipase A_2 known as platelet activating factor acetylhydrolase that can utilize oxidized (but not intact) LDL phosphatidylcholine as a substrate (44). Some lipid oxidation products found in oxidized LDL such as oxysterols, oxidized phospholipids and lyso-phosphatidylcholine have been shown to be important mediators or regulators of many biological effects associated with atherosclerosis, and will be more fully discussed below.

Endothelial cells, smooth muscle cells, lymphocytes, monocytes and macrophages have also been shown to be capable of enhancing the rate of in vitro oxidation of LDL (21). The role of the cells in oxidation of LDL appears to be simply to accelerate the rate of peroxidation at low

(nanomolar) free metal ion concentrations, either by providing thiols which can reduce transition metal ions to a catalytically active form (45-46), or by "seeding" LDL in the medium with lipid hydroperoxides to initiate a peroxidation reaction (47). In fact, identical changes occur when LDL is oxidized in the absence of cells by incubation with micromolar concentrations of transition metal ions such as copper and iron (21,40). Both cellular and transition metal ion oxidation of LDL typically result in full depletion of endogenous antioxidants, more than 70% depletion of its linoleic and arachidonic acid content, nearly half of the phosphatidylcholine converted to lysophosphatidylcholine, and more than 30% of lysine residues derivatized by lipid peroxide decomposition products, resulting in an electrophoretic mobility at least 2.5 times that of native LDL. Oxidation of LDL, however, does not occur in the presence of serum, suggesting that LDL does not become oxidized in the circulation. In atherosclerotic arteries, oxidation of LDL is more likely to occur within the arterial wall, out of reach of the antioxidant activity of plasma and where increased levels of copper and iron ions are generally found (48).

1.3   The Biological Effects of Oxidized LDL

Several lines of evidence have implicated oxidized LDL as a potential causal factor in atherosclerosis including the demonstration that oxidatively modified LDL exists in atherosclerotic lesions in vivo in both experimental animals and humans (49-52) and that probucol and other antioxidant drugs can retard progression of this disease in experimental animals (53-59). These observations prompted a number of investigators to explore possible mechanisms by which oxidized LDL might contribute to atherogenesis. As described below, several investigators have found oxidized LDL to exhibit a number of interesting biologic properties not present in native (unmodified) LDL. However, it should be emphasized that a given biologic action of oxidized LDL may vary depending on the nature and extent of oxidative modification of LDL. In some studies, the extent of LDL oxidation was not controlled and/or inadequately characterized, thus making it difficult to present a simple catalogue of actions that

have been attributed to oxidized LDL. On the other hand, however, most reports describing the biological effects of oxidized LDL have used LDL that has been extensively oxidized by means of transition metal ion incubation (as discussed above). Therefore, for the purpose of comparing the findings of this report to those of literature, our discussion of oxidized LDL and its atherogenic properties will be focused on LDL that has been extensively oxidized.

Extensively oxidized LDL has been shown to have many potentially atherogenic actions in vitro that may account for several of the features associated with the progression of atherosclerotic lesions. Proatherogenic properties associated with oxidized LDL include accelerated uptake in macrophages via scavenger receptors (39, 60-61), foam cell formation (61-62), cytotoxicity (63-68), increased susceptibility to form aggregates (69), resistance to lysosomal degradation (70), enhanced binding to collagen (71-72), activation of T lymphocytes (73), increased monocytic cell expression of interleukin-8 (74), increased monocytic cell release of interleukin-1β (75), inhibition of platelet-derived growth factor (PDGF) and tumor necrosis factor-α expression (76-78), inhibition of lipopolysaccharide-induced interleukin-1β expression (79), increased growth factor gene expression in endothelial cells (80-81), and the promotion of DNA synthesis, cell cycle entry and proliferation of vascular smooth muscle cells (82-85).

Oxidized LDL has also been shown to promote monocyte chemotaxis and inhibition of migration of differentiated macrophages (86-89), the induction of VCAM-1 and ICAM-1 expression in endothelial cells (80, 90-93), and the stimulation of leukocyte adherence to the microvascular endothelium in vivo (94-95). Such events would tend to increase the number of macrophages in the arterial intima at sites of lesion formation. These findings suggest that oxidized LDL may play an important role in the development of early stage atherosclerotic lesions by leading to increased monocyte/macrophage accumulation, and an enhanced rate of foam cell formation.

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Oxidized LDL and Atherosclerosis
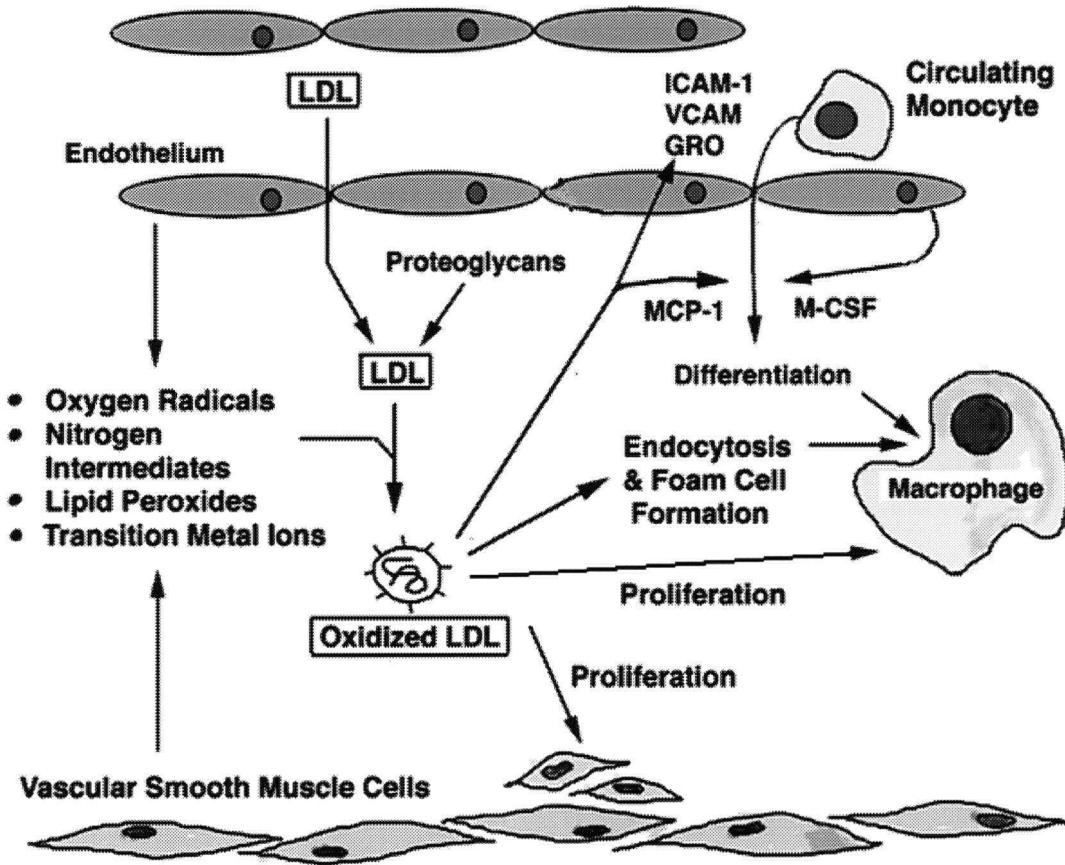
Figure 1.1   The role of oxidized LDL in the development of atherosclerosis. Once in the intimal space, LDL becomes trapped by proteoglycans and oxidized by a transition metal catalyzed, free radical mediated reaction that converts unsaturated fatty acids to reactive lipid hydroperoxides and aldehydes. These reactive lipid substances then further accelerate oxidation of the LDL particle, leading to the depletion of polyunsaturated fatty acids and antioxidant activity, and the fragmentation of the apo B protein. Oxidized LDL stimulates the expression of cell adhesion molecules (VCAM, ICAM-1 and GRO) and the secretion of MCP-1 and M-CSF from endothelial cells, causing monocytes to enter the arterial intima and differentiate into macrophages. Oxidized LDL also becomes endocytosed by scavenger receptors and oxidized LDL receptors of macrophages, resulting in the formation of foam cells. Oxidized LDL has also been shown to promote chemotaxis of vascular smooth muscle cells from the tunica media, as well as stimulate cellular proliferation of both smooth muscle cells and macrophages.

1.4   Oxidized LDL Induced Macrophage Proliferation

An additional mechanism that could also increase the number of macrophages within the intimal space is cell proliferation. Aside from its role in foam cell formation, oxidized LDL has also been shown to induce the proliferation of both mouse peritoneal macrophages (96-98) and human monocyte-derived macrophages (99). Until recently, it has been thought that smooth muscle cells are the major proliferating cell type found in atherosclerotic lesions. However, recent immunocytochemical studies of human atherosclerotic lesions have shown that macrophages are the predominant cell type expressing proliferating cell nuclear antigen, even in lesions containing cells derived mainly from smooth muscle cells (100-101). Based on these observations, it is reasonable to expect that oxidized LDL induced macrophage foam cell proliferation may play an important role in lesion formation in vivo.

To date, only one group has investigated the proliferative effects of oxidized LDL in macrophages. In these studies, the mitogenic effect of oxidized LDL was attributed to scavenger receptor mediated internalization of lysoPC (97-98), a phospholipid which is generated during oxidation of LDL through enzymatic hydrolysis of oxidized PC (as mentioned above). However, when lysoPC was stripped from oxidized LDL with HDL, macrophage growth was not abolished. Further experimentation by this group also showed that oxidized LDL induced cell growth was effectively inhibited by anti-GM-CSF antibodies, suggesting that oxidized LDL could stimulate macrophages to secrete GM-CSF, leading to growth via autocrine/paracrine mechanisms (97). In addition, it was also shown that oxidized phospholipids are able to augment macrophage cell growth (102). These findings suggest the possibility that components other than lysoPC are also involved in macrophage growth by oxidized LDL.

Recently, oxidized LDL has also been shown to be mitogenic for bovine and human vascular smooth muscle cells (85). In these cells, the mitogenic effect of oxidized LDL was attributed to an oxidized phospholipid that contained PAF-like bioactivity, and blocked with platelet-activating factor (PAF) receptor antagonists, suggesting that oxidized LDL may also

stimulate growth through PAF receptor activation. Oxidation of LDL results in the generation of a number of PC oxidation products that contain short-chain polar acyl fragments in the sn-2 position which exhibit PAF-like bioactivity and activate the PAF receptor (85, 103-104). These compounds have been shown to be high affinity substrates for PAF- acetylhydrolase, which selectively cleaves these phospholipids at the sn-2 position to form lysoPC. However, during the process of LDL oxidation, PAF-acetylhydrolase becomes irreversibly inhibited (105), resulting in the accumulation of oxidatively fragmented PC molecules on the surface of oxidized LDL. These compounds are believed to be resposible for the mitogenic effect of oxidized LDL in vascualr smooth muscle cells through activation of the PAF receptor, however, it is not clear whether internalization of the LDL particle is required for this effect. Several other studies support the theory that oxidized LDL exerts biological effects through PAF receptor stimulation. Oxidized LDL has been shown to induce immune cell activation, an effect that can be inhibited by PAF receptor antagonists (106) as well as downregulate PAF receptor expression in monocytes and macrophages through PAF receptor activation (107). These findings provide modest evidence for a link between oxidized LDL and PAF receptor signaling, and strengthen the possibility that oxidized LDL induced macrophage growth is mediated via PAF receptor mechanisms.

1.5 Oxidized LDL and PAF Receptor Mediated Signal Transduction.

Several lines of evidence have linked PAF receptor mediated signal transduction to mitogenesis. PAF receptor signaling has been shown to activate guanine nucleotide regulatory proteins (G-proteins) (108), and increase intracellular calcium and phospholipid turnover through the activation of PLC (109), PLA2 (110), and PLD (111). In addition, PAF receptor stimulation also activates protein tyrosine kinases (PTK's) (108, 112), PI 3-kinase (113), protein kinase C (PKC) (114), and mitogen activated protein kinase (MAPK) (115), all of which are commonly involved in growth factor receptor-mediated signaling. These early biochemical

events are followed by specific gene expression. For example, PAF receptor signaling has been shown to increase the expression of the growth related oncogenes *c-fos and c-jun* in neuroblastoma cells, leading to neurite outgrowth (116). PAF receptor stimulation has also been shown to activate proliferative and differentiating signals in several non-inflammatory cells, including the upregulation of proliferation in lymphocytes and fibroblasts (117-119).

Similarly, exposure of macrophages to modified LDL's (oxidized LDL and acetyl LDL) has also been reported to activate signaling events which are consistent with those mediated by growth factor and PAF receptor activation. In macrophages, oxidized LDL has been shown to increase intracellular calcium and phosphoinositide turnover, and activate PLC (120), PLD [1] (111), the cytoplasmic tyrosine kinase p53/p56 Lyn (121), PKC (122), and MAP kinase (123-124). Although the signaling mechanism(s) by which oxidized LDL stimulates cell proliferation remain unclear, the above findings strengthen the possibility that oxidized LDL transduces its mitogenic signal through the PAF receptor in macrophages. If PAF receptor activation is involved in oxidized LDL induced macrophage cell growth, the downstream signaling events coupled to this response need be more fully explored.

Aside from the intracellular signaling mediators mentioned above, both PAF receptor and growth factor receptor mediated signaling commonly involve the activation of phosphatidylinositol 3-kinase (PI 3-kinase) (113, 125-127). This enzyme acts as a direct biochemical link between a novel phosphatidylinositol pathway and a number of proteins containing tyrosine kinase activity (128). The PI 3-kinase enzyme is comprised of 2 subunits, a 110kD (p110) catalytic subunit and an 85kD (p85) regulatory subunit (129). It can become activated following interaction of its SH2 domain with activated membrane-associated cytoplasmic tyrosine kinases (130-131), or by direct interaction with the SH3 domains of activated receptor tyrosine kinases, followed by association of the regulatory p85 and catalytic p110 subunits (128-129). Several potential SH2 phosphorylation sites are present in the p85 subunit (129), however, phosphorylation of this subunit is not always required for PI 3-kinase

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[1] A. Gómez-Muñoz and U. Steinbrecher, unpublished observations.

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activation (132). PI 3-kinase has recently received much attention since its main physiological product, phosphatidylinositol (3,4,5) triphosphate (PI 3-P), appears be a second messenger involved in many cellular functions such as membrane ruffling and glucose transport (108,127). More importantly, PI 3-kinase is also thought to be an important regulator of cell growth and proliferation. Recent studies have shown that the mitogenic effects of interleukins and haematopoetic growth factors on B-cells and macrophages directly involve PI 3-kinase dependent signaling mechanisms (125, 128, 133). Since PAF receptor mediated signaling has been shown to involve activation of PI 3-kinase, and macrophage growth by oxidized LDL may require PAF receptor activation, it is possible that PI 3-kinase activation may play a pivotal role in the proliferative effect of oxidized LDL.

1.6   Rationale

Oxidized LDL has been shown to promote many potentially atherogenic actions, including the ability to stimulate macrophage (96-98) and vascular smooth muscle cell growth (85). In studies performed by Sakai and colleagues in macrophages, the mitogenic effect of oxidized LDL was attributed to SR-AI/II mediated internalization of lysoPC. However, the data indicate that lysoPC could not account for the full mitogenic effect of oxidized LDL, suggesting that other components of oxidized LDL could also stimulate growth. In contrast, Heery and co-workers have shown that oxidized LDL stimulates vascular smooth muscle cell proliferation, and that this effect is attributed to oxidized phospholipids and can be completely inhibited with PAF receptor antagonists (85). These findings suggest the possibility that macrophage growth by oxidized LDL may also be mediated by oxidized phospholipids either through direct or indirect activation of the PAF receptor, in addition to a partial effect mediated by lysoPC. In order to more fully understand the mechanism(s) by which oxidized LDL transmits its proliferative effect, the nature of the discrepant findings reported by Heery et al. and Sakai and coworkers need to be addressed and resolved.

Although Sakai and colleagues have concluded that the mitogenic effect of oxidized LDL is attributable to lysoPC, several inconsistencies in their research have been identified that compromise the validity of their findings. Firstly, fundamental differences in oxidized LDL preparations, specifically in chemical composition and extent of LDL oxidation have been shown to exist when compared to preparations from our lab and other investigators. In studies by Sakai and colleagues, 24 hour oxidation of LDL (200 μg/ml) by incubation with 5 μM copper at 37°C results in an relative electrophoretic mobility (REM) of approximately 2.4 fold that of native LDL upon agarose gel electrophoresis (96, 134). In contrast, our lab and other investigators have found that oxidation of LDL under identical conditions results in a REM approximately 4 fold that of native LDL (105, 135-136). Sakai and colleagues have also shown LDL to contain approximately 1.2 -1.4 umoles of phospholipid / mg LDL protein (97, 99) approximately 2 times that previously described by our lab and other groups (135). These differences suggest that the LDL preparations of Sakai and colleagues differ from those of our lab not only in their susceptibility to oxidation, but also in the extent of apo B modification and phospholipid chemistry and composition. These differences may account for part of the discrepant results observed with respect to oxidized LDL induced cell growth.

Secondly, some of the data presented in the studies of Sakai et al. directly contradict previous findings by our lab, raising questions as to the importrance of lysoPC in the mitogenic effect of oxidized LDL macrophages. In one of the studies performed by Sakai and colleagues, acetyl LDL, which does not possess discernible amounts of lysoPC, was found to stimulate a four fold increase in thymidine incorporation in murine peritoneal macrophages, an effect nearly 50% that observed with oxidized LDL (96). However, more recent studies by the same group show acetyl LDL to have negligible growth stimulating activity under similar conditions unless it is treated with PLA$_2$ (97). In addition, our lab has demonstrated 3 and 5 fold higher levels of binding and uptake of oxidized LDL respectively in macrophages compared to the results of Sakai and colleagues (70, 98, 137). This findings is interesting as it suggests that oxidized LDL prepared by Sakai and colleagues delivers ~75% less lysoPC to macrophages than that of

oxidized LDL prepared by our lab, further questioning the conclusion that lysoPC is the component of oxidized LDL responsible for its proliferative effect.

Because of the reports describing variable mechanisms for oxidized LDL induced cell growth and questions surrounding the significance of lysoPC in this effect, the role of oxidized LDL in macrophage cell growth needs to be re-examined. In doing so, the relative importance of lysoPC and oxidized PC needs to be studied more closely using rigorously controlled oxidation conditions and adequate characterization of LDL to more clearly define the mitogenic effect of oxidized LDL on macrophages.

The purpose of this study was to further characterize the mechanism(s) by which oxidized LDL stimulates macrophage growth. To meet this objective, we will determine the relative importance of both lyso and oxidized PC in the effect of oxidized LDL, investigate the role of both scavenger and PAF receptors in macrophage growth by oxidized LDL, and further explore the signal transduction mechanisms that regulate oxidized LDL induced macrophage growth.

Possible Mechanism of Macrophage Growth by Oxidized LDL

Oxidized LDL

oxidized PC

PAF Receptor

Scavenger
Receptor

Oxidized LDL
Receptors

lysoPC

G-proteins

oxidized PC

Oxidized LDL

PTK

Oxidized LDL

PI 3-K

PLCγ

PKC ◄— DAG, IP3

MAPK

Increased c-jun,
c-fos expression

• DNA Replication ◄—(∿∿) Nucleus
• Proliferation
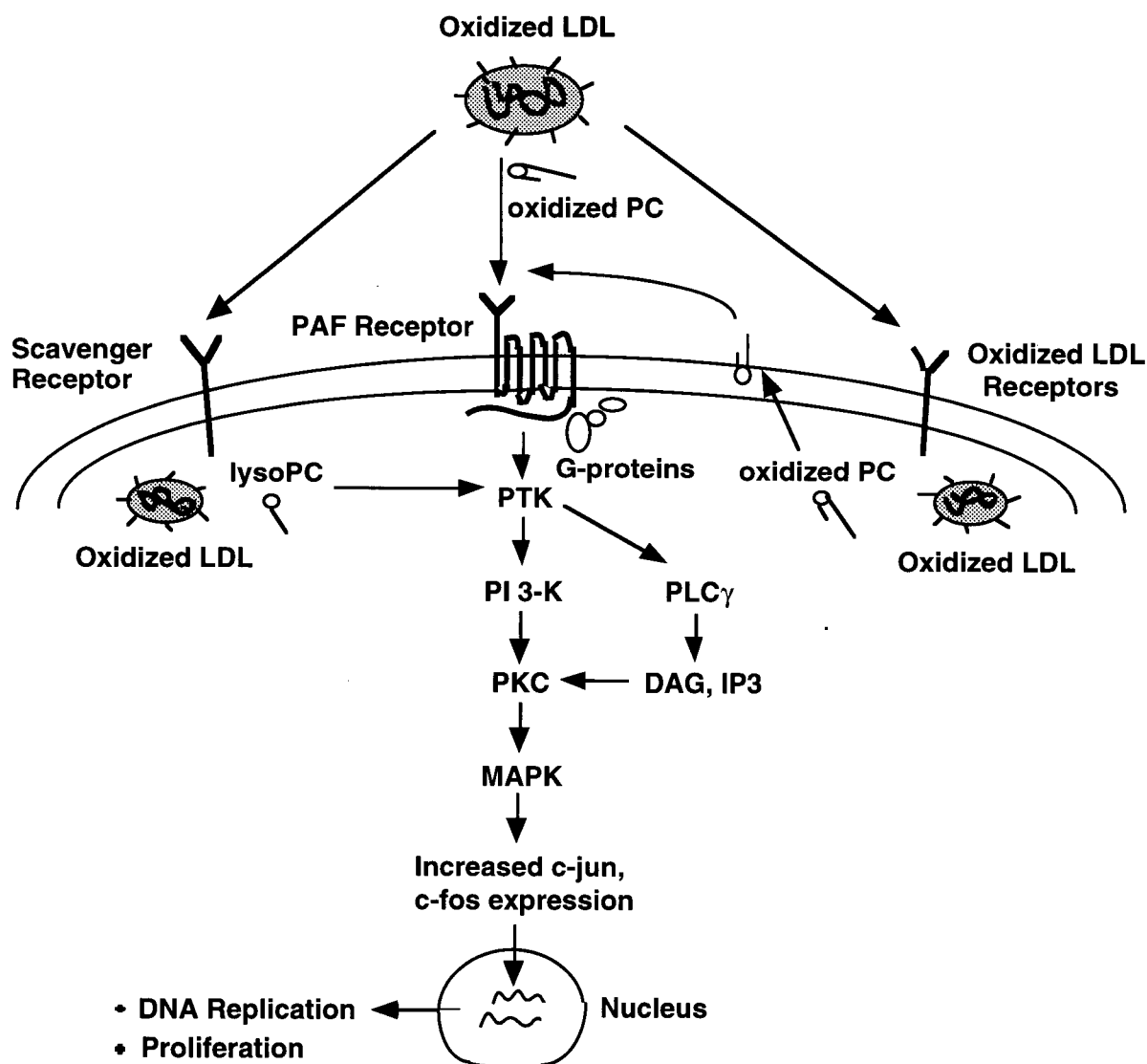
Figure 1.2   Possible mechanism of macrophage cell growth by oxidized LDL. Macrophage growth by oxidized LDL is likely to involve several mechanisms that may require internalization of the LDL particle mediated by oxidized LDL or SR-AI/II receptors, or extracellular activation of the PAF receptor. Following internalization and lysosomal degradation of oxidized LDL, phospholipids such as oxidized PC and lysoPC are released to the cytosol and may lead to the activation of PTK's such as p53/p56 Lyn. Cytosolic oxidized phospholipids may also become incorporated into the cytoplasmic membrane, and, in addition to a possible direct extracellular effect of oxidized phospholipids on the surface of oxidized LDL, stimulate activation of the PAF receptor. PAF receptor stimulation has been shown to involve PTK activation (p53/p56 Lyn), leading to downstream activation of PLCγ, PI 3-kinase, PKC and MAPK. MAPK activation results in a rapid and transient expression of early response genes, leading to DNA replication and cellular proliferation. See page ix for abbreviations.

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1.7   Objectives and Hypotheses

The main objective of this study was to further elucidate the role of oxidized LDL in macrophage cell growth. To achieve this objective, the following specific hypotheses were addressed:

Macrophage growth by oxidized LDL:

1.   depends on the extent of oxidation of LDL.

2.   is dependent on an effect mediated by the generation of oxidized phospholipids and/or lyso PC in oxidized LDL.

3.   involves PAF receptor activation.

4.   involves the activation of PI 3-kinase.

Chapter 2   Materials and Methods

2.1   Chemicals and Reagents.

Reagents for enhanced chemiluminescence, and L-alpha phosphatidylinositol were purchased from Amersham International (Oakville, Ontario, Canada). Protein A-agarose was purchased from Biorad Laboratories (Mississauga, Ontario, Canada). Goat anti-mouse horseradish peroxidase (HRPO) was from Cedarlane (Hornby, Ontario, Canada). Anti-phosphotyrosine mAb (4G10, 05-321), and anti-phosphoinositide 3-kinase (PI 3-kinase), N-SH2 mAb (UB93-3) were purchased from Upstate Biotechnology, Inc. (UBI; Lake Placid, NY). RPMI 1640 medium was from Gibco BRL Products, (Burlington, Ontario). Hyclone defined fetal bovine serum was supplied by Professional Diagnostics (Edmonton, Alberta, Canada). Lipopolysaccharide (LPS) (Escherichia coli 0127:B8) was from Difco Laboratories (Detroit, MI, USA), and was solubilized in RPMI 1640 containing 10% fresh, human AB^+ serum. Crotalus atrox venom PLA_2 (phospholipase A_2), PMSF (phenylmethylsulfonyl-fluoride), XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide), PMS (N-methyldibenzopyrazine methyl sulphate salt) and wortmannin were purchased from Sigma Chemical Co.(Mississauga, Ontario, Canada). LY294002 was obtained from BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA. Endotoxin test kit (Limulus Amebocyte Lysate assay) was from BioWhittaker Inc. (Walkersville, MD, USA). The PAF receptor antagonist L-659,989 was a gift from Dr. John Chabala of Merck, Sharp & Dohme Research (Rahway, NJ). Other chemicals were the highest grade available from Fisher Scientific (Vancouver, British Columbia, Canada) or VWR Canlab (Edmonton, Alberta, Canada).

2.2   LDL Preparation.

2.2.1   LDL Isolation.

LDL (d=1.019-1.063 g/ml) was isolated by sequential ultracentrifugation of EDTA-anticoagulated plasma from fasting normolipidemic human volunteers by the method of Havel et al. (138). Before ultracentrifugation, the density of the plasma was adjusted with sodium bromide solutions containing 10 mM EDTA. The concentration of EDTA in the isolated LDL preparations was then reduced by two dialysis washes against Dulbecco's PBS containing 10uM EDTA at pH 7.4 at 4°C, followed by storage of the LDL at 4°C in the dark. This concentration of EDTA was sufficient to inhibit spontaneous oxidation on storage, but low enough to permit reproducible oxidation upon addition of 5 μM $CuSO_4$.

2.2.2   LDL Modification.

Conditions for oxidation LDL were incubation of 200 μg/ml LDL in PBS with 5 μM $CuSO_4$ at 37°C for 1-24 h (21, 39). Further oxidation was arrested by addition of 100 μM EDTA and 40 μM butylated hydroxytoluene. To prepare BSA-treated oxidized LDL, oxidized LDL (200 μg/ml) was incubated with fatty acid-free BSA (10 mg/ml) for 18 h at 20°C in sterile PBS (pH 7.4). A control incubation with oxidized LDL was performed with the same volume of PBS without BSA. Oxidized LDL samples were then reisolated by ultracentrifugation for 20 h at 10°C (d< 1.210) (139). Acetylation of LDL was performed by sequential addition of acetic anhydride (16). Seven aliquots each of 1 μl of acetic anhydride were added at 15 minute intervals to 4 mg of LDL in 2 ml of ice-cold 50 % saturated sodium acetate. These conditions for both oxidized and acetyl LDL typically resulted in a 3.5 to 4.5 fold increase in electrophoretic mobility relative to unmodified native LDL on agarose gels. PLA_2 treated native and acetyl-LDL were prepared as previously described (87). Briefly, for each experiment, 0.2 ml of 0.1 M Tris-HCL, pH 7.4, 10

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mM $CaCl_2$ containing 25 units of PLA_2/ml was added to 1 ml of PBS containing 1.5 mg of native or acetyl-LDL, and incubated at 37°C for 2 h. This reaction was stopped by the addition of 10 mM EDTA and subsequent cooling of the solution. Lipoproteins were then separated from PLA_2 by repeated washings with sterile PBS using ultrafiltration membrane cones as described below. Pretreatment of LDL with PMSF prior to oxidation was also performed as previously described (21). For these experiments, LDL (1.5 mg/ml) was incubated with 5 mM PMSF for 1 h at 37°C, before oxidation. This treatment consistently resulted in a 95% inhibition of PAF-acetylhydrolase activity. LDL's were then cone washed with sterile PBS without calcium, concentrated to about 750 µg/ml using ultrafiltration membrane cones (Centricon CF 25, Amicon), sterile filtered through 0.2 um filters, and stored at 2-4° C for no longer than 2 weeks.

2.2.3 Characterization of LDL.

Lipoprotein electrophoresis was performed using a Corning apparatus and Universal agarose film in 50 mM barbital buffer (pH 8.6) according to manufacturer's instructions. Bovine serum albumin was added to lipoprotein samples to ensure reproducible migration distances. Lipoprotein bands were visualized by staining with Fat red. Protein was assayed by the method of Lowry (140) in presence of 0.05% sodium deoxycholate to minimize turbidity with bovine serum albumin used as the standard. Endotoxin (LPS) levels in LDL's were consistently shown to be less than 100 pg/mg LDL protein as determined by limulus amoebocyte lysate assay.

2.3   Cell Culture.

For analysis of oxidized LDL induced cell growth, murine resident peritoneal macrophages were collected from male CD-1 mice (25-30 g) by peritoneal lavage with ice-cold Ca^{2+}-free Dulbeco's PBS. Cells were resuspended in RPMI 1640 supplemented with 10% fetal

bovine serum, and gentamicin (50 µg/ml). Cell suspensions were then adjusted to 1 x 10^5/ml for XTT assay, 5 x 10^5/ml [^{3}H] thymidine incorporation assay and 5 x 10^4/ml for cell counting. For XTT and thymidine incorporation assays, 0.1 ml of cell suspension was then added to each well of 96-well tissue culture plates, while for cell number determinations, 1.0 ml of cell suspension was added to 24-well tissue culture plates (Falcon, Lincoln Park, NJ), and incubated for 12 h at 37° C in a humidified atmosphere of 5% CO_2 in air. Non-adherent cells were then removed by gentle washing with medium. Macrophages were then cultured in either 0.1 ml (XTT and [^{3}H] thymidine incorporation assays) or 1.0 ml RPMI medium containing 5% fetal bovine serum and 50 µg/ml gentamicin together with lipoproteins or inhibitors for the number of days indicated without a medium change. Scavenger receptor class A type I/II knockout mice were obtained from Dr. T. Kodama (University of Tokyo). The description of the construct and the phenotypic characterization in homozygous knock out mice have been reported elsewhere (27, 141-142). For experiments using SR-AI/II deficient macrophages, the cells were obtained and cultured in the exact manner as described above. The THP-1 cell line was obtained from the American Type Culture Collection (Rockville, MD.). These cells, derived from a patient with acute monocytic leukemia, are phagocytic and posses other characteristics of monocytes including the expression of Fc and C3b receptors (143).

For the analysis of tyrosine phosphorylation and PI 3-kinase activity, THP-1 cells were grown in HEPES-buffered RPMI 1640 medium containing 50 µM 2-mercaptoethanol, 10% FBS and 0.5 mg/ml gentamicin. To induce differentiation of THP-1 cells to a macrophage-like phenotype, 5 x 10^6 cells were dispensed into each culture dish in 5 ml medium containing 100 ng/ml PMA and incubated for 24 hours at 37°C. Before experiments, cells were washed three times with medium and then incubated in 5 ml of serum-free medium for 3 h to induce "quiescence". Cells were then stimulated with different concentrations of oxidized or native LDL, or an equivalent volume of sterile PBS for varying times. In each experiment, 1 µg/ml LPS was used as a positive control for tyrosine phosphorylation and PI 3-kinase activation.

2.4   Experimental Assays.

2.4.1 XTT Growth Assay.

Murine peritoneal macrophage growth was determined by the XTT formazan method. This assay is based on the cellular reduction of XTT by mitochondrial dehydrogenases to an orange formazan product that can be measured spectrophotometrically, and has been shown to correlate well with cell number under these conditions (Figure 3.1) (144-145). Briefly, 50 μl of XTT solution (1 mg/ml XTT, 25 μM PMS in RPMI 1640) was warmed to 37°C, added to each well, and then incubated for 4.5 h at 37°C. Following incubation, the absorbance at 450 nm was then measured with a multiwell spectrophotometer. For the cell type used in these studies, there was a linear correlation between cell number and XTT formazan formation in the range 2 x 10^3 cells/well to 5 x 10^4 cells/well (Figure 3.1).

2.4.2 [^{3}H] Tritiated Thymidine Incorporation Assay.

Peritoneal macrophage growth was also determined by the incorporation of [^{3}H] thymidine into the DNA of cells. Briefly, 10 μl of 20 μCi/ml [methyl-^{3}H] thymidine (80 Ci/mmol, Amersham Life Sci., Clevland, Ohio) was added to each well of 96 well plates for the last 24 hours of each experiment. The medium was then aspirated, and cells were washed twice with ice-cold 10% TCA to precipitate DNA and remove unincorporated label. Cells were then dissolved in 0.5 % SDS with 0.3 N NaOH to hydrolyse the acid-insoluble material. Samples were then transferred to scintillation vials, and radioactivity was analyzed using a liquid scintillation counter.

2.4.3 Cell Counting Assay.

Macrophage cell number was determined using two independent methods. During culture, cell number was measured using inverted phase-contrast microscopy by counting the number of cells within 4 random fields of view (0.40 mm^2) from two separate wells. To determine the number of cells following 10 days of culture, the medium was aspirated, cells were washed with medium, the adherent cells lysed with Triton X-100, and the number of napthol blue-black stained nuclei counted using a hemocytometer as previously described by Nakagawara (146).

2.4.4 Morphological Observations.

Morphological observations of cultured macrophages were made by inverted phase-contrast microscopy.

2.4.5 Anti-Phosphotyrosine Immunoblotting.

Following incubation with oxidized LDL, THP-1 cells were washed 3 times with Hanks buffered saline solution (HBSS) and incubated for 15 minutes on ice with lysis buffer (20 mM Tris-HCL buffer, pH 8.0, containing 137 mM NaCl, 10 % v/v glycerol, 2 mM EDTA, 1 % v/v Triton-X 100, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM molybdate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysates were then spun in a microfuge at 14,000 rpm for 20 minutes at 4° C and the supernatants containing soluble proteins were collected. For analysis of protein-tyrosine phosphorylation in whole cell lysates, 50 μg of protein from each sample was loaded and then separated by SDS-polyacrylamide gel electrophoresis (7.5%). Gels were calibrated using prestained high range molecular weight markers (Rainbow, Amersham). Proteins were then transferred to nitrocellulose paper, incubated overnight with 2% BSA, 0.01 NaN_3, and then

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washed three times with Tween buffer (20 mM Tris HCl pH 7.6, 137 mM NaCl, and 0.1 % Tween-20). Blots were probed with 4G10 murine anti-phosphotyrosine antibody for 2 h, and washed three times with Tween buffer. Bound primary antibody was then visualized with horseradish peroxidase-conjugated goat anti-mouse IgG for 1 h at 20°C and imaged on Kodak XR film using an enhanced chemiluminescence system (Amersham) with 5 minutes exposure time.

2.4.6 Immunoprecipitation and PI 3-Kinase Assay.

Following incubation with oxidized LDL, THP-1 cells were washed 3 times with Hanks buffered saline solution (HBSS) and incubated for 15 minutes on ice with lysis buffer (20 mM Tris-HCL buffer, pH 8.0, containing 137 mM NaCl, 10 % v/v glycerol, 2 mM EDTA, 1 % v/v Triton-X 100, 1 mM Na_3VO_4, 1 mM PMSF, 1 mM molybdate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysates were then spun in a microfuge at 14,000 rpm for 20 minutes at 4°C and the supernatants containing soluble proteins were collected. Aliquots of cell lysates normalized for protein content (~ 350 μg protein) were incubated overnight at 4°C with monoclonal antibody (mAb) to PI 3-kinase. The immune complexes were then collected on protein A-agarose beads for 2 h at 4°C, washed twice with lysis buffer containing 50 μM vanadate, and three times with 10 mM Tris, pH 7.4. Immunoprecipitates were then resuspended in 100 μl of 20 mM HEPES, 1 mM EDTA, pH 7.4 and placed on ice. To assay PI 3-kinase activity, 20 μg of phosphatidylinositol was dried under N_2, resuspended in 20 μl of 30 mM HEPES buffer, pH 7.4, and dispersed by sonication. Twenty μl of sonicated lipid was then added to the immunoprecipitate, followed by 30 μl of buffer containing 10 uCi of [gamma-^{32}P] ATP, 200 μM adenosine, and 50 μM ATP. Reactions were stopped by the addition of 0.1 ml 1N HCl and 0.2 ml chloroform: methanol (1:1, v/v). Lipids were separated on oxalate-treated silica thin layer chromatography plates using a solvent system of chloroform : methanol : water : 28% ammonia (45:35:7.5:2.5, v/v/v/v). Plates were then exposed to X-ray film for 18 h at

-70°C, and incorporation of radioactivity into the lipids was quantified by excising the corresponding portions of the TLC plate followed by liquid scintillation counting.

2.5 Analytical Techniques.

2.5.1 Phospholipid Analysis.

For the determination of lysoPC, sphingomyelin and PC, lipids were extracted from lipoproteins (100 μg) using chloroform/methanol (2:1,v/v), and the solvents were evaporated under nitrogen. The phospholipids were then resuspended in chloroform, separated by TLC on silica gel G with chloroform/methanol/water (65:35:7, v/v/v) and visualized with iodine vapor. LysoPC, sphingomyelin and PC bands were then scraped from the plates and assayed for phosphorus content according to the method described by Rouser et al. (147). Briefly, silica fractions containing phospholipids were dissolved in perchloric acid by heating at 180°C for 30 minutes, and subsequently treated with 2.5 ml H_2O, and 500 μl each of 1% ammonium molybdate and 10% ascorbic acid. Samples were then incubated in a 37°C water bath for 15 minutes, and the absorbances read at 820 nm using a spectrophotometer. The amount of phosphate in each phospholipid sample was determined in nmoles/mg of LDL protein.

2.5.2 Limulus Assay.

For the determination of endotoxin (LPS) levels in lipoproteins, random native and modified lipoprotein preparations were selected and subjected to a sensitive chromogenic limulus lysate assay using LPS prepared by Westphal phenolic extraction from *E. Coli* 0111:B4 endotoxin. By this method, endotoxin concentrations in LDL preparations were consistently found to be less than 100 pg/ml LDL solution or 1.0 pg/μg LDL protein.

2.6 Statistical analysis.

All data were expressed as the mean ± standard error of the mean (SEM). Significance of differences between experimental groups was analyzed using either Student's unpaired t-test, or by two-factor analysis of variance as appropriate. Differences were judged to be significant at a level of $p < 0.05$.

Chapter Three   Results

3.1   Oxidized LDL Induced Macrophage Cell Growth.

The effects of various LDL's on the growth of mouse resident peritoneal macrophages was examined using XTT reduction, thymidine incorporation and cell counts. The XTT assay is sensitive enough to be applied to cells grown in 96-well plates, and was found to correlate well with macrophage cell number (Figure 3.1) (145).
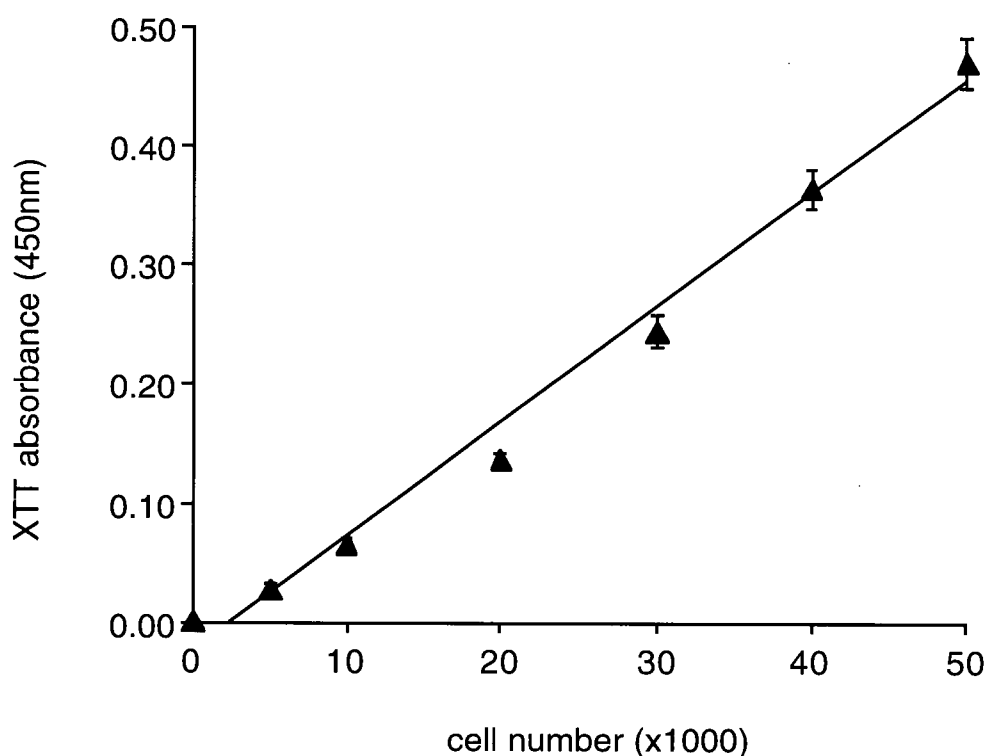
Figure 3.1. Correlation between macrophage cell number and formation of XTT formazan product. Resident mouse peritoneal macrophages were cultured in 96 well microplates at the indicated cell densities (ranging from 5 x 10^3 to 5 x 10^4 cells/well) in a final volume of 0.1 ml in 10% FBS in RPMI 1640 for 6 h to allow adherence to plates. Fifty μl of XTT solution, (50 μg XTT, 0.38 μg PMS dissolved in RPMI 1640) was then added to each well, followed by an additional 4.5 h incubation. Absorbances were then read with a microplate spectrophotometer at 450 nm to detect the colored formazan product. The values shown represent the mean ± standard error of quadruplicate determinations from five experiments.

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Macrophage growth was not significantly increased by native or acetyl LDL, but was increased up to 15-fold with 30 μg/ml oxidized LDL (Figure 3.2). It was also observed that concentrations of oxidized LDL as low as 2.5 μg/ml produced significant growth activity, while concentrations of oxidized LDL greater than 60 μg/ml appeared to be cytotoxic to macrophages, and were associated with blebbing, cell detachment, and a decrease in the proportion of cells that excluded trypan blue (data not shown).
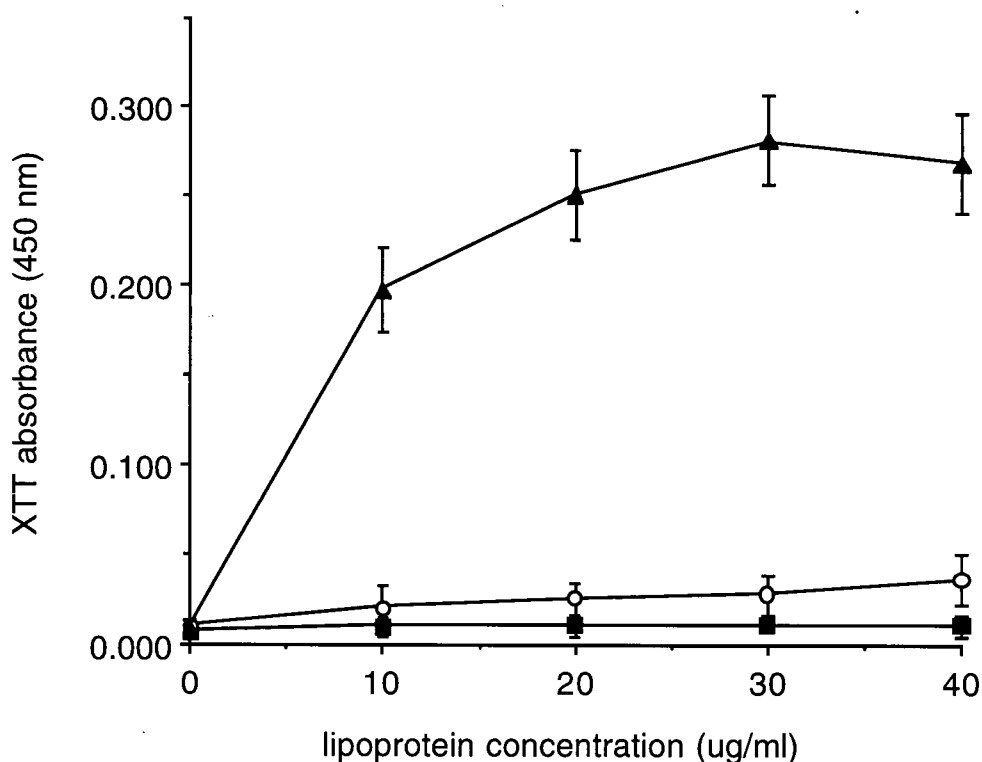
Figure 3.2. Dose dependence of oxidized LDL induced macrophage cell growth. Mouse resident peritoneal macrophages were plated at a density of 1.0 x 10^4 cells /well in 0.1 ml of RPMI 1640 supplemented with 10% fetal calf serum for 12 hours. After washing with medium, cells were then incubated in RPMI 1640 with 5% FBS with the indicated concentrations of native LDL (□), acetyl LDL (O), or oxidized LDL (▲) for 4 days without a medium change. Cell growth was then measured by XTT assay as described in "methods". The values shown represent the mean ± standard error of quadruplicate determinations from three experiments.

When cell growth was examined using thymidine incorporation assay, 30 µg/ml oxidized LDL

resulted in a 4 to 5 fold increase in thymidine incorporation over control on day 6, while native

LDL produced negligible effects (Figure 3.3).
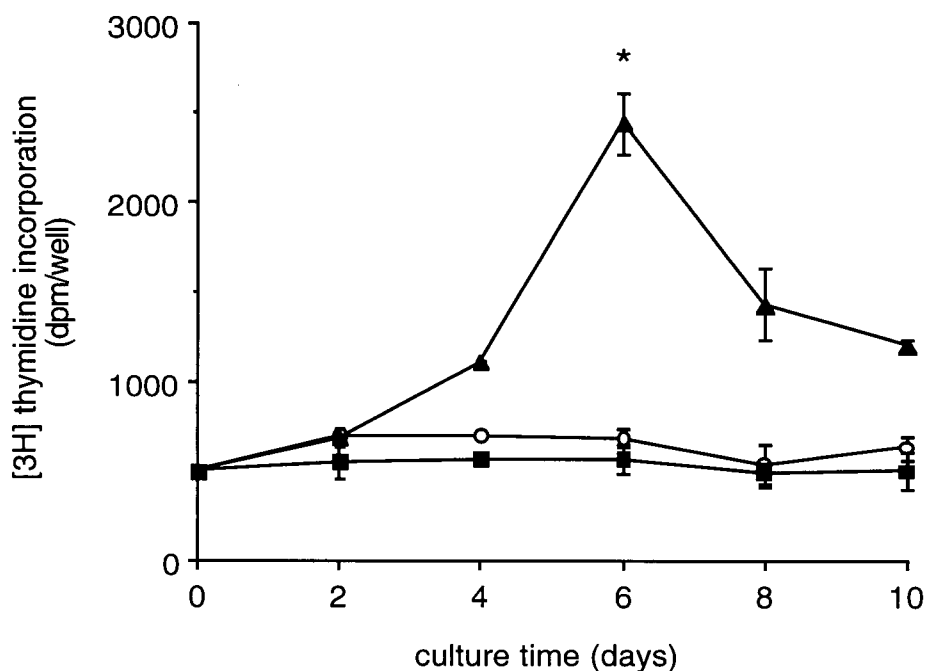
Figure 3.3. Time course of oxidized LDL induced macrophage cell growth. Resident macrophages (2.0×10^4 cells /well) in a final volume of 0.1 ml , were incubated with either 30 µg/ml of native LDL (■) oxidized LDL (▲) or medium alone (O) in RPMI medium containing 5% FBS for the number of days indicated without a medium change. 24 h before the end of each experiment, 10 µl of 10 µCi/ml [^{3}H] thymidine was added to each well. The radioactivity incorporated into the cells was then determined by liquid scintillation counting as described in "methods". The values shown represent the mean ± standard error of quadruplicate determinations from three experiments. *$p < 0.01$ compared with native LDL or medium only control by Student's unpaired t-test.

To verify that the increased reduction of XTT and thymidine incorporation into DNA by oxidized

LDL was accompanied by cell division, we performed cell counts every 2 days up to 10 days in

culture, followed by counting of solubilized nuclei on day 10. Compared to control, both

oxidized LDL and GM-CSF led to significant and progressive increases in cell number in culture

(Figure 3.4), and a 3-4 fold increase in the number of solubilized nuclei (Figure 3.5). In both cases, neither native nor acetyl LDL significantly altered macrophage cell number. In these experiments, 10 ng/ml GM-CSF served as a positive control for macrophage proliferation and was found to stimulate comparable macrophage growth to that of oxidized LDL (Figure 3.5).
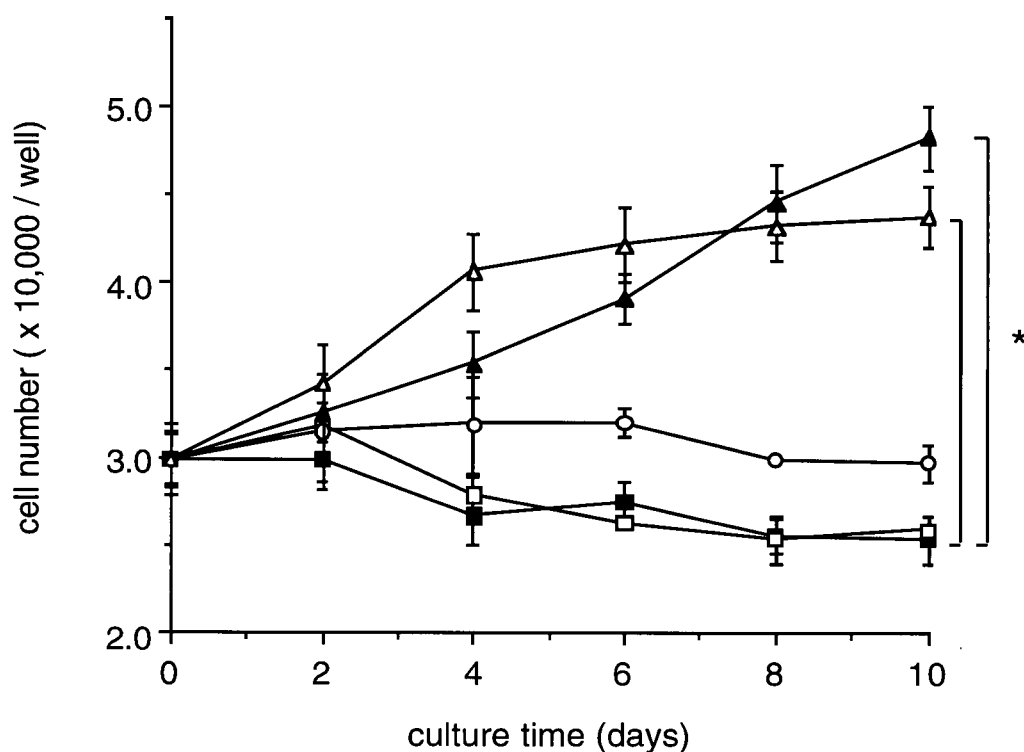
Figure 3.4. The effects of oxidized LDL on macrophage cell number in culture. Resident macrophages (5 x 10^4 cells / well) were cultured in 24 well plates in a final volume of 1.0 ml with either 30 μg/ml of native LDL (□), acetyl LDL (○), oxidized LDL (▲), 10 ng/ml GM-CSF (Δ), or with medium with 5% FBS alone (■) for 10 days without a medium change. Every 2 days, cell numbers in culture were determined as described in methods. On day 10, cells were washed with medium, adherent cells were lysed with triton X-100, and the number of napthol blue-black stained nuclei counted on a hemocytometer. The values shown represent the mean ± standard error of quadruplicate determinations from two experiments. *p < 0.001 compared to medium only control (by two-factor ANOVA with repeated measures).

Macrophages incubated with oxidized LDL also showed a substantial increase in size and change in morphology, leading to the formation of elongated, spindle-shaped cells that exhibited greater adherence to tissue culture plates over control cells. Cells incubated with either native LDL, acetyl LDL, or medium alone remained small, rounded, and without projections (Figure 3.6).
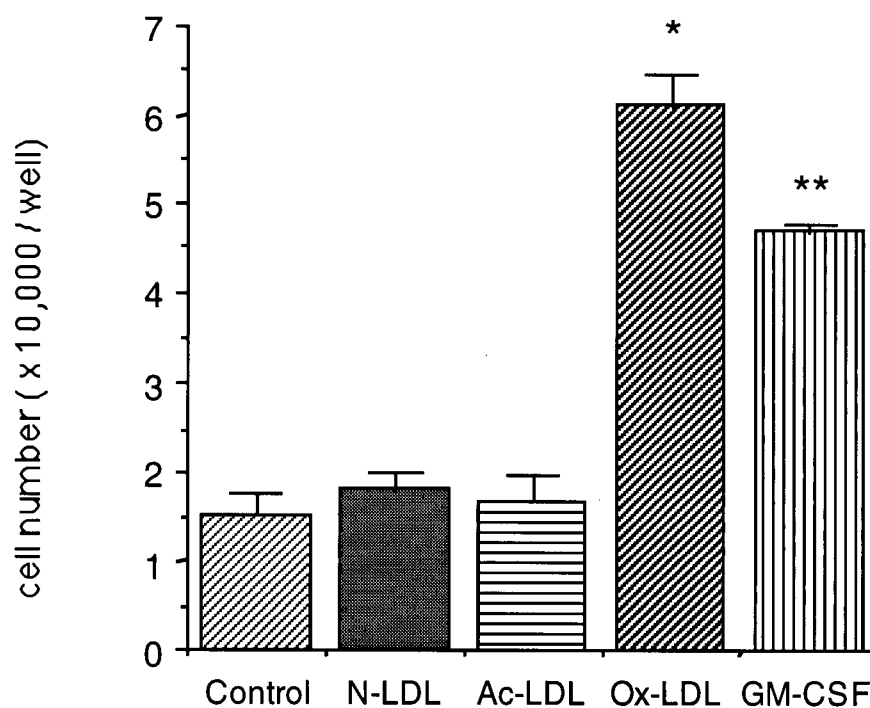
Figure 3.5. The effects of oxidized LDL on macrophage cell number by counting solubilized nuclei. Resident macrophages (5×10^4 cells / well) were cultured in 24 well plates in a final volume of 1.0 ml with either 30 μg/ml of native LDL (N-LDL), acetyl LDL (Ac-LDL), oxidized LDL (Ox-LDL), 10 ng/ml GM-CSF or with medium with 5% FBS alone (Control) for 10 days without a medium change. On day 10, cells were washed with medium, adherent cells were lysed with triton X-100, and the number of napthol blue-black stained nuclei counted on a hemocytometer as described in "methods". The values shown represent the mean $\pm$ standard error of quadruplicate determinations from two experiments. * $p < 0.001$ and ** $p < 0.01$ compared to medium only control by unpaired Student's t-test.
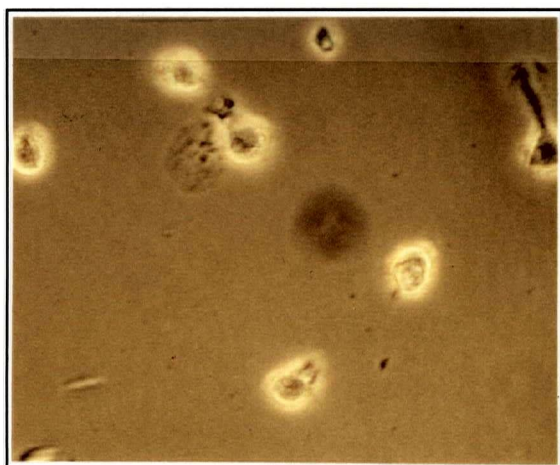
Control
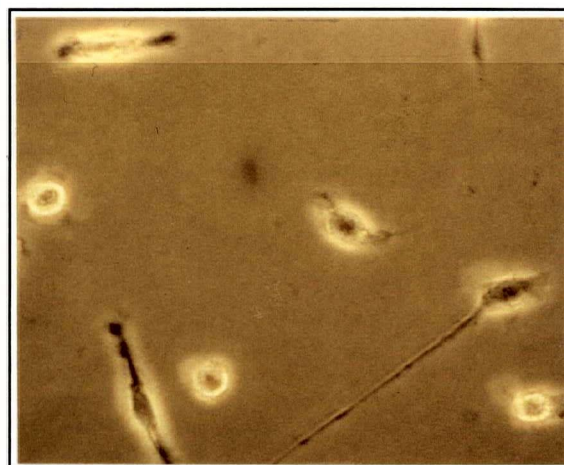
Native LDL
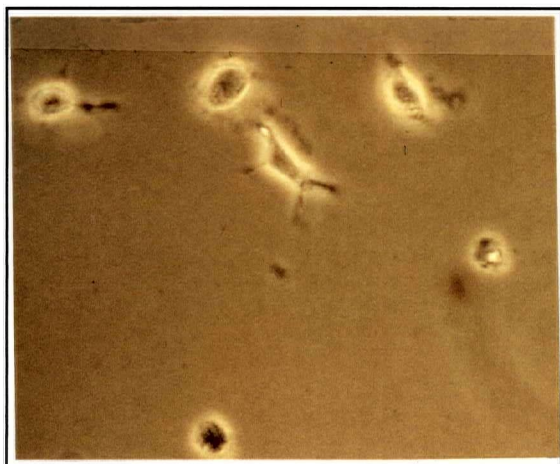
Acetyl LDL
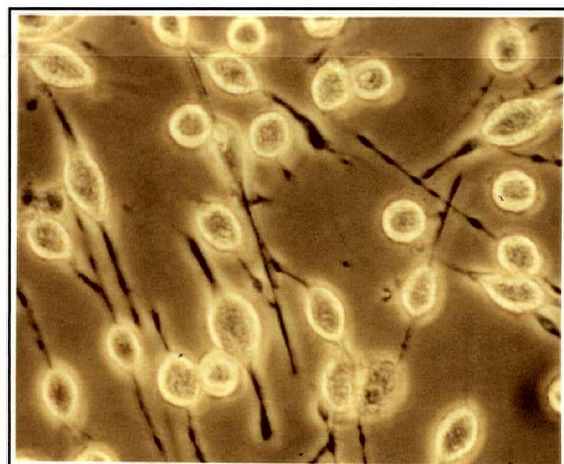
Oxidized LDL

Figure 3.6. Photomicrographs of Macrophage Cells Cultured with Modified LDL's. Resident macrophages (1.0×10^6 cells / well) were cultured on sterile coverslips in 6 well plates in a final volume of 2.0 ml with either 30 ug/ml of native LDL, acetyl LDL, oxidized LDL, or with medium supplemented with 5% FBS alone for 6 days without a medium change. On day 6, cells were washed with PBS, coverslips inverted on glass slides, and photographs taken at 40x magnification. These photomicrographs are representative of the morphological features of cells from all growth experiments as judged by inverted phase-contrast microscopy.

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3.2 Macrophage Growth is Dependent on the Degree of LDL Oxidation.

We next examined the effects of LDL with varying degrees of oxidative modification on macrophage cell growth. LDLs with defined, incremental degrees of oxidation were prepared by oxidizing 200 µg/ml LDL with 5 µM $CuSO_4$ for various times from 1-24 hours. As shown below in Table 3.1, the PC content of LDL decreases progressively during oxidation, falling to one-half of the initial value after 24 hours oxidation. Sphingomyelin content remains constant under these conditions. About 70% of the lost PC is converted to lysoPC, and the rest presumably remains as oxidized PC. Two factors contribute to the incomplete conversion of oxidized PC to lysoPC: first, some oxidized PC species contain long-chain polar acyl derivatives and are poor substrates for the enzyme (44), and second, PAF acetylhydrolase is inactivated during LDL oxidation and very little activity remains beyond 10 hours of oxidation. Interestingly, however, LDL that was oxidized for 15 hours could only

Table 3.1. PC and lysoPC Contents of LDLs with Differing Degrees of Oxidation Modification.

	PC	lysoPC
	nmol PO$_4$ / mg protein	
Native LDL	709 ± 70	14 ± 4
2 h Oxidation	657 ± 45	68 ± 37
5 h Oxidation	500 ± 12	126 ± 20
10 h Oxidation	442 ± 46	186 ± 19
15 h Oxidation	378 ± 64	229 ± 15
24 h Oxidation	360 ± 64	255 ± 23

Native LDL was subjected to copper oxidation for 2, 5, 10, 15, and 24 hours at 37°C. At the end of each incubation period, oxidation was stopped as described in "methods". The PC and lysoPC content of each lipoprotein was then determined by lipid extraction and separation of each lipid component by TLC, followed by phosphate analysis as described in "methods". Each value represents the mean ± standard error of duplicate determinations from three experiments.

stimulate 30-40% of the growth effect produced by fully oxidized LDL (24 hour oxidation) (Figure 3.7), even though the apo-B100 protein had undergone sufficient oxidative modification (REM of 3.05 relative to native LDL) to allow near maximal binding and uptake of the oxidized LDL particle [2] (Figure 3.8). These observations suggest that changes to LDL lipids or apoB associated with very extensive oxidation appear to be important in growth stimulation.
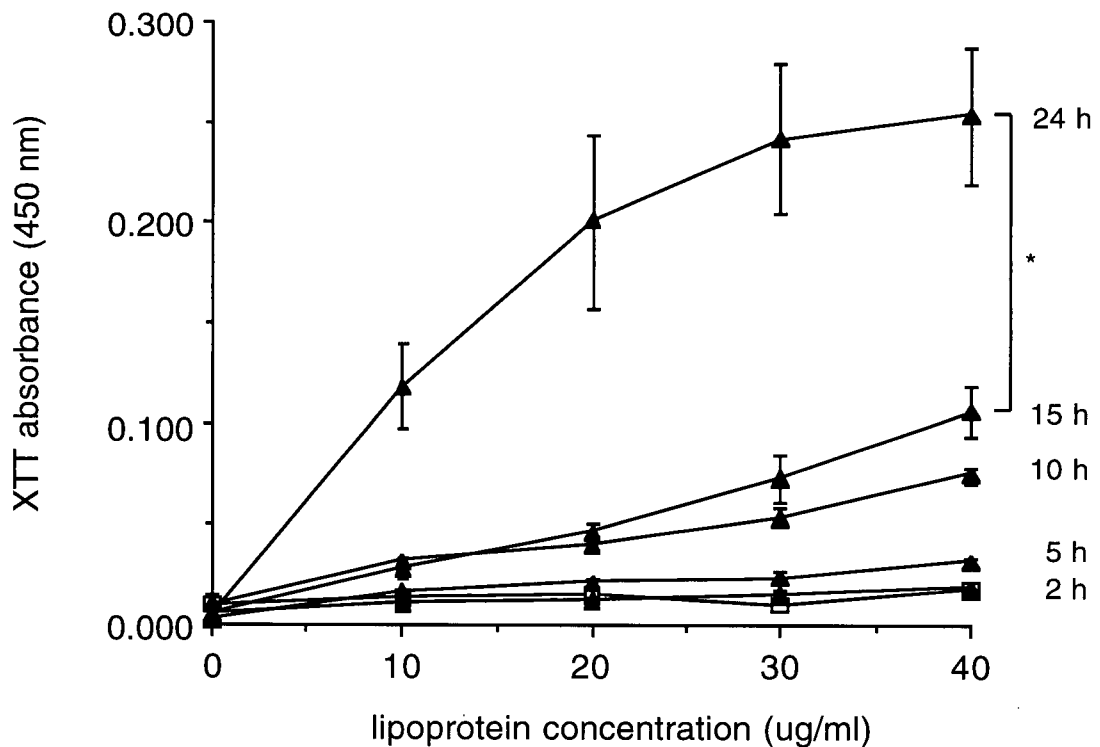
Figure 3.7. The effects of varying degrees of LDL oxidation on macrophage cell growth. Resident peritoneal macrophages (1.0×10^4 cells /well) were cultured in medium with 5% FBS with the indicated concentrations of native LDL ($\square$) or oxidized LDL ($\blacktriangle$) for 4 days without a medium change. Cell growth was then measured by XTT assay. In this experiment, LDL was oxidized in the presence of copper for 2, 5, 10, 15, and 24 hours as described in "methods". The duration of LDL oxidation for each sample is indicated beside the corresponding growth response on the graph. The values shown represent the mean ± standard error of quadruplicate determinations from three experiments. *$p < 0.0001$ by ANOVA.

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[2] M. Lougheed and U. Steinbrecher, unpublished observations.

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| Rf (BSA) | 0.24 | 0.27 | 0.42 | 0.61 | 0.70 | 0.88 |
| Rf (LDL) | 1.00 | 1.17 | 1.83 | 2.70 | 3.05 | 3.84 |

| LDL | 2h Ox | 5h Ox | 10h Ox | 15h Ox | 24h Ox |
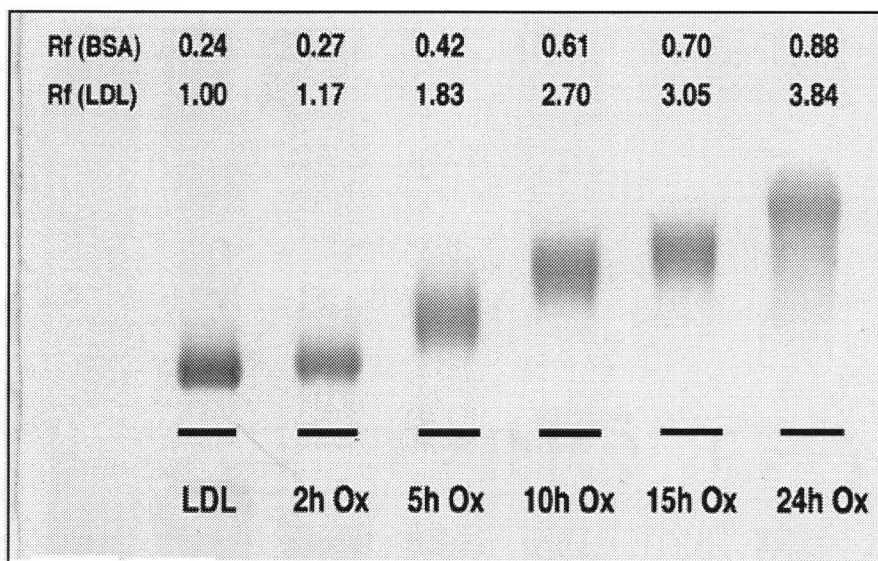
Figure 3.8. Electrophoretic mobilities of LDL with varying degrees of oxidative modification. Freshly isolated native LDL (200 µg/ml) was incubated with 5 µM Cu^{2+} in sterile PBS containing 10 mM calcium for the indicated periods of time at 37°C as described in "methods". Aliquots of each sample were run on agarose gel electrophoresis (1-2 µg / lane) and stained with Fat Red. The electrophoretic mobility of oxidized LDL is expressed relative to the migration distance of both BSA (first row of values), and unmodified Native LDL (second row of values). Data are representative of two separate experiments.

3.3 The Role of lysoPC in Oxidized LDL Induced Macrophage Cell Growth.

To address the hypothesis advanced by Sakai and colleagues that lysoPC accounts for all of the growth stimulatory effect of oxidized LDL (97), we tested the effect of oxidized LDL that had been depleted of lysoPC by incubation with fatty acid-free bovine serum albumin. As shown in Figure 3.9, macrophage growth stimulation by oxidized LDL after treatment with albumin was about 80% compared to that of control oxidized LDL even though albumin treatment had removed more than 97% of the lysoPC (Table 3.2). This result indicates that lysoPC cannot account for more than a small part of the growth stimulatory effect of oxidized LDL, but does not exclude the possibility that lysoPC might be capable of stimulating growth

Table 3.2. Extraction of lysoPC from oxidized LDL by incubation with fatty acid-free BSA.

	PC	lysoPC
	nmol PO_4 / mg protein	
Native LDL	600 ± 45	27 ± 12
Oxidized LDL	263 ± 15	250 ± 10
Oxidized LDL exposed to BSA	210 ± 13	32 ± 3
Oxidized LDL exposed to PBS	245 ± 23	195 ± 29

The accumulation of lysoPC during oxidation of LDL was removed by incubating oxidized LDL (200 µg/ml) with 10 mg of fatty acid-free bovine serum albumin per ml for 24 h at 20°C and reisolating the LDL by preparative ultracentrifugation (d < 1.210). The PC and lysoPC content of each lipoprotein was then determined as previously described. Each value represents the mean ± standard error of duplicate determinations from three experiments.
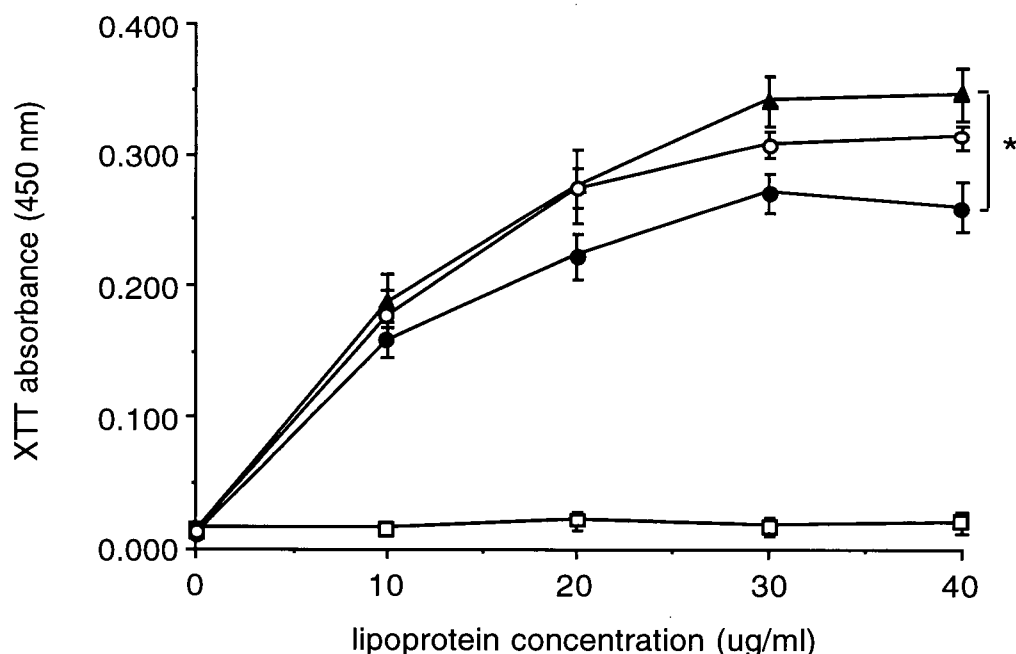
Figure 3.9. Oxidized LDL retains its ability to induce macrophage growth following extraction of lysoPC. Resident macrophages were plated at a density of 1.0×10^4 cells /well and incubated for 4 days with the indicated concentrations of native LDL (□) oxidized LDL (▲), oxidized LDL reisolated after incubation with fatty acid-free BSA (d < 1.210) (○), or oxidized LDL reisolated after incubation with PBS (d < 1.210) (●) without a medium change. Cell growth was then measured by XTT assay. Each value represents the mean ± standard error of quadruplicate determinations from four experiments. * $p < 0.05$ by ANOVA.

Table 3.3. PC and lysoPC contents of native and acetyl LDL following incubation with PLA_2.

	PC	lysoPC
	nmol PO_4 / mg protein	
Native LDL	632 ± 49	51 ± 7
PLA_2 treated Native LDL	50 ± 66	527 ± 57
Acetyl LDL	607 ± 62	58 ± 6
PLA_2 treated Acetyl LDL	39 ± 64	514 ± 49
Oxidized LDL	310 ± 18	227 ± 16

Native or acetyl-LDL (1.5 mg) was incubated with 5 units/ml PLA_2 for 2h at 37°C, separated from PLA_2 by repeated ultrafiltration cone washes with PBS, and the PC and lysoPC content of each lipoprotein was then determined as described in "methods". Each value represents the mean ± standard error of duplicate determinations from three experiments.

under some conditions. To test this, we treated both native LDL and acetyl LDL with phospholipase A_2, and then determined their effects on macrophage growth. Table 3.3 shows that treatment of native or acetyl LDL with PLA_2 resulted in conversion of more than 90% of PC to lysoPC. Oxidized LDL contained 60% less lysoPC than PLA_2-treated acetyl LDL but was 7 to 10-fold more potent than PLA_2-treated acetyl LDL in inducing macrophage growth (Figure 3.10). When expressed as the amount of lysoPC delivered to the cytosol, the difference is even greater because the rate of uptake of acetyl LDL and extensively oxidized LDL by macrophages is the same, but degradation of oxidized LDL is much less efficient than that of acetyl LDL (137). PLA_2-treated native LDL had no effect on growth, indicating that lipoprotein internalization, and not simply transfer of lysoPC to plasma membrane, was required for growth stimulation. Taken together, the above results demonstrate that lyso PC cannot account for the stimulation of macrophage growth by oxidized LDL.
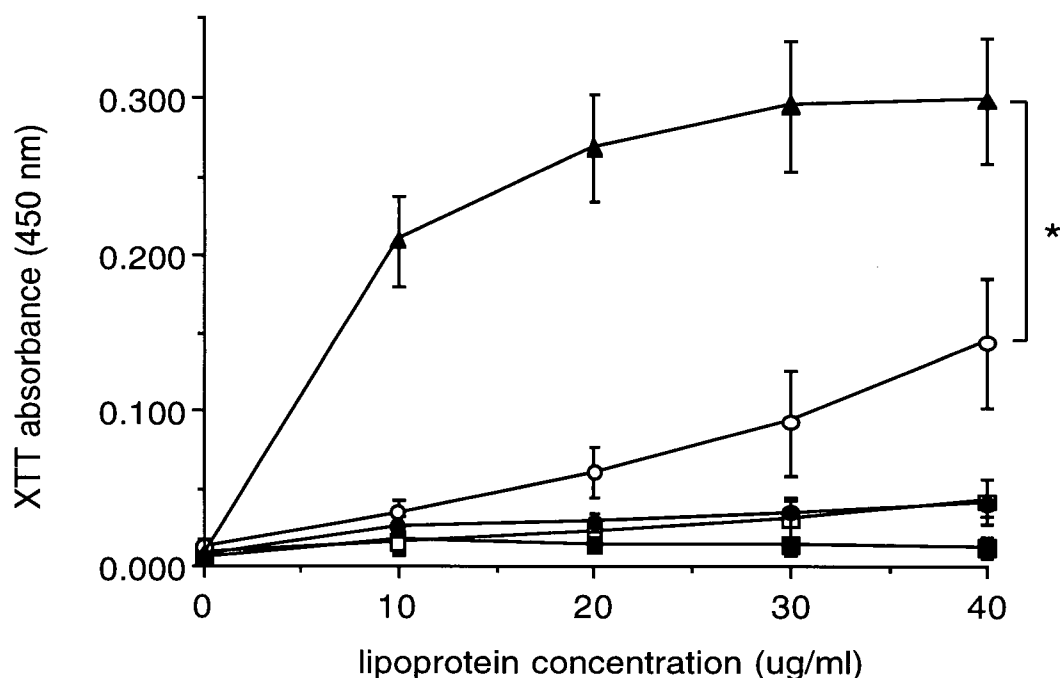
Figure 3.10. Effect of PLA2 treated native and acetyl LDL on macrophage cell growth. Resident macrophages (1.0 x 10^4 cells /well) were incubated for 4 days with the indicated concentrations of native LDL (■), acetyl LDL (●), oxidized LDL (▲), or with native (□) or acetyl LDL (○) following a 2 hour incubation with PLA2 at 37°C as described under "methods". Cell growth was then measured by XTT assay. Each value represents the mean ± standard error of quadruplicate determinations from four experiments. *P < 0.0001 by ANOVA.

3.4   The Effects of LDL Pretreatment with PAF-Acetylhydrolase Inhibitors Prior to Oxidation on Macrophage Cell Growth.

To determine whether growth induction by oxidized LDL may involve the formation of oxidatively fragmented PC phospholipids, we pretreated LDL with PMSF prior to oxidation, and then determined its ability to stimulate cell growth. PMSF is a serine esterase inhibitor that has been shown to inhibit the PLA$_2$ activity of the LDL associated PAF-acetylhydrolase enzyme by approximately 95% (44, 105). Consistent with this, our results in Table 3.4 show that when LDL is pretreated with PMSF prior to oxidation, the formation of lysoPC was less than 10% compared to control oxidized LDL. We have also shown that incubation of LDL with PMSF has

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Table 3.4 LDL pretreatment with PMSF prior to oxidation abolishes the generation of lysoPC.

	PC	lysoPC
	nmol PO$_4$ / mg protein	
Native LDL	678 ± 97	20 ± 7
Acetyl LDL	606 ± 54	44 ± 8
Oxidized LDL	355 ± 99	239 ± 46
PMSF pretreated Oxidized LDL	387 ± 14	48 ± 6

Native LDL (1.5 mg) was incubated with 5 mM PMSF for 1 hour at 37°, oxidized at 200 μg/ml, and the PC and lysoPC content of each lipoprotein determined as described in "methods". Values represent the mean ± standard error of duplicate determinations from three experiments.

little effect on the REM of oxidized LDL (REM of 3.55 versus 3.84 for PMSF treated and untreated oxidized LDL respectively compared to native LDL, data not shown), consistent with the findings of other investigators (105). It has been previously shown that following inhibition of PAF-AH with PMSF, oxidation of LDL results in the accumulation of oxidized PC compounds that contain short-chain polar acyl fragments in the sn-2 position, and exhibit PAF like bioactivity (85, 104-105, 148). Most of these oxidized PC compounds migrate between PC and lysoPC on thin-layer chromatography. The results in Table 3.4 suggest that nearly 40% of the original PC content of PMSF-pretreated oxidized LDL exists as oxidized PC. When the lipoproteins described in Table 3.4 were tested for their ability to stimulate macrophage growth, it was shown that PMSF pretreated oxidized LDL was 30-40% more effective than untreated oxidized LDL (Figure 3.11), suggesting that growth stimulation by oxidized LDL may be attributable to oxidized PC.
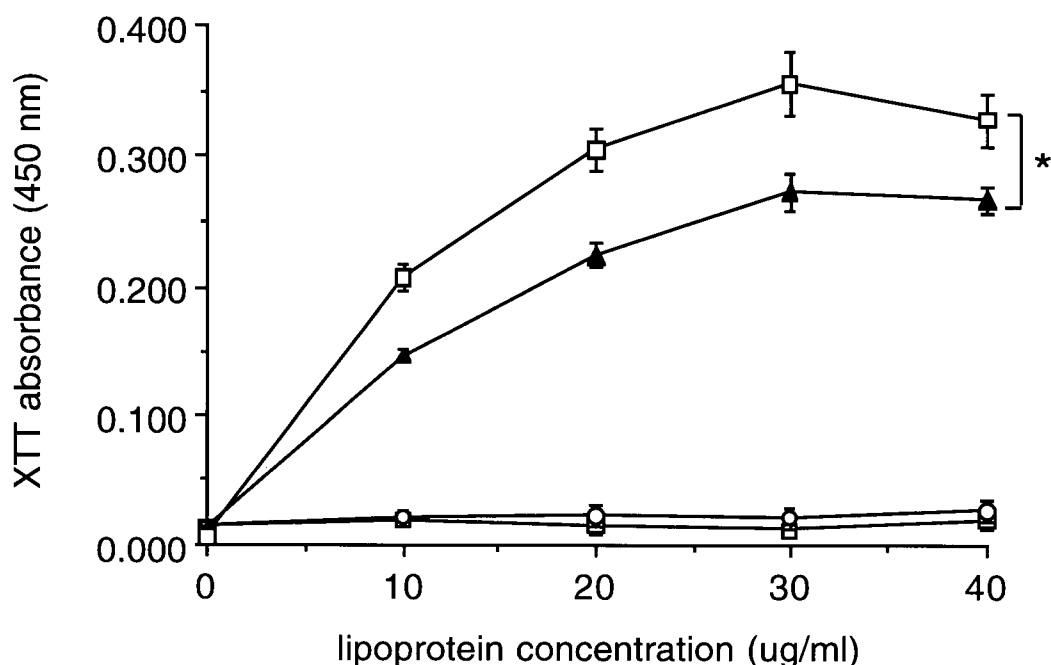
Figure 3.11. Pretreatment of LDL with PMSF prior to oxidation enhances macrophage cell growth. Resident macrophages (1.0×10^4 cells /well) were incubated for 4 days with the indicated concentrations of native LDL (■), acetyl LDL (O), oxidized LDL (▲), or with LDL that was pretreated with 5 mM PMSF for 1 hour at 37°C prior to oxidation (□) as described under "methods". Cell growth was then measured by XTT assay. Each value represents the mean ± standard error of quadruplicate determinations from four experiments. * $p < 0.001$ by ANOVA.

3.5   Macrophage Growth by Oxidized LDL Involves PAF Receptor Activation.

Heery and colleagues previously reported that the growth of smooth muscle cells is stimulated by oxidized PC through activation of the PAF receptor (85). Because the results described above implicated oxidized PC in growth stimulation in macrophages, the PAF receptor seemed to be an obvious candidate to test as a mediator of this effect. Accordingly, macrophages were treated with the PAF receptor inhibitor L-659,989 prior and during incubation with oxidized LDL, and their proliferative response examined. Figure 3.12 shows that L-659,989
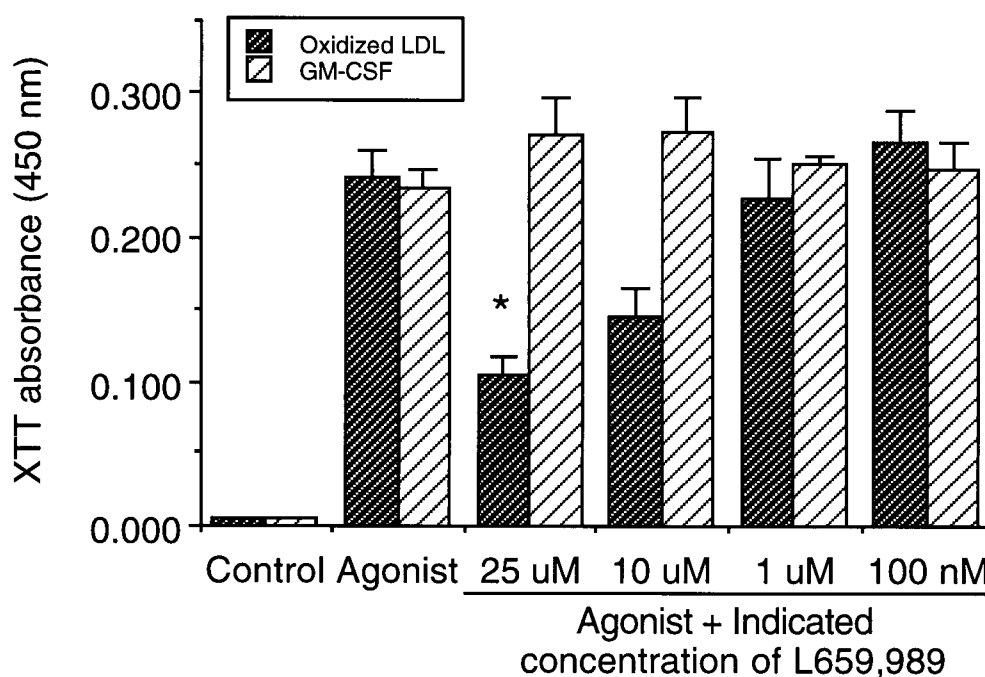
Figure 3.12. Macrophage growth by oxidized LDL is partially blocked by an antagonist to the PAF receptor. Resident macrophages (2.0 x 10^4 cells /well) were preincubated for 30 min. with varying concentrations of the PAF receptor antagonist L 659,989 and then incubated for 4 days in medium with 5% FBS with either 30 μg/ml oxidized LDL or 10 ng/ml GM-CSF (which generate a similar mitogenic response). Control and agonist conditions received vehicle only (0.1% DMSO) during preincubation. Cell growth was then measured by XTT assay. The results are expressed as a percentage of values obtained with oxidized LDL alone. Each value represents the mean ± standard error of quadruplicate determinations from three experiments. *$P < 0.005$ between oxidized LDL (agonist) and oxidized LDL (25 μM L 659,989) conditions (by unpaired Student's t-test).

blocked most of the macrophage growth stimulation by oxidized LDL. There was no morphologic evidence of cytotoxicity with up to 25 μM L-659,989 and this drug had no effect on macrophage growth stimulation by 10 nM GM-CSF. Based on these observations, we suggest that the principal mediator of the growth promoting effect of oxidized LDL on macrophages is not lysoPC, but an oxidized phospholipid that transmits its proliferative signal via activation of the PAF receptor.

3.6 The Role of Scavenger Receptor-AI/II in Stimulation of Macrophage Growth by Oxidized LDL

The scavenger receptor AI/II (SR-AI/II) is responsible for about 30% of the uptake of oxidized LDL by murine macrophages (27). Hence, if oxidized LDL uptake is essential for growth stimulation in macrophages, one would expect a proportionate decrease in growth stimulation in SR-AI/II deficient macrophages compared to control cells. On the other hand, if uptake was not required or at least was not rate-limiting, then one would expect no difference
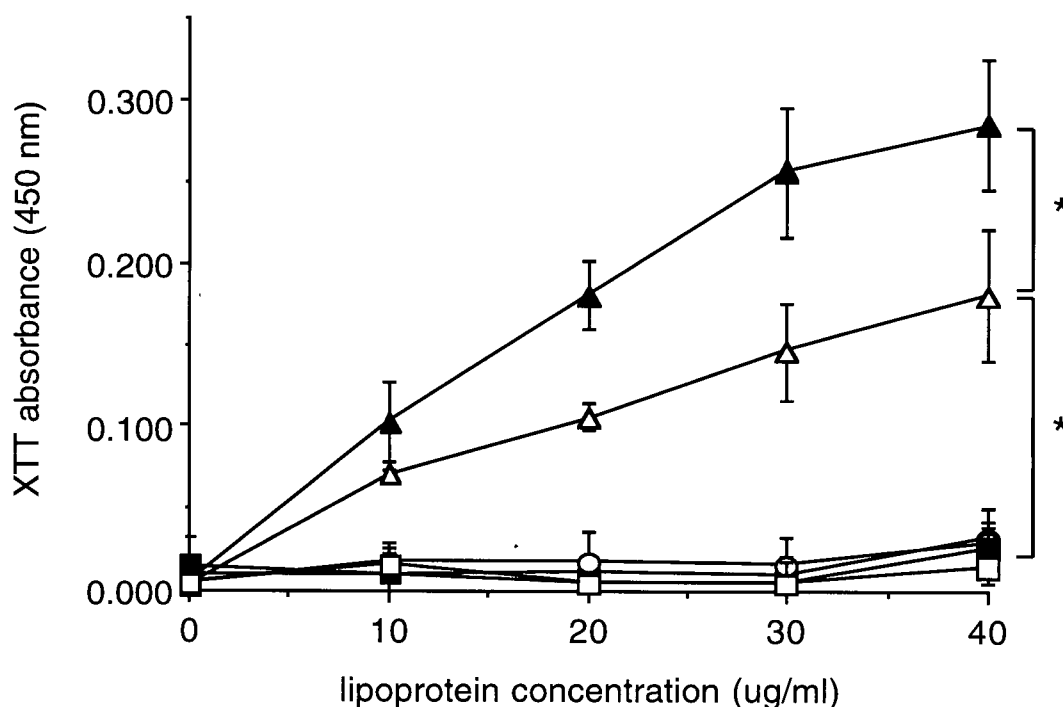
Figure 3.13. The growth stimulating effects of oxidized LDL on SR-AI/II knockout macrophages. Resident macrophages were obtained from Swiss CD-1 or SR-AI/II knockout mice, plated at a density of 1.0×10^4 cells /well, and incubated for 4 days in medium with 5% FBS with the indicated concentrations of native LDL (■), acetyl LDL (●), or oxidized LDL (▲) without a medium change. Closed and open symbols represent cells from CD-1 and SR-AI/II knockout mice. Cell growth was then measured by XTT assay. Each value represents the mean ± standard error of quadruplicate determinations from three experiments. *$P < 0.01$ by ANOVA.

between control and SR-AI/II knockout macrophages with respect to stimulation by oxidized LDL. Figure 3.13 shows that there was indeed less growth stimulation by oxidized LDL in SR-AI/II-deficient macrophages compared to controls. The magnitude of the decrease observed in growth stimulation (about one-third) is similar in magnitude to the decrease in oxidized LDL uptake by these cells. Hence, it appears that SR-AI/II is not essential for growth stimulation but simply provides an additional pathway for the internalization of oxidized LDL. Qualitatively similar results were reported by Sakai et al. (98).

3.7   Protein-Tyrosine Phosphorylation in Oxidized LDL Treated Macrophages.

In response to the finding that macrophage growth by oxidized LDL may involve PAF receptor activation, we next examined the ability of oxidized LDL to promote protein-tyrosine phosphorylation, an important event in PAF receptor mediated signal transduction. For these experiments, differentiated THP-1 macrophages were exposed to 40 μg/ml of either native or oxidized LDL and cell lysates were assayed for total tyrosine-directed protein phosphorylation by immunoblotting with 4G10 anti-phosphotyrosine antibody. As shown in Figure 3.14, phosphorylation of cellular proteins was increased with exposure of cells to oxidized LDL, becoming maximal after 10 minutes, and returning to basal levels by 15 minutes. Stimulation of cells with LPS is shown here as a positive control for tyrosine-directed protein phosphorylation. Exposure of cells to native LDL had no effect (data not shown). In response to either LPS or oxidized LDL, increased tyrosine phosphorylation is evident in a variety of protein bands with apparent molecular weights of 85 kDa, 100 kDa, and 110 kDa. Phosphorylation of proteins in the 55-65 kDa range is also seen to increase after only 5 minutes of incubation with oxidized LDL. Increased phosphorylation of the 85kDa band suggested the possibility that oxidized LDL may stimulate PI 3-kinase activation in macrophages by phosphorylation of its p85 regulatory subunit. Figure 3.15 shows that the patterns of increased phosphorylation with oxidized LDL and LPS are similar but not identical as evidenced by densitometric quantification of the bands.

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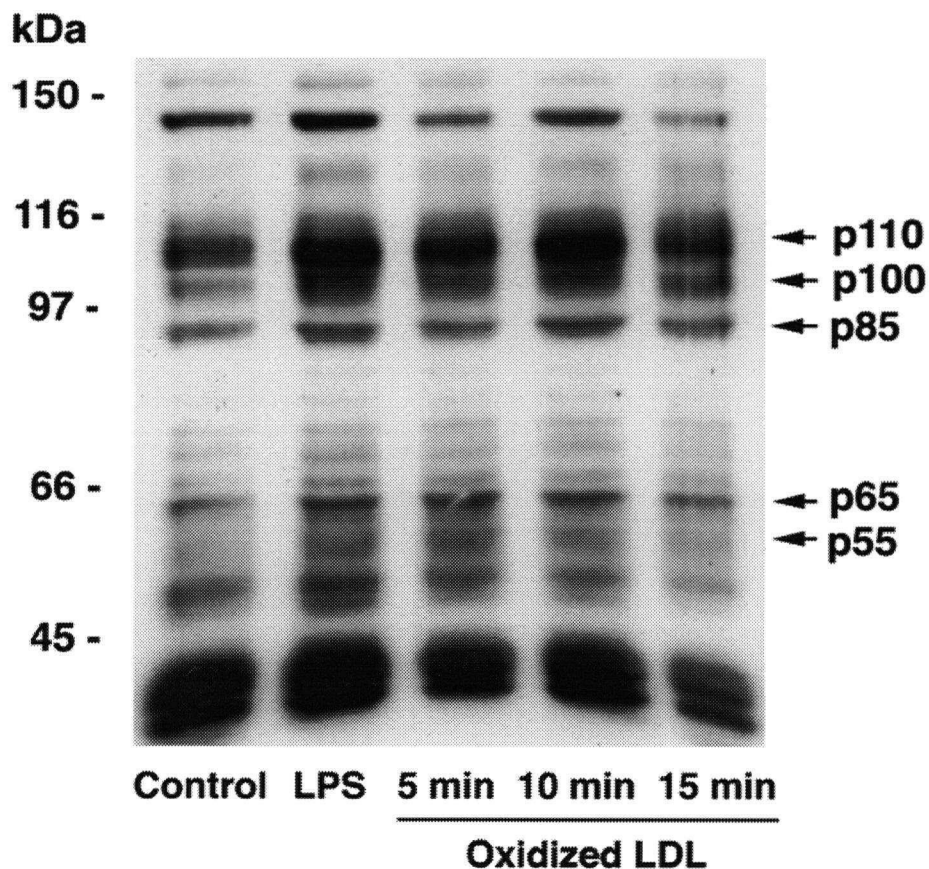
Figure 3.14. Effects of oxidized LDL on protein-tyrosine phosphorylation in THP-1 macrophages. THP-1 cells (5×10^6 cells/dish) were induced to differentiate by incubation for 24 hr with 10 nM PMA. Cells were then incubated in either medium alone (control), 1 µg/ml LPS for 15 min., or with 40 µg/ml oxidized LDL for the times indicated. Cell lysates (50 µg protein) were then analyzed by immunoblot using phosphotyrosine-specific monoclonal antibody. The results shown are from one of three independent experiments that yielded similar results. Increased tyrosine-directed phosphorylation is evident in proteins with corresponding molecular weights of approximately 55-65 kDa, 85 kDa, 100 kDa, and 110 kDa when compared with lysates from untreated controls. Native LDL (40 µg/ml) had no effect on protein tyrosine phosphorylation (not shown).

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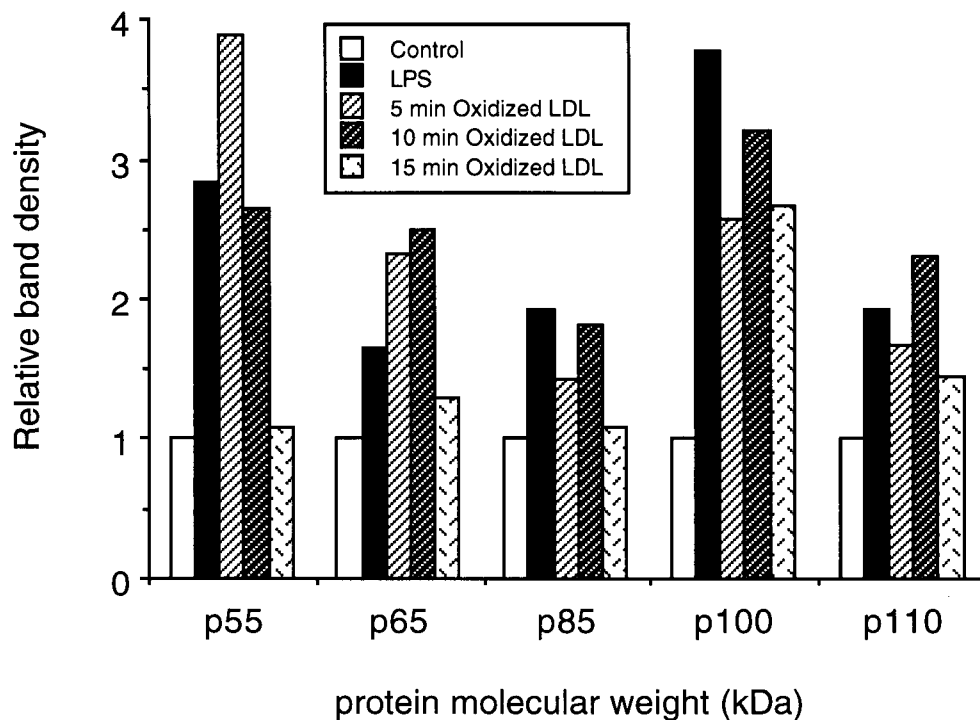
Figure 3.15. Band densitometry of LPS and oxidized LDL induced protein-tyrosine phosphorylation. Band densitometry was performed on the 4G10 immunoblot from figure 16 on bands in the regions of 55-65 kDa, 85 kDa, 100 kDa, and 110 kDa to show relative differences in phosphorylation intensity between LPS and oxidized LDL stimulated samples. The patterns of tyrosine phosphorylation by these two agonists were similar, but not identical. The results shown are from one of three independent experiments with similar results.

3.8 Phosphatidylinositol 3-Kinase Activity in Macrophages Treated with Oxidized LDL.

To further explore the intracellular signaling events triggered by oxidized LDL in macrophages, we next tested the ability of oxidized LDL to activate phosphatidyltinositol 3-kinase, an important regulator of both PAF and growth factor mediated signal transduction (121-122, 127). For these experiments, THP-1 macrophages were incubated with either native or oxidized LDL, and whole cell lysates were examined for PI 3-kinase activity. The time course
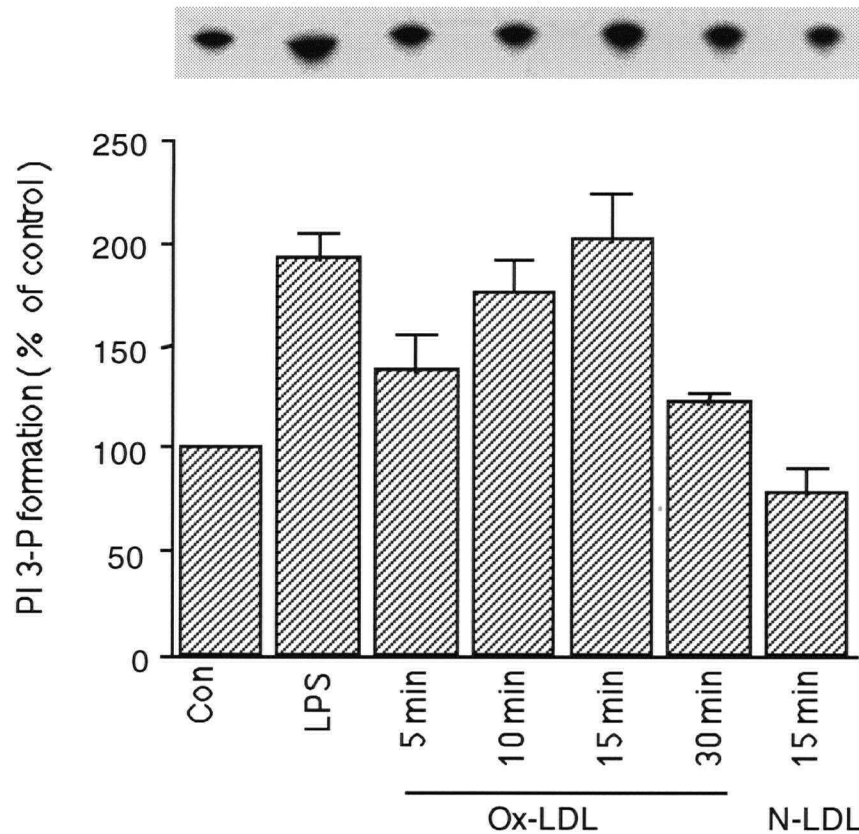
Figure 3.16. Time course of PI 3-kinase activation in THP-1 macrophages treated with oxidized LDL. THP-1 cells (5×10^6 cells/dish) were induced to differentiate by incubation for 24 hr with 10 nM PMA, and were then treated with PBS alone (control), with 1ug/ml LPS for 15 min. or with 40 μg/ml oxidized LDL or native LDL for the indicated times. PI 3-kinase was immunoprecipitated from equivalent amounts of cell lysates, incubated with [gamma-^{32}P] ATP and phosphatidylinositol, and labeled PI 3-P was detected by thin-layer chromatography and autoradiography (top panel). To quantify PI 3-kinase activity, spots corresponding to PI 3-P were scraped and counted in a liquid scintillation spectrometer (lower panel). Results are expressed as percent of activity in untreated cells. The values shown represent the mean ± standard error of three independent experiments, each using fresh LDL samples from different donors. Compared to control, $p < 0.05$ for oxidized LDL incubated for 10 or 15 minutes (by unpaired Student's t-test).

of activation of PI 3-kinase by oxidized LDL is shown in Figure 3.16. After 15 minutes of incubation with oxidized LDL, PI 3-kinase activity as significantly increased to a maximum of approximately 2-fold that of control, and returned to near basal levels by 30 minutes. Incubation of macrophages with native LDL for 15 minutes had no stimulatory effect on PI 3-kinase activity
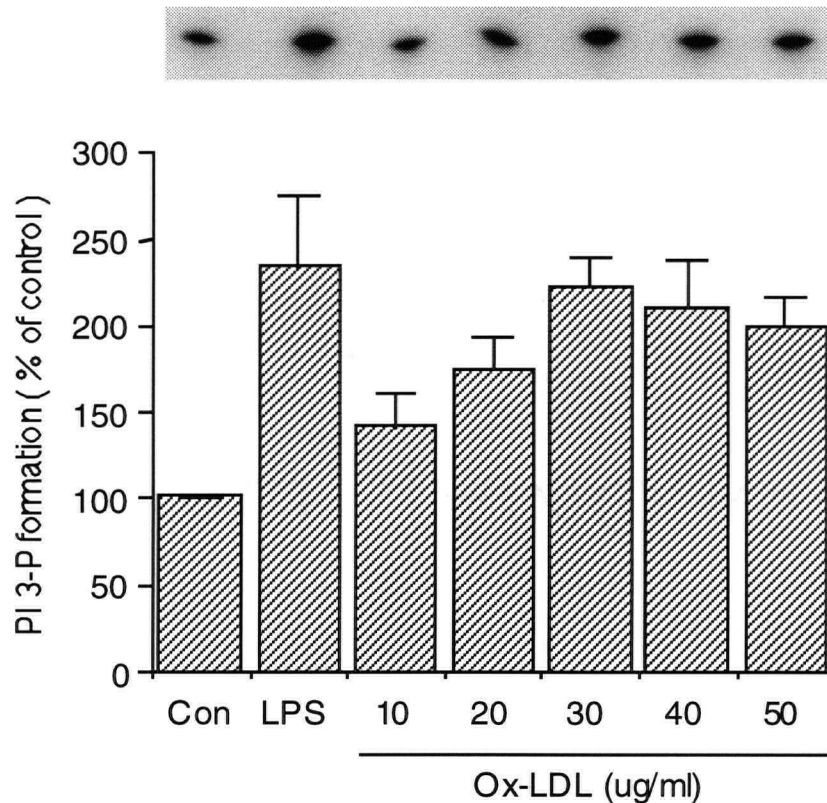
Figure 3.17. Dose dependence of PI 3-kinase activation in response to oxidized LDL in THP-1 cells. PMA-differentiated THP-1 cells ($5x10^6$) were treated with PBS alone (control), with 1ug/ml LPS for 15 min. or with the indicated concentrations of oxidized LDL for 15 min. Equivalent amounts of cell lysates were assayed for PI-3K activity, as described in the legend to Figure 2. The top panel shows autoradiogram of the PI-3P zone, and the lower panel shows radioactivity in PI-3P expressed as percent of control. The values shown represent the mean ± standard error of three independent experiments, each using fresh LDL samples from different donors. Compared to control, $p < 0.05$ for all concentrations of oxidized LDL (by unpaired Student's t-test).

compared to control. Figure 3.17 shows the effects of different concentrations of oxidized LDL on activation of PI 3-kinase. Increased PI 3-kinase activity was detectable at oxidized LDL concentrations as low as 10 µg/ml, and was maximal with 30 µg/ml oxidized LDL. To determine if the increased in PI 3-kinase activity was accompanied by increased phosphorylation of the p85 subunit, as suggested by the increased phosphorylation of an 85 kDa protein observed in figure 3.14, cell lysates were immunoprecipitated with a monoclonal antibody to PI 3-kinase, and then parallel immunoblots were performed with anti-phosphotyrosine and anti-PI 3-kinase antibodies.

As illustrated in figure 3.18, the intensity of the p85 band on anti-PI 3-kinase immunostaining was shown to be constant, indicating the presence of similar amounts of p85 protein (B). However, the increase in tyrosine phosphorylation of an 85 kDa protein observed (A) was not found to be associated with increased tyrosine phosphorylation of p85 from PI 3-kinase (C).
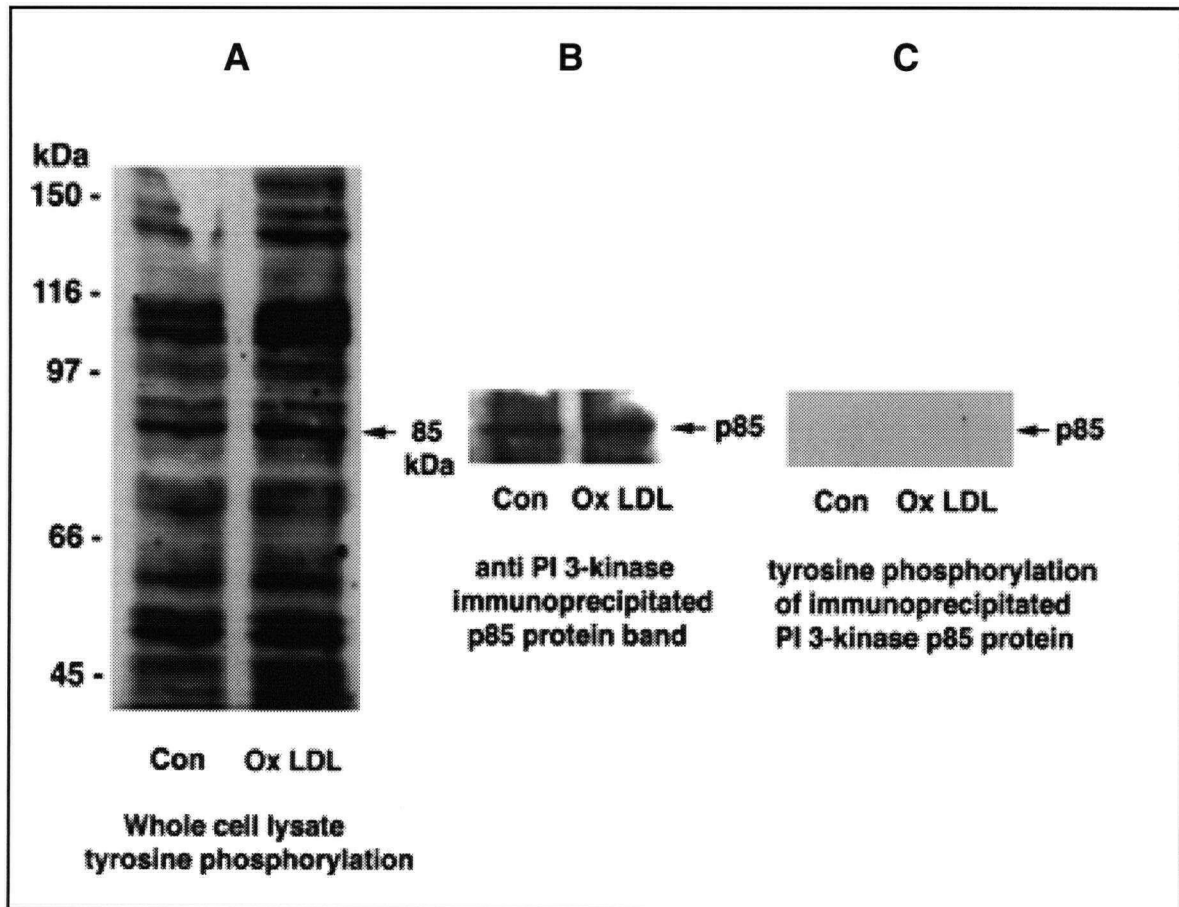
Figure 3.18. Oxidized LDL induced activation of PI 3-kinase does not require p85 subunit phosphorylation. THP-1 cells ($2x10^6$ cells/dish) were induced to differentiate by incubation for 24 hr with 10 nM PMA. Cells were then incubated with PBS alone (control) or with 40 μg/ml oxidized LDL for 15 minutes. Cell lysates (50 μg protein) were then analyzed for protein tyrosine phosphorylation by immunoblot using a 4G10 phosphotyrosine-specific monoclonal antibody (A), or immunoprecipitated with monoclonal antibody to PI 3-kinase, and parallel immunoblots performed using monoclonal antibodies specific for the p85 subunit of PI 3-kinase, or phosphotyrosine (B and C respectively). The results shown are from one of two independent experiments with similar results.

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These findings suggest that tyrosine phosphorylation of the regulatory subunit of PI 3-kinase is not involved in activation of the enzyme in response to oxidized LDL. This conclusion is consistent with the prevailing view that activation of PI 3-kinase in response to growth factors and cytokines does not involve tyrosine phosphorylation of p85 (132). To exclude the possibility that the stimulatory effect of oxidized LDL on PI 3-kinase might simply be an artifact of LPS contamination of LDL preparations, endotoxin levels in oxidized LDL were determined by sensitive limulus lysate assay, and tested for their ability to stimulate PI 3-kinase. Endotoxin levels in both native and oxidized LDL preparations were consistently shown to be less than 100 pg/ml (in LDL samples concentrated to 1 mg/ml). Control experiments indicated that similar concentrations of LPS alone (100 pg/ml), or 10 fold higher concentrations of LPS (1 ng/ml) in
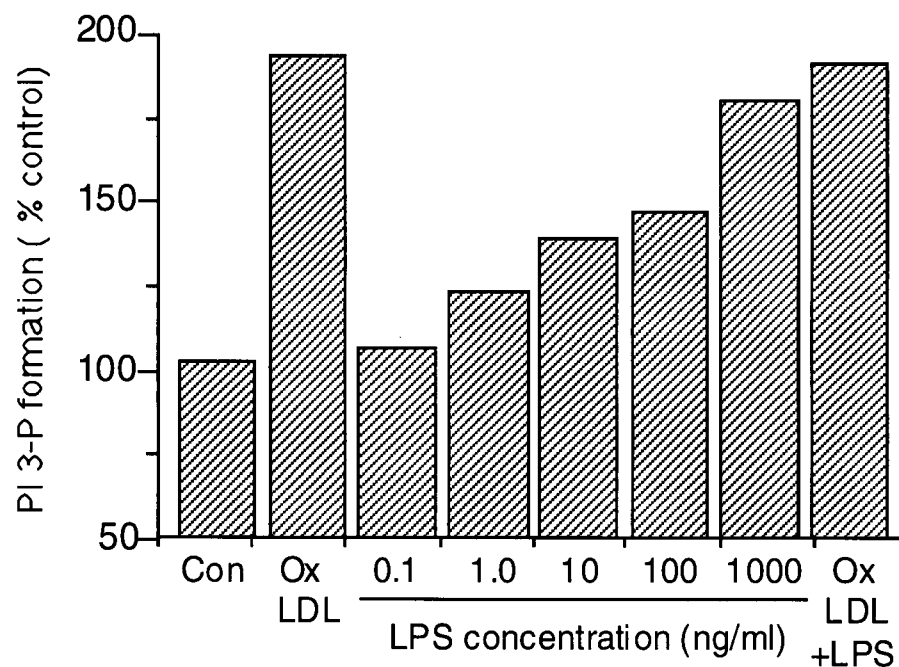
Figure 3.19. LPS does not account for oxidized LDL induced activation of PI 3-kinase. THP-1 macrophages (5x10^6) were treated with PBS alone (control), or with 30 µg/ml oxidized LDL, varying concentrations of LPS (100 pg/ml to 1 µg/ml), or 30 µg/ml oxidized LDL supplemented with 10 ng/ml LPS for 15 minutes. Equivalent amounts of cell lysates were then assayed for PI 3-kinase activity, as described in the legend to Figure 18. The results are expressed as percent of radioactive PI-3P formation and are from one of two independent experiments with similar results, each using fresh LDL samples from different donors.

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the presence of oxidized LDL had no additional stimulatory effect on PI 3-kinase activity (Figure 3.19). These findings suggest that the stimlatory effect of oxidized LDL on PI 3-kinase is not the result of LPS contamination of oxidized LDL preparations.

3.9 Effect of PI 3-Kinase Inhibitors on Oxidized LDL Induced Macrophage Cell Growth.

To determine if the increase in PI 3-kinase activity observed was required for the induction of macrophage cell growth by oxidized LDL, mouse peritoneal macrophages were pretreated with the PI 3-kinase inhibitors wortmannin and LY294002 and then incubated with 30 µg/ml oxidized LDL. As shown in Figure 3.20, pretreatment of cells with 100 nM wortmannin or 20 µM LY294002 inhibited oxidized LDL induced macrophage growth by approximately 40 and 50% respectively. There was no further inhibition observed with 2-fold higher concentrations of inhibitors, and wortmannin was without toxic effects to cells at concentrations up to 1 µM, as judged by trypan blue dye exclusion assay. Although we did not measure PI 3-kinase activity throughout the 4-day incubation period, these concentrations of inhibitors completely block PI 3-kinase activity in human monocyte-derived macrophages (131), in human and murine B-cells (133), and in several other cell types (149-151). Therefore, it seems unlikely that incomplete inhibition of growth is due to incomplete inhibition of PI 3-kinase.
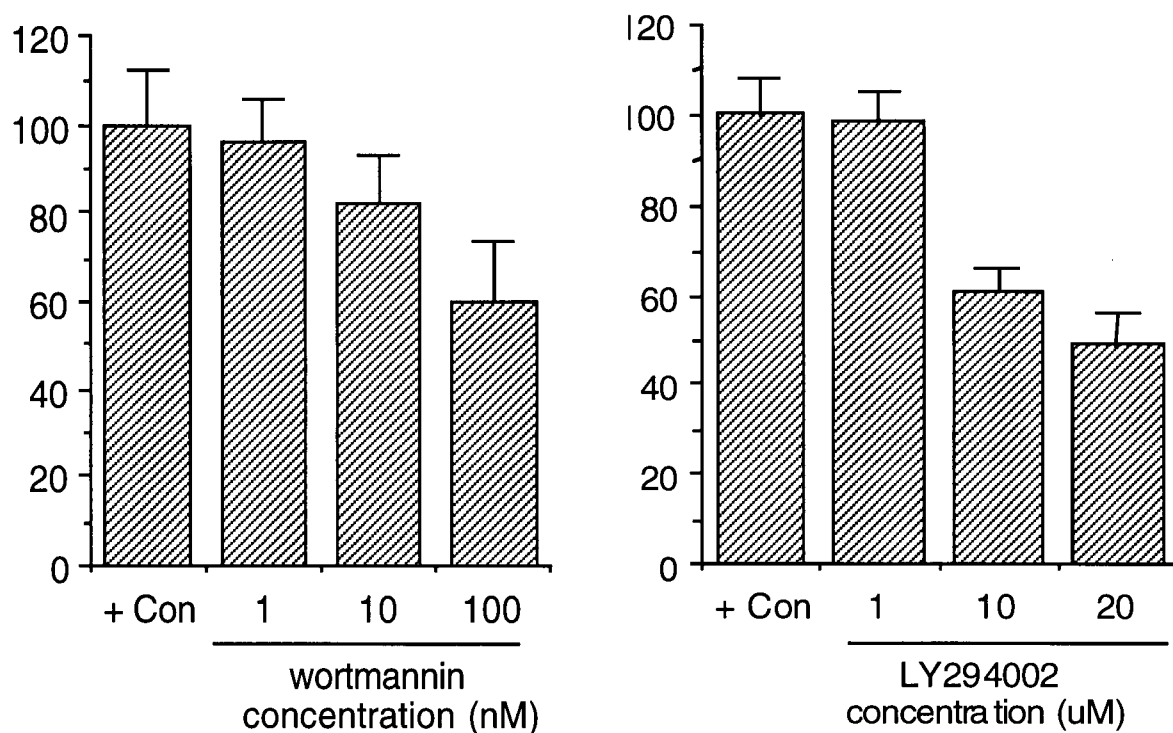
Figure 3.20. PI 3-kinase inhibitors wortmannin and LY294002 attenuate oxidized LDL induced macrophage cell growth. Resident mouse peritoneal macrophages were plated at a density of 1.0×10^4 cells/well. Cells were then preincubated with the indicated concentrations of wortmannin (A) or LY294002 (B) in RPMI medium containing 5% fetal bovine serum. Control wells received vehicle only (0.1% DMSO). After a 20 min. preincubation, 30 µg/ml oxidized LDL was added and cells were incubated for 4-days. Cell growth was then measured using XTT assay. The results are expressed as a percentage of values obtained with oxidized LDL alone. The values shown represent the mean ± standard error of quadruplicate determinations from four independent experiments. Compared to control, $p < 0.05$ for 10 and 100 nM wortmannin, and 10 and 20 µM LY294002 (by unpaired Student's t-test).

Chapter Four   Discussion

Oxidized LDL has recently been shown to be mitogenic towards several types of cells including murine peritoneal macrophages, human monocyte-derived macrophages, and vascular smooth muscle cells (85, 96-97). In macrophages, the mitogenic effect has been attributed to lysophosphatidylcholine, and requires scavenger receptor-mediated internalization of oxidized LDL for growth stimulation to occur (98). In smooth muscle cells, the mitogenic effect was found to be mediated by oxidized phospholipids that interacted with the PAF receptor (85). With respect to these studies, it is not clear whether the divergent conclusions can be explained by oxidized LDL acting through different receptor pathways in smooth muscle cells and macrophages, or if both lysophosphatidylcholine and oxidized phospholipids can activate similar signaling pathways leading to mitogenesis in these cells.

The objective of the present study was to determine the relative importance of both lyso and oxidized PC in the effect of oxidized LDL, investigate the role of both scavenger and PAF receptors in macrophage growth by oxidized LDL, and further examine the intracellular signaling pathways that may be involved in the induction of macrophage growth by oxidized LDL. The main findings in this report are first, that the stimulation of macrophage growth by oxidized LDL depends on structural changes to the LDL particle associated with very extensive oxidation, second, that oxidized phospholipids and not lysoPC account for the growth effect of oxidized LDL, third, that this effect can be blocked by approximately 60% by a PAF receptor antagonist, and fourth, that PI 3-kinase is involved in the induction of macrophage growth by oxidized LDL. These findings suggest an important role for PAF receptor mediated signaling in the mitogenic effect of oxidized LDL in macrophages.

Sakai and colleagues were the first to describe the stimulation of macrophage growth by oxidized LDL. This group concluded that the effect of oxidized LDL was due to lysoPC and required lipoprotein internalization by SR-AI/II (97-98). Although these interpretations appear to conflict with ours, the actual data are in general agreement. Specifically, we can confirm their

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findings that PLA$_2$-treated acetyl LDL can mimic the effect of oxidized LDL, and that macrophages lacking scavenger receptors are less responsive to oxidized LDL than control cells. However, we showed that the effect on growth of differently oxidized LDL preparations does not correlate well with their content of lysoPC, that removal of more than 97% of lysoPC from oxidized LDL does not greatly reduce its effect on growth stimulation, and that oxidized LDL is almost an order of magnitude more potent than PLA$_2$-treated acetyl LDL in its ability to stimulate macrophage growth. Hence, the effect on growth cannot be accounted for by lysoPC. The difference in findings with SR-AI/II-deficient mice appear to be quantitative - Sakai and colleagues find a greater proportion of oxidized LDL uptake to be mediated by the SR-AI/II than we have noted, and hence it is not surprising that there is also a greater effect on growth. This discrepancy most likely is a reflection of differences in oxidized LDL preparations between laboratories (as previously discussed), as the same strain of SR-AI/II-deficient transgenic mice were used in both cases (141).

Heery and coworkers have analyzed of the effects of oxidized LDL on growth of arterial smooth muscle cells (85). It was found that oxidized LDL (but not native LDL) induced smooth muscle cell growth, and that this effect was mimicked by PAF and could be blocked by the PAF receptor antagonist WEB 2086. Furthermore, it was shown that the growth stimulation was associated with a lipid fraction intermediate in polarity between PC and PAF, consistent with oxidized PC. This biological activity of this component was eliminated by PAF acetylhydrolase and in fact, active fractions could only be recovered from LDL that had been pretreated with diisopropylfluorophosphate to inactive PAF acetylhydrolase prior to oxidation. These observations in smooth muscle cells are congruent with our findings in macrophages that growth stimulation is inhibited by a PAF receptor antagonist and is increased by inactivation of PAF acetylhydrolase. Although we were unable to recover growth-inducing activity in a lipid extract of oxidized LDL, it seems likely that similar species of oxidized PC are responsible for PAF-receptor mediated growth stimulation in both cell types.

To date, there has been no detailed characterization of the structure of the oxidized phospholipids in oxidized LDL that are responsible for PAF receptor activation. Prescott's group identified 1-palmitoyl-2-[5-oxovaleroyl] PC in oxidized LDL and showed that this compound is a substrate for PAF acetylhydrolase (103, 148), and can increase thymidine incorporation in smooth muscle cells (85). However, the maximal effect of 2-[5-oxovaleroyl] PC was only a 33% increase over control, whereas PAF and oxidized LDL more than doubled thymidine incorporation. This suggests that there may be compounds more potent than 2-[5-oxovaleroyl] PC that account for most of the growth stimulation. Our finding that the active components could not be removed by preincubation of oxidized LDL with albumin suggests that they are not highly polar PC derivatives, and hence may have a residue longer than 5-oxovaleroate at the 2-position. One such PC oxidation product could be F_2-isoprostanoyl PC, which has been shown to be formed during nonenzymatic oxidation of LDL (152). It remains unclear how such compounds (with an ester rather than an ether in the 1-position and a long-chain acyl derivative in the 2-position) could be capable of activating the PAF receptor but they appear to be substrates for PAF acetylhydrolase, which has a similar substrate specificity as the PAF receptor (152).

The evidence implicating the PAF receptor in these studies is based mainly on the growth inhibitory effect of the PAF receptor antagonist L659,989. An obvious concern is that this compound might have a nonspecific growth-inhibitory effect. However, this drug had no effect on growth stimulation of macrophages with a dose of GM-CSF that resulted in very similar growth stimulation to that seen with oxidized LDL. Furthermore, Heery and colleagues showed that this compound, and a structurally unrelated PAF receptor blocker WEB 2086, both blocked the activation of the PAF receptor by oxidized LDL to a similar extent (85).

There are several previous reports that PAF receptor activation can promote cell growth either directly (116-117), or by potentiation of the effect of PDGF (153). The signal transduction pathways involved in these systems have not been clearly defined. However, it is known that PAF receptor stimulation can lead to the activation of a number of signaling pathways involved

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in mitogenesis, including activation of protein tyrosine kinases (108, 112), PI 3-kinase (113), phospholipase D (PLD) (154) protein kinase C (PKC) (114), and mitogen activated protein kinase (MAPK) (115). Interestingly, many of the same intracellular messengers involved in PAF receptor mediated signaling have been shown to become stimulated by oxidized LDL in a variety of cell types. Our results show that oxidized LDL induces tyrosine phosphorylation of several different proteins in THP-1 macrophages. The main substrates detected have apparent molecular weights of 55-65 kDa, 85 kDa, 100 kDa, and 110 kDa. Maximal phosphorylation of the 55-65 kDa proteins was noted 5 min. after addition of oxidized LDL, while the higher molecular weight proteins showed maximal phosphorylation after 10 min. The patterns of increased phosphorylation between oxidized LDL and LPS appeared to be similar, but were shown not to be identical by band densitometry. We also show that PI 3-kinase is rapidly and transiently activated by oxidized LDL in a dose and time dependent manner, that this increased activity was not the result of LPS contamination of oxidized LDL. When we examined the role of PI 3-kinase in oxidized LDL induced macrophage growth, we observed that the growth stimulating effect of oxidized LDL was attenuated by approximately 50% when cells were pretreated with concentrations of PI 3-kinase inhibitors that completely block enzyme activity (100 nM wortmannin and 20 µM LY294002). Hence, it is attractive to speculate that at least part of the growth stimulation of macrophages by oxidized LDL is a result of activation of PI 3-kinase by the PAF receptor.

These findings are consistent with previous evidence supporting a role for PI 3-kinase in mitogenic signaling. For example, Iwama et al. have shown that association of PI 3-kinase with the PDGF receptor of vascular smooth muscle cells appears to be necessary for PDGF induced cellular mitogenesis (128). Yusoff et al. concluded that PI 3-kinase activation may be involved in growth stimulation of bone marrow-derived macrophage by hematopoietic growth factors CSF-1 and GM-CSF (125). In these studies, CSF-1 and GM-CSF stimulated a dose-dependent activation of PI 3-kinase, while concavalin A, phorbol myristyl acetate, and formyl-methionyl-

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leucyl-phenylalanine had no mitogenic activity in these cells and did not significantly increase PI 3-kinase activity.

Several other studies have also shown that incubation of macrophages with modified LDL's leads to rapid tyrosine phosphorylation of several intracellular proteins, including a member of the Src tyrosine kinase family, p53/p56 Lyn (121). Activated tyrosine kinases such as p53/p56 Lyn have been shown to physically interact with and activate PI 3-kinase in human monocytes and B-lymphocytes (130-131). Association of intracellular PI 3-kinase with activated tyrosine kinases is thought to be mediated by either SH3 domains of tyrosine kinases (155), or via SH2 domains of the p85 regulatory subunit of PI 3-kinase (132) leading to increased PI 3-kinase activity. It has been suggested that either or both of these mechanisms may account for PI 3-kinase activation, however, it is not clear whether phosphorylation of the p85 subunit of PI 3-kinase is required for its activation (131). Several reports have suggested that PI 3-kinase activation can occur without SH2 domain phosphorylation of the p85 subunit, while, in contrast, several phosphorylation sites have been found in the p85 subunit, and phosphorylation of one or more of these sites has been suggested as a mechanism leading to PI 3-kinase activation (129, 132). Although our results indicate an increase in protein-tyrosine phosphorylation of an 85 kDa protein band with oxidized LDL stimulation, no detectable increase in phosphorylation of the p85 subunit of PI 3-kinase was observed.

While the findings of this study indicate that the growth promoting effects of oxidized LDL are PI 3-kinase dependent, it is also evident that additional pathways independent of PI 3-kinase are also likely involved. Another potential target for oxidized LDL-induced mitogenic signaling is protein kinase C (PKC). Sakai and colleagues have suggested a role for PKC in oxidized LDL-induced mitogenesis as they found that exposure of murine resident peritoneal macrophages to oxidized LDL results in rapid calcium influx and a sustained increase in intracellular calcium concentrations (97). Exposure of vascular smooth muscle cells to oxidized LDL has also been shown to increase PKC activity as well as to enhance PDGF-AA production, PDGF receptor mRNA expression, and DNA synthesis (83). In contrast, inhibition of PKC

with staurosporine decreased oxidized LDL-induced DNA synthesis (83). These findings suggest that PKC activation may contribute to the growth stimulation of both murine macrophages and vascular smooth muscle cells by oxidized LDL.

The effects of oxidized LDL on other enzymes known to be involved in mitogenesis has also been investigated . Recently, Deigner et al. demonstrated MAP kinase activation in U937 cells stimulated with oxidized LDL, independent of PKC activation (123). However, incubation of these cells with native LDL induced an even greater increase in MAP kinase activity. Kusuhara and colleagues examined the effects of native or oxidized LDL on MAP kinase activity in smooth muscle cells, endothelial cells, and macrophages. This group also found that both oxidized LDL and native LDL stimulated MAP kinase in a PKC-dependent manner, although in macrophages and smooth muscle cells, the effect oxidized LDL was significantly greater than that of native LDL. Although the role of MAP kinases in oxidized LDL induced macrophage cell growth has not been addressed, it is likely that these enzymes are directly involved in the mitogenic effect of oxidized LDL. Phospholipase D (PLD) activation has also been observed in vascular smooth muscle cells stimulated with oxidized LDL (111). PLD activation results in the generation of phosphatidic acid and lysophosphatidic acid, both of which are known to be mitogenic (156-157). However, at present there is no direct evidence linking PLD activation to the mitogenic effect of oxidized LDL.

The results of previous studies on growth stimulation by oxidized LDL suggest that a phospholipid component of oxidized LDL was required (85, 97). However, it is also possible that the modified apoB of oxidized LDL may interact with membrane proteins and stimulate growth by a process analogous to integrin-mediated signaling (158). Oxidized LDL binds to the scavenger receptor AI/II, and it has been suggested that this receptor may lead to tyrosine phosphorylation (121). However, acetyl LDL, which is an excellent ligand for the scavenger receptor AI/II does not stimulate macrophage growth, indicating that mere ligation of this receptor is not sufficient to induce growth. Oxidized LDL also binds to membrane proteins that do not interact well with acetyl LDL, including CD36, FcγRII, MARCO and macrosialin/CD68

(27). The possibility that selective binding of oxidized LDL to these or other plasma membrane proteins may initiate the activation of mitogenic signal trasduction cascades warrants further consideration.

Chapter Five.   Summary

Preliminary experiments to characterize the growth stimulating effects of lipoproteins on mouse peritoneal macrophages showed that extensively oxidized LDL induced significant increases in XTT reduction, thymidine incorporation and cell number over control while native LDL and acetyl LDL produced no such effect. Growth stimulation by oxidized LDL was dependent on degree of LDL oxidation, requiring at least 10 hours of copper incubation to achieve significant levels of growth stimulation.

To evaluate the role of lysoPC in oxidized LDL induced macrophage cell growth, oxidized LDL was incubated with fatty acid-free BSA to selectively remove lysoPC. This treatment removed nearly all lysoPC phospholipids from oxidized LDL, but only reduced its mitogenic activity by approximately 20%. In similar experiments, both native and acetyl LDL were treated with phospholipase A_2 to increase lysoPC formation and tested for their ability to stimulate macrophage growth. This treatment resulted in more than a 90% conversion of each lipoproteins PC component to lysoPC, but only enhanced the ability of acetyl LDL to stimulate growth by 30% compared to oxidized LDL.

To examine the importance of oxidized phospholipids in oxidized LDL induced macrophage growth, and to provide further evidence that lysoPC was not responsible for this effect, LDL was pretreated with PMSF to inhibit PAF-acetylhydrolase prior to oxidation. This treatment resulted in a 70-80% reduction in the hydrolysis of oxidized phospholipids to lysoPC during the oxidation of LDL, and produced a further 30% increase in growth stimulating activity over untreated oxidized LDL.

To determine the importance of the PAF receptor and SR-AI/II in macrophage growth stimulation by oxidized LDL, growth experiments were performed in the presence of PAF receptor antagonists or with SR-AI/II deficient macrophages. Here, PAF receptor inhibition with 25 µM L-659,989 reduced oxidized LDL induced growth by approximately 60-70%, while growth of SR-AI/II deficient cells by oxidized LDL was reduced by only 30% compared to

oxidized LDL incubated with control macrophages. These findings suggest that most of the mitogenic effect of oxidized LDL in macrophages is attributable to PAF receptor activation, while approximately 30% of the effect may be mediated through SR-AI/II dependent mechanisms.

In experiments designed to examine the intracellular signaling pathways activated by oxidized LDL in macrophages, oxidized LDL was found to increase protein-tyrosine phosphorylation of several protein species with molecular weights ranging from 55-110kDa, and stimulate a 2-fold increase in PI 3-kinase activity in macrophages compared to controls. In these studies, activation of PI 3-kinase by oxidized LDL was shown to occur without p85 subunit phosphorylation. In these experiments, similar concentrations of native LDL had no stimulatory effect.

Finally, to determine if oxidized LDL induced growth stimulation required activation of PI 3-kinase, cells were preincubated with PI 3-kinase inhibitors (100 nM wortmannin and 20 μM LY294002), stimulated with oxidized LDL, and cell growth examined. Following pretreatment with PI 3-kinase inhibitors, the growth stimulating activity of oxidized LDL was reduced by 40-50%. These results suggest that the mitogenic effect of oxidized LDL in macrophages is in part, mediated by the activation of PI 3-kinase.

Chapter Six.   Conclusions

The data in this study present compelling evidence that suggest oxidized phospholipids, not lysoPC, are responsible for the induction of macrophage growth by oxidized LDL, and that PAF receptor activation likely plays an important role in this process. In addition, the data also provide strong evidence for a direct link between oxidized LDL-induced PI 3-kinase activation and macrophage growth. These findings not only increase our understanding of how oxidized LDL transmits its proliferative signal in macrophages, but also give insight into the signaling pathways that may be involved in some of the other biological functions of oxidized LDL. To fully understand how oxidized LDL stimulates macrophage growth, further studies with more detailed analyses of the lipid and protein components of oxidized LDL are required in order to identify the components responsible for growth stimulation, and to define interactions with or effects mediated by other signaling pathways. The ultimate importance of in situ proliferation of macrophages in the artery wall during the development of atherosclerotic lesions is unknown and will be a challenge to ascertain. However, the present findings provide impetus to look for evidence of macrophage replication in atheromas, and to test the effects of PAF receptor antagonists in the early stages of atherogenesis in animal models.

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