Function of Lipoprotein Lipase and Endothelial Lipase in Human Macrophages

by

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ABSTRACT

Lipoprotein lipase (LPL) and endothelial lipase (EL) are expressed in atherosclerotic lesions, mainly in macrophages. However, the functional roles of LPL and EL in macrophages are not well characterized. In the present thesis, the effects of these lipases on cholesterol efflux, low density lipoprotein (LDL) catabolism, and proinflammatory cytokine secretion in human macrophages were investigated.

Lentivirus transduction successfully induced EL suppression or over-expression in macrophages. LPL suppression was mediated by lentivirus transduction whereas dexamethasone was used to stimulate LPL expression. Apolipoprotein AI- (apoAI-) mediated cholesterol efflux was modestly reduced after LPL and EL suppression, but significantly increased in lipase-overexpressing macrophages as well as transfected 293 cells. This effect was partially inhibited after the elimination of either catalytic or non-catalytic lipase function, but completely abolished when both functions were blocked. The observed effect on cholesterol efflux was mediated partially by an increased apoAI binding, an effect dependent on cell surface lipase. Lipase expression was inversely associated with phosphatidylcholine and sphingomyelin levels, but positively with lysophosphatidylcholine production, the later was shown to promote apoAI-mediated cholesterol efflux dose-dependently.

EL expression was positively correlated with both native and oxidized LDL binding and association via non-catalytic function as observed in both EL-suppressed and over-expressed macrophages. By contrast, the catalytic activity of EL did not have a significant role in oxidized LDL metabolism with the exception of a positive correlation with native LDL association, which also partially depended on the LDL receptor.

The concentration of interleukin-1ß and 6, macrophage chemoattractant protein-1, and tumor necrosis factor-α was reduced after LPL and EL suppression. The lipase suppression also amplified the inhibitory effect of oxidized LDL in macrophages. Microarray analysis indicated that >50 genes, mainly proinflammatory ones, had marked expression changes after lipase suppression.
Atorvastatin treatment reduced LPL and EL expression as well as Rho, the liver X receptor (LXR), and nuclear factor-κB (NF-κB) levels. Mechanistic studies identified LXR and NF-κB to be involved in atorvastatin-induced suppression of LPL and EL, respectively.

In summary, by promoting apoAI-mediated cholesterol efflux, lipoprotein binding and uptake, and proinflammatory cytokine expression in macrophages, EL and LPL may influence the atherogenic potential of macrophages.
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<td>FBS</td>
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<td>FDP</td>
<td>Fibrin(ogen) Degradation Product</td>
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<td>HUVEC</td>
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<tr>
<td>KO</td>
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<td>LCAT</td>
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<td>LDL(-c)</td>
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<td>LDLR</td>
<td>Low Density Lipoprotein Receptor</td>
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<td>Leukemia Inhibitory Factor</td>
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<td>Lectin-like Oxidized Low-Density Lipoprotein Receptor</td>
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<td>Lipoprotein (a)</td>
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<td>Lipoprotein Lipase</td>
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<td>LP-PLA2</td>
<td>lipoprotein-associated phospholipase 2</td>
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<td>Liver X Receptor</td>
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<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
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<td>Nicotinamide Adenine Dinucleotide Phosphate (Reduced form)</td>
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<td>NF-κB</td>
<td>Nuclear Factor Kappa Beta</td>
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<td>Nitric Oxide Synthase</td>
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<td>Oxidized Low Density Lipoprotein</td>
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<td>PAF</td>
<td>Platelet-Activating Factor</td>
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<td>1-palmitoyl-2-arachidonoyl-3-sn-phosphatidylcholine</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PARC</td>
<td>Pulmonary and Activation-Regulated CC Chemokine</td>
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<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
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<td>Phosphatidylcholine-Specific Phospholipase C</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PDGF</td>
<td>Platelet-Derived Growth Factor</td>
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<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<td>PECAM</td>
<td>Platelet-Endothelial Cell Adhesion Molecule</td>
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<td>Prostaglandin</td>
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<td>Protein Kinase C</td>
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<td>1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
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<td>PPAR</td>
<td>Peroxisome Proliferator-Activated Receptor</td>
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<td>RAP</td>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<td>shRNA</td>
<td>small hairpin Ribonucleic Acid</td>
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<td>SHR-SP</td>
<td>Stroke-prone Spontaneously Hypertensive Rat</td>
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<tr>
<td>siRNA</td>
<td>small interfering Ribonucleic Acid</td>
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<tr>
<td>SMC</td>
<td>Smooth Muscle Cell</td>
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<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<tr>
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<td>Specificity Protein</td>
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<td>secretory Phospholipase A2</td>
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<tr>
<td>SR</td>
<td>Scavenger Receptor</td>
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<td>Abbreviation</td>
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<td>--------------</td>
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<tr>
<td>SRA</td>
<td>Scavenger Receptor A</td>
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<td>Scavenger Receptor Class B, Type 1</td>
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<td>Thymus- and Activation- Regulated Chemokine</td>
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<tr>
<td>TC</td>
<td>Total Cholesterol</td>
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<tr>
<td>TG</td>
<td>Triglyceride</td>
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<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
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<tr>
<td>THL</td>
<td>Tetrahydrolipstatin</td>
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<td>TIMP</td>
<td>Tissue Inhibitors of Metalloproteinase</td>
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<td>Tumor Necrosis Factor</td>
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<td>TXA2</td>
<td>Thromboxane A2</td>
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<td>VCAM</td>
<td>Vascular Cell Adhesion Molecule</td>
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<td>Vascular Endothelial Growth Factor Receptor 2</td>
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<tr>
<td>VLDL</td>
<td>Very Low Density Lipoprotein</td>
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<tr>
<td>V_{max}</td>
<td>Maximal Rate of the Enzyme Reaction</td>
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<td>vWF</td>
<td>von Willebrand factor</td>
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<tr>
<td>WBC</td>
<td>White Blood Cell</td>
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<td>WHHL</td>
<td>Watanabe Heritable Hyperlipidemic Rabbit</td>
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ACKNOWLEDGEMENTS

I am extremely grateful to my graduate supervisor, Dr. John Hill, for his tremendous support during my study. The guidance, encouragement and inspiring discussions he provided made this project proceed as well as it did. Moreover, I highly appreciate his patience and help in preparing manuscripts and this thesis.

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Finally, I would like to acknowledge the Heart and Stroke Foundation of B.C. and Yukon for providing me a Doctoral Research Award.
DEDICATION

I dedicate this thesis

To my wife, Weiqi Wang, this thesis would not be possible without the loving support from you, you bring me joys and life goals, and you are still my strength and purpose of life.

To my mother and father, your moral support and unconditional love cross the ocean, giving me huge encouragement and strength so that I can complete this thesis. Also to my mother-in-law, how grateful I am for your selfless and countless help.

To the principle investigators and staff in the ASL lab and Healthy Heart Program, I have learned much from you during my training, and I owe you a lot.
Chapter 1. Introduction

1.1 Atherosclerosis

1.1.1 General Facts
Atherosclerosis is defined as the narrowing and hardening of large and medium-sized arteries, with the formation of atheromatous plaques containing cholesterol and lipids in the center. This insidious but progressive pathological process will lead to the disruption of artery blood flow due to either the chronic plaque volume expansion or abrupt plaque rupture and consequent thrombosis. Atherosclerosis is responsible for most cardiovascular diseases, including coronary heart diseases (CHD) such as angina pectoris and myocardial infarction, and ischemic stroke. Epidemiological studies have revealed that atherosclerosis-related cardiovascular disease is the leading cause of death (37.3 percent of all deaths) in most industrialized countries, with a prevalence of 17 per 1000 in the general population of the USA. This disease also accounts for 20,000 hospitalizations and 14,979 deaths per year. Patients afflicted with atherosclerosis have an average loss of 7.5 life years (2001 Heart and Stroke Statistical Update, American Heart Association).

1.1.2 Pathology of Atherosclerosis
Atherosclerosis typically begins in major arteries in the early years of life, and slowly proceeds over years, yet is often asymptomatic until it occludes >50% of the vascular lumen. The pathological classification (Figure 1-1) is deduced from a series of postmortem examinations of many persons who died at different ages, although the distinctions between two successive pathological stages are ambiguous in most cases. Aorta, coronary artery, and ostia of branch vessels are the predisposing sites for atherosclerosis.

1.1.2.1 Intima Thickening
The intima is defined as the region of the arterial wall from and including the endothelial surface at the lumen to the internal elastic lamina, the latter is the luminal margin of the media. Intima thickening is an adaptive reaction and regarded to be the initial lesion leading towards atherosclerosis. This lesion can be found as early as in infancy and childhood. The histological changes of this initial lesion are minor. In general, extracellular matrix, most of which is
Figure 1-1. The classification of atherosclerotic lesions. Atherosclerosis usually develops insidiously with ambiguous distinction between two consecutive categories. An initial intima thickening featuring the deposition of amorphous material and few lipoproteins progresses into yellow-appearing fatty streak when typical foam cells start to accumulate. Foam cells are derived mainly from infiltrating macrophages and to a lesser degree, smooth muscle cells. With the formation of fibrous tissue, aggregation of foam cells, and small lipid pools, an intermediate lesion develops. The persistence of risk factors drives intermediate lesion into a new stage of atheroma lesion characterized by a full-fledged fibrous cap, one or multiple necrotic lipid cores, and abundant cellularity. Advanced lesions are the continuation of atheromas, bulging inward to narrow vascular lumen. When lesions fissure or rupture, surface thrombosis surmounts with the consequence of partial or complete occlusion of vessel. Mural thrombi are the major cause of ischemic stroke of brain. (modified from Thromb Haemost 2002; 88:554-67)

Amorphorous material composed of biglycan, decorin, and chondroitin sulfates, deposits in the subendothelial space, whereas the content of heparan sulfate and hyaluronic acid are reduced.3,4 There is only a minor increase in cellularity in the intima at this stage. In the first 8 months of life, 45% of infants have macrophages in their coronary arteries.5,6 Smooth muscle cells (SMCs) in the intima may be the main source responsible for the production of extracellular matrix (ECM).7 Macrophages and smooth muscles are sparsely distributed, typical “foam cells” and
extracellular lipid deposition are seldom observed. The internal elastic lamina remains undisrupted at this stage. This initial stage of atherosclerosis can only be detected microscopically.

1.1.2.2 Fatty Streak
If atherosclerosis progresses, the next stage is termed fatty streaks, which are yellow streaks, spots, or patches on gross inspection. Fatty streaks are inclined to occur at sites of eccentric intimal thickening, and stain red with Sudan III or Sudan IV. The lesion is usually characterized by the enrichment of macrophage foam cells and increased subendothelial lipid contents. A feature is the stratified aggregates of macrophage "foam cells" which is distinctive from that in the stage of intima thickening when isolated macrophages do not assume the appearance of foam cells. Foam cells are so-described because of their appearance resulting from the numerous internal lipid vesicles. Smooth muscle cells (SMCs) increase in number in the intima, also contribute to the foam cell formation after excessive lipid internalization. T lymphocytes are detected at this stage as well, but less numerous than macrophages and SMCs. Lipid droplets, which primarily consist of cholesteryl esters (CE), free cholesterol (FC), and phospholipids (PL), start to accumulate in the extracellular space. Extracellular matrix, especially collagen type I and III, increases noticeably. Another noteworthing feature for this stage is the disruption of endothelial lining and internal elastic lamina. Fatty streaks may regress with either therapeutic interventions or the removal of risk factors. A progression-prone lesion is typically characterized by a large number of macrophages and SMCs, abundant extracellular matrix and extensive lipid deposition.

1.1.2.3 Intermediate Lesion (Type III)
A type III lesion is applied to the pathological change which forms the bridge between fatty streaks and atheromas; therefore, its pathological findings overlap adjacent lesion stages. In general, confluent, well-delineated lipid pools in the extracellular space are evident, with the accumulation of more macrophages and SMCs. A fibrous cap, composed of a collagen layer, is evident on the luminal surface.
1.1.2.4 Atheroma (Type IV)
The features of a typical atheroma are lipid core, mature fibrous cap, and deformation of vascular structure. The excessive lipid deposition defined as the lipid core is believed to result from the continued insulation of lipoproteins from plasma and lipids released from necroxed foam cells. The enlargement of the lipid core markedly thickens and deforms the intima. Macrophages and SMCs are filled with lipid droplets in the cytoplasm; consequently, apoptotic/necrotic processes frequently take place in these cells. The basal part of the lesion is abundant in cellular components compared to the luminal surface which is covered by a fibrous cap. Connective tissue is also abundant in shoulder regions. More compellingly, the internal elastic lamina is largely destroyed or even disappears. The lesions at this stage usually bulge out from vascular surface; nevertheless, they often fail to narrow the vascular lumen due to the compensatory dilatation of the vascular wall.

1.1.2.5 Advanced Atheroma (type V)
At this stage, the vascular lumen area is noticeably narrowed by the lesion expansion so that blood flow is compromised. Based on the amount of lipid content, this lesion can be subdivided into fibrous-lipid (fibroatheroma) plaque and fibrous (fibrotic) plaque. Fibrous-lipid plaques are richer in lipid content compared with fibrous plaques, and often take on a multilayered appearance of lipid-fibrous-lipid arrangement. In addition, cellular components such as macrophages and SMCs are more abundant than in fibrous plaques, in which lipid-laden cells are relatively rare and fibrous tissues are predominant. Fibrous-lipid plaques are more prone to develop complications. In addition, the third type lesion featuring extensive calcification is also included in this category.

1.1.2.6 Complicated Lesions (type VI)
These lesions always occur on the basis of type IV and V lesions, and largely contribute to the morbidity and mortality from atherosclerosis. The lesions can be subdivided into: 1) intraplaque hemorrhage due to the erosion of neovaculature; 2) fissures, rupture, and ulceration, which typically occur on lesions rich in macrophages and lipid contents, the direct outcome of plaque rupture or fissure is the activation of platelets and coagulation, which leads to 3) thrombosis. Procoagulative components (tissue factor, collagen) are exposed towards platelets and coagulation factors following plaque rupture or fissure, as a consequence, a cascade of
coagulation process ensues in which vessels can be completely occluded. Recanalization can occur to resume the blood supply to some degree if patients survive. 4) embolism, thrombi or the remnants of thrombi are frequent findings on the surface of advanced atheromas. Those thrombi are vulnerable to shed, and lead to ischemic clinical encounters like cerebral strokes. 5) aneurysm: With the degradation of extracellular matrix in the media, mainly collagen type I, the atherosclerotic segment of vessels may bulge outwards, producing an aneurysm. Aneurysms often contain mural thrombi, both recent and old.

Although the exact cause of atherosclerosis is not well-elucidated; epidemiological studies in large populations have identified various anatomical, physiological and behavioral risk factors for atherosclerosis. These risk factors can be divided into nonmodifiable/congenital and modifiable/acquired. Aging, family history, and other genetic makeups are nonmodifiable factors, whereas dyslipidemia, hypertension, and unhealthy lifestyles are categorized into modifiable factors as aggressive interventions can reduce their deleterious effects.

1.1.3 Conventional Risk Factors
1.1.3.1 Age and Gender
The risk of atherosclerosis increases with age. In an Italian multicenter study, thirty-six percent of the patients under age 45 had a normal angiogram compared with 17% of the patients over 45 years.11 Age is associated with multiple modifiable risk factors, as pro-atherogenic lipid concentration tends to increase with age, so does the blood pressure, sugar level, homocysteine level etc.12,13 Men are more likely to have atherosclerosis than women before age 60. However, the risk is equal for men and women after 60. Differences in lipid profile and sex hormones may account for the disparity of atherosclerosis prevalence between sexes before age 60.14-17 Salutary effects of estrogen also protect women from atherosclerosis, as increased postmenopausal androgenicity in women is associated with an unfavorable cardiovascular risk profile, which may contribute to the resurgence of atherosclerosis in postmenopausal women.18-21

1.1.3.2 Tobacco Smoking
The Framingham study found smoking to be an independent factor for atherosclerosis and related complications.22,23 There are sex and age differences of smoking effect on atherosclerosis and its outcomes. The risk increase by smoking is only evident in men ages 45 to
64 but not for older men and women. Smoking men have a higher mortality than smoking women, cardiovascular mortality was 47% in men who smoked and only 10% in women who smoked in The Nutrition Canada Survey.

1.1.3.3 Sedentary Life-Style
About half of the population are physically inactive in modern society. Sedentary lifestyle is associated with increased cardiovascular events, and about 22% of global cases of CHD are attributed to physical inactivity. Physical inactivity is thought to double the risk of heart disease (WHO World Health Report, 2002 & World Heart Federation Fact-Sheet, 2002). The atherogenic lipid profile is more prevalent in the sedentary population than in the physically active one. Higher total cholesterol (TC), apolipoprotein B (apoB), and atherogenic index was observed in sedentary men. Physical inactivity also increases oxidative stress in the human body. Vascular nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase expression and the production of reactive oxygen species (ROS) are enhanced with consequently increased vascular lipid peroxidation, impaired endothelium-dependent vasorelaxation, and accelerated atherosclerotic lesion development. By contrast, moderate and vigorous exercise substantially reduces the incidence of cardiovascular events in a series of prospective epidemiological studies. Moreover, physical activity is associated with lower levels of atherogenic lipid profile, inflammatory, and coagulation markers [i.e., C-reactive protein (CRP), serum amyloid-A (SAA), interleukin (IL)-6, tumor necrosis factor (TNF) α, white blood cell (WBC) counts, and fibrinogen (FIB)]. Physical training also improves endothelial function. Physically active lifestyle improves collateral circulation and myocardial perfusion in patients with coronary artery disease and prevents the progression of carotid and coronary atherosclerosis.

1.1.3.4 Diets
A high-fat, animal based western-style diet is associated with an increased risk of atherosclerosis. Small increase in the consumption of animal-based foods is associated with increased disease risk. Preliminary results of "China Study II" disclose that the switch from traditional plant-based, high-fiber Chinese diet to a Western-style one, may account for the increasing prevalence of cardiovascular diseases (http://www.ctsu.ox.ac.uk/~china/monograph/). Epidemiological evidence and secondary prevention trials suggest that the intake of omega-3
polyunsaturated fatty acids from fish oils offers protection from CHD, reduces overall mortality and mortality secondary to myocardial infarction, and reduces sudden death in patients with coronary heart disease. In addition, antioxidants may give rise to anti-atherogenic effects. Consumption of vitamin E, C, and beta-carotene could reduce the risk of CHD. Polyphenols from plant and fruit juice and lycopene from tomato can improve endothelial function, suggesting their anti-atherosclerosis capability.

1.1.3.5 Family History and Genetic Makeup.
The risk for atherosclerosis is greater if there is a family history of premature coronary heart disease defined as coronary heart disease with onset age of <55 years for men and <65 years for women. In the four community-based cohorts of the ARIC Study, relatives of 3,034 African Americans and 9,048 white probands aged 45 to 64 years were assessed. Family risk score was associated positively with mean carotid artery intima-media wall thickness (IMT) in white and African-American women and white men. There are high levels of haemostatic variables (fibrinogen, factor VIIc, factor VIIIc, von Willebrand factor (vWF), antithrombin III (AT III), protein C) in both women and men who have a positive family history of atherosclerosis.

Quantitative trait locus analysis have shown that there are more than 80 genes participating in the regulation of plasma levels for high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and triglycerides. Those genes are potential genetic modifiers of atherosclerosis. In certain families and isolated communities, the effect of a single candidate gene upon atherosclerosis susceptibility may be profound, such as mutations in low-density lipoprotein receptor, which produce familial hypercholesterolemia.

More commonly, single gene polymorphisms (SNPs) cause intermediate phenotypes, and their direct effect on atherosclerosis may be difficult to define. When more than two candidate genes are defective concurrently, the risk for atherosclerosis then increases considerably because of the accumulative effect of intermediate phenotypes. The phenotype expression of the genetic makeup may not culminate until the human body is challenged under certain conditions related to the developmental stage, age, chronic and acute dietary influence, drug or toxin, the aggravating physiological state of organs. This is the basis of the gene-environment interaction hypothesis.
1.1.3.6 Dyslipidemia

Elevated serum cholesterol has been long recognized as the risk factor of atherosclerosis. More severe and widely distributed atherosclerotic lesions are found in patients with familial hypercholesteremia compared to normolipidemic patients.\textsuperscript{47} Cholesterol exists in conjunction with lipoproteins, high LDL-cholesterol (LDL-c) level is directly related to atherosclerosis, whereas HDL-cholesterol (HDL-cholesterol) is negatively associated with atherosclerosis. An elevated ratio of LDL-c to HDL-cholesterol is a common finding in patients with atherosclerosis.

The level LDL is well correlated with coronary atherosclerotic lesions, the reduction of LDL cholesterol levels by diet and combined drug regimens can induce the regression of atherosclerotic lesions in patients with familial hypercholesterolemia.\textsuperscript{48,49} High LDL-c promotes atherosclerosis by a wide range of mechanisms, including impairing endothelial function,\textsuperscript{50} promoting foam cell formation, stimulating SMC proliferation and ECM production.\textsuperscript{51} Further modified LDL such as small-sized LDL, oxidized LDL (oxLDL), and glycated LDL are even more potent in biological activities. However, reduced levels have a reduced capacity to prevent the detrimental process so that atherogenesis is accelerated.

The effect of triglycerides on atherosclerosis is increasingly recognized. A stronger association between plasma triglyceride levels and coronary heart disease is clearly demonstrated in patients with total cholesterol levels less than 5.7 mmol/L, or HDL-cholesterol levels less than 1 mmol/L. The reduction of plasma triglyceride levels significantly decreased coronary heart disease mortality by 36\% in general and by 60\% in patients whose triglyceride levels fell greater than 30\%.\textsuperscript{52-54} Hypertriglyceridemia may affect atherogenesis through the increased production of atherogenic chylomicrons (CM) and very low density lipoprotein (VLDL) remnants, with the concurrent decreased HDL level.

1.1.3.7 High Blood Pressure

Hypertension is associated with a 2 to 3 fold increase in risk for atherosclerosis-related cardiovascular events.\textsuperscript{55} In animal models, hypertensive mice and rabbits have significantly more extensive atherosclerosis in the abdominal aorta, brachial, iliac-femoral, and carotid
arteries.\textsuperscript{56-58} Prolonged existence of high blood pressure destroys endothelial integrity, induces SMC proliferation, and promotes atherosclerotic plaque progression.\textsuperscript{59} Proinflammatory cytokine expression associated with hypertension further aggravates the atherogenic process.\textsuperscript{60}

1.1.3.8 Diabetes or Impaired Glucose Tolerance

There is a wealth of clinical data supporting that impaired glucose tolerance, and of course type 1 and type 2 diabetes mellitus are positively correlated to atherosclerosis and related clinical complications. According to National Diabetes Information Clearinghouse USA, rates of stroke and CVD death are 2 to 4 times higher among adults with diabetes than in those without the disease (http://diabetes.niddk.nih.gov/). Hyperinsulinemia may be the first and genuine marker connected to the clinical manifestations of atherosclerosis.\textsuperscript{61} A variety of other mechanisms participate in the accelerated development of atherosclerosis in diabetics. Glycated end products and high-level insulin promote pro-inflammatory cytokine expression, SMC proliferation, lipid accumulation, and lesion development.\textsuperscript{62-65} Altered lipid metabolism, especially hypertriglyceridemia and low HDL level, also plays a contributory role to atherosclerosis.

1.1.3.9 Obesity

The Framingham Heart Study in the United States has consistently shown that increased degree of obesity is accompanied by greater rate of CHD.\textsuperscript{66} Obesity can be divided into two types: central/abdominal and gluteal ones. The risk level for atherosclerosis is much higher in patients with abdominal/central obesity in comparison with those with gluteal obesity. The indicators for central obesity such as waist-to-hip ratio and waist circumference are associated with accelerated atherosclerosis in common carotid artery in a 4-year observation period in men.\textsuperscript{67} In addition, the intravascular coronary ultrasound study reveals that obesity is independently associated with coronary atherosclerosis in patients with angiographically normal or mildly diseased coronary arteries.\textsuperscript{68,69} In young men, body mass index (BMI) is positively associated with both fatty streaks and raised atherosclerotic lesions in coronary arteries, however, the effect of obesity on atherosclerosis is less significant in women than in men.\textsuperscript{69} Further investigations disclosed the association of obesity with other conventional and emerging risk factors including atherogenetic lipid pattern, pro-thrombotic and hypofibrinolytic pattern, proinflammatory status, and insulin resistance.\textsuperscript{70}
1.1.3.10 Psychological Factors
Psychosocial stress or depression also influences the development and progression of atherosclerosis.\textsuperscript{71,72} The prevalence of carotid lesions among men in the highest stress quintile was 36\% compared with 21\% among men in the lowest quintile, an increased IMT was also observed in men in the highest quintile compared to the lowest.\textsuperscript{73} Conversely, stress reduction with the Transcendental Meditation program decreases coronary heart disease risk factors and cardiovascular mortality in general populations as well as in African Americans.\textsuperscript{74,75}

1.1.4 Emerging Risk Factors
1.1.4.1 Homocysteine
Homocysteine is an amino acid, derived from the metabolism of methionine. An elevated homocysteine level in blood is usually due to congenital enzyme defects or nutritional influences.

High level of blood homocysteine is increasingly being recognized as an important risk factor for atherosclerosis. Wicken et al. first found that patients <50 years old with angiographically proven coronary artery disease had a higher homocysteine mixed disulfide than in control subjects using the methionine loading test.\textsuperscript{76} High level of blood homocysteine was also found in patients with premature vascular disease and stroke.\textsuperscript{77,78} There is a linear relationship between the severity of coronary artery disease and cerebrovascular disease with homocysteine levels, higher homocysteine level is always associated with more severe atherosclerotic diseases.\textsuperscript{79} The association between fasting plasma homocysteine and CHD is also evident in other ethnic groups such as the Indian and Chinese populations.\textsuperscript{80}

In the cellular biological levels, homocysteine has been proven to stimulate SMC proliferation,\textsuperscript{81} injure the endothelial function,\textsuperscript{82} and enhance monocyte margination.\textsuperscript{83,84} Homocysteine also increases the oxidative stress and lipid peroxidation,\textsuperscript{85-87} interferes with coagulation and fibrinolysis, and stimulates platelet activation and aggregation.\textsuperscript{88-90} Homocysteine levels can be reduced by low-dose folic acid combined with vitamins B\textsubscript{6} and B\textsubscript{12} as well as cereal consumption.\textsuperscript{91,92} Vitamin supplementation in addition to grain fortification to all men aged 45 years or older will be considered to be the preferred strategy in the primary and secondary prevention of coronary heart disease.\textsuperscript{93}
1.1.4.2 Lipoprotein (a)
The size and structure of lipoprotein (a) [Lp(a)] is very similar to LDL, except that Lp(a) contains a unique protein called apolipoprotein (a) [apo(a)], which is covalently bound to apoB100. Apo(a) is structurally homologous to plasminogen molecule so that Lp(a) may be involved in thrombotic process. Lp(a) tends to be more easily oxidized than LDL and increases its atherogenicity.

High level of Lp(a) is an independent risk factor for atherosclerosis. Patients with coronary heart disease have a higher level of Lp(a) than controls. In general, a concentration of >30 mg/dl of Lp(a) in serum is associated with a 2- to 6-fold increase in risk, depending on the presence of other risk factors. Patients with increased Lp(a) levels are at increased risk of unstable angina and myocardial infarction. An increased level of Lp(a) was associated with increased risk of cardiac death in patients with acute coronary syndrome. Moreover, rapid progression of coronary atherosclerotic lesions and restenosis after angioplasty and coronary bypass procedures were observed in patients with increased Lp(a) concentrations. The serum Lp(a) level also has a close correlation with angiographic progression, a much higher median Lp(a) concentration was found in the progression group than those in the no-change and regression groups.

1.1.4.3 CRP
C-reactive protein (CRP) is an acute phase reactant in the response to acute injury, infection, or other inflammatory stimuli. CRP is generally produced from inflammatory cells including monocytes, neutrophils, and lymphocytes, and has been recognized as a marker of chronic inflammation. Pathological investigation has disclosed that those inflammatory cells exist in atheromatous plaques, and that the recruitment of those cells is one of the earliest event in atherogenesis.

Studies have shown a positive association between CRP and coronary artery disease. The odds ratio for coronary heart disease in participants in the top third percentile of CRP values was 1.45 in comparison with those in the bottom third percentile. High-sensitivity CRP (hs-CRP) level is also associated with the number and score of aortic plaques, even after the adjustment for age,
sex, smoking status, and additional atherosclerosis risk factors.\textsuperscript{107,108} As the plaque rupture appears to occur in the regions like the shoulder area which is abundant in inflammatory cells, thus the release of acute phase reactants such as CRP has been proposed as a potential marker of unstable atheromatous plaque and consequent ischemic cardiac events.\textsuperscript{109} Indeed, an elevated plasma CRP level increases the relative risk of initial myocardial infarction at every level of the ratio of plasma TC/HDL-cholesterol in apparently healthy men in the Physicians’ Health Study.\textsuperscript{110}

Multiple mechanisms have been proposed as a mechanistic link between CRP and cardiovascular diseases. High CRP is associated with profound endothelial dysfunction and disrupted nitric oxide (NO) production.\textsuperscript{111,112} CRP at high concentrations (\textgreater{} or \textless{} 15 \( \mu \)g/ml) significantly inhibits endothelial progenitor cell (EPC) differentiation with decreased expression of the endothelial cell-specific markers vascular endothelial growth factor receptor 2 (VEGFR2)/Tie-2, endothelial cell-lectin, and vascular epithelium-cadherin, also reduces EPC cell number, increases EPC apoptosis, and impairs EPC-induced angiogenesis.\textsuperscript{113} CRP may also contribute to plaque vulnerability by inducing SMC apoptosis and expression of matrix metalloproteinase (MMP) 2.\textsuperscript{114,115} CRP stimulation can markedly increase ROS production in macrophages to increase oxidative stress.\textsuperscript{116} CRP may also stimulate the adhesion molecule expression by endothelial cell to attract more monocytes,\textsuperscript{117,118} promoting the formation of macrophage-derived foam cells by increasing LDL internalization.\textsuperscript{119,120}

1.1.4.4 Fibrinogen and Prothrombotic Status

The cascade of coagulation and thrombosis, where fibrinogen is regarded as the central process, is closely involved in the pathogenesis of atherosclerosis.\textsuperscript{121} The occurrence of fibrinogen/fibrin I, fibrin II, and fibrin(ogen) degradation products (FDP) in fibrous and advanced plaques indicates their critical role in the development of atherosclerosis.\textsuperscript{122}

The case-control analysis of the ARIC Study demonstrated a significant association between plasma fibrinogen concentration and early atherosclerosis in the carotid arteries,\textsuperscript{123} this association was also found for femoral, and aortic plaque and coronary calcium deposit.\textsuperscript{124} Elevated fibrinogen is also associated with the progression of atherosclerosis, and predicts the risk of cardiovascular events. Adjusted hazard ratios for carotid atherosclerosis progression with
increasing quartiles of baseline fibrinogen were 1.83, 2.09, and 2.45, respectively, compared with the lowest quartile. Increased FDP level was associated with both the frequency of complications and the mortality in patients with acute myocardial infarction.

1.1.4.5 Microorganism Infection

The fact that many microorganisms have been detected in the atherosclerotic raises the infection hypothesis in atherogenesis. Viral infection as a potential cause of atherosclerosis was first proposed by Benditt EP and Melnick JL et al. in 1983 as they showed that herpes simplex virus (HSV) and cytomegalovirus (CMV) infection were detectable in atherosclerotic specimens. In a case-control study, a high CMV antibody titer was associated with an adjusted odds ratio of 5.3 for atherosclerosis compared with a low CMV antibody titer. In-vitro studies show that HSV infection stimulates SMC proliferation and lipid accumulation, increases the prothrombotic activity, and promotes the inflammatory cell recruitment. However, the causative relation between HSV infection and atherosclerosis remains unconfirmed.

The association between Chlamydia infection and atherosclerosis was first reported in patients with coronary heart disease in 1993. The C. pneumoniae index based on the relative amount of immune complex-derived antibodies and free antibodies was significantly higher compared with control subjects. The direct evidence of Chlamydia pneumoniae infection in atherosclerotic lesions has already been demonstrated by polymerase chain reaction (PCR) assay, immunocytochemistry, electron microscopy, and in situ hybridization, and this pathogen is specifically localized in macrophages and SMCs. Chlamydia infection is also associated with asymptomatic atherosclerosis according to the Atherosclerosis Risk in Communities Study. The causative role of Chlamydia in atherogenesis is also confirmed in an animal model where Chlamydia infection accelerates the development of atherosclerosis and treatment with azithromycin prevents atherosclerosis.

It is also reported that pathogens for periodontal diseases could be linked to atherosclerosis. Subjects infected with Campylobacter rectus and Peptostreptococcus micros were more likely to have higher IMT scores (OR 2.9). Porphyromonas gingivalis and Streptococcus sanguis are two major odontopathogens that have been detected in atherosclerotic plaques.
1.2 Macrophage and Atherogenesis

Macrophages are an essential component of body immune defense, assuming critical functions in both native and acquired immunity. In innate immunity, macrophages interact with the surface molecular pattern of pathogens, trigger phagocytosis and further processing in lysosomes to inactivate or detoxify them. In acquired immunity, phagocytosis of antigen by macrophages is enhanced by antigen-bound immunoglobulin G. Macrophages can also collaborate with T cells through cell-cell network and cytokine-mediated machinery to coordinate inflammatory responses.\textsuperscript{143}

Many hypotheses for atherogenesis have been formulated, including response-to-injury hypothesis, inflammation hypothesis, oxidative stress hypothesis, and endothelial dysfunction hypothesis, however, the central process inevitably involves macrophages (Figure 1-2).\textsuperscript{105,144} The atherogenic process is believed to begin when endothelial cells become damaged. As a response to injuries, circulating white blood cells, especially monocytes, are attracted and adhere to the vessel wall, migrate underneath the surface layer, and initiate the inflammatory reaction. Even if the initial appearance of macrophages seems to be protective against vascular damage such as clearance of lipoproteins and increasing cholesterol efflux into HDL, the overall long-term effect is destined to be proatherogenic. For example, hypercholesterolemic mice become much more resistant to atherosclerosis after macrophage depletion through breeding with macrophage-deficient mice.\textsuperscript{145}

1.2.1 Macrophage Recruitment and Migration

The prerequisite for monocyte recruitment is the disruption of endothelial integrity so that adhesion molecules are over-expressed on the surface. So far, a variety of risk factors have been proven to be detrimental to endothelia and stimulate the production of adhesion molecules on endothelial lining.\textsuperscript{146-149}
Figure 1-2. The role of macrophage in atherogenesis. The overexpression of adhesion molecules and chemokines on atherosclerosis-prone sites under the abusing factors will attract and recruit monocytes into intima where they differentiate into macrophages. Activated macrophages obtain the enhanced ability to internalize lipoproteins to become lipid-laden foam cells. Also, macrophages are active in the secretion of various cytokines and bioactive substances which can magnify the reaction in a malicious feedback loop by stimulating SMC proliferation, impairing endothelial function, and increasing inflammatory and oxidative stresses. The massive degradation of extracellular matrix will lead to plaque rupture and serious outcomes.

1.2.1.1 Adhesion Molecules

When injured, endothelial cells increase their expression of adhesion molecules such as selectins, integrins, intercellular adhesion molecule (ICAM) -1, vascular cell adhesion molecule (VCAM) -1. These adhesion molecules have relatively high affinity for monocytes, regarded as major players in early enrollment of monocytes. In ICAM-1 deficient mice, monocyte deposition in intima decreases with the accompaniment of reduced atherosclerosis. Selectins are also playing an important role in the recruitment of macrophages. Animals lacking P- or E-selectins have a decreased tendency to form atherosclerotic plaques.
Generally, the margination of monocytes into intima can be divided into 3 phases, namely, rolling, anchoring/firm adhesion, and migration. The rolling step is thought to be mediated by P-, L-, and E-selectins as over-expression of those selectins in endothelial cells is associated with enhanced adherence of monocytes.\textsuperscript{153-156} Thereafter, this attachment is fortified by the sequential participation of integrins, VCAM-1, and ICAM-1.\textsuperscript{157} The blockage of P-selectin or VCAM-1 significantly inhibits monocyte attachment and margination.\textsuperscript{158}

1.2.1.2 Chemokines

Chemoattractants such as monocyte chemoattractant protein (MCP) -1/CCL2 (CC-motif chemokine ligand 2), IL-8, and CXC-motif chemokines are also important during monocyte infiltration. Like adhesion molecules, the expression of chemokines is elevated by risk factors, and is associated with an increased number of monocytes in the intima.\textsuperscript{159-161} Chemotactic receptors such as CCR-2 (CC-motif chemokine receptor 2) and CXCR-2 (CXC motif chemokine receptor 2) for MCP-1 and IL-8 respectively are found on the monocyte surface. Deficiency of MCP-1 or CCR-2 markedly decreased atherosclerotic lesion formation and limited the progression and destabilization of established atherosclerosis in the genetic background of apoE or low density lipoprotein receptor (LDLR) knockout as well as apoB transgenic mice.\textsuperscript{162-166}

Concentration gradient of chemoattractants such as MCP-1 and IL-8 between epical and basal sides of endothelial layer is the driving force of monocyte migration through endothelial barrier into intima.\textsuperscript{167,168} Although less well established, other chemokines such as RANTES (Regulated on Activation, Normal T-cell Expressed and Secreted), macrophage inflammatory protein (MIP) -1α and MIP-1β, MCP-4, EBI1 ligand chemokine (ELC) and pulmonary and activation-regulated CC chemokine (PARC) have also been implicated in atherosclerotic lesion formation.\textsuperscript{169}

1.2.2 Monocyte Differentiation and Activation

Undifferentiated monocytes are granted less capacity to internalize lipids in comparison with differentiated monocytes/macrophages. The differentiation is actually taking place during migration rather than an isolated event. Upon interaction with surface receptors, adhesion molecules and chemokines immediately trigger a series of complicated intracellular biochemical
reactions. For example, mitogen-activated protein kinases (MAPKs) like extracellular signal-regulated kinase (ERK), Janus kinase (JAK), c-Jun N-terminal kinase (JNK) and p38 are activated by MCP-1, so are intracellular cyclic guanosine monophosphate (GMP) and cyclic GMP-dependent protein kinase, and calcium mobilization. The cascade activation of intracellular signaling molecules leads to the activation and translocation of nuclear transcription factors such as nuclear factor-kappa B (NF-κB).

1.2.3 Macrophage and Proinflammatory Response

Activated macrophages can de novo synthesize and release a large variety of cytokines (i.e., IL-1, IL-1ra, IL-6, IL-8, IL-10, IL-12, TNF-alpha, interferon (IFN) α, IFN-γ, MCP-1, MCP-3, macrophage migration inhibitory factor (MIF), macrophage colony stimulating factor (M-CSF), granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), MIP-1, MIP-2, leukemia inhibitory factor (LIF), oncostatin M (OSM), transforming growth factor (TGF) -β. Table 1-1).

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<th>Cytokines and its receptors</th>
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<tr>
<td>Chemokines: MCP-1,2,3, IL-8, RANTES, ELC, PARC, MIP-1a,b, macrophage-derived chemokine (MDC), thymus- and activation-regulated chemokine (TARC)</td>
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<td>Colony stimulating factors: M-CSF, G-CSF, GM-CSF</td>
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<td>Growth factors: platelet-derived growth factor (PDGF), TGF</td>
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<td>Interferons: IFN-α, IFN-γ</td>
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<td>Interleukins: IL-1β, 4, 6, 8, 10, 12, 13, 15, 18</td>
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<th>Adhesion molecules and receptors</th>
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<td>Oxidative stress related: secretory phospholipase A2 (sPLA2), cyclooxygenase, lipoxygenase, NADPH oxygenase,</td>
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Some cytokines can upregulate the production of other cytokines in macrophages for self-amplification of the inflammatory reaction. These cytokines and chemokines are involved in a...
variety of biological and pathological processes, modulate many macrophage functions, lead to monocyte recruitment, increased lipid oxidation and uptake, compromised cholesterol efflux, SMC proliferation, and ECM remodeling.

1.2.4 Macrophage and Foam Cell Formation

Foam cell formation is characteristic of atherosclerosis. Macrophages express several lipoprotein receptors that are responsible for lipid uptake. Although macrophages have constitutional expression of LDLR and LDL receptor related protein (LRP), the contribution of these to overall intracellular lipid deposition is relatively negligible. Most important receptors involved in lipid internalization are scavenger receptor (SR) A, CD36, and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1). Upon oxidation, the affinity of lipoproteins for receptors significantly increased in proportion to oxidized phospholipid content. Progressive decreases in oxidized phospholipids associated with apoB in oxLDL decreases the ability of the protein to compete for macrophage scavenger receptors and also decreases its reactivity with antibodies against oxLDL. Scavenger receptor BI (SR-BI) generally is colocalized with and regulated by caveolin-1 on the cell membrane. SR-BI was initially thought to be the HDL receptor and implicated in the selective cholesterol uptake in hepatocytes and steroidogenic tissues, recent findings support that SR-BI may serve as the receptor for oxLDL and hypochlorite-modified LDL in human macrophages to increase the cholesterol uptake.

1.2.5 Macrophage and Oxidative Stress

Accumulating evidence supports that the modification of lipoproteins is the essential step in its conversion towards atherogenic particles and sequential lipid accumulation in macrophages. The well-studied mechanism of lipoprotein modification is oxidation. Oxidation occurs mainly in intima as well as on the endothelial surface. The entrapment of lipoproteins in ECM is critical for the oxidation to be completed, because engineered LDL particles defective for binding to heparan sulfate proteoglycans are less atherogenic in LDLR-/- mice. Activated macrophages produce a large quantity of oxygen free radicals, free nitric and superoxide radicals, and reactive nitrogen species, which have been implicated for lipoprotein oxidation. Lipoxygenase, myeloperoxidase, and NADPH oxidase, are found to be highly expressed in macrophages and are regarded as primary enzymes in lipoprotein oxidation. Macrophages also produce
inducible nitric oxide synthase (iNOS) which may also elicit an oxidation reaction. However, its role is still controversial, as the overexpression of iNOS demonstrated protective effect in mouse models.\textsuperscript{189,190}

Paraoxonase-1, bilirubin and heme oxygenase are enzymes thought to counteract excessive oxidative stress.\textsuperscript{191-193} Paraoxonase-1 activity is impaired in atherosclerosis-related diseases.\textsuperscript{194} However, even with overexpression, the antioxidative capacity of heme oxygenase-1 and bilirubin are still not strong enough to eliminate the reactive oxygen and nitrogen species during atherogenesis.\textsuperscript{196}

1.2.6 Macrophage and Endothelial Dysfunction

Endothelial cells can produce a plethora of cytokines, chemokines, cell surface receptors, adhesion molecules, and other bioactive substances, playing an important role in thrombosis and coagulation, inflammation, vasodilation, and oxidation. Disrupted structural and functional integrity results in pathological states as exemplified by atherosclerosis. Overactivation of macrophages can impair the structural integrity of endothelia, resulting in high permeability of endothelial barrier.\textsuperscript{197} Elevated oxidative stress after macrophage activation further aggregates endothelial dysfunction, of which serum myeloperoxidase levels can serve as a strong and independent predictor.\textsuperscript{198} In addition, macrophage incubation with endothelial cells upregulates the expression of adhesion molecules,\textsuperscript{199} which recruit more inflammatory cells, and then trigger a malicious feedback loop. Impaired equilibriums of coagulation and fibrinolysis, vasodilation and vasoconstriction are also induced with the increased expression of plasminogen activator inhibitor-1, angiotensin II, and endothelin-1, the expression of which is closely related to macrophage activation.\textsuperscript{200}

1.2.7 Macrophage and Plaque Vulnerability

ECM proteins such as collagens encircle the necrotic core. The intensity and thickness dictate the plaque vulnerability. Atherosclerotic plaque vulnerable to rupture is characterized by high cellularity (especially macrophages), thin fibrous cap, and large lipid-rich necrotic core. The shoulder region of plaque where rupture usually occurs is correlated with the enrichment of macrophages.
Matrix Metalloproteinases (MMPs) and their tissue inhibitors of matrix metalloproteinases (TIMPs) are the enzymes responsible for the maintenance of ECM equilibrium. Enhanced regional expression of vascular MMPs has been detected in atherosclerotic plaques, which contributes to the thinning of matrix and favors plaque rupture. Matrix-degrading enzymes are constitutionally produced by macrophages, and their expression is enhanced by macrophage activation. Statin regimens have been proven to stabilize atherosclerotic plaque, and this effect may be partially due to their ability to suppress the expression of MMPs in human macrophages.

ECM degradation is also intimately related to the formation of pseudo-aneurysm. In apo E knock-out mice also deficient in TIMP-1, the internal elastic lamina is apparently destroyed and inflicted regions are prone to dilation. ECM degradation is also involved in intraplaque hemorrhage since a close association of macrophage activation with intraplaque microvessel hemorrhage was revealed by immunostaining in human endarterectomy samples.
1.3 Lipid Metabolism

1.3.1 Lipoprotein Classification

Cholesterol and triglycerides are insoluble in aqueous plasma, therefore, circulating lipids are incorporated into the form of lipoproteins, and delivered to various tissues for energy utilization, lipid deposition, steroid hormone production, and bile acid formation. A typical lipoprotein is composed of esterified and unesterified cholesterol, triglycerides, phospholipids, and protein contents, the latter are known as apolipoproteins and surround lipoprotein particle.

Apolipoproteins are lipid-binding proteins that have α-helical structures in common. Due to their amphipathic properties, apolipoproteins are capable of solubilizing the hydrophobic lipid constituents of lipoproteins, and transport dietary lipids through the bloodstream from the intestine to the liver, and endogenously synthesized lipids from the liver to tissues. Also, apolipoproteins can serve as either enzyme co-factors or receptor ligands, participating in the regulation of lipoprotein metabolism.

Lipoproteins can be classified into several classes according to their densities (Table 1-2), as such, they can be isolated using density gradient centrifugation. In addition, the density of lipoproteins is inversely related to their size, as chylomicron has the largest diameter but lowest density, and HDL is smallest in size but highest in density (figure 1-3).

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Density g/ml</th>
<th>TG %</th>
<th>CE %</th>
<th>FC %</th>
<th>PL %</th>
<th>Apolipoprotein</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM</td>
<td>&lt;0.95</td>
<td>85~88</td>
<td>3</td>
<td>1</td>
<td>8</td>
<td>B48, E, AI, AII, AIV, CI, CII, CIII, H</td>
</tr>
<tr>
<td>VLDL</td>
<td>0.95~1.006</td>
<td>50~55</td>
<td>12~15</td>
<td>8~10</td>
<td>18~20</td>
<td>B100, E, CI, CII, CIII</td>
</tr>
<tr>
<td>IDL</td>
<td>1.006~1.019</td>
<td>25~30</td>
<td>32~35</td>
<td>8~10</td>
<td>25~27</td>
<td>B100, E, CI, CII, CIII</td>
</tr>
<tr>
<td>LDL</td>
<td>1.019~1.062</td>
<td>10~15</td>
<td>37~48</td>
<td>8~10</td>
<td>20~28</td>
<td>B100, E, CI, CII, CIII</td>
</tr>
<tr>
<td>HDL</td>
<td>1.063~1.20</td>
<td>3~15</td>
<td>15~30</td>
<td>2~10</td>
<td>26~46</td>
<td>AI, AII, AIV, E, CI, CII, CIII, D</td>
</tr>
</tbody>
</table>

(TG: triglyceride, CE: cholesteryl ester, FC: free cholesterol, PL: phospholipids, CM: chylomicron, VLDL: very low density lipoprotein, IDL: intermediate density lipoprotein, LDL: low density lipoprotein, HDL: high density lipoprotein.)
The classification of lipoproteins by size. Chylomicrons are largest in size and contain >90% triglycerides, very low density lipoprotein (VLDL) is 10 times smaller in size than chylomicrons with less triglyceride but higher cholesterol and phospholipid contents. Intermediate and low density lipoproteins (IDL and LDL) are derived from VLDL. Compared to VLDL, IDL and LDL have a progressive decrease in size and triglyceride content but relatively higher cholesterol percentage. High density lipoprotein (HDL) is smallest in size and rich in phospholipids and protein.

1.3.2 Lipoprotein Metabolism
Lipoprotein metabolism can be divided into exogenous and endogenous pathways (Figure 1-4 and 1-5). The exogenous pathway starts with the intestinal absorption of dietary lipids and formation of chylomicrons in enterocytes, and ends with the removal of chylomicron remnants by liver. The endogenous pathway is represented by hepatic synthesis of VLDL and its metabolism.

1.3.2.1 Exogenous Pathway
Dietary lipids include triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acids, and sterols (most are animal-origin cholesterol). Triglycerides contribute to >90% energy supply from fats. Most of phospholipids and bile salts in the gut are provided by biliary secretion. In order to translocate dietary lipids from the intestinal lumen into enterocytes, dietary lipids must undergo a series of physicochemical processes such as emulsification, lipolysis, and solubilization, also called the intraluminal phase of digestion.
Dietary lipids are virtually insoluble in aqueous solution, thus large aggregates of dietary lipids must be broken down physically and held in suspension, this process is called emulsification. Bile salts play a critical role in lipid emulsification. Bile salts are derived from cholesterol and have both hydrophobic and hydrophilic domains. The hydrophobic groups enable bile salts to interact with hydrophobic surface of dietary lipids, and the hydrophilic groups are arranged outwards. This coating process will lead to the breakdown of lipid aggregates into small lipid droplets, and suspend them in aqueous environment. Emulsification maximizes the efficiency of lipid hydrolysis, which is predominantly accomplished by pancreatic lipase. Pancreatic lipase hydrolyzes triglycerides at 1- and 3- positions to release two free fatty acids and one 2-monoacylglycerol. The liberated fatty acids and residual monoacylglycerol are associated with bile salts, and incorporate phospholipids to form a spherical structure called micelles. Dietary sterols like cholesterol are also integrated into micelles.

Figure 1-4. Exogenous pathway of lipoprotein metabolism. Dietary triglycerides are emulsified by bile salts, then hydrolyzed by pancreatic lipase in intestine, the hydrolytic products, phospholipids, and cholesterol are solubilized in micelles, then absorbed into enterocytes where triglycerides are resynthesized. Chylomicrons (CM) are formed from absorbed phospholipids, cholesterol, and resynthesized triglycerides in enterocytes, transported into lymphatic vessels, and eventually enter the circulation. Chylomicrons lose triglycerides after lipoprotein lipase (LPL) hydrolysis in peripheral tissues like adipose tissues and skeletal muscles to become chylomicron remnants which are removed from blood by liver via a receptor-mediated pathway. Hepatic lipase (HL) may accelerate the CM remnant removal by hydrolysis and enhancing CM binding to hepatocytes.
Absorption takes place on the brush border of enterocytes. Free fatty acids and monoacylglycerol are able to cross the enterocyte membrane by free diffusion. Cholesterol is absorbed in the jejunal and ileal surface by both free diffusion and active transportation, ABC transporters are involved in this process.\textsuperscript{213, 214} Also, the esterification of cholesterol in enterocytes can accelerate its absorption.\textsuperscript{215, 216} The absorbed free fatty acids and monoacylglycerol are reassembled into triglycerides in smooth endoplasmic reticulum and then transported into Golgi apparatus, where they are packaged with cholesterol and apolipoproteins into chylomicrons. The synthesized chylomicrons are then secreted by exocytosis at the basolateral side of enterocytes. Chylomicrons eventually enter the lymphatic vessels in the microvilli of intestinal epithelia and transported through the thoracic duct into the circulation.

Chylomicrons are associated with a variety of apolipoproteins, particularly apoB48. Enterocytes also produce apoAI, AII, and AIV, and those apolipoproteins are also incorporated into newly synthesized chylomicrons in the Golgi apparatus. After entering the blood, chylomicrons acquire apoE, apoCl, CII, CIII from HDL. Meanwhile, lipolysis, initiated mainly by lipoprotein lipase (LPL), occurs in tissues such as skeletal muscles and adipose tissues. The released fatty acids are absorbed and utilized for either energy supply or storage. With the decrease in size, chylomicrons undergo a remodeling process during which excess apoAs, apoCs, and phospholipids are transferred back to HDL. These remodeled chylomicrons are called chylomicron remnants, and contain primarily cholesterol, apoE, and apoB48. Chylomicron remnants will be eventually removed by liver through LDL receptor or LDL receptor related protein (LRP).\textsuperscript{217} A chylomicron remnant receptor, which interacts with apoE, also participates in the removal of chylomicron remnants.\textsuperscript{218}

1.3.2.2 Endogenous Pathway
The metabolic pathway of VLDL represents the endogenous pathway of lipid metabolism (Figure 1-5). VLDL is primarily produced by hepatocytes in liver, carrying endogenous triglycerides and to a lesser degree, cholesterol. The major apolipoprotein associated with VLDL is apoB100. Upon secretion into the blood, VLDL acquires cholesteryl ester in exchange for triglycerides, apoCl, CII, CIII, and apoE from HDL. VLDL is hydrolyzed in a similar way as chylomicrons, LPL decreases the VLDL size by hydrolyzing triglycerides, transforms VLDL...
into IDL. Components of apoC are shed off and return to HDL during the remodeling from VLDL to IDL. With the further loss of triglycerides, IDL converts into a dense and small-sized particle LDL. The LDL particle predominantly consists of cholesteryl ester, apoB100, and apoE. LDL has been proven to be proatherogenic and is considered to be one of the most important risk factors for atherosclerosis. Another well-known proatherogenic lipoprotein is Lp(a) which is derived from LDL with apo(a) covalently bound to apoB100. LDL can be chemically modified into various forms such as oxidized LDL and glycated LDL, and the latter are even more proatherogenic, as they can be rapidly transported by macrophage scavenger receptors.

![Endogenous pathway of lipoprotein metabolism](image)

Figure 1-5. Endogenous pathway of lipoprotein metabolism. VLDL, which is synthesized by hepatocytes, loses its triglycerides by LPL hydrolysis in peripheral tissues to provide free fatty acids for energy consumption in skeletal muscles or storage in adipose tissues. With the progressive loss of lipid content, the remnant VLDL is transformed into IDL and then LDL, which are cleared by the liver via receptor-mediated pathway.

VLDL and its derivatives IDL and LDL deliver triglycerides and cholesterol to tissues, where triglycerides are hydrolyzed to release free fatty acids for energy utilization and storage. Cholesterol, mainly in an esterified form, is also taken up by tissues for the purposes of hormone and steroid synthesis.
IDL and LDL are generally removed from the blood via the LDL receptor and LRP, both of which interact with apoB and/or apoE components in IDL and LDL. The mutation of LDLR, as occurs in familial hypercholesterolemia, will result in elevated cholesterol levels in blood due to the defective clearance of LDL. Compared to native LDL, modified LDLs have higher affinity for scavenger receptors (i.e., SRA, CD36, CD68, and LOX-1). Upregulation of these receptors in cells or target tissues cause excessive lipid retention and atherosclerosis.\textsuperscript{219}

1.3.3 Metabolism of HDL

1.3.3.1 HDL Classification

The major apolipoprotein in HDL is apoAI, which is synthesized in the liver and small intestine.\textsuperscript{220,221} Most circulating apoAI is recycled from the transformation of lipoproteins. Chylomicrons are a significant carrier of intestine-synthesized apoAI. ApoAI can be transferred to HDL during chylomicron remodeling consequent to LPL lipolysis. When subjected to LPL catabolism, triglyceride-rich VLDL and LDL can be additional donors for apoAI.\textsuperscript{222} Similarly, redundant apoAI will be released during the transition of HDL\textsubscript{3} to HDL\textsubscript{2}.\textsuperscript{223} Regenerated free apoAI can be reincorporated back into HDL.\textsuperscript{224,225}

HDL formation requires lipidation of free apoAI with cholesterol and phospholipids intracellularly or extracellularly.\textsuperscript{226} Extracellular lipidation takes place in two distinct pathways, an ATP-binding cassette transporter A1 (ABCA1) -mediated and a non-ABCA1 mediated pathway. Free apoAI is a good lipid acceptor from peripheral tissues, predominantly through interacting with ABCA1. The mutation of ABCA1 in the case of Tangier disease results in compromised lipidation and virtually undetectable plasma HDL levels.\textsuperscript{227} After initial binding of phospholipids, apoAI is transformed into discoidal pre-β HDL. This subsequently interacts with SR-BI, ABCG1, or caveolins to acquire cholesterol and become a mature spherical HDL particle.

Pre-β HDL is associated with various apolipoproteins including apoAI, AII, CI, CII, CIII, D, and E. This nascent discoidal HDL has a phospholipid bilayer and two or more apoAI molecules. Because Lecithin:cholesterol acyltransferase (LCAT) can quickly convert discoidal HDL into spherical HDL, the concentration of pre-β HDL particle is considerably low.\textsuperscript{228} Spherical HDL
accounts for most circulating HDL in normal plasma, HDL₂ and HDL₃ are two major spherical HDL particles (Table 1-3), of which the hydrophobic core is composed of cholesteryl ester and a small portion of triglycerides, the periphery is surrounded by a monolayer of phospholipids, unesterified cholesterol, and apolipoproteins.

1.3.3.2 HDL Maturation and Related Enzymes

LCAT plays an important role in the maturation of nascent HDL to mature HDL. Unesterified cholesterol is docked on the surface of discoidal pre-β HDL, where LCAT catalyzes the transfer of a fatty acid from phosphatidylcholine to cholesterol. Upon esterification, cholesterol will be translocated to the interior of HDL due to its hydrophobic character. In LCAT deficient states, this process for HDL maturation is blocked, apoAI and AII containing-HDL are rapidly catabolized, thus, a marked reduction of HDL in blood is observed.²²⁹,²³⁰

Table 1-3. HDL category²³¹,²³²

<table>
<thead>
<tr>
<th>Category</th>
<th>Shape</th>
<th>Gel electrophoresis</th>
<th>Density</th>
<th>ApoAI molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid-free apoAI</td>
<td></td>
<td>Pre-β migration</td>
<td>&gt;1.159</td>
<td>Single</td>
</tr>
<tr>
<td>Pre-β HDL</td>
<td>Discoid</td>
<td>Pre-β to pre-alpha migration</td>
<td>1.159</td>
<td>2 or 3</td>
</tr>
<tr>
<td>HDL₃</td>
<td>Spherical</td>
<td>Alpha migration</td>
<td>1.125&lt;d&lt;1.21 g/ml</td>
<td>≥3</td>
</tr>
<tr>
<td>HDL₂</td>
<td>Spherical</td>
<td>Alpha migration</td>
<td>1.063&lt;d&lt;1.125 g/ml</td>
<td>≥3</td>
</tr>
</tbody>
</table>

With cholesterol esterification and further uptake of phospholipids, discoidal pre-β HDL becomes spherical HDL₃. Lipid redistribution among lipoproteins occurs due to the action of cholesteryl ester transport protein (CETP) and phospholipid transport protein (PLTP).

CETP is mainly synthesized by liver and adipose tissues, and circulates in blood in an associated form with lipoproteins. Due to the relative abundance of cholesteryl ester (CE) in immature HDL, cholesteryl ester flows towards apoB-containing lipoproteins such as VLDL, LDL, and IDL, as well as chylomicrons under the aid of CETP. In exchange, triglycerides are concurrently transported to HDL particle. It’s not clear whether or not CETP-processed HDL is more efficient in functionality, however, CETP stimulates apoAI turnover and reduces HDL levels. In addition, CETP increases LDL-cholesterol implying a proatherogenic potential. The importance
of CETP in HDL maturation is well demonstrated in a CETP-deficient Japanese cohort, whose HDL level is strikingly increased. Conversely, transgenic expression of CETP in mice decreased HDL levels to a large degree. Furthermore, CETP deficiency has been reported to accelerate the clearance of LDL.

Conversely, PLTP transfers phospholipids from apoB-containing lipoproteins to HDL. PLTP is positively related to HDL level. The disruption of PLTP in mice results in reduced levels of HDL and apoAI. By contrast, overexpression of PLTP increases pre-β HDL and apoAI. The remodeling of HDL with phospholipid integration by PLTP promotes HDL-and-cell interaction so as to improve its ability to further remove cholesterol.

With the incorporation of phospholipids, triglycerides and removal of CE, HDL₃ becomes the large and more buoyant HDL₂ through particle fusion, redundant apoAI is released during this process.

1.3.3.3 HDL Clearance

HDL is mainly catabolized in liver, kidney and steroidogenic tissues, where HDL can be either removed of cholesteryl ester (termed selective cholesterol uptake) or endocytosed as a whole (termed holoparticle uptake). SR-BI, a member of the superfamily of scavenger receptors, has high affinity for HDL. This receptor is rich in liver and other steroidogenic tissues (ovary, adrenal glands), and mediates selective cholesterol uptake from HDL. In mouse model, overexpression of SR-BI accelerates HDL and apoAI clearance. Holoparticle clearance of HDL mainly occurs in liver and kidney and lysosomal enzymes are involved in that process. In kidney, the filtered HDL or apoAI are internalized into renal tubular epithelia via the cubilin/megalin system.

1.3.4 Pleiotropic Role of HDL

HDL carries not only apolipoproteins but also many enzymes and other bioactive substances including serum amyloid A (SAA), ceruloplasmin, transferrin, LCAT, paraoxonase 1 (PON1), platelet activating factor acetylhydrolase (PAF-AH/LP-PLA2). These components may give biological functions to HDL distinct from lipid metabolism.
1.3.4.1 Anti-atherogenic Property
A high level of HDL is associated with low risk of atherosclerosis. The overexpression of apoAI in mice has significantly reduced the extent of atherosclerosis. ApoAI deficiency increases atherosclerosis in both animal models and human. As far as antiatherogenic properties of HDL are concerned, the reverse cholesterol transport of cholesterol from peripheral tissues to liver has received the most attention. In this pathway, HDL acts as the recipient of cholesterol in peripheral tissue, transports the cholesterol directly or through LDL indirectly to the liver for recycling or excretion to bile. HDL-associated SAA can stimulate ABCA1-dependent cholesterol efflux in fibroblasts, as well as, promoting SR-BI-dependent cholesterol efflux towards HDL.

1.3.4.2 Anti-oxidative Property
There is substantial evidence to support that HDL has antioxidative properties. For example, apoAI can neutralize the peroxidation of LDL lipids. One of HDL components, PON 1, can also protect lipids and tissues from oxidation injury by hydrolyzing lipid peroxides, hydrogen peroxides and hydroperoxides. Furthermore, PAF-AH can remove oxidized lipids and prevent the accumulation of oxLDL. LCAT level is positively related to PAF-AH and PON-1 activities. The gene transfer of LCAT significantly decreases atherosclerosis by elevating the HDL-associated antioxidant enzyme PON1. HDL also carries two additional antioxidants ceruloplasmin (copper-binding protein) and transferrin (iron-binding protein), which chelate plasma copper and iron respectively, and may attenuate the oxidative modification of lipids and other molecules.

1.3.4.3 Anti-inflammatory Property
Atherogenesis is considered to be an inflammatory process, therefore, many inflammatory markers and cytokines have been implicated in this process. CRP, an emerging cardiovascular risk factor, has been found to be negatively associated with HDL levels. In addition, HDL inhibits the expression of adhesion molecules, including VCAM-1, ICAM-1, and E-selectin through NOS upregulation. Serum HDL level also influences IL-6 release, high HDL was associated with low IL-6 in patients undergoing surgical operations. The inverse correlation between TNF-α with HDL was also reported.
1.3.4.4 Anti-thrombotic Property

HDL has been attributed with anti-thrombotic properties. Patients with low HDL levels have a higher risk for venous thrombosis. Mechanistically, PAF can be degraded by HDL-associated enzyme PAF-AH, thus, platelet activation and subsequent thrombosis is confined. In addition, the synthesis of PAF by endothelial cells is also inhibited by HDL. HDL is also a selective inhibitor of platelet 12-lipoxygenase, so the generation of 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) is suppressed. Furthermore, HDL is able to upregulate NOS to inhibit platelet aggregation. HDL can also modulate platelet function through regulating platelet membrane cholesterol content. The concentration of HDL particles is negatively correlated with platelet membrane cholesterol. The depletion of cholesterol reduces membrane rigidity and inhibits platelet aggregation. Moreover, the association of GpIb and FcγRII, which are critical for platelet activation and aggregation, is decreased upon incubation of platelets with HDL.
1.4 Reverse Cholesterol Transport

Cholesterol plays a critical role in membrane biogenesis, hormone synthesis, lipoprotein assembly, and cell growth. The functional integrity of mammalian cells requires the homeostasis of cellular cholesterol. Excess cholesterol is compartmentalized in the cell for either estification and storage or export to maintain the desired intracellular cholesterol level. The cholesterol on cell membranes can be picked up by cholesterol carriers such as apolipoproteins and lipoproteins; this process is termed cholesterol efflux. These cholesterol acceptors deliver the cholesterol to the liver for excretion with bile.

1.4.1 Source, Storage, and Transport of Intracellular Cholesterol

Intracellular cholesterol can be derived from either de novo synthesis or uptake of exogenous cholesterol (Figure 1-6). Cholesterol is synthesized in the smooth endoplasmic reticulum. β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) reductase is the rate-limiting enzyme for de novo cholesterol synthesis, statins can competitively inhibit HMG-CoA reductase, and are extensively used to treat patients with hypercholesteremia. After synthesis, cholesterol is channeled to the Golgi apparatus and packaged into lipid vesicles; the latter are then sorted to different organelles or compartments. Receptor-mediated lipoprotein internalization is another source of intracellular cholesterol. Internalized LDL will be hydrolyzed in lysosomes, released cholesterol is esterified and then stored in lipid droplets or utilized for membrane or hormone production. Intracellular cholesterol can also be obtained from HDL particles, and residual HDL particles are released back to extracellular compartments.

Intracellular cholesterol is stored in an esterified form because free cholesterol is toxic to cells. To prevent the toxic effect, free cholesterol is converted into esterified cholesterol by the enzyme acyl coenzyme A cholesterol acyl transferase (ACAT). There are two isoforms of ACAT, ACAT1 and ACAT2. ACAT1 is present within endoplasmic reticulum of many cells, including macrophages, Kupffer cells in the liver, neurons, and steroidogenic cells in the adrenal. ACAT2 is present exclusively in hepatocytes and intestinal cells. The expression of ACAT1 in macrophages significantly increases cholesterol storage and transforms macrophages into lipid-laden foam cells. Conversely, the inhibition of ACAT by specific inhibitors mobilizes cholesterol from lipid droplet, decreases cholesterol accumulation in macrophages and prevents
the progression of atherosclerotic lesions. A different enzyme, cholesteryl ester hydrolase (CEH), acts in the opposite direction by liberating free cholesterol from CE. The overexpression of CEH results in enhanced free cholesterol efflux from human THP1-macrophages. Hormone sensitive lipase (HSL), has a similar function as CEH, and can also hydrolyze cholesteryl ester into free cholesterol.

The cholesterol transport among cellular organelles is fulfilled by vesicular transport. These vesicles can bud from lysosomes, Golgi apparatus, and lipid droplets. The proteins intercalated in vesicular membrane direct cholesterol trafficking. Caveolins, which are mainly found in cholesterol rich domains on cell membrane, are also present in the plasma membrane of lipid vesicles. Caveolin can integrate heat-shock protein 56, cyclophilin 40, cyclophilin A, and cholesterol into a heat-shock protein-immunophilin chaperone complex. This compound transports cholesterol to cell membrane and the deficiency of caveolin causes the incapability of transporting newly synthesized cholesterol. Niemann-Pick type C protein (NPC) is also found in vesicle membrane. In Niemann-Pick disease where NPC is mutated and non-functional, the transport of cholesterol from late endosomes to various destinations, including plasma membrane, is defective. Other lipid transfer proteins such as sterol carrier protein 2 are also closely related to intracellular cholesterol transport.

1.4.2 Cholesterol-rich Domains in Cellular Membranes
In order to release excess cholesterol from intracellular compartments, cholesterol must be first distributed on the membrane. The cell membrane is characteristic of uneven distribution of phospholipids in adaptation to cell functions, exofacial/exoleaflet membrane is predominant in phosphatidylcholine and sphingomyelin, whereas endofacial aspect is rich in phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol.

1.4.2.1 Cholesterol-rich Rafts
Cholesterol and sphingomyelin aggregation in the plane of the membrane is termed a cholesterol raft. Cholesterol-rich rafts are resistant to the non-ionic detergent (Triton X-100) action as a result of the tight packing of lipids and strong van der Waals forces. Rafts are also branded detergent resistant membranes (DRM) or detergent-insoluble glycolipid-enriched complexes.
Figure 1-6. The source, storage, trafficking of intracellular cholesterol. Intracellular cholesterol can be either de novo synthesized or acquired via receptor-mediated lipoprotein internalization. Cholesterol can be de novo synthesized in smooth endoplasmic reticulum, then esterified by ACAT in Golgi apparatus and transported to lipid vesicles. Internalized lipoproteins are degraded in lysosomes and their cholesterol can be either stored in lipid droplets in an esterified form or channeled to membrane. Cholesteryl ester (CE) in lipid vesicles can be transformed under the action of cholesteryl ester hydrolase (CEH) and hormone sensitive lipase (HSL) into free cholesterol, the latter is then delivered to membrane for release. Caveolin and Niemann-Pick type C protein (NPC) participate in cholesterol trafficking in cells.

(DIGs). Besides sphingomyelin, phosphatidylinositol 4,5-bisphosphate is also enriched in DRMs. Lipid rafts are also associated with specific proteins such as glycosylphosphatidylinositol (GPI)-anchored and acylated proteins which are typically situated on the outer leaflet and inner leaflet, respectively. These proteins may also participate in the regulation of cholesterol efflux.

1.4.2.2 Caveolae
Caveoli are special rafts on the cellular membrane, which invaginate inward to form 50-70nm plasma membrane pits. This membrane invagination is coated with caveolin, a 22-kDa protein, instead of clathrin. Caveolin is palmitoylated at multiple sites. Caveoli are active cell organelles, involved in material potocytosis and transcytosis. Recent findings demonstrate that caveolin is also associated with a variety of downstream signaling molecules, including Src-
family tyrosine kinases, p42/44 MAPK, and endothelial nitric oxide synthase (eNOS), suggesting its important role in signal transduction.\(^2\) Equally important, caveolin can bind one mole of cholesterol per mole of protein,\(^2\) the expression of caveolin 1 in a caveolae-null cell causes the enrichment of FC in caveolae region,\(^3\) this implicates caveolae in the regulation of cholesterol trafficking and efflux.\(^4\)

### 1.4.2.3 Two Distinct Kinetic Pools of Membrane Cholesterol

Membrane cholesterol can be divided into two pools: a fast pool and a slow pool. The cholesterol in the fast pool is always orientated on the external leaflet of membrane. However, the slow pool of cholesterol could be the cholesterol associated with rafts/caveolae or the cholesterol which is located the inner leaflet of membrane. With the progressive removal of the fast pool of cholesterol, the slow-pool cholesterol will move into the fast pool. Sphingomyelin and phosphatidylcholine are major phospholipids for the maintenance of two distinct kinetic pools of cholesterol. The treatment of membrane with sphingomyelinase or phospholipase C dramatically increases cholesterol efflux from the fast pool. This increase is not mediated by the lipid reorganization and randomization after the hydrolysis of phospholipids, but rather a shift of cholesterol from the slow pool to the fast pool.\(^5\)

### 1.4.3 Cholesterol Efflux

The process of cholesterol liberation from membrane into medium is called cholesterol efflux. Several mechanisms have been described, including free diffusion, receptor-facilitated efflux, and ABC transporter-mediated efflux (Figure 1-7).

#### 1.4.3.1 Free Diffusion

Passive aqueous free diffusion is driven by the concentration gradient from high to low concentration. The cholesterol which spontaneously desorbs from cell membranes into the aqueous environment is incorporated into lipoproteins such as HDL as well as apolipoproteins. It has been reported that a considerable proportion of total cholesterol efflux to human serum is mediated by free diffusion. One experiment has shown that ~70–90% of cholesterol efflux is not mediated by either SR-BI or ABC transporter pathways.\(^6\) Moreover, plasma proteins other than apoAI and HDL like cyclodextrins and albumins can also serve as cholesterol acceptors to induce cholesterol efflux by free diffusion.\(^7\)
Reverse cholesterol transport. Apolipoprotein AI can be synthesized by liver or regenerated during the metabolism of chylomicron and HDL. Lipid-free apoAI and nascent HDLs (pre-β HDL and HDL₃) are preferred acceptors for cholesterol released from peripheral tissues via free diffusion, SR-BI, and ABC transporter pathways. LCAT converts pre-β HDL into HDL₃ by esterifying cholesterol. Nascent HDL₃ exchanges cholesteryl ester for phospholipids from chylomicron and LDL under the aid of CETP and PLTP. The cholesterol in mature HDL₂ is removed by liver and eventually secreted into bile via ABCB and ABCG transporters. The LDL cholesterol obtained from HDL in exchange with phospholipids can also be removed and excreted by liver.

The rate of aqueous free diffusion is highly dependent on the structure of acceptor particles as well as the lipid composition of the membrane. Large HDL particles are less efficient as cholesterol acceptors. Phosphatidylcholine and its metabolite lysophosphatidylcholine have been reported to influence cholesterol efflux since 16% of the cholesterol molecules are directly hydrogen bonded to oxygen atoms in phosphatidylcholine. Lysophosphatidylcholine increases the non-apoAI-mediated cholesterol efflux in mouse macrophage foam cells. HDL-associated enzyme PON1 was found to increase cholesterol efflux, which may be ascribed to the
increased production of lysophosphatidylcholine.\textsuperscript{299} As mentioned before, cholesterol is clustered in sphingomyelin domains, thus, the degradation of sphingomyelin can cause cholesterol perturbation and subsequently influence cholesterol efflux. It is reported that the sphingomyelin metabolite ceramide is able to promote the cholesterol outflow in the fetal rat astrocytes and meningeal fibroblasts.\textsuperscript{300} Phosphatidylethanolamine and phosphatidylserine inhibits the formation of hydrogen bonds between cholesterol and sphingomyelin, thereby increasing the desorption of cholesterol.\textsuperscript{301}

1.4.3.2 SR-BI-Mediated Cholesterol Efflux

The receptor facilitated cholesterol efflux is primarily mediated by SR-BI. In Chinese hamster ovary cells (CHO), overexpression of SR-BI by stable transfection resulted in a 3–4 fold increase of cholesterol efflux to HDL.\textsuperscript{302} More convincingly, when SR-BI was blocked by specific antibody, or SR-BI was mutated to lose its ability of HDL binding, the cholesterol efflux to HDL was markedly impaired.\textsuperscript{303,304} SR-BI is also able to bind LDL by the interaction with apoE and apo B, so that SR-BI could efflux cholesterol to lipidate LDL. For example, SR-BI overexpression increased LDL cholesterol level in rabbits.\textsuperscript{305,306} The extracellular domain of SR-BI is crucial for bidirectional flux of cholesterol.\textsuperscript{307} The SR-BI mediated cholesterol efflux is a function of PC content of the acceptor, larger reconstituted HDL binds to SR-BI much better than small HDL particle, and promotes more cholesterol efflux than smaller particle.\textsuperscript{308} Enrichment of HDL with phospholipids appreciably promotes cholesterol efflux, and depletion by incubating HDL with phospholipase A2 otherwise decreases SR-BI mediated efflux.\textsuperscript{309,311} Moreover, the HDL particle modified by endothelial lipase loses its phospholipid contents as well as the capability to induce cholesterol efflux.\textsuperscript{312}

The SR-BI mediated cholesterol transport is concentration-driven and bidirectional. The net movement of cholesterol via SR-BI depends on the direction of cholesterol gradient.\textsuperscript{313} One experiment, which was done in SR-BI-expressing cells loaded with different cellular cholesterol mass, clearly addressed the influence of cholesterol content on the cholesterol transport direction. When cells were cholesterol-depleted, SR-BI promotes net cholesterol influx from HDL\textsubscript{3}; On the other hand, incubation of cholesterol-rich SR-BI-expressing cells with HDL\textsubscript{3} resulted in net efflux of cell cholesterol.\textsuperscript{313} Nevertheless, the direction of cholesteryl ester transport by SR-BI is unidirectional. In adrenocortical cells where cholesterol is required for
steroid synthesis, SR-BI delivers cholesteryl ester into the steroidogenic pathway. SR-BI also helps the clearance of cholesteryl ester from blood by the liver.

The mechanism by which SR-BI facilitates cholesterol efflux still remains to be elucidated. It has been proposed that SR-BI promotes cholesterol efflux by enhancing free diffusion. In addition, SR-BI may change the distribution of cholesterol on membrane. It has been shown that SR-BI expression shifts FC from the slow pool into the fast pool, and the latter is more accessible for cholesterol efflux.

Interestingly, SR-BI is co-localized with caveolin in cholesterol-rich caveolae domains of membrane, and SR-BI is also shown to be able to stabilize caveolin, suggesting caveolin may play some role in SR-BI mediated cholesterol efflux. Caveolin assumes a critical role in cholesterol trafficking from intracellular compartments to cell membrane. Caveolin transfection in hepatic cells led to a 40% increase in the amount of plasma membrane cholesterol in cholesterol-rich domains (caveolae and/or rafts) and a 67% increase in the rate of cholesterol trafficking from intracellular compartments to these domains. In NIH/3T3 cells, expression of caveolin effluxed cholesterol more rapidly to HDL. In contrast, caveolin blockage by antisense DNA reduced cholesterol incorporation into HDL. In view of their colocalization, this evidence implies that caveolin may play a role in SR-BI mediated cholesterol efflux.

1.4.3.3 ABC-Mediated Cholesterol Efflux

The members of ATP-binding cassette (ABC) transporter family share a similar structure and nucleotide-binding domain, which enables them to bind and hydrolyze adenosine triphosphate (ATP) and use this energy to pump compounds across the membrane or to flip molecules from the inner to outer leaflet of the membrane. ABC transporter family can be divided into at least 8 subfamilies from A to H. ABCA and ABCC subfamilies are full transporters composed of two subunits. ABCD, ABCG, and ABCH subfamilies are half transporters consisting of one subunit. ABCB subfamily exists as either half or full transporter. In exception, ABCE and ABCF subfamilies have no transmembrane domains, no transport function has been described for those 2 subfamilies. Each subfamily can also be subdivided into several classes, each class is different in functions. ABC transporter family participates in various biological functions, one
well-known function is its anti-tumor drug transportation, which results in the drug resistance of tumors, therefore, some ABC genes are coined with the synonym - multiple drug resistance (MDR) genes. Furthermore, research advances prove that some ABC family members, for example, A, B, and G subfamilies, are involved in cholesterol transport.

1.4.3.3.1 ABCA1

The role of ABCA1 in cholesterol efflux was first observed in Tangier disease and familial HDL deficiency (FHD). These mutations cause the inability of cholesterol efflux and subsequent impaired apoAI lipidation, leading to a consequent low or undetectable HDL levels in plasma. Moreover, fibroblasts isolated from Tangier disease and FHD are defective to induce cholesterol efflux and generation of nascent HDL particles, upregulation of ABCA1 by cAMP promotes heterogeneous HDL particle formation in J774 macrophages. These results indicate that ABCA1 is the key gatekeeper protein for cholesterol efflux.

ABCA1 seems to interact with apoAI specifically, because ABCA1 expression markedly increased cellular cholesterol efflux to apoAI but has only minor effects on lipid efflux to HDL, furthermore, the direct evidence of apoAI binding to ABCA1 has been demonstrated. Lipid-rich apoAI or nascent HDL particles have a much lower affinity for ABCA1 in comparison with apoAI. Multiple amphipathic helices in the C-terminal domain of apoAI are responsible for the interaction between ABCA1 and apoAI. Within those helices, several tyrosine residues have been identified to be critical for apoAI-ABCA1 binding since the peroxidation (nitration and chlorination) of tyrosine residues in apoAI by myeloperoxidase markedly impaired apoAI and ABCA1 interaction and subsequent cholesterol transport. A C-terminal VFVNFA motif of ABCA1 is responsible for its interaction with apoAI. Recently, it has been postulated that ABCA1-induced efflux is not restricted to apoAI, other apolipoproteins like apoAII, apoAIV, apoCI, apoCII, apoCIII, apoE can also be the acceptors for cholesterol via ABCA1 pathway.

Upon apoAI docking, ABCA1 transports lipid moieties onto apoAI via its lipid translocase driven by ATP consumption. Two hypotheses, one-step and two-step mechanism, have been proposed to explain the mechanism. According to the one-step pathway, after the interaction between apoAI and ABCA1, ABCA1 directly translocates phospholipids and unesterified
cholesterol from cell membrane to apoAI.\textsuperscript{337} By contrast, the two-step hypothesis suggests that ABCA1 exports phospholipids to apoAI first to convert apoAI particle into pre-β-migrating, discoidal HDL, then this pre-β HDL sequentially accepts free cholesterol by free diffusion.\textsuperscript{338-340} The two-step hypothesis is supported by several experiments. First, the phospholipid content in HDL particles is disproportionately lower than that of cholesterol in cells with an ABCA1 mutation.\textsuperscript{341} Secondly, apoAI conditioned in the medium with SMC induced higher rates of cholesterol efflux than non-primed apoAI.\textsuperscript{339} It is also worth mentioning that ABCA1-apoAI mediated cholesterol efflux pathway does not involve sphingomyelin-rich raft domains in membrane as the raft domain depletion do not disrupt apoAI mediated cholesterol transport.\textsuperscript{342}

Several other ABCA members are also implicated in cholesterol efflux. ABCA7 contributes to nascent HDL formation by efflux lipids.\textsuperscript{343} However, ABCA7 differs from ABCA1 in terms of lipid efflux and HDL remodeling. HDL remodeling by ABCA7 mainly produces small HDL particles other than large HDL particle by ABCA1 pathway.\textsuperscript{344} Recently, ABCA7 was shown to transport only phospholipids and not cholesterol to apoAI.\textsuperscript{345,346} ABCA2, which is predominantly expressed in brain and neural tissues, shares high homology with ABCA1 and ABCA7, its expression in macrophages is induced during cholesterol loading, indicating that ABCA2 is a cholesterol-responsive gene.\textsuperscript{347,348} ABCA2-overexpressing reduce cholesterol esterification in CHO cells.\textsuperscript{349} Lack of ABCA3 causes defective secretion of phospholipids (primary component of surfactants) by type II alveolar epithelia, and is causatively related to fetal respiratory distress syndrome.\textsuperscript{350} This gene could also be involved in cholesterol transport, although more work has to be done to address this question.\textsuperscript{351}

1.4.3.3.2 ABCB

The knowledge of ABCB subfamily in cholesterol transport is largely drawn from the mechanistic exploration of bile secretion, their effects in other organs or tissues whereas remain uncovered (the ABCB role in bile secretion is described in “reverse cholesterol transport”).

1.4.3.3.3 ABCG

This ABC transporter subfamily is composed of at least 5 members, G1, G2, G4, G5, and G8. Except for G2, all members engage in the regulation of cholesterol transport.\textsuperscript{352} In 2000,
Klucken et al reported that the ATP-binding cassette (ABC) transporter ABCG1 was expressed in monocyte-derived macrophages, and inhibition of ABCG1 by antisense DNA caused decreased HDL₃-mediated cholesterol influx. In some tissues, the role of ABCG1 in cholesterol transport outweighs that of ABCA1, for example, it is ABCG1 but not ABCA1 responsible for lipid outflow in cerebellar astroglia. ABCG4 was also found to mediate the efflux of cellular cholesterol to HDL. ABCG5 and ABCG8 are predominantly expressed in enterocytes and hepatocytes, and their localization on apical/luminal aspect of the membrane suggests a role in lipid absorption and bile secretion.

The main acceptors for cholesterol from ABCG1 and ABCG4 are lipiddated apoAI and HDL. In transfected 293 cells, ABCG1 and ABCG4 stimulate cholesterol efflux to both HDL₃ and HDL₂ subclasses but not to lipid-poor apoAI. The capacity of acceptors to induce ABCG1-mediated efflux is strongly correlated with their total phospholipid content, suggesting that acceptor phospholipids drive ABCG1-mediated efflux. Moreover, ABCA1 may synergize with ABCG1-mediated cholesterol export through lipiddating lipid-free apoAI into pre-β-HDL.

1.4.3.4 Apo E induced Cholesterol Efflux.
ApoE-mediated but ABCA1-independent cholesterol efflux was demonstrated in several lines of experiments. First, adenoviral-mediated expression of apoE in dermal fibroblasts isolated from ABCA1(-/-) mice significantly increased both sterol and phospholipid efflux. Second, expression of human apoE in a macrophage cell line increased sterol efflux, and this increment in efflux was not reduced after ABCA1 suppression. Third, reduction of apoE expression using an apoE small interfering RNA (siRNA) significantly reduced sterol efflux from ABCA1(-/-) mouse peritoneal macrophages. However, the recycling of apoE back into cells can be potentiated by ABCA1 expression and apoAI. This repeated cycle of secretion and recycling may maximize cholesterol outflow from cells, impaired recycling of apoE can lead to intracellular cholesterol accumulation.

1.4.4 Reverse Cholesterol Transport in Blood
The transportation of cholesterol from peripheral tissue to liver, followed by bile secretion, is defined as reverse cholesterol transport (Figure 1-7). This function is assumed largely by HDL, and to a lesser extent by LDL.
1.4.4.1 Cholesterol Transport by HDL in Blood

The capability of HDL as cholesterol carrier can be regulated at the cholesterol efflux level as well as HDL maturation level. The regulation of SR-BI and ABC transporter mediated cholesterol efflux is discussed above; herein, the emphasis is placed on HDL maturation and remodeling.

HDL maturation and remodeling involves cholesterol esterification and lipid exchange. The cholesterol which is effluxed from cells is unesterified, and associated on the surface of nascent/pre-β HDL. In order to maintain the concentration gradient between HDL surface and cell membrane to drive more cholesterol efflux, unesterified cholesterol on HDL surface must be removed. LCAT serves this purpose by esterifying free cholesterol into cholesteryl ester which moves into the core of HDL. The overexpression of LCAT considerably enhances cholesterol efflux and subsequent HDL cholesterol transport rate.\(^{302,362}\) The accumulation of cholesteryl ester in the HDL core will limit HDL ability to receive more cholesterol, so the cholesteryl ester will be removed by CETP. Although CETP tends to decrease overall HDL-cholesterol level, CETP-modified HDL particles are more efficient to induce cholesterol efflux.\(^{363}\) However, recent research regards CETP as proatherogenic factor, as HDL secondary to CETP-deficiency has enhanced ability to promote cholesterol efflux from macrophages, and CETP overexpression aggravates atherosclerosis in APOE*3-Leiden mice.\(^{364}\) HDL is also remodeled by PLTP, the latter has been proven to increase HDL-mediated cholesterol transport.\(^{238,365}\) PLTP is also found to promote HDL remodeling and pre-β HDL regeneration, and stabilize ABCA1.\(^{366,367}\)

Reverse cholesterol efflux ends with the uptake and secretion by liver. The HDL receptor SR-BI in liver accounts for selective cholesterol uptake. With the removal of cholesterol, residual HDL particles will return into circulation.\(^{180,303,368}\) Overexpression of SR-BI in liver promotes the cholesterol clearance and accelerates macrophage reverse cholesterol transport despite the decreased level of HDL in circulation.\(^{180,239}\) Thus, it is clear that SR-BI in liver is critically important to HDL metabolism and reverse cholesterol efflux.
1.4.4.2 Cholesterol Transport by LDL in Blood

A certain portion of HDL cholesterol can be transferred to LDL in exchange for triglycerides by CETP. In a human study, HDL labeled with cholesteryl ester tracer was injected into circulation, a considerable portion of labeled cholesteryl ester was transferred to LDL, and eventually appeared in bile.\(^ {369}\) The liver is rich in LDL receptors, so cholesteryl ester transferring from HDL to LDL is regarded as another pathway for reverse cholesterol transport.

1.4.5 Biliary Secretion of Cholesterol

The cholesterol taken up by liver will be ultimately secreted into bile in forms of bile salts and free cholesterol. ABCB and ABCG subfamilies are two important players for bile secretion. Mutation of either ABCB4 (MDR-3) or ABCB11 (bile salt export pump) gives rise to progressive familial intrahepatic cholestasis,\(^ {370,371}\) which is characterized by defective secretion of both phosphatidylcholine and associated cholesterol.\(^ {372,373}\) Interestingly, overexpression of ABCB11 in mice increased cholesterol secretion in biliary tree and consequent increase in the rate of cholesterol gall stone formation.\(^ {374}\) ABCG5 and ABCG8 also participate in the regulation of sterol secretion. The mutation of ABCG5 and ABCG8 causes sitosterolemia which features increased level of blood cholesterol and impaired cholesterol secretion by liver.\(^ {375,376}\) Because ABCG subfamily members are half transporters, they usually dimerize to become homodimer or heterodimer in order to function as a transporter.\(^ {377,378}\)
1.5 Lipase Gene Family

Lipids such as triglycerides and phospholipids play a central role in a variety of physiological functions, including energy supply and storage, maintenance of cell membrane integrity, hormone production, and vitamin supply. From lipid absorption in intestine to lipid metabolism in tissues, various enzymes are involved, of which several are grouped into a lipase gene family due to the similarity in both protein and gene structures. This gene family is composed of at least 7 members. Among them, pancreatic lipase (PL), lipoprotein lipase (LPL), endothelial lipase (EL), and hepatic lipase (HL) are critical enzymes in human lipid metabolism.

Sequence analysis has revealed remarkable homology among these lipases. The amino acid sequence of porcine pancreatic lipase was first determined in 1981 and so became the prototype of this lipase gene family. Tryptic digestion revealed that the peptic segments of bovine LPL share a close homology with porcine pancreatic lipase in most instances, indicating that LPL belongs to the same family as pancreatic lipase. When human lipoprotein lipase cDNA and protein were compared to those of rat and porcine pancreatic lipases, extensive homologies among the enzymes were revealed as well. As expected, LPL from different species also share a high homology, there is 94% homology in amino acids between mouse and human LPL, and 92% between human and bovine LPL. Human hepatic lipase, which is 85% identical to the protein sequence of pig pancreatic lipase and 70% identical to dog pancreatic lipase, has a same exon-intron arrangement as LPL. Recently, EL was cloned from endothelial cells in 1999, sequence analysis revealed a 45% homology with LPL, 40% with HL, and 27% with pancreatic lipase.

Although these lipases are thought to be derived from the same ancestral gene, they may have diverged into different evolutionary pathways to suit different environments. In the intestine, pancreatic lipase assumes a primary role in lipid digestion for the subsequent absorption of fatty acids by enterocytes. Pancreatic lipase is a strict triglyceride-hydrolyzing enzyme, secreted by the pancreas to digest triglycerides into 2 free fatty acids and 1 molecule of monoacylglycerol; the hydrolytic products are then absorbed by intestine. There are two other lipases produced by pancreatic cells called pancreatic lipase related protein (PLRP) 1 and 2. These two lipases also belong to the same lipase gene family. Among them, PLRP-2 is well known to be able to digest
intestinal lipids, with a substrate preference for phospholipids. PLRP-1, which was once thought to be “inactive”, may play a certain role in lipid digestion as well.

Lipoproteins are lipid carriers in the circulation, delivering lipids throughout human body. As far as lipid utilization is concerned, three major enzymes including hepatic lipase, LPL, and EL are implicated. LPL, which is extensively expressed in muscles and adipose tissues, liberates free fatty acid from triglycerides for energy consumption in muscles and energy storage in adipose tissues. Hepatic lipase, mainly expressed in the liver, can hydrolyze both triglycerides and phospholipids, and plays a role in the selective uptake of HDL cholesterol. The expression of hepatic lipase is also found in steroidogenic tissues such as adrenal gland, ovary, and testes. With the aid of hepatic lipase, these tissues can utilize cholesterol for steroid hormone synthesis. EL preferentially hydrolyzes phospholipids, and is regarded as a modulator of HDL concentration. Besides their catalytic activities, these three lipases also bear heparin binding function through which lipases can provide substrates to proteoglycan-bearing tissues for metabolism; this is termed a “bridging” function.

1.5.1 LPL
1.5.1.1 Gene Structure
Human genomic clones that span the entire LPL gene have been isolated and used to determine LPL structure. The LPL gene is mapped in human chromosome 8p22, approximately 30 kilobase (kb) pairs in length, containing 10 exons and 9 introns. Exons 1-9 have an average size of 105-243 base pairs whereas exon 10 is 1948 basepairs in length, which encodes the entire 3' noncoding sequence. Exon 1 codes for the signal peptide as well as the first two amino acid residues in mature LPL, exons 2 to 4 contain the protein domains responsible for lipoprotein binding, and exons 6 to 9 code for carboxyl terminal (C-terminal) sequence that is relatively rich in basic amino acids and therefore likely involved in anchoring of the enzyme to the acidic domain of heparan sulfates. The eighth exon codes for a domain containing another N-linked glycosylation site. The LPL gene appears to be regulated in a tissue-specific manner. Several motifs in the LPL gene may be responsible for the tissue-specific expression of LPL. First, there are four transcription initiation sites at the 5' terminal end. In addition, two potential enhancer sequences in the 5' upstream region are also observed, one is the response element to intracellular Ca$^{2+}$ mobilization, and another one is related to the expression in adipocytes.
There is 70% homology in cDNA among different species-derived LPLs, and more than 90% homology in protein structure is predicted.

1.5.1.2 Structure-Function Relationships

1.5.1.2.1 Protein Translation and Glycosylation

LPL mRNA is translated into a 475 amino acid protein precursor, which is then translocated into endoplasmic reticulum, where the signal peptide of 27 amino acids is cleaved. Meanwhile, the LPL undergoes N-linked glycosylation in rough endoplasmic reticulum. The oligosaccharide chain accounts for approximate 10% molecular weight of mature LPL. Ong et al. examined the effects of glycosylation on rat LPL activity and maturation. They found that the non-glycosylated rat LPL was 49 kiloDaltons (kD) in molecular weight rather than 55 kD of the fully glycosylated structure. Furthermore, non-glycosylated LPL is unable to be secreted and dimerized in cells. When glycosylation was blocked by either tunicamycin or glucose deprivation, secreted rat LPL activity was reduced by 90%. There are two N-glycosylated sites on the LPL protein. Using site-specific mutagenesis, asparagines at positions 43 and 359 of human LPL were mutated, causing the elimination of N-linked glycosylation and consequent intracellular accumulation of inactive protein and marked decrease in secreted LPL activity. Normal glycosylation is also required for LPL homodimerization and heparin binding as the abolishment of N-linked glycosylation dramatically decreased enzyme activity and heparin binding ability. Oligosaccharide chain trimming, during which the mannose is removed after glycosylation, is also critical for LPL maturation.

1.5.1.2.2 Homodimer Structure

Pancreatic lipase is the first member of this family to be crystallized, then a complete 3-dimentional model of protein structure of human pancreatic lipase was determined. In this model, pancreatic lipase contains a large N-terminal domain typical of α/β structure dominated by a central parallel β-sheet, and a C-terminal domain which is formed by two layers of β-sheets. A triad of Ser152-His263-Asp167 is situated in N-terminal domain and recognized to be the catalytic site. The catalytic site is covered by a surface loop between the disulphide-bridge residues 237 and 261. Binding to substrates appears to result in a conformational change of the surface loop and neighboring structures so that the active site will be accessible to substrates.
The C-terminal domain in a β-sandwich organization is responsible for the binding of colipase, which anchors pancreatic lipase to the lipid/water interface.\(^{401}\)

Two different crystal structures from pancreatic lipase and pancreatic lipase-procolipase-phospholipid complex were applied by Tilberugh et al. to implement molecular modeling for LPL.\(^ {402}\) Due to the high homology between LPL and PL, most structure motifs are conserved in LPL, for example, the α/β structure is abundant in N-terminal domain. The conservation of the β-sandwich analogue is also noticed in the C-terminal domain. Surface loop and catalytic triad are also highly conserved in LPL. However, LPL differs from pancreatic lipase in several aspects, for example, the active form of LPL is in a homodimer organization, and have the ability to bind heparan sulfates (Figure 1-8).

![Figure 1-8. Schematic demonstration of the dimeric structure of LPL. The homodimer structure is generated by homology modeling using pancreatic lipase as a template. The cluster of hydrophobic residues Trp390, Trp393, and Trp394 (yellow) at C-terminus (blue) are postulated to be responsible for lipoprotein binding. The catalytic site (Ser132-Asp156-His241, red) resides in N-terminal domain (brown) and is close to the lipid binding domain in another monomer. The lid structure (green, in an open form) controls the accessibility of active site to substrates. Four clusters (purple) of basic amino acids in both C- and N-terminal domains are proposed to be involved in HSPG binding. (Modified from Eur J Biochem, 2002:4701)\(^{55}\)](image)

When a head-to-tail association was observed in a crystallographic dimer of the open pancreatic lipase-colipase-phospholipid complex, this dimerization mode is also proposed for LPL through homology modeling (Figure 1-8).\(^ {402}\) Since the C-terminus has been implicated in lipoprotein binding and the N-terminus for lipid hydrolysis, this head-to-tail orientation can facilitate the substrate transfer from C-terminus to N-terminus across lipase subunits. The homodimerization
of LPL is necessary for lipolytic activity as well as heparin binding. The dissociation of the LPL dimer leads to rapid loss of lipolytic activity. Interestingly, the existence of Ca$^{2+}$ rapidly converts LPL monomers into competent dimers. An LPL dimer has a higher affinity for heparin than LPL monomers and elutes from a heparin-Sepharose column at higher NaCl concentration.

1.5.1.2.3 Active Site and Lid Structure

Based on the sequence alignment between LPL and pancreatic lipase, the homologous catalytic triad in human LPL corresponds to Ser132-Asp156-His241. The proposed active triad was confirmed from patients with LPL deficiency as well as by site-directed mutagenesis. The mutation of Asp156→Gly was found in patients with familial type I hyperlipoproteinemia and this mutant is devoid of enzyme activity when expressed in COS cells. Furthermore, there are 8 serines in LPL protein, but only the mutation of serine at position 132 results into a complete loss of enzymatic activity of LPL. The substitution of His241 by several different residues resulted in the expression of an enzyme lacking both triolein and tributyrin esterase activities. The presence of a mutation in the neighborhood of the catalytic triad also influences catalytic activity. For example, a substitution of Pro157 for Arg was found in a proband of Dutch subject who was deficient in LPL activity. Asn291→Ser substitution in LPL gene was also reported in patients with LPL deficiency and hyperlipidemia.

This well-conserved catalytic triad (Ser132-Asp156-His241) is situated in a groove in the heart of an LPL dimer, and shielded by a surface loop/lid structure (residues 216~239). The surface loop is thought to control LPL activity by conformational changes between closed and open forms which correspond to inactive and active forms of LPL, respectively. Compared to the closed conformation, the open form stretches the surface loop and exposes the catalytic triad-hosting groove, which makes the latter more accessible to substrates. A helix-turn-helix motif with two short amphipathic helices in loop structure is required for substrate binding/hydrolysis. Site-directed mutagenesis which leads to reduced amphipathic property of the loop but does not change the predicted secondary structure of the loop deprives LPL of the ability to hydrolyze emulsified, long chain fatty acid triglycerides. Besides, trioleinase activity is still retained after replacing the loop of LPL with amphipathic loop of hepatic lipase, furthermore, the substitution of the LPL loop by a short four amino acid peptide abolishes the
ability of LPL to hydrolyze substrates. Meanwhile, the partial deletion or mutation of the middle/apical section of the loop reserves normal lipolytic activity, however, the mutation at the proximal section of the loop leads to LPL deficiency, suggesting the proximal and distal parts of the loop are critical for substrate hydrolysis.

The lid structure controls not only substrate binding but also substrate specificity. This structure-function relation was elucidated by lid switching between LPL and other members of lipase family. For example, chimeric LPL containing the lid of HL had reduced triolein hydrolyzing activity, but increased phospholipase activity in 1,2-di-oleoyl-sn-glycero-3-phosphocholine (DOPC) vesicle, DOPC proteoliposome, and DOPC-mixed liposome assay systems. In contrast, chimeric HL containing the LPL lid was more active in hydrolyzing triolein than DOPC. Furthermore, the replacement of the LPL lid with the corresponding EL sequence also partially shifted the substrate specificity of LPL from triglycerides to phospholipids, and an EL chimera with an LPL lid shifted substrate specificity from phospholipids to triglycerides.

ApoCII is the cofactor of LPL, activating LPL substantially after binding to LPL. A specific region (residues 65–86) of LPL was identified to interact with apoCII, especially the 11 amino acid residues at position 65–68 and 73–79 of the N-terminus domain. On the other hand, another essential domain responsible for LPL binding was mapped to the C-terminus of apoCII. The residues 63, 66, 69, and 70 of apoCII have been implicated in binding to LPL, but no single amino acid residue seems to be absolutely critical.

1.5.1.2.4 Lipid Binding Domain

The relative hydrophobicity of the C-terminal domain of LPL facilitates the interaction with hydrophobic lipids. Lookene et al. constructed a LPL molecule lacking a C-terminus, this truncated LPL lost its ability to bind chylomicrons or milk fat globules. Antibody specific for the C-terminal segment of LPL was also able to influence trioleinase activity, possibly through interfering with substrate recognition site. This lipid binding site was pinpointed to a tryptophan cluster (Trp390-Trp-393-Trp-394) in the C-terminal domain. This interaction of LPL with lipoproteins like VLDL and CM is fairly complicated, involving electrostatic and hydrophobic forces. An accepted hypothesis is that LPL interacts with
apolipoproteins on the surface of lipoproteins. For example, expression of an N-terminal fragment of apoB significantly increased LPL binding to cell surface, and antibodies against the N-terminal region of apoB blocked LPL interaction with LDL.\textsuperscript{424-426} However, this hypothesis has been challenged by several observations, suggesting LPL binds to phospholipids because the binding of LPL with lipoproteins occurs even in the absence of apoB\textsubscript{100}.\textsuperscript{427} Also, partial delipidation of LDL markedly decreases its binding to LPL, whereas, phosphatidylcholine-containing liposomes efficiently compete with LDL for binding to LPL.\textsuperscript{427} These findings suggest that a lipid-LPL interaction also plays a critical role in lipoprotein-LPL association.

1.5.1.2.5 Heparin Binding Domain

The evidence of an ionic binding of LPL to heparin was demonstrated by Olivecrona in 1971.\textsuperscript{428} A 220-kD heparan sulfate proteoglycan on endothelial cell surface was identified as a receptor for LPL.\textsuperscript{429} Due to the electrostatic nature and abundance of negative charges in heparin, the key heparin-binding residues were hypothesized to be the aggregates of positively charged residues such as arginine and lysine. Two consensus sequences X-B-B-X-B-X and X-B-B-B-X-X (B is basic amino residue and X represents a neutral residue) have been postulated as candidates for heparin binding.\textsuperscript{430,431} Four clusters of lysine and arginine residues are present at the back of lipoprotein lipase by homology modeling, those alkaline amino acid clusters including Arg 263-Arg279-Lys280-Arg262, Arg294-Lys296-Arg297-Lys300, Lys319-Lys403-Arg405-Lys407-Lys414-Lys415, are dispersed in both N- and C-terminal domains.\textsuperscript{402,405,432,433} The mutation of alkaline residues in cluster 1 (aa279-282) or 2 (aa292-304) into neutral ones strikingly reduces LPL affinity for heparin.\textsuperscript{405,433,434} Recent findings also support the role of the C-terminus in heparin binding. The chimeric construct of the N-terminal domain of LPL with the C-terminal domain of HL has a lower affinity for heparin, suggesting the substitution of cluster 4 in the C-terminus leads to the change of affinity for heparin.\textsuperscript{435} This finding is also substantiated by an experiment using site-directed mutagenesis, where the mutation of residues Lys 321, Arg 405, Arg 407, Lys 409, and Lys 416 resulted in a decrease in the affinity of LPL for heparin.\textsuperscript{436}

1.5.1.2.6 Receptor Binding Domain

LPL also has been found to be capable of binding to lipoprotein receptors including the LDL receptor, LRP, and Glycoprotein 330 (a member of low density lipoprotein receptor family).
Glycoprotein 330 can form a saturable, divalent cation-dependent binding to LPL with high affinity. In a competition assay, LPL effectively displaces alpha 2-macroglobulin and 39-kD alpha 2M receptor-associated protein (RAP) from LRP in cultured mutant fibroblasts. LPL binding to highly purified LRP was also demonstrated in a solid-phase assay, furthermore, polyclonal antibody against LRP blocked cellular degradation of LPL in a dose-dependent manner. In addition, a high affinity of LPL to purified VLDL receptor was also demonstrated in an in-vitro binding experiments. The binding sites to cell surface receptors have been confirmed on the C-terminal domain of LPL. A fragment of human LPL containing the C-terminal residues 313-448 is able to bind LRP, and mutation of Lys407→Ala drastically reduces the affinity of LPL for LRP by 10-fold. Some other sites are also identified to contribute to the LPL affinity for LRP. The residues 378-423 at C-terminus of LPL bind to purified and cellular LRP, are also able to competitively inhibit the binding of LPL and the lipase-mediated binding of lipoproteins to LRP. Residues 313-448, 380-384, and 404-414 are also vital for LPL binding to LRP.

1.5.1.3 Regulation of Lipase Expression

1.5.1.3.1 Tissue Expression of LPL

LPL plays a central role in triacylglycerol metabolism. It is most abundantly expressed in adipose and muscular tissues, where free fatty acids supplied by LPL are actively utilized for either energy storage by re-esterification or oxidation for energy supply, respectively. The mammary gland is another important source of LPL, but the synthesized enzyme is secreted in association with milk fat droplets. LPL is also detected in vascular smooth muscle cells in large arteries. LPL is found on the endothelial surface, but endothelial cells are not the primary source for LPL production. Surface-bound LPL is believed to be synthesized by parenchymal cells and transferred to the endothelium. In addition, activated macrophages produce LPL at high level, and this may aggravate the development of atherosclerosis. LPL expression is observed in adrenal, ovary, testes, lung, spleen, kidney, liver, and brain, the role of LPL in those tissues is not well-characterized. After secretion, LPL is docked on membrane-anchored proteoglycans, most of which are heparan sulfate proteoglycans (HSPGs). Small extracellular proteoglycans located in subendothelial layer also bind LPL.
1.5.1.3.2 Physiological Regulation of LPL Expression

LPL expression is regulated by a variety of factors in order to meet the physiological demands. For example, lactation markedly increases LPL level in mammary gland with corresponding decrease in adipose tissue, and fasting leads to the mobilization of LPL in adipose tissue and inactivation in cardiac muscle.\textsuperscript{450} The expression of LPL in adipose tissues was investigated in very obese subjects before and after weight reduction which was achieved by low-calorie diet. After weight loss, the expression level of LPL increased in all patients.\textsuperscript{451} Cold exposure elicits LPL elaboration in brown fat, so that normal cordial body temperature can be maintained.\textsuperscript{452}

Food deprivation, cold environment, and stress all lead to blood level changes of hormones such as insulin, glucocorticoids, and adrenaline. Therefore, the effects of nutrition and other physiological factors on LPL are believed to be mediated primarily through hormone action. Growth hormone regulates in a positive way the transcription of LPL in preadipocyte Ob1771 cells;\textsuperscript{453} isoproterenol and insulin upregulate LPL gene expression in rat adipocytes, but through different mechanisms. Catecholamines decrease LPL expression at both transcriptional and post-transcriptional levels, however, insulin increases LPL expression by stabilizing LPL mRNA without affecting gene transcription.\textsuperscript{454, 455} Thyroid hormone has opposite effects on LPL expression in muscular and adipose tissues where LPL expression is up-regulated in adipocytes but down-regulated in cardiac myocytes.\textsuperscript{456, 457} By contrast, glucocorticoids increase LPL expression in both cardiac myocytes and adipocytes.\textsuperscript{458-461}

1.5.1.3.3 Regulation of LPL Expression in Macrophages

Immunohistochemistry has colocalized LPL expression with macrophages in atherosclerotic lesions.\textsuperscript{462} It is evident that the switch of monocytes to macrophages is accompanied with a spike of LPL expression. It is well-known that inflammatory cytokines and a variety of growth factors and proteases are involved in macrophage activation and differentiation. For example, M-CSF stimulates monocyte growth and differentiation towards macrophages, and enhances LPL secretion in human monocyte-derived macrophages.\textsuperscript{463}
Some risk factors have been investigated for their influences on LPL expression. Several lines of evidence indicate that oxidative stress plays a role in the pathogenesis of atherosclerosis. Treating cultured murine macrophages with hydrogen peroxide is associated with striking increases in both LPL activity and mRNA levels. Moreover, this stimulation offsets TNF-α induced LPL suppression. Exposure of macrophages to high glucose concentrations results in a dramatic upregulation of LPL at both mRNA and protein levels. High glucose levels in vivo also produce advanced glycation end products, the latter are proven to be able to potentiate the stimulatory effect of glucose on macrophage LPL expression. Homocysteine induces lipoprotein lipase expression in macrophages through protein kinase C activation and c-Fos upregulation.

LPL expression is also regulated by several cytokines. Interleukins such as IL-1, IL-2, IL-6, and IL-11, which are involved in inflammatory processes, have an inhibitory impact on LPL expression in macrophages. Despite the stimulatory effect of LPL on TNF-α expression in macrophages, TNF-α inhibits LPL expression. One potential mechanism is the indirect action of TNF-α on LPL through NOS. The treatment of macrophage with TNF-α raises inducible NOS and subsequent NO production, and NO supplementation attenuates the LPL expression. In addition, a NOS inhibitor relinquishes TNF-α of suppressive action on LPL. IFN-γ is also shown to inhibit LPL expression in macrophages, with a synergism with TNF-α. Similarly, exposure of macrophages to LIF and IFN-γ or IL-6 and LIF or INF-γ and TNF-α results in a synergistic suppression of LPL activity. Lipopolysaccharides (LPS) suppress in a dose- and time-dependent manner the heparin-induced secretion of LPL from the macrophage-like tumor cell line J774.1 and bone marrow derived mononuclear phagocytes. The combination of LPS with INF-γ also synergistically suppresses LPL production. By contrast, PDGF-BB stimulates LPL production via protein kinase C (PKC) activation in macrophages, and PDGF-BB induced LPL expression is reversed by the immunoneutralization with anti-PDGF-BB antibody.

The mechanisms of LPL regulation have been investigated in several studies. There are numerous cis-acting elements found in the LPL promoter and nearby region: (1) CT element; (2) sterol regulatory element; (3) interferon-gamma responsive element; (4) peroxisome proliferator activated receptor (PPAR) responsive element; (5) oxysterol liver X receptor (LXR) responsive element; (6) nuclear factor-1 receptor; and (7) activator protein 1 (AP-1) or AP-1 like element.
The activation of PPAR-γ represses LPL expression,\(^{481,482}\) however, a contradictory report states that PPAR-α and γ agonists upregulate LPL expression in human macrophages.\(^{483}\) Cytokines can elicit either dissociation or association of these DNA binding proteins with specific regions in LPL gene so as to regulate LPL expression. For example, TNF-α disrupts the association of nuclear factor Y and an octamer-binding protein with the promoter of LPL gene. As a consequence, LPL gene expression is dampened.\(^{484}\) Additionally, IFN-γ reduces the steady state level of Specificity protein (Sp) 3 protein and decreases the DNA binding activity of Sp1.\(^{485}\) Post-transcriptional regulation of LPL may also occur by influencing mRNA stability, translation, protein degradation, processing, secretion, translocation to the site of action, as well as competitive inhibition by the catalytic products.\(^{478,486}\)

1.5.1.4 Catalytic Function and Lipid Metabolism

1.5.1.4.1 Biochemistry of LPL

LPL is a primary triglyceride hydrolase, whose activity relies on intact homodimerization and apoCII presence. The LPL monomer has a lower affinity for heparin-sepharose than LPL homodimer, the dissociation of homodimer leads to a minimal activity of triglyceridase.\(^{487,488}\) The presence of apoCII increases the LPL activity by more than 10 fold with corresponding decrease of the \(K_m\).\(^{489,490}\) Intestine-synthesized chylomicrons and liver-derived VLDLs are predominant substrates of LPL because the primary lipid content of those lipoproteins is triglycerides. After contacting endothelial surface where LPL is anchored, the hydrolysis of triglycerides to free fatty acids takes place. Interestingly, the hydrolytic product free fatty acids in return suppress the LPL activity.\(^{491}\)

LPL also has a small amount of phospholipase activity, amounting to <2% of triacylglycerol hydrolase activity.\(^{492}\) LPL purified from bovine milk readily hydrolyzed chylomicron phosphatidylcholine to lysophosphatidylcholine, but the proportion and amount of phosphatidylcholine hydrolyzed is always less than that of triacylglycerols.\(^{493}\) Hydrolytic activity of LPL on phospholipids is showed to be of the phospholipase A\(_1\) as LPL cleaves phosphatidylcholine at 1-acyl ester bond.\(^{493}\)

The phospholipase A\(_1\) activity of LPL relies on the presence of apoCII. The addition of apoCII is associated with a time-dependent release of lysolecithin in a concentration-dependent manner.
By contrast, apoCIII causes a strong inhibition of triglyceridase activity of LPL, however, the phospholipase A₁ activity of LPL is not altered and even slightly stimulated after apoCIII addition.⁴⁹⁰,⁴⁹⁴,⁴⁹⁵ Paradoxically, the association between LPL and phospholipids decreases after the addition of apoCIII.⁴⁹⁶ The phospholipase A₁ activity is also influenced by the composition of substrates or the type of lipoproteins. The hydrolysis of phospholipids by LPL is most efficient in VLDL followed in descending order by IDL, HDL, and LDL.⁴⁹⁷ When VLDL is exposed to LPL, 85%-90% of the triacylglycerols are hydrolyzed to fatty acids and 25%-35% of phosphatidylcholine to lysophosphatidylcholine. In comparison, only 4% of phosphatidylcholine in HDL is hydrolyzed even after prolonged incubation of HDL with LPL.⁴⁹⁸ Moreover, LPL hydrolyzes phospholipids containing unsaturated fatty acyl chains 5-10 times faster than saturated lipids, and the length of 16 carbons for fatty acyl chains is optimal.⁴⁹⁹

1.5.1.4.2 LPL and Metabolism of Triglyceride-rich Lipoproteins
Chylomicrons and VLDL are abundant in triglycerides and represent favorable substrates for LPL. In a rat liver perfusion model, addition of LPL caused lipolysis of chylomicron triacylglycerols as evidenced by increased release of fatty acids in the perfusate. The removal of chylomicron core particle was also enhanced, and inhibition of lipolytic activity of LPL delayed chylomicron removal by the liver.⁵⁰⁰ In human, inhibition of LPL by specific antibodies retarded the clearance of chylomicrons from plasma and decreased their uptake by the liver.⁵⁰¹ Furthermore, several mutations, like glycine 142 → glutamic acid, proline207 → leucine, Trp86→Arg, and Asp250→Asn, have been found in LPL gene which lead to a catalytic inhibition and consequent familial chylomicronemia (type I hyperlipoproteinemia).⁵⁰²-⁵⁰⁵

1.5.1.4.3 LPL and Metabolism of ApoB Containing Lipoproteins
LPL is also involved in the metabolism of apoB containing lipoproteins. With the progressive loss of triglycerides and the enrichment of esterified cholesterol, VLDL remnants or IDL are remodeled into LDL. In vivo turnover studies have revealed that heterozygous LPL knockout mice have impaired VLDL clearance.⁵⁰⁶ In patients without functional LPL, the levels of LDL, VLDL, and triglyceride increase; however, the fractional secretion rate for apoB is significantly lower in the patients than those in controls.⁵⁰⁷ Furthermore, LPL can diminish apoB output by the liver to regulate the levels of apoB-containing lipoproteins in the blood.⁵⁰⁸ Compared to lipoproteins in the absence of LPL, apoB-containing lipoproteins associated with LPL have
significantly greater and faster clearance by the liver, and the clearance of apoB₄₈-containing lipoproteins are also enhanced after association with LPL.⁵⁰⁹

1.5.1.4.4 LPL and HDL Metabolism

Although HDL is not the preferred lipoprotein for the lipolytic action of LPL, the HDL level is also influenced by LPL. Modest but significant changes are observed in carriers of N9 and S291 mutations in comparison with normal alleles.⁵¹⁰ In the Copenhagen City Heart Study performed in the Danish general population, most genetic variants of LPL were associated with lower HDL and apoAI levels.⁵¹¹ This correlation is also substantiated by animal studies. The knockout of LPL led to undetectable HDL level at 18 hours after birth in mice.⁵⁰⁶ When LPL was inhibited, blood HDL levels in monkeys decreased, and apoAI catabolism in the kidney was correspondingly increased.⁵¹² CETP is involved in the regulation of HDL by LPL. The overexpression of LPL in CETP transgenic mice is associated with higher HDL levels compared with LPL-overexpressing littermates not carrying the CETP gene.⁵¹³ In addition, the genotype of LPL influences the response of HDL to statin treatments. Certain haplotypes are associated with a decreased increment in HDL-cholesterol following statin treatment, however, some other haplotypes were associated with increased HDL-cholesterol response to therapies.⁵¹⁴

1.5.1.5 Non-Catalytic function of LPL and Lipoprotein Metabolism

1.5.1.5.1 Accelerated Catabolism of Lipoproteins by Catalytically-inactive LPL

Lipolytic modification of lipoproteins does not appear to be necessary for increased catabolism because the effect of LPL is not prevented by enzymatic inhibitors p-nitrophenyl N-dodecylcarbamate and phenylmethylsulfonyl fluoride.⁵¹⁵,⁵¹⁶ HSPGs are involved in LPL-mediated lipoprotein binding and uptake, as heparinase treatment sharply abrogates the enhancing effect of LPL on LDL and VLDL binding to cells.⁵¹⁷ Lipoproteins such as chylomicrons, VLDL, and LDL can bind to HSPG due to the presence of apoE and B, this binding affinity for HSPG is increased by up to 40-fold by LPL.⁵¹⁷-⁵¹⁹ In vivo studies demonstrate that the transgenic expression of catalytically inactive LPL in muscles enhances triglyceride hydrolysis as well as whole particle internalization and selective cholesteryl ester uptake.⁵²⁰,⁵²¹ The impairment of LPL binding to HSPGs leads to a defective delivery and clearance of lipids to the liver, interfering with normal lipoprotein metabolism.⁵²²

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1.5.1.5.2 Lipoprotein Receptor-Dependent Pathway

The LPL-enhanced metabolism of lipoproteins can be mediated by lipoprotein receptors. For example, LPL increases VLDL binding to the VLDL receptor via apoE.\textsuperscript{523, 524} It is also reported that bovine and human LPLs were able to increase the specific binding of chylomicrons to LRP by up to 30–40-fold in human fibroblasts.\textsuperscript{518} Similarly, LDLR is involved in LPL-facilitated LDL uptake. The uptake of LDL into wild type (LDLR+/+) primary aortic endothelial cells was almost doubled after the addition of LPL, however, there was virtually no LPL-mediated change of LDL uptake into LDLR-/- cells.\textsuperscript{525} It is assumed that LPL can facilitating docking of LDL/VLDL or chylomicrons on cell surface via HSPGs, and then those bound lipoproteins are more susceptible for receptor-mediated internalization.\textsuperscript{515, 526} Also, LPL may directly transfer lipoproteins to receptors since LPL has been found to have the affinity for both receptors and lipoproteins.\textsuperscript{423, 437, 438, 440, 527}

1.5.1.5.3 Lipoprotein Receptor-Independent Pathway

LPL can also facilitate lipoprotein clearance through non-receptor mediated pathways. For example, in vascular smooth muscle cells expressing LRP and the VLDL receptor but not the LDL receptor, the inhibition of VLDL receptor and LRP did not completely abolish the internalization and degradation of LPL-associated beta-VLDL, suggesting there is a receptor-independent pathway.\textsuperscript{528} Furthermore, the increased uptake of LDL by LPL is observed in LDLR and LRP deficient cell lines, further supporting that the LPL facilitated LDL uptake is independent of LDLR and LRP pathways.\textsuperscript{529} This non-receptor dependent pathway is postulated to be the direct endocytosis via HSPG.\textsuperscript{530} By immunoelectron microscopy, a difference between receptor-dependent and independent lipoprotein uptake has been described. In general, the receptor-dependent pathway is associated with a rapid internalization with the formation of central, lysosome-like vesicles in cells, however, the receptor-independent pathway is a slow endocytotic process with small, widely distributed intracellular vesicles.\textsuperscript{531}

1.5.1.6 LPL and Atherosclerosis

1.5.1.6.1 LPL Expression in Atherosclerotic Lesions

Several lines of evidence indicate that LPL is intimately involved in the pathogenesis of atherosclerosis. LPL expression in macrophages is correlated with risk factors for
atherosclerosis. In patients with type 2 diabetes, increased levels of LPL mRNA, activity, and protein were detected in macrophages when compared to macrophages from healthy control subjects. Similarly, macrophages from patients with familial hypercholesterolemia showed a significant increase in LPL production when compared with macrophages of control subjects. The higher expression of LPL in macrophages was also found in atherosclerosis-susceptible C57BL/6J mice in comparison to atherosclerosis-resistant C3H/HeN mice.

Direct evidence of LPL upregulation in atherosclerotic tissue has been described in several in vivo studies. During the neointimal formation following balloon aortic denudation or placement of silastic collar around the aorta in normolipidemic rabbits and rats, striking LPL immunostaining was displayed, and the expression level was parallel to the extent of neointimal thickening. Northern blot analysis confirmed that macrophage-derived foam cells are the major source of LPL production. The upregulation of macrophage LPL synthesis in atherosclerosis is also evident in coronary arteries in hearts from patients undergoing cardiac allografts.

1.5.1.6.2 Paradoxical Role of Systemic LPL Expression in Atherosclerosis

Contradictory results have been obtained in animal models where LPL was either overexpressed or knocked out to observe the outcomes in atherogenesis.

In rabbits fed with a cholesterol-rich diet, systemic overexpression of LPL inhibited diet-induced hypercholesterolemia, and dramatically suppressed the development of aortic atherosclerosis. In apoE KO mice, overexpression also decreases the development of atherosclerosis, as the LPL transgenic mice had 2-fold smaller fatty streak lesions in the aortic sinus compared to the control apoE-/- mice.

However, when human LPL was successfully expressed in apoE-/- mice, the extent of occlusion in the aortic sinus region of male LPL transgenic mice increased by 51% compared with control mice after 8 weeks on Western diet. Moreover, transgenic Watanabe heritable hyperlipidemic (WHHL) rabbits overexpressing human LPL developed two-fold greater aortic atherosclerosis than non-transgenic WHHL rabbits. In heterozygous (LPL+/-) mice with LDLR-/- background, a worse lipid profile was noted after 3-month atherogenic diet, but these mice did
not develop more atherosclerosis, as compared to LPL+/+ mice. It was suggested that the
decreased expression of LPL in the atherosclerotic lesion may have beneficial effects by
preventing the retention of atherogenic lipoproteins. The discrepancy of the LPL effect in
animal models may originate from the use of different animal species and experimental
strategies.

In human, LPL deficiency or insufficiency has been described in several mutations in the LPL
gene and associated with increased incidence of atherosclerosis. In the quantitative coronary
angiographic clinical trial REGRESS, carriers with Asp9→Asn substitution in the LPL enzyme
more often had a positive family history of cardiovascular disease and more progression of
coronary atherosclerosis than non-carriers. Also, low enzymatic activity in patients with LPL
gene mutations is associated with severe angina pectoris, suggesting an increased incidence of
coronary atherosclerosis. In a 14 to 30-year follow-up of four patients with familial
chylomicronemia, serial evaluations for carotid, peripheral, and coronary atherosclerosis
revealed premature peripheral or coronary atherosclerosis (or both) before age 55 for all four
patients. Furthermore, postheparin LPL mass and activity in patients are well correlated with
the volume of total calcific atherosclerosis and coronary artery calcific atherosclerosis.

Clinical data from LPL deficiency/insufficiency substantiate the anti-atherogenic character of
LPL, this effect can be ascribed to a beneficial lipid profile associated with LPL. For example,
LPL activity is positively related to HDL level, and helps the clearance of apoB-containing lipoproteins.

It is clear that lipolytic products of lipoproteins by LPL can release LPL from HSPGs. With
decreased LPL catalytic activity, there may be an increased retention of lipids on HSPGs in
peripheral tissues, subsequently leading to increased LDL uptake. For example, catalytically
dysfunctional LPL due to the D9N polymorphism caused 4.6 fold enhanced binding and 2.6 fold
increased internalization of LDL in comparison to wild-type LPL. Moreover, monocytes are
more inclined to adhere to cells expressing catalytically inactive LPL than wild-type dimer.
1.5.1.6.3 Pro-atherogenic Role of LPL Expression in Macrophage

As described before, there is an increased expression of LPL in atherosclerotic tissues, especially in macrophages. Despite the low level of plasma LPL in diabetic patients, macrophages isolated from those patients produce more LPL than those from normal controls. These interesting findings raise the question of a potential pro-atherogenic role of macrophage LPL. Several animal models with macrophage-specific expression of LPL were generated to address this question.

LPL knock-out in macrophages decreases atherosclerosis in animals. Lethally irradiated C57BL/6 mice transplanted with fetal liver cells or bone marrow from LPL-/- mice to produce macrophage specific knockout of LPL. After 19 weeks on the atherogenic diet, mice with macrophage LPL knockout developed less severe atherosclerosis in aorta with ~50% reduction in mean lesion area.\(^{447,538,546}\) Similarly, in LDLR(-/-) mice lacking macrophage LPL, the mean lesion area in the proximal aorta was significantly reduced compared with mice with macrophage LPL expression after 8 weeks on Western diet. A dose-dependent effect of macrophage LPL on mean aortic lesion area was also revealed by en face analysis of the aorta in an ascending order of LPL(-/-), LPL(-/+), and LPL(+/+).\(^{547}\)

Increased atherosclerosis has been described in rabbits engineered to express human LPL under the control of the human scavenger receptor enhancer/promoter, which specifically drives macrophage-specific expression of human LPL gene. Atherosclerotic lesions were significantly increased in these transgenic rabbits compared to non-transgenic littermates after 16 weeks of a diet containing 0.3% cholesterol. A 1.4-fold increase in total aortic en face atherosclerotic lesions and a 2-fold increase in intimal lesions were also observed.\(^{548}\)

In those animal models, there were no apparent differences in plasma post-heparin LPL activity and lipoprotein metabolism between two groups. These experimental results highly support a pro-atherogenic role of macrophage-derived LPL.

1.5.1.6.4 Potential Pro-atherogenic Mechanisms of Macrophage LPL

Several mechanisms have been proposed to address the proatherogenicity of macrophage LPL (Figure 1-9). LPL on the macrophage cell surface may facilitate the binding and uptake of LDL...
to promote foam cell formation.\textsuperscript{447, 547, 549, 550} By producing lipolytic products and NO, LPL increases the permeability of endothelium, so that lipoproteins can more readily infiltrate through the endothelial barrier into the intima.\textsuperscript{551, 552} LPL diffused in the intima may accelerate the anchoring of penetrating lipoproteins to ECM, promoting their internalization by macrophages.\textsuperscript{551, 553, 554} LPL can also cause lipoproteins to aggregate, increasing the atherogenicity of LDL.\textsuperscript{555} With cell surface HSPGs acting as extracellular chaperones, LPL can act as an adhesion molecule to recruit more monocytes into atherosclerosis-prone sites.\textsuperscript{556, 557} Furthermore, LPL has already been proven to be able to act as a mitogen to stimulate SMC proliferation.\textsuperscript{558} This proliferative effect may be mediated by free fatty acids released from

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Figure 1-9. The proatherogenic mechanisms of macrophage LPL. Macrophage-expressed LPL is the major source of LPL in atherosclerotic lesions. Secreted LPL can adhere to endothelial surface to promote the LDL binding (1) and subsequent retention in intima. Released free fatty acid can increase the endothelial permeability (2) to increase LDL and macrophage infiltration. LPL can increase the LDL atherogenicity by anchoring LDL on extracellular matrix (ECM) for modification (3) and aggregation (4). Meanwhile, LPL can act as adhesion molecule (5) to recruit monocytes into local atherosclerotic sites. Furthermore, LPL promotes the LDL internalization by macrophages and smooth muscle cells (SMC) to form foam cells (6).
lipoproteins by LPL lipolysis. Finally, LPL can magnify the inflammatory response of macrophages, as TNF-α and PDGF production in macrophages is upregulated after LPL stimulation.

1.5.2 EL
1.5.2.1 Gene Locus and Structure
The EL gene was discovered in 1999 by two independent research groups using different strategies. The human genome database revealed that the EL locus is on chromosome 18 (18q21.1), and has a homology of 45% with LPL, 40% with HL, and 27% with pancreatic lipase. Importantly, critical structural characteristics consistent with the lipase gene family are conserved in this gene. Comparison of the cDNA sequence with the human genome sequence indicates that EL contains at least 11 exons, within which the first exon is not translated and its function is unclear; the second exon codes for a signal peptide of 18 amino acid residues; the last exon has an untranslated region of 2172 nucleotides. The full open reading frame of EL gene is 1500 nucleotides in length encoding a highly conserved protein of 500 amino acids.

1.5.2.2 Protein Structure-Function Relationships
1.5.2.2.1 Protein Sequence
The mature human EL protein consists of 482 amino acids. Several truncated EL isoforms are also found due to varying splicing sites in mRNA; those truncated isoforms not functional and not secreted. The predicted molecular weight of mature EL protein is approximately 55 kD. There are also significant amounts of smaller EL species with a molecular weight of 40 kD, which is believed to be the product of specific proteolytic cleavage at the amino acid sequence of RNKR, a known recognition sequence for the proprotein convertase family. Ten cysteine residues which are found in all lipase family members are also conserved in EL to form disulfide bonds in order to create the 3-dimensional conformation necessary for enzymatic activity.
1.5.2.2.2 Protein Glycosylation

Reduction in molecular mass of EL after treatment with glycosidases or treatment of EL-expressing cells with the glycosylation inhibitor tunicamycin suggests that EL is a glycosylated protein. Each putative glycosylation site of asparagine was examined by site-directed mutagenesis. Mutation at Asn60 markedly reduced the secretion and slightly increased the specific activity of EL; however, mutation of Asn116 increased the specific activity but not the secretion. Mutation of both Asn60 and Asn-116 resulted into an decreased apparent K_m and increased apparent V_max. Asn373 mutation significantly reduced the specific activity with a decreased apparent V_max whereas the secretion was not influenced. Neither the secretion nor the specific activity was changed after the mutation at Asn471. Asn449 seemed not to be involved in glycosylation as mutation of this site resulted in no change in secretion, activity, or molecular mass. Among those glycosylation sites, only the Asn373 mutant demonstrated a 3-fold decrease in bridging function compared with wild-type EL, suggesting this glycosylation site also plays a role in the EL bridging function.

1.5.2.2.3 Catalytic Triad and Surface Loop

Alignment of the amino acid sequence of human LPL with HL reveals the conservation of the GXSXG lipase motif in EL, with serine at position 169, aspartic acid at 193, and histidine at 274. This motif functions as a catalytic center to hydrolyze specific substrates. Similar to LPL and HL, 19 residues from two stretches of hydrophobic amino acids at positions 163-172 and 272-281 form a conserved lid structure. The protein sequences among EL, LPL, and HL are almost identical in the regions bordering the lid, whereas the lid domain contains the greatest sequence divergence in these lipases. On this occasion, the EL lid region is three residues shorter and less amphipathic compared with LPL and HL. This difference may be responsible for different substrate preferences. The lid structure covers the catalytic center in 3-dimensional models and presumably controls the interaction of EL with lipid substrates. Mutation of specific residues of the EL lid (G241R, 245R, or E250Q) causes a moderate increase in the triglyceridase/phospholipase ratio compared with wild-type EL. In particular, the 245R mutation is more efficient in hydrolyzing triglycerides. By exchanging the EL lid domain with that of LPL, the substrate specificity of phospholipids in the newly formed EL chimera was greatly compromised. This study also demonstrated that the substrate specificity was determined by the
EL lid rather than C-terminal domain. Meanwhile, the lid domain could help substrate binding to EL C-domain.\textsuperscript{416}

1.5.2.2.4 C-terminus and Substrate Specificity

As well-recognized, the substrate specificity is usually conferred by the lid structure in N-terminus. Recent findings show that the C-terminal domain may play a role in the determination of substrate tropism. For example, inter-generic (rat-human) or human chimeras of HL(C-terminal)-LPL(N-terminal) or LPL(C-terminal)-HL(N-terminal) displays the similar triglyceride esterase activity as HL or LPL, respectively; moreover, LPL and HL antibodies against C-terminal domains reduce the enzymatic activity to a great degree.\textsuperscript{435, 566, 567} As for EL, the substitution of EL C-terminal domain for that of LPL switches substrate preference of wild-type EL from phospholipid-rich HDL to triglyceride-rich lipoproteins.\textsuperscript{568} These evidences suggest that the C-terminal domain of EL can be also involved in the determination of substrate specificity.

1.5.2.2.5 Heparin-Binding Domain

EL is also a heparin binding protein. The secreted enzyme is bound on cell surfaces and can be displaced by heparin in vitro and in vivo. Alignment of human LPL sequence with other lipase family members demonstrates the conservation of heparin binding domains in EL in C-terminus. These heparin-binding amino acid residues are proposed to be cluster 1: Arg327-Lys329-Arg330-Lys333; cluster 2: Arg312-Lys313-Arg315; cluster 3: Gly184-Arg188; and cluster 4, Lys352-Arg450-Lys452-Lys459.\textsuperscript{386} The heparin binding property of EL can be modulated by glycosylation.

1.5.2.2.6 Homodimerization of EL Molecules

LPL and HL undergo homodimerization within cells before secretion. The binding of two identical monomers can stabilize the protein in extracellular milieu and is necessary to preserve enzymatic activity. There is no direct evidence to demonstrating EL homodimerization, however, a clue obtained from an \textit{in vitro} cell model may imply that EL is able to form dimers. In this experiment, Cos-7 cells were stably co-transfected with both human and mouse EL. Human and
mouse EL are highly homologous so that dimerization between them is possible. Compellingly, human EL was coimmunoprecipitated with mouse EL by using an anti-mouse EL antibody, whereas mixing conditioned media from cells expressing either mouse or human EL alone did not produce coimmunoprecipitation of both human and mouse EL. Therefore, it was deduced that human EL may dimerize with mouse EL prior to secretion.569

1.5.2.3 EL Expression and Regulation

1.5.2.3.1 Tissue Expression of EL

Endothelial cells do not produce LPL or HL, so the lipase found in endothelial cells such human umbilical vein endothelial cells, human coronary artery endothelial cells, and murine endothelial-like yolk sac cells with a hydrolytic preference on phospholipids is termed endothelial lipase.385,386 However, EL expression is not restricted to endothelial cells, as EL expression in the placenta, liver, lung, kidney, testis, thyroid, and corpus luteum of the ovary was also observed by in situ hybridization analysis, PCR, and Northern blot in several studies.385,386,570 Small intestine, mammary gland, adipose tissue, and the adrenal gland are also able to produce endothelial lipase.571 In addition, the expression of EL is reported in several macrophage cell lines such as human THP-1 and mouse RAW 294.7 cells, as well as primary macrophages.385,386 The EL expression at specific tissue sites is regulated by both 5’ and 3’ flanking regions. Regulatory elements within 11.4 kb of 5’ and 9.9 kb of 3’ human EL flanking region control the expression of EL in the small intestine, ovary, testis, mammary gland, brain, lung, aorta, adipose tissue and the adrenals, whereas kidney-specific EL expression is under the control of regulatory sequences between 27.4 and 11.4 kb of 5’ or 9.9 and 48.7 kb of 3’ human EL flanking regions.571

1.5.2.3.2 Atherosclerosis Risk Factors and EL Expression

As mentioned above, EL expression was significantly increased in THP-1 cells after oxLDL treatment.385 Secondly, in spontaneously hypertensive rats (SHR-SP) and Ang II-induced hypertensive rats, multiple tissues were excised and analyzed by RNase protection assays for EL expression. This study showed that EL mRNA levels were upregulated in tissues including the aorta, heart, and lung from both hypertensive rats, compared to the control, with highest EL expression in the aorta.572 Furthermore, the effect of diabetes mellitus on EL expression was
examined in placenta taken from patients with type 1 diabetes mellitus. A higher expression level of EL was detected in placental tissues from these patients when compared to normal controls. In addition, the difference was even more pronounced in poorly controlled diabetics compared to well-controlled diabetics. Elevated inflammatory status triggered by lipopolysaccharide injection markedly increased EL mRNA and protein levels in diverse tissues from mice. Finally, shear stress and cyclic stretch have been found to induce 2~3 fold increase of EL in endothelia from human umbilical veins and coronary arteries.

1.5.2.3.3 Cytokines and EL Expression

As a new member of lipase gene family, the information regarding the regulation of EL by cytokines is relatively limited.

Though EL mRNA is expressed at low levels in quiescent rat aortic SMCs, the treatment with angiotensin II and phorbol 12-myristate 13-acetate (PMA) significantly increased EL mRNA levels by 2.9- and 3.3-fold in a time-dependent manner, with a maximum expression at 24 hours.

TNF-α and IL-1β are two well-known enhancers for EL expression. In HUVECs and human coronary artery endothelial cells, a dose-and time-dependent upregulation of EL secretion ensues after the treatment with either TNF-α or IL-1β.

One recent study reported an inhibitory effect of angiopoietin-like protein 3 (ANGPTL 3) on EL expression. ANGPTL 3, is a liver-specific secretory factor that has been shown to increase plasma triglyceride via LPL inhibition. The plasma level of ANGPTL 3 is found to be well correlated with plasma HDL cholesterol and phospholipid levels in human. In vivo, there is higher EL activity and resultant lower HDL levels in ANGPTL 3 knockout mice; the supplementation of adenovirus-ANGPTL 3 in turn decreases EL activity and reverses HDL level back to the normal. Furthermore, ANGPTL 3 suppresses the phospholipase activity of EL in vitro, this action is thought to be mediated by the interaction between EL and the heparin-binding domain in the N-terminus of ANGPTL 3.
Some nuclear receptors and transcriptional factors could be involved in the regulation of EL expression. Jin et al. had examined the effect of various signaling pathways on EL expression using SN50 (an NF-κB pathway inhibitor), SB 20358 (a p38 MAPK–specific inhibitor), PD 98059 (a p42/44 MAPK–specific inhibitor), GF 109203X (a PKC inhibitor), and D609 (a PC-PLC–activity inhibitor). None of these compounds had any effect on EL expression in human umbilical vein endothelial cells (HUVECs) after IL-1β and TNF-α stimulation except SN50, which diminished EL mRNA induction by above cytokines. PPARs and LXR, two important gene expression regulators, also affect EL expression. Treatment of brain capillary endothelial cells with 24(S)OH-cholesterol (LXR agonist), bezafibrate (PPAR-α agonist), or pioglitazone (PPAR-γ agonist) led to the EL down-regulation at mRNA and protein levels.

1.5.2.4 EL and Lipoprotein Metabolism

1.5.2.4.1 Substrate Specificity

EL exhibits a preference in hydrolyzing phospholipids, this phospholipase activity is inhibited by apoCII when using dioleoylphosphatidylcholine liposomes as substrates. In contrast to 24.1 for HL and 139.9 for LPL, the ratio of triglyceridase to phospholipase activity for EL is 0.65, suggesting that EL is in favor of phospholipids as its substrates.

EL has both phospholipase A₁ and A₂ activities. The liberation of either saturated or unsaturated fatty acids were detected by gas chromatography analysis in the reaction mixtures of EL with reconstituted discoidal HDLs (rHDLs) that contained free cholesterol, apolipoprotein AI, and either 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), 1-palmitoyl-2-linoleoylphosphatidylcholine (PLPC), or 1-palmitoyl-2-arachidonoyl-phosphatidylcholine (PAPC). The cleavage of sn-1 fatty acids is the prerequisite for the cleavage of sn-2 acyl chain because EL-mediated deacylation does not occur in rHDLs containing 1-O-1'-hexadecenyl-2-arachidonoylphosphatidylcholine, which is not cleavable due to a nonhydrolyzable alkyl ether linkage at sn-1 position. The lack of phospholipase A₂ activity of EL in lysophosphatidylcholine consolidates that conclusion. In addition, hydrolysis rate at sn-1 site is higher than that at sn-2 site of phospholipids. Even for phospholipids, EL displays different preferences on phospholipids, typically, in a descending order of (PAPC)rHDL ~ (PLPC)rHDL > (POPC)rHDL > (PDPC [1-palmitoyl-2-docosahexaenoyl-phosphatidylcholine])rHDL.
EL-released free fatty acids can be an alternative source for lipid synthesis in liver as well as energy storage in adipose tissues. When HepG2 hepatocytes with EL overexpression were incubated with [14C]-HDL-PC, and cellular lipids were analyzed by thin layer chromatography, there was an increased amount of cellular [14C]-lipids compared to non-EL-expressing hepatocytes. These EL-liberated fatty acids were mainly incorporated into phospholipids and triacylglycerols in hepatocytes. EL is generally expressed in adipocytes at a very low level. In contrast, EL expression and phospholipase activity are considerably increased in mouse adipose tissue as well as isolated adipocytes after LPL is knocked out. Since LPL deficiency does not cause abnormal adipose distribution, it is assumed that the EL overexpression compensates for LPL deficiency to produce fatty acids which are absorbed by adipocytes and incorporated into fat.

1.5.2.4.2 EL Activity and Apolipoprotein AII

Mature HDL is the favored lipoprotein for EL due to the abundance of phospholipids on HDL particles. ApoAII can compete with and displace apoAI from lipoprotein particles due to the higher lipid affinity than apoAI. ApoAII can modulate HDL metabolism via the inhibition of various enzymes including LCAT, CETP, and PLTP. Similarly, the enzymatic activity of EL is also inhibited by apoAII. Human EL was expressed in human apoAI or apoAI/AII transgenic mice by the injection of low-dose EL-encoding adenoviral vectors. Despite similar levels of EL protein expression, the apoAI/AII double transgenic mice had lower plasma phospholipase activity and less reduction of HDL-CHOLESTEROL, phospholipid, and apoAI levels than the apoAI single transgenic mice; apoAII also reduced EL-mediated HDL binding to HSPGs. In vitro, EL did not hydrolyze reconstituted HDL containing only apoAII and phospholipids in one study, but the hydrolysis in rHDL containing both apoAI and AII was greater than in rHDLs containing apoAI only in another study.

1.5.2.4.3 Regulation of HDL Metabolism

An accumulating body of evidence indicates that EL is a major determinant of plasma HDL levels. In wild-type C57BL/6 mice, expression of human EL via intravenous injection of EL-encoding adenovirus reduced plasma HDL level significantly. The HDL level was undetectable
in the first 14 days after adenovirus injection and this effect lasted at least 41 days. Similar results were also observed in human apoAI transgenic mice where HDL cholesterol and apoAI were reduced after EL overexpression. Using the same strategy, >80% decrease of HDL cholesterol was observed in chow-fed LDL receptor-deficient mice as well. Strikingly, HDL cholesterol, phospholipids and total cholesterol was increased 24-48 hours post intravenous administration of EL polyclonal antibodies in three mouse models: wild-type, hepatic lipase knockout, and human apoAI transgenic mice. In C57B1/6 background mice, fasting plasma HDL cholesterol was increased in EL(-/-) mice and EL(+/-) mice, whereas EL transgenic mice decreased HDL cholesterol level by 19%, when compared with syngeneic controls. Moreover, the turnover time of HDL was much longer in EL knockout mice than wild-type ones. In humans, 584C→T polymorphism of EL is relatively common, and this polymorphism could be associated with low phospholipase activity. An investigation has demonstrated that there is an allele-dependent variation in HDL cholesterol as well as apoAI, with the ranking order of TT > CT > CC. These findings support that the genetic variation in the EL gene can influence plasma HDL cholesterol levels.

The phospholipase activity of EL is sufficient to remodel HDLs into small particles. HDL particles after inhibition of EL by antibodies in wild-type, HL/-, and human apoAI transgenic mice were noticeably larger in size and richer in phospholipid contents, similar results were also obtained from EL knockout mice. It is also reported that apoAI is not shed during EL-mediated HDL remodeling. Spherical rHDL particles containing apoAI or apoAI/AII were incubated with EL, and the diameter decreased with the reduction of phospholipid contents. However, this change did not affect the conformation of apoAI, and neither apoAI nor apoAII dissociated from rHDLs.

The functional domains responsible for the cell surface binding of EL are separate from the catalytic site. As well-documented before, the non-catalytic function of LPL is shown to participate in the metabolism of lipoproteins, as such, the non-catalytic function of EL can also contribute to HDL metabolism.

In order to test this hypothesis, the HDL binding and metabolism were investigated using an in vitro cell culture model. When the enzymatically inactive EL-S149A was expressed in CHO cells, the facilitation of HDL binding by EL-S149A was comparable to that by native EL.
addition, the inhibition of EL catalytic activity by tetrahydrolipstatin (THL) increased EL-mediated HDL binding and selective cholesterol uptake by hepatocytes. Lipase-released free fatty acids can displace lipases from cell surface. Due to the reduced production of free fatty acids consequent to hydrolytic incapability of EL-S149A, EL bound HDL is then more inclined to adhere to cell surface. Unlike LDL, of which the majority is internalized after lipase bridging, 70% of bound HDL through EL is liberated back to medium in CHO cells.

Cell surface HSPG is an obligatory component for the bridging function of EL. Associated with HSPG by electrostatic interaction, EL can mediate lipoprotein binding and internalization. The dissociation of EL from HSPGs resulted in decreased HDL and LDL binding by 3–4.4 fold compared to control conditions. Similarly, the abrogation of proteoglycan sulfation by either sodium chlorate or heparin eliminated EL-mediated HDL and LDL binding.

Wild-type EL or catalytically inactive EL (AdELS149A) was delivered into wild-type, apoAI transgenic, and HL knockout mice by adenovirus. Both wild-type and catalytically-inactive EL were bound to HSPGs at a high expression level in mice. Overexpression of wild-type EL decreased levels of total cholesterol, HDL cholesterol, phospholipids, and apoAI in all 3 mouse models. Expression of catalytically-inactive EL did not decrease lipid or apoAI levels in wild-type and apoAI transgenic mice, whereas reduced total cholesterol, HDL cholesterol, and phospholipids levels in HL-deficient mice, but the magnitude of reduction was less than that in HL-/- mice overexpressing wild-type EL. This experiment shows the evidence that catalytically-inactive EL has some ability to mediate HDL binding/uptake.

EL may also regulate other regulatory factors in HDL metabolism. Despite increased levels of hepatic LCAT mRNA and plasma protein in EL” mice, the endogenous esterification rate of LCAT in these mice was significantly impaired (50–60%), suggesting EL may modulate LCAT activity. HL and LPL are also upregulated after EL knockout, whereas, PLTP is downregulated. These changes after EL expression also could affect HDL metabolism.

1.5.2.4.4 EL and ApoB Containing Lipoprotein Metabolism

The level of apoB-containing lipoproteins is regulated by EL although to a lesser extent than HDL. In chow-fed LDLR/- mice, introduction of human EL by adenovirus reduced
VLDL/LDL cholesterol by approximately 50%. In C57Bl/6 mice, the EL expression level is positively correlated with plasma LDL and cholesterol levels with an ascending order of EL-/-, EL+/-, wild-type, and EL transgenic mice. The above findings were also confirmed in apoE-deficient, LDLR-deficient, and human apoB transgenic mice with hepatic expression of human EL. An marked decrease in VLDL/LDL cholesterol, phospholipid, and apoB levels was detected, as well, the catabolism of LDL apolipoprotein and phospholipid was increased. The deficiency of EL also elevates the level of other lipoproteins like chylomicrons, VLDL, remnants, VLDL, and IDL.

EL-mediated apoB-containing lipoprotein metabolism is dependent on its catalytic activity to a large extent, which is supported by a fact that a catalytically inactive form of human EL (ELS149A) did not reduce but increased plasma lipids in the above 3 mouse models. The role of noncatalytic function of EL in apoB-containing lipoprotein metabolism is still unaddressed in mouse models. However, an in vitro study may suggest a role of noncatalytic function of EL in LDL metabolism. In that study, an enzymatically inactive EL-S149A was equally effective in facilitating LDL binding to CHO cells as native EL, and 90% of the bound LDL was internalized by CHO cells.

1.5.2.5 EL and Atherosclerosis

1.5.2.5.1 EL Expression in Atherosclerotic Lesions
Since EL can be synthesized by macrophages, EL expression in atherosclerotic lesions was then investigated. Ten autopsy specimens from coronary arteries were examined for EL immunohistochemically. Endothelial cells and SMCs in media constitutively expressed EL even in non-atherosclerotic arteries. However, in atheroma, EL is more highly expressed, and colocalized with infiltrated macrophages, SMCs, and endothelial cells. This supports the involvement of EL in the development of atherosclerosis. In cultured macrophages, oxLDL treatment raises EL production, further suggesting the implication of EL in atherosclerosis.

1.5.2.5.2 EL Polymorphism and Atherosclerosis
Several EL gene polymorphisms have been found to be associated with plasma HDL levels, and the T111I missense polymorphism in exon 3 is the most common allele in humans.
In the Quebec family study where 281 women and 216 men aging 17 to 76 participated, the I allele was significantly correlated with higher apoAI and HDL cholesterol levels in women when compared with the T allele. The relationship of EL genetic variant $T_1I_1I_1$ and HDL level was again confirmed in the Lipoprotein and Coronary Atherosclerosis Study (LCAS) in a population of 372 individuals. Patients with the TT allele had a 14% higher mean HDL cholesterol level compared with those with the CC allele. However, the clinical outcomes and coronary atherosclerotic progression were not significantly different regardless of $T_1I_1I_1$ variation. So this raises the question whether the increased HDL consequent to low EL activity is functionally protective? In the same study, EL $T_1I_1I_1$ variation changed the HDL composition, $I_1$ homozygote had 10% higher HDL$_3$ than TT homozygotes in women. In general, HDL$_3$ is more efficient than HDL$_2$ for the removal of peripheral cholesterol. However, elevated HDL level secondary to low EL activity is predominant in HDL$_2$ particles, the latter have the decreased capacity to induce reverse cholesterol transport, which may explain the lack of difference in clinical outcomes among different genotypes in the LCAS study.

1.5.2.5.3 EL Expression and Atherosclerosis Risk Factors

Despite the ambiguous relation between EL gene polymorphism and atherosclerosis, the level of EL expression is elevated in patients with risk factors for atherosclerosis. In a population of moderately obese men, plasma EL levels were increased, and positively correlated to an inflammatory score calculated from CRP, IL-6, and secretory phospholipase A2 concentrations. Moreover, a positive correlation between postheparin plasma EL levels and body mass index, visceral adipose tissue accumulation, and a proatherogenic lipid profile was also established in a sample of 80 healthy sedentary men.

Family history of atherosclerosis-related diseases is another risk factor for atherosclerosis. In the Study of the Inherited Risk of Atherosclerosis which recruited a population of 858 healthy individuals with a family history of premature coronary heart disease, post-heparin EL mass concentration was approximately 3 fold higher than in controls. Both pre- and post-heparin plasma EL was significantly correlated with all metabolic syndrome factors: waist circumference, blood pressure, triglycerides, HDL cholesterol, and fasting glucose. After adjusting for age, gender, waist circumference, vasoactive medications, hormone replacement
therapy (women), and established cardiovascular risk factors, EL mass concentration in both routine and post-heparin plasma was associated with coronary artery calcification score. Hypertension is also associated with high expression of EL. In stroke-prone spontaneously hypertensive rats (SHR-SP) and Ang II-induced hypertensive rats, EL expression was upregulated in aorta, heart, and lung, as analyzed by RNase protection assays. Thus, it is speculated that EL expression might be increased in hypertensive human subjects.

1.5.2.5.4 EL and Monocyte Recruitment

Monocytes are important in early stage of atherosclerosis, where they may trigger cascades of inflammatory reactions in the intima. Like LPL, EL can act as an adhesion molecule to facilitate monocyte adherence to endothelial cell surface. More adherent monocytes on aortic strips from EL transgenic mice were demonstrated by ex vivo adhesion assays, and less monocytes on aortic strips from EL knock-out mice as compared with wild-type mice. Furthermore, overexpression of EL in COS7 or Pro5 cells significantly enhanced monocyte binding to EL-expressing cells; the EL effect on monocyte adhesion depends on its bridging function as heparin or heparinase treatment inhibited EL-mediated increase of monocyte adhesion in a dose-dependent manner. By facilitating monocyte recruitment, EL could contribute to the atherogenic process.

1.5.2.5.5 EL Expression and Atherosclerosis in Animal Models

The EL knockout animals have been generated in order to investigate the role of EL in atherosclerosis. By crossbreeding EL-null mice with apoE knockout mice, homozygous double knockout animals were generated. When compared with apoE knockout mice, EL/apoE double knockout mice had a relatively higher HDL cholesterol level. Levels of VLDL, IDL, and LDL cholesterol in apoE/EL double knockout mice were also greater than those in apoE knockout animals. Despite this atherogenic lipid profile, these mice with apoE/EL double knockout developed ~70% less atherosclerotic lesions than apo E knockout mice on regular, chow, and high-fat diets.
However, a different observation has been described by Ko et al.. In that study, the influence of EL on atherosclerosis was investigated in apoE knockout mice on the C57BL/6 background. After 26 weeks of chow diet, quantitative morphometric cross-sectional analysis of aortic atherosclerotic lesions displayed no difference between EL/apoE double knockout and apoE knockout mice. They repeated the same investigation in LDLR-/- mice fed a Western diet. Morphometric analysis again revealed no difference in atherosclerotic lesion area between two groups. By far, no more information from animal studies is available, and the role of EL in atherosclerosis remains unclear.
1.6 Rationale

Macrophages play a critical role in the development and progression of atherosclerosis. Upon activation, macrophages obtain an enhanced ability of lipid internalization and accumulation, a process worsened by an impaired ability to efflux cholesterol. This imbalanced equilibrium between lipid uptake and efflux eventually leads to the formation of foam cells, a signature feature of atherogenesis. Concomitant with this process is an increased production of proinflammatory cytokines that augment inflammatory responses and accelerate atherogenesis.

As a newly found lipase member, knowledge about the role of EL in atherosclerosis is very limited and results from animal studies by systemic EL knockout are inconsistent. Similar to EL, systemic expression of LPL gives rise to conflicting outcomes in mice, however, LPL expression in macrophages is consistently proatherogenic. It is possible that EL expression in macrophages may also be proatherogenic, but no studies have addressed this specific issue. The present thesis seeks to reveal new information regarding the specific attributes of EL in human macrophage function including influences on lipid binding/uptake, cytokine expression, and cholesterol efflux.

Although LPL has been extensively investigated with regard to structure-function relationships as well as its role in lipoprotein metabolism, thus far, there is little information about its role in cholesterol efflux and cytokine expression in macrophages. The investigation of the effect of LPL expression on these two major events during atherogenesis will provide further understanding of the mechanisms responsible for the proatherogenic nature of macrophage-derived LPL.

Statins which competitively inhibit HMG-CoA reductase to reduce cholesterol production have been extensively prescribed to treat patients with dyslipidemia. Although many effects in addition to lipid lowering of statins have been reported in previous studies, there are few reports about lipase regulation by statins. In the present thesis, I have investigated the specific role of atorvastatin on lipase expression which provide further insights on pathways of lipase regulation in human macrophages.
1.7 Hypotheses and Objectives

1.7.1 Overarching Hypotheses
The expression of lipases (LPL and EL) in macrophages will alter their functions in cholesterol efflux, lipoprotein binding and uptake, and proinflammatory cytokine expression, where differential roles are attributed to catalytic and non-catalytic functions of lipases. Furthermore, atorvastatin treatment will regulate the expression of lipases (LPL and EL) in macrophage through changes in signaling pathways.

1.7.2 Main Objectives
1. To establish macrophage cell models with lipase suppression or overexpression
2. To explore the effect of lipase suppression or overexpression on cholesterol efflux, lipoprotein binding and uptake, and pro-inflammatory cytokine expression in macrophages and related mechanisms
3. To investigate the effect of atorvastatin on lipase expression in macrophages and related mechanisms.
1.8 Experimental Design

The study design is illustrated in figure 1-10.

Figure 1-10. The experimental design. The EL expression in macrophages will be modulated by lentivirus containing either shRNA for loss of function or EL cDNA for gain of function. The LPL expression will be upregulated by dexamethasone treatment or suppressed by lentivirus-mediated RNA interference. Thereafter, the functional changes of apoAI-mediated cholesterol efflux, lipoprotein binding and uptake, and pro-inflammatory gene expression in macrophages will be evaluated and related mechanisms will be also explored as well. Furthermore, the lipase expression after atorvastatin treatment and implicated signaling pathways will also be investigated.
1.9 Organization of Research Work

Chapter 2. This chapter centers on the effect of LPL and EL on apoAI-mediated cholesterol efflux. The potential mechanisms such as ABCA1 expression, apoAI binding, and phospholipid metabolism have been investigated and a related model was proposed.

Chapter 3. In this chapter, the effect of EL on the binding and uptake of both native and oxidized LDLs was investigated, and the contributory role of catalytic and non-catalytic functions of EL was differentiated.

Chapter 4. After LPL and EL suppression, the production of proinflammatory cytokines IL-1β, 6, 8, MCP-1, and TNF-α was measured in macrophages treated with or without oxidized LDL. Furthermore, the expression of atherosclerosis-related genes was analyzed by microarray.

Chapter 5. The expression of LPL and EL was investigated in atorvastatin-treated macrophages, and major signaling pathways such as Rho protein, LXR, and NF-κB were also explored to elucidate their relationships with LPL and EL regulation by atorvastatin.

Chapter 6. The research findings were summarized in this chapter, and a general discussion related to all research topics was provided.
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Chapter 2. The Expression of Endothelial Lipase and Lipoprotein Lipase Promotes Cholesterol Efflux in THP-1 Derived Human Macrophages

2.1 Introduction and Rationale

Atherosclerosis is a multifactorial disease which indolently progresses into a symptomatic phase, causing severe sequelae such as myocardial infarction and stroke. Recently, a number of lines of research have demonstrated that atherosclerosis is an inflammatory process, where blood leukocytes are recruited into atherosclerosis-prone sites. Among these leukocytes, macrophages predominate in number, and play a pivotal role in the development and progression of atherosclerosis.\(^1\)\(^-\)\(^4\)

The formation of macrophage-derived foam cells is a characteristic of atherogenesis.\(^3\) Cellular lipid homeostasis is dominated by the balance between lipid accumulation and efflux. It is well known that, when activated, macrophages acquire an enhanced ability to internalize lipoproteins and accumulate cholesterol and ultimately transform themselves into lipid-laden foam cells. Lipid efflux is a regulatory pathway which responds to an increased intracellular lipid content. The impairment of lipid efflux in macrophages may aggravate the lipid accumulation and accelerate the progression of atherosclerosis.

Cholesterol is mosaiced in the phospholipid bilayer of cell membranes with an uneven distribution between exo- and endofacial membranes.\(^5\) The interaction of cholesterol with phospholipids such as phosphatidylcholine and sphingomyelin is critical for the maintenance of cholesterol integrity in membrane.\(^6\)\(^,\)\(^7\) Free diffusion, receptor-facilitated efflux, and ATP-binding cassette (ABC) transporters are the principal pathways for cholesterol efflux in macrophages. The active removal of cholesterol via ABC transporters, mainly ABCA1, assumes a major role in cholesterol efflux.\(^8\) For example, in ABCA1 mutated macrophages, cholesterol efflux was impaired and an increased accumulation of intracellular cholesterol followed.\(^9\)\(^,\)\(^10\) Extracellular apolipoprotein AI (ApoAI) is recognized as the primary acceptor of ABCA1-mediated cholesterol efflux.

\(^8\) A version of this chapter will be submitted for publication. Qiu, G. and Hill, J.S. The expression of endothelial lipase promotes apoAI-mediated cholesterol efflux from human macrophages.

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Lipoprotein lipase (LPL) and endothelial lipase (EL) are two major lipases secreted by human macrophages, and their expression in macrophages have been found in human atherosclerotic lesions.\textsuperscript{11-13} Evidence from animal studies supports that LPL expression in macrophages is proatherogenic.\textsuperscript{14,15} Although the proatherogenic role of LPL has been largely ascribed to its facilitating role in LDL uptake by macrophages,\textsuperscript{15,16} it is still possible that LPL may modify the lipid deposition by altering cholesterol efflux. Thus far, the effect of LPL on macrophage cholesterol efflux has not been investigated.

EL has also been implicated in atherosclerosis. For example, increased levels of plasma EL has been associated with visceral obesity, metabolic syndrome, inflammation, and premature coronary heart disease.\textsuperscript{17-19} However, inconsistent outcomes have been described in two animal studies. One study in apoE knockout mice found that EL deficiency significantly decreased atherosclerosis,\textsuperscript{20} whereas no significant changes were observed after EL knockout in another study in which both apoE and LDLR knockout mice were investigated.\textsuperscript{21} Therefore, the role of EL in atherogenesis still remains unclear, and investigation of the effects of EL on macrophage function such as cholesterol efflux may provide useful information to help clarify the role of macrophage-derived EL in atherosclerosis.

LPL and EL share common structural features including a large N-terminal domain responsible for lipid hydrolysis and a small C-terminal domain involved in ligand binding (bridging function). Also, two lipase monomer subunits are necessary to form a catalytically active dimer (probably in a head-to-tail manner).\textsuperscript{22} Despite varying substrate preferences, both LPL and EL are capable of hydrolyzing phospholipids,\textsuperscript{23-25} implying that LPL and EL may influence the membrane cholesterol integrity by altering phospholipid composition. Moreover, the intrinsic ability of these lipases to bind to apolipoprotein B100 (apoB\textsubscript{100}) suggests they might also enhance the apoAI interaction with the membrane,\textsuperscript{26-28} thereby altering cholesterol efflux. However, the effects of LPL and EL in apoAI-mediated cholesterol efflux have not been investigated.
2.2 Hypotheses and Specific Aims

2.2.1 Hypotheses

The expression of lipases (LPL and EL) will impair the apoAI-mediated cholesterol efflux in macrophages with specific roles attributed to their catalytic and non-catalytic functions.

2.2.2 Specific Aims

1. To establish lipase suppressed or overexpressing macrophage models.
   a. Lipase suppression by RNA interference through the transduction of lentivirus containing shRNA specific for each lipase
   b. LPL overexpression in macrophages by the treatment with dexamethasone
   c. EL overexpression in macrophages by lentivirus transduction of EL cDNA
   d. EL overexpression in FLP-IN 293 cells by stable transfection

2. To analyze apoAI-mediated cholesterol efflux in above cell models.
   a. Cholesterol efflux in lipase suppressed or overexpressing macrophages and 293 cells
   b. Cholesterol efflux in macrophages after the addition of exogenous bLPL and EL

3. To clarify the relative role of catalytic and non-catalytic function of LPL and EL in cholesterol efflux
   a. Cholesterol efflux after the catalytic inhibition by tetrahydrolipstatin in lipase-overexpressing macrophages
   b. Cholesterol efflux after the inhibition of lipase bridging function by heparin in lipase-overexpressing macrophages
   c. Cholesterol efflux in lipase-overexpressing macrophages after the treatment of both THL and heparin

4. To investigate LPL and EL effects on ABCA1 expression
   a. ABCA1 expression after lipase suppression
   b. ABCA1 expression after lipase overexpression

5. To analyze apoAI binding in macrophages after lipase suppression and overexpression
   a. ApoAI binding in lipase-suppressed macrophages
   b. ApoAI binding in lipase-overexpressing macrophages and 293 cells
   c. The role of catalytic and non-catalytic function of lipases on apoAI binding
6. To analyze lipid composition after lipase suppression and overexpression
   a. Analysis of lipid composition in lipase-suppressed macrophages
   b. Analysis of lipid composition in lipase-overexpressing macrophages
   c. Analysis of lipid composition in lipase-overexpressing 293 cells
2.3 Material and Methods

2.3.1 Cell Culture

THP-1 human monocytes (ATCC number TIB-202) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic (Invitrogen, 15240-096), 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 1.0 mM sodium pyruvate.  293T human embryonic kidney (HEK) cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS, and 1% antibiotic-antimycotic. Flp-In™ 293 Cells (Invitrogen, R750-07) were cultured in DMEM with 10% FBS, 1% antibiotic-antimycotic, and 100µg/ml Zeocin. All cells were incubated in humidified incubator at 95% air, 5% carbon dioxide, and 37°C and used within 20 passages.

2.3.2 Candidate shRNA Selection and Incorporation into pSHAG Vector

Several web-based small interfering RNA (siRNA) design tools (Invitrogen, Qiagen, Dhharmacon, Ambion, GeneScript, and RNAi Central) were utilized for the selection of candidate small hairpin RNA (shRNA) sequences, based on the sequence reproducibility in their outputs, and the accessibility of target sites (S-fold). Scrambled shRNA without homology with any known genes served as a control (shRNA sequences are listed in table 2-1).

Table 2-1. Sequence of shRNA oligonucleotides for LPL, EL and control constructs.

<table>
<thead>
<tr>
<th>Name</th>
<th>Target site</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPL-shRNA-Forward</td>
<td>470</td>
<td>ACATTGGAGTCTGGTTCTCTGTACAGAAAGCTTGTGTATAAGAGGAGGCCAGATTCATTCATTTTTTTT</td>
</tr>
<tr>
<td>LPL-shRNA-Reverse</td>
<td>470</td>
<td>GATCAAAAAATGACATTTGGAATCTGGCTCCCTTTATACACAAGCTTCGTACAGAAGCTTGGATGTAGTT</td>
</tr>
<tr>
<td>EL-shRNA-Forward</td>
<td>598</td>
<td>GGAGCCAGTCAACCACAACCATCATTGGGAGAAGCTGTCAGTGAGCTTTGTTGGCTCCTCTCCCTTTTT</td>
</tr>
<tr>
<td>EL-shRNA-Reverse</td>
<td>598</td>
<td>GATCAAAAAAGGAGGAAGCGAGGCAACCAAACACAACGACTGAGACAGCTTTGGGATGTTGGCTCCTGG</td>
</tr>
<tr>
<td>Scramble-shRNA-Forward</td>
<td>None</td>
<td>TGGTTTACATATGTCTGTACAGTGAGCTTGTACTGCTGTAGGAGCCAGACAGTACGGAGCCGAGAGCAGTT</td>
</tr>
<tr>
<td>Scramble-shRNA-Reverse</td>
<td>None</td>
<td>GAGGAAAAAACGTTACAGTGCTCGCCTGCAGCAGTACAGGCTTTCAGAGCAGCAAGAGGAGCCGAGAGCAGTT</td>
</tr>
</tbody>
</table>

Paired shRNAs (Qiagen OPERON) were diluted to a final concentration of 50uM in the annealing buffer (1 mM MgCl₂, 20 mM Tris-HCl, pH 8.0). The mixture was then incubated at 95°C for 3 minutes, and cooled down naturally to room temperature. Thereafter; the annealed
shRNA diluted to 50nM in annealing buffer was used for ligation. Entry vector pSHAG (kind gift from Dr. Greg Hannon, Cold Spring Harbor Laboratory), was digested by restrict endonucleases BseRI and BamHI, and gel-purified (QIAquick gel extraction kit, 28704, QIAGEN). Annealed shRNAs were ligated into sticky-ended pSHAG at the molar ratio of 3–5:1 (50–100ng:1ug of shRNA:pSHAG) at room temperature for 1 hour using T4 ligase of rapid ligation kit (Roche Applied Science, 11635379001). Heat-inactivated (10 minutes at 65°C) ligation mixture (5 μL) was used to transform Top10 one-shot chemically competent E. coli (Invitrogen, C4040-03) following the factory’s instructions. The transformation mixture (50 μL) was streaked on kanamycin+ agar medium and positive colonies were isolated for DNA (QIAprep Spin Miniprep Kit, 27106, Qiagen) which was thereafter digested with HindIII for the confirmation of shRNA incorporation (Figure 2-1). pSHAG with shRNA (designated pSHAG-shRNA) was sequenced to assure the fidelity of shRNA sequence using U6 primer (5’-GGACTATCATATGCTTACCG-3’, Qiagen OPERON, Appendices 1~3).

Figure 2-1: Hind III digestion of pSHAG to confirm the shRNA inserts. shRNA oligo bears a Hind III site at the center so that pSHAG vectors containing shRNA insert will be digested by HindIII enzyme as shown by the upward size shift (constructs 1, 2, 3, 4, 7, 8, and 10) on 2% agarose gel compared to pSHAG vectors without insert (constructs 5, 6, and 9).

2.3.3 Integration of shRNA Expression Cassette into Lentiviral Vector

Lentiviral vector pHR-CMV-EGFP (kind gift from Dr. Alice Mui, UBC) engineered with gateway system (Invitrogen) in the ClaI site located at the downstream of 5'-LTR and upstream of CMV-EGFP by Dr. Alice Mui’s research group was maxpreped using QIAfilter Plasmid Maxi Kit (12262, Qiagen) and served as the destination vector. shRNA expression cassette (U6 promoter-shRNA) was transferred from pSHAG-shRNA into pHR-CMV-EGFP at attR1/2 sites by Gateway reaction using LR Clonase Enzyme (Invitrogen, 11791-019). Briefly, 300ng of pSHAG-shRNA and 300ng of pHR-CMV-EGFP were mixed with 1X LR clonase reaction buffer in TE buffer (pH 8.0). After the addition of 4ul of LR clonase enzyme mixture, the reaction was carried out at 25°C for 60 minutes and terminated by incubating with 2ul of
proteinase K solution at 37°C for 10 minutes. Top10 E. coli were transformed according to manual instructions, and plated on ampicillin+ agar medium overnight, positive colonies were isolated for DNA which was thereafter subjected to DNA sequencing for the confirmation of the insertion of shRNA expression cassette using U6 primer. pHR-CMV-EGFP inserted with shRNA expression cassette was designated as pHR-shRNA.

2.3.4 Lentivirus Production
The production of lentivirus was illustrated in figure 2-2. Two additional vectors (pCMVΔR8.2 and pMD.G), which encode viral integrase, protease, reverse transcriptase, capsid and matrix proteins, and vesicular stomatitis virus G protein, were used for lentiviral packaging and pseudotyping. Briefly, 40μl of lipofectamine 2000 (Invitrogen, 11668-019) was diluted in 1.5ml of OPTI-MEM I medium (31985, Invitrogen) for 5 minutes, and then combined with another 1.5ml OPTI-MEM I medium containing DNA mixture of 10μg pHR-shRNA, 7.5μg pCMVΔR8.2, and 2.5μg pMD.G for 30 minutes. The lipofectamine 2000 and DNA mixture was added to poly-L-lysine (P4832, Sigma) coated culture dishes with 5ml of 10%-FBS containing OPTI-MEM I medium, and then 5ml of 293T HEK cells at 2.0×10⁶/ml were added. Medium was replaced with 6ml of DMEM medium with 10% FBS and 1% antibiotics-antimycotic next day. Viral supernatants were then collected for 4 consecutive days, cell debris were removed by centrifugation at 1500 rpm for 10 minutes and subsequent filtration through 0.45μm syringe filter. All viral supernatants were pooled, and concentrated 25-fold using Centricon Plus-20 Centrifugal Filter Units (cutoff molecular weight of 100KD, Millipore, UFC2BHK08), aliquoted and stored at -80°C.

2.3.5 Lentivirus Production for EL Overexpression
A new lentiviral expression system (lentiviral vector pWPI plus two packaging vectors psPAX2 and pMD.2G, TronoLab) was used for EL overexpression. EL cDNA was separated from pDNA5-FRT-EL (kind gift from Dr. Howard Wong, UCLA) after PME I digestion. Gel-purified EL cDNA was then ligated into PME I site of lentiviral vector pWPI using T4 ligase of the rapid ligation kit (Roche Applied Science). Top 10 E. Coli were then transformed and streaked on ampicillin+ agar medium, positive clones were isolated and designated as pWPI-EL. The direction and sequence fidelity of EL cDNA were confirmed by DNA sequencing using
A. Cotransfection of 293T cells for lentiviral production

B. Concentration and titration of lentivirus

C. Lentiviral transduction of monocytes

Figure 2-2. Illustration of Lentivirus production and transduction of monocytes for lipase suppression and overexpression
forward primer CTCCTTGGAATTTGCCCT (at the site of 3201) and reverse primer TCAACAGACCTTGCAATT (at the site of 3690) (Appendix 5). The procedure for lentiviral production was the same as for shRNA.

2.3.6 Lentiviral Titration
293T HEK cells ($5 \times 10^5$ cells/well) were seeded in 6 well plates for 1 day, and then transduced with lentivirus by replacing with DMEM culture medium where stock virus was serially diluted by 1:100, 1:1000, 1:10,000, and 1:100,000. 293T cells were also counted in number at the same day for the titer calculation. Three days after transduction, 293T HEK cells were collected after trypsinization and re-suspended in culture medium, flow cytometry was applied to obtain the percentage of EGFP-positive 293T HEK cells. The lentiviral titers were calculated as follows: transduction units/ml = (average cell number at the time of transduction × % of GFP-positive cells)/100 × dilution factor.

2.3.7 Lentiviral Transduction of Monocytes/Macrophages
THP-1 monocytes ($5 \times 10^5$/well) were seeded in 12-well plate, and transduced at the same day with lentivirus at various multiplicity of infection (MOI). After 48 hours, monocytes were collected for FACS analysis to evaluate the transduction efficiency. Meanwhile, monocytes were also differentiated with 100nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, P1585) for additional 72 hours. Fluoroscopy was used to assess EGFP expression in mature macrophages. The optimal MOI was chosen for subsequent experiments.

2.3.8 Construction of EL-Expressing FLP-IN 293 Cell Line
EL cDNA was transferred from pcDNA6/EL (A kind gift from Dr. Howard Wong, UCLA) into Pme I site of pcDNA5/FRT, and confirmed by sequencing (CMV forward primer: 5'-CGCAAAATGGGCGGTAGGCGTG-3', and BGH reverse primer: 5'-TAGAAGGCACAGTCGAGG-3', Appendix 5). A stable EL-expressing FLP-IN 293 cell line was constructed using the Flp-In™ System (Figure 2-3, Invitrogen). Briefly, one day before transfection, FLP-IN 293 cells ($3 \times 10^6$) were cultured in growth medium without antibiotics in a 10cm culture dish. 10μg DNA (9μg pOG44 and 1μg pcDNA5/FRT/EL) in 1.5ml of Opti-MEM® I Reduced Serum Medium were combined with 20μl of lipofectamine 2000 and
incubated at room temperature for 20 minutes, then added to 293 FLP-IN cell culture. Medium was replaced at 24 hours, and cells were passaged at 1:10 the next day under the selection pressure of 50μg/ml hygromycin. Selection medium with hygromycin was replaced every 3~4 days until cell foci were identified. 5~10 foci were isolated and expanded in new culture dishes. Conditioned culture medium was evaluated for trioleinase activity. Monoclonal FLP-IN 293 cells with highest trioleinase activity were selected for EL expression (named FLP-IN 293 EL cells).

Expression of lacZ and Zeocin fusion gene

1. pFRT/lacZeo is stably transfected to generate Zeocin-resistant Flp-ln 293 cells.
2. The pcDNA5/FRT vector containing EL cDNA is cotransfected into FLP-IN 293 cells with pOG44 vector expressing Flp recombinase.
3. Integration of the expression construct allows transcription of EL and confers hygromycin resistance and zeocin sensitivity to Flp-ln 293 cells.

Figure 2-3. The generation of FLP-IN 293 cells stably expressing EL. EL cDNA was extracted from pcDNA6/EL and inserted into PmeI sites in pcDNA5/FRT, the latter was then cotransfected with pOG44 into FLP-IN 293 cells. The transfected cells were selected by hygromycin to obtain EL-overexpressing FLP-IN 293 cell line. (Modified from the manual of FLP-IN system for stable cell line generation, Invitrogen.)

2.3.9 EL Purification

AKTA FPLC system (GE Health) was used for EL purification. Heparin-challenged conditioned Opti-MEM medium from FLP-IN 293 EL cells was collected for consecutive 5~7 days, centrifuged and 0.45μm filtered to remove the cell debris, and then diluted with the equal
volume of sample buffer (50mM Tris-HCl, 0.15M NaCl, 20% glycerol, pH 7.4). HiTrap heparin column (GE Health, 5ml) was equilibrated with five volumes of equilibration buffer (50mM Tris-HCl, 0.15M NaCl, 10% glycerol, pH 7.4). Diluted sample was loaded onto column at the speed of 5ml/min, heparin-bound proteins were then eluted with 5 column volumes in gradient concentration from 0% to 100% of elution buffer (50mM Tris-HCl, 2.0M NaCl, 10% glycerol, pH 7.4) at the speed of 2ml/min (Figure 2-4). Fractionates were desalted using PD-10 columns (GE Health), and assessed for trioleinase activity and protein concentration.

Figure 2-4. EL purification from heparin-challenged conditioned medium in EL-overexpressing FLP-IN 293 cells. Fractionates were analyzed for trioleinase activity, with highest activity of 16.4nmol/min/ml at the UV peak of 137.40mAU. Pooled EL preps from fractionates 7~11 had a trioleinase activity of 4.6nmol/min/ml, compared to 0.05nmol/min/ml in control preps from FLP-IN 293 cells.

2.3.10 Up-regulation of Endogenous Lipoprotein Lipase in Macrophages
Dexamethasone (DXM) has been shown to upregulate the LPL gene expression in THP-1 cells. After 24 hours of PMA stimulation, THP-1 macrophages were treated with 0.1μM dexamethasone for 48 hours in order to upregulate endogenous LPL expression.

2.3.11 Trioleinase Activity Assay
LPL preferentially catabolizes triglyceride to release free fatty acids, and its triglyceridase activity is apoCII-dependent and salt-sensitive. Trioleinase activity was measured in heparin-challenged conditioned medium for LPL activity using a triolein emulsion containing
radiolabeled triolein as described previously. Briefly, 100μl of 7.5mg/mg triolein (Sigma), 100μl of 1.0mg/ml phosphatidylcholine (Sigma), and 50uCi [³H]-triolein (Amersham) were combined and dried under nitrogen gas stream. For total trioleinase activity, dried lipids were added with 2.1ml of low salt-buffer (0.2M Tris-HCl, 0.15M NaCl, and pH 8.2) plus 0.4ml of 1% BSA in low salt buffer, and emulsified by sonication at 50% pulse and lowest power setting for 8 minutes (Sonifier Cell Disruptor 350, Branson Sonic Power Co.). Thereafter, 0.5ml of 4% BSA in low salt buffer and apoCII at final concentration of 2μM were added to the lipid emulsion. The salt-resistant trioleinase activity was measured in lipid emulsion where the same lipid composition was emulsified in high salt buffer (0.2M Tris-HCl, 1M NaCl, and pH 8.8) without apoCII supplementation. 20μl of heparin-challenged conditioned medium was added to 80μl of either low-salt or high-salt buffer, and then 100μl of substrate in low-salt or high-salt buffer were added to corresponding tubes and incubated at 37°C for 30 minutes. The reaction was terminated by adding 3.25ml of chloroform:methanol:heptane (1.25:1.41:1). The phases were separated by the addition of 1.05ml of 0.1M H₂CO₃ and 0.1M K₂CO₃, pH 10.5 and centrifugation at 1500×g for 10 minutes after 15 seconds of vigorous vortexing. 1ml of the upper phase was aliquotted into scintillation tubes, mixed with 4ml of ACS scintillation fluid (Amersham), and counted for radioactivity. The trioleinase activity was calculated as:

\[
\text{Trioleinase activity (nmol/min/ml)} = \text{coefficient} \times \frac{\text{radioactivity (cpm)}}{\text{volume} \times \text{time}}.
\]

The LPL activity was represented by the apoCII-dependent and salt-sensitive portion of total trioleinase activity.

2.3.12 Phospholipase Activity Assay

EL preferentially hydrolyzes phospholipids more than triglycerides, so an in-well phospholipase activity assay modified from literatures was utilized to evaluate the EL activity. Briefly, self-quenched fluorescent substrate bis-BODIPY FL C₁₁-PC (Invitrogen, B7701) sonicated in PBS was added into 1ml of cell culture at final concentration of 4μg/ml for 1 hour at 37°C in the presence of 10 units of heparin. 200μl of culture medium was transferred into a 96-well microplate and measured at excitation/emission wavelength of 488/530 nm for the fluorescence intensity, which was normalized for the total cellular protein.
2.3.13 Real-Time One-step Quantitative Reverse Transcription PCR (qRT-PCR)
Total RNA was isolated from macrophages using RNAqueous®-4PCR (Ambion, 1914). 25μl of the reaction system for real-time one-step qRT-PCR was assembled with 0.5μl of Superscript III RT/platinum Taq mix, 12.5μl of 2X reaction mix, (SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity, Invitrogen, 12574-035), 10μl of DEPC-treated ddH2O, 1μl of RNA sample, and 1μl of Assays-on-Demand primer set of either 18S rRNA (Hs99999901_sl), LPL (Hs00173425_ml), or EL (Hs00195812_ml). The reaction was carried out on ABI PRISM® 7900HT system with the parameter setting as followings: 50°C for 30 minutes, 95°C for 15 minutes, followed with 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. The standard curves were made using serially diluted RNA samples. 18S rRNA served as the internal control.

2.3.14 μMACS Microbead-based Immunoprecipitation
Macrophages (5 × 10^6) were washed with ice-cold PBS twice, and then lysed in RIPA buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich, P8340) on ice for 15 minutes. Lysates were scraped into microtubes and passed through small pipette tip several times, and then centrifuged at 10,000 rpm and 4°C for 5 minutes. The supernatants were collected, mixed with 1~2ug of primary antibody (Rabbit anti-EL polyclonal antibody, Cayman Chemical; Mouse anti-LPL monoclonal antibody 5D2, kind gift from Dr. John Brunzell) and 50μl of protein A microbeads (Miltenyi Biotec, 130-071-001), and incubated on ice for 30 minutes. Thereafter, lysate-microbead mixture was loaded into μ-columns which were mounted on μMACS separator and pre-equilibrated with 200 μl lysis buffer, letting the solution flow through by gravity. μ-columns were then rinsed with 4 × 200 μl of RIPA lysis buffer, and 1 × 100 μl of low salt wash buffer (1% NP-40, 50mM Tris-HCl, pH 8.0). Bound analyates were eluted by adding 20μl of 95°C 1× SDS loading buffer (50mM Tris-HCl, 50mM DTT, 1% SDS, 0.005% bromophenol blue, 10% glycerol) for 5 minutes, followed by additional 50μl of 95°C 1× SDS loading buffer.

2.3.15 Western Blot
For the regular Western blot, macrophages (5×10^6) were lysed in RIPA lysis buffer supplemented with protease inhibitor cocktail (Sigma) and scraped into microtubes. Cell lysates
were obtained by collecting the supernatants after centrifugation at 10,000 rpm at 4°C for 5 minutes. Cell lysates were quantitated for protein concentration, and equal amounts of total protein (20 μg) were mixed in 1× Laemmli sample buffer (2× Laemmli sample buffer for 100ml: 40ml of 10% SDS, 12.5ml of 1M Tris, pH6.8, 30ml of glycerol, 1ml of β-mercaptoethanol, 20μg of bromophenol blue, and 16.5ml of H2O) and boiled for 5 min. For electrophoresis, cell lysate samples and μMACS eluates were loaded onto 5% or 7.5% SDS-PAGE gel and electrophoresed in SDS running buffer (3.03g Tris base, 14.4g glycine, 1.0g SDS in1L H2O) at 200v for 45–60 min. Gel-sized PVDF membrane (Millipore Immobion-P) was wetted in methanol for 30 seconds, then soaked in transfer buffer (14.4g Glycine, 3.0g Tris base, 0.75g SDS, 200ml Methanol, and 800ml H2O). The resolving gel was sandwiched in the order of (anode) sponge-filter paper-PVDF membrane-resolving gel-filter paper-sponge (cathode), and electrotransferred at 100v and 4°C for 1–1.5 hour. The PVDF membrane was blocked in Superblock buffer (Pierce) for 1 hour, and then incubated with primary antibodies (1:1000 dilution) with gentle shaking at 4°C overnight. After 3 times washes with TBS-Tween 20, the blot was incubated with secondary HRP-conjugated antibody (1:1000 dilution, either anti-Rabbit or anti-Mouse) with gentle shaking for 1 hour. The PVDF membrane was washed again with TBS-Tween 20 for 3 times, and then Supersignal West Femto Maximum Sensitivity substrate (Pierce, 34095) was added. The chemiluminescent signal was captured and quantitated by ChemiGenius² system (SYNGENE, Frederick, MD USA).

2.3.16 ApoAI Mediated Cholesterol Efflux
THP-1 (5×10⁵) cells stimulated with 100nM PMA in 12-well plates and FLP-IN 293 EL (2×10⁵) cells in 6-well plates were cultured for 48 hours, and then labeled with 1μCi/ml ³H-cholesterol in base medium (For macrophages: RPMI 1640 containing 1% antibiotics-antimycotic, 2% bicarbonates, 1% pyruvate, 0.2% BSA, and 1×10⁻⁷ M PMA; For FLP-IN 293 cells: DMEM medium containing 1% antibiotics-antimycotic, and 0.2% BSA) for 24 hours. After two-hour equilibration with base medium, cholesterol efflux was induced with 10μg/ml apoAI in base medium for 4 hours. Exogenous bLPL or EL was added during the efflux stage. Cholesterol efflux is represented as the percentage of medium cpm to total cpm (medium plus cellular cpm), and apoAI-mediated cholesterol efflux is calculated as the total cholesterol efflux minus basal cholesterol efflux which is measured in the absence of apoAI. During cholesterol efflux, 10units/ml heparin and 50μg/ml tetrahydrolipstatin (THL, kind gift from Hoffmann La Roche)
were added into cholesterol efflux medium alone or in combination to evaluate the contributory roles of lipolytic and non-catalytic functions.

2.3.17 ApoAI Binding Assay
ApoAI was labeled with fluorescent dye Alexa Fluor 532 (Invitrogen) following manufacturer's instruction. Briefly, 100μl of 1M sodium carbonate was added into 1ml of apoAI solution (concentration < 2mg/ml) to raise the pH value, and then the mixture was transferred into reaction vial containing fluorescent dye Alexa Fluor 532. Reaction vial wrapped in aluminum foil was placed on a magnetic stirrer and stirred at room temperature for 1 hour. The mixture was then dialyzed against two changes of 500 ml of PBS at room temperature for 1 hour. Optimal labeling degree of 4 moles dye per mole protein was obtained. The apoAI binding protocol was modified from literature, 33 10μg/ml fluorescence-labeled apoAI was added into cell culture in base medium for 4 hours, then washed with 0.2% BSA-PBS and PBS twice each. Cells were lysed in RIPA buffer, 200μl supernatants were measured for fluorescence at the excitation/emission wavelength of 531/554nm, and bound apoAI was calculated from a standard curve, and normalized for total cellular protein.

2.3.18 Characterization of ABCA1 Expression
ABCA1 expression was evaluated at mRNA and protein levels. mRNA was isolated and quantitated by real-time qRT-PCR (Assay-on-demand ABCA1 primer set: Hs00194045_ml). Cell lysates were electrophoresed and transferred onto PVDF membrane, then detected by ABCA1 antibody (Abcam). The detailed procedures are described above.

2.3.19 Analysis of Cell Membrane Lipid Composition
Lentivirus-transduced macrophages (5×10^6) were detached using 1% EDTA-PBS solution and collected in a glass tube. Trypsinized 293-FLP-IN cells were collected as well. After a single PBS wash, cells were resuspended in 3 ml of chloroform/methanol (1:2) plus an additional 0.8 ml of distilled water, and vortexed vigorously for 30 seconds. After 30 minute incubation at room temperature, the mixture was centrifuged at 3000 rpm for 5 minutes, and the organic phase on the bottom was carefully transferred by glass pipettes into a filter paper-lined funnel. The filtrates collected in glass tubes were dried under a nitrogen gas stream, and then re-dissolved in 80μL of chloroform/methanol (1:2) and transferred onto an HPLC column with the addition of
50μL of betulin reference standard, and dried under nitrogen. The dried sample was re-dissolved in HPLC solvent (4:6:1:1 of chloroform/methanol/hexane/acetone in volume ratio) for analysis. Lipid composition was quantified using a method as described by Sheila M. Innis and Roger A. Dyer. 34

2.3.20 Statistical Analysis
All parametric data are presented as mean ± standard error of the mean. Data were analyzed with Prism 4 for Windows (GraphPad Software, Inc.) using either student’s t test or two-way ANOVA, p value less then 0.05 was considered to be significant.
2.4 Results

2.4.1 Lentiviral Titration and Transduction
Lentivirus containing either shRNA or EL cDNA was produced and titrated in 293T HEK cells, with a typical titer of $2\sim4\times10^8$ transduction units/ml when concentrated by 25 fold. Lentivirus has been reported to have high transduction efficiency in a variety of cells including several terminally differentiated or non-dividing cells. However, the experience in monocytes/macrophage transduction is still limited, and differentiated macrophages are generally difficult to transduce, so THP-1 monocytes were transduced with lentivirus before PMA stimulation in order to increase the transduction efficiency. Two days after lentiviral transduction, monocytes were analyzed by FACS for EGFP expression. The results showed that 83% of monocytes expressed EGFP at MOI of 10, and 100% of monocytes were positive for EGFP at MOI of 20, meanwhile, the mean fluorescent intensity increased by 5 fold from MOI of 10 to MOI of 20 (Figure 2-5 A~C).

Figure 2-5. The quantitation of transduction efficiency by FACS and fluoroscopy. Monocytes were transduced by lentivirus for 48 hours and evaluated by FACS for EGFP positivity; meanwhile, monocytes were differentiated into macrophages by PMA stimulation for 3 days, and assessed for EGFP expression under fluoroscope. Panel A. FACS and fluoroscope images at MOI of 0; Panel B. FACS and fluoroscope images at MOI of 10; Panel C. FACS and fluoroscope images at MOI of 20. (X axis: logarithm of fluorescence, Y axis: cell counts)
The EGFP expression was also verified by fluoroscopy in macrophages after three days of PMA stimulation. Consistent with FACS results, the percentage of EGFP positive macrophages under the fluoroscope increased accordingly with the increase of MOI value, and almost all macrophages were expressing EGFP with higher fluorescent intensity at MOI of 20 in comparison to that at MOI of 10 (Figure 2-5 B and C). Therefore, the MOI of 20 was chosen as the optimal lentiviral concentration for later experiments.

2.4.2 Lipase Knockdown by shRNA Lentivirus

A morphologic observation showed that macrophages were fully differentiated 3 days after PMA stimulation, meanwhile, the expression of LPL and EL after PMA stimulation showed that the mRNA and protein/activity levels of LPL and EL reaches the peak at day 2 and day 3, respectively (Figure 2-6 A and B). Therefore, heparin-challenged conditioned medium and mRNA were collected at day 3, and analyzed by lipase activity/protein and real-time qRT-PCR, respectively.

Compared to the lentivirus containing a scrambled shRNA sequence, shRNA-lentivirus targeting LPL caused an 83% decrease in mRNA level (Figure 2-7A). LPL-shRNA lentivirus also led to a 70% decrease in apoC-II dependent, salt-sensitive trioleinase activity in heparin-challenged medium (Figure 2-7A). Correspondingly, the LPL protein level was decreased by more than 70% as determined by immunoprecipitation-Western blotting (Figure 2-7C). EL-shRNA lentivirus reduced EL mRNA by 76% in comparison to scrambled shRNA lentivirus as analyzed by qRT-PCR (Figure 2-7D). An in-well phospholipase assay, a method for the measurement of all-source phospholipase activity, was implemented in the culture condition.
with the heparin addition. A reduction of 35% in total phospholipase activity was detected in EL-shRNA lentivirus (Figure 2-7D). The suppression of >70% EL was also evident on Western blot (Figure 2-7F).

Figure 2-7: The suppression of LPL and EL by lentivirus containing shRNA specific for each lipase. Monocytes were transduced by shRNA lentivirus for 48 hours prior to PMA stimulation, and total RNA and heparin-challenged conditioned medium at day 3 of PMA stimulation were collected for real-time qRT-PCR, lipase activity, and Western blotting analyses. Panel A. LPL mRNA, trioleinase activity, and phospholipase activity; Panel B. EL mRNA expression in LPL suppressed macrophages; Panel C. LPL protein in Western blot; Panel D. EL mRNA, total phospholipase activity, and trioleinase activity; Panel E. LPL mRNA expression in EL suppressed macrophages. Panel F. EL protein in Western blot (the representative of 3 individual blots). Statistical comparisons between control and lipase suppression (LPL-LOF or EL-LOF) are indicated as: * p<0.05, ** p<0.01.

In order to exclude the cross-reactivity between LPL and EL shRNAs, each lipase other than shRNA target was evaluated by qRT-PCR and lipase activity. The results showed that there was no suppression or compensatory upregulation of the non-targeted lipase in each case (Figure 2-7
EL suppression did not cause significant decrease in trioleinase activity (Figure 2-7D), in contrast, the phospholipase activity in LPL-shRNA lentivirus transduced macrophages was moderately decreased by ~20% (Figure 2-7A), suggesting LPL may contribute to a considerable portion of total phospholipase activity.

2.4.3 Lipase Overexpression in Macrophages and FLP-IN 293 Cells

DXM has been reported to be able to increase LPL expression in macrophages, thus, macrophages were treated with DXM to create an LPL-overexpressing cell model. The treatment of macrophages with 48-hour DXM significantly increased LPL mRNA by 12.8 fold, with a corresponding 3.6 fold increase in apoCII-dependent, salt-sensitive trioleinase activity (Figure 2-8A). Despite the decreased expression of EL mRNA after the LPL overexpression by DXM, there was no significant change in total phospholipase activity (Figure 2-8 A and B).

Lentivirus containing EL cDNA (EL lentivirus) was constructed with the similar transduction efficiency as lentivirus used for lipase suppression. At MOI of 20, EL lentivirus markedly increased EL mRNA by >100 fold (Figure 2-9A). Meanwhile, the EL protein was increased by 4.4 fold as shown on Western blot (Figure 2-9C). The drastic increase of EL mRNA transcription may overwhelm the mRNA translation machinery, which may explain the disproportionate increase in EL protein compared to mRNA expression. Accordingly, total phospholipase activity after EL-cDNA introduction by lentivirus was elevated by 50% (Figure 2-9A). Interestingly, LPL mRNA was decreased by 57% after EL overexpression, however, the total trioleinase activity was not affected (Figure 2-9 A and B), suggesting that EL overexpression compensates for the loss of triglyceridase activity associated with LPL down-regulation.

Utilizing the facility of Flp-In™ system, we generated stable FLP-IN 293 EL cells under the selection pressure of hygromycin. In a monoclonal FLP-IN 293 EL cell line, EL mRNA was markedly increased by more than 2000 fold when compared to the untransfected FLP-IN 293 cells, and the total phospholipase activity and EL protein increased by >4- and 6-fold, respectively (Figure 2-10 A and B). In addition, EL overexpression also led to a 1.9 fold increase in the trioleinase activity (Figure 2-10A). The 293 cell line expresses LPL at very low level, so the increased trioleinase activity was most likely due to the EL overexpression.
Figure 2-8: The LPL overexpression in macrophages. Monocytes were differentiated into macrophages by PMA stimulation for 3 days with simultaneous addition of DXM for 48 hours. LPL mRNA, trioleinase activity and protein levels were analyzed. Panel A. LPL mRNA (n=4), trioleinase activity (n=7), and phospholipase activity (n=4) after DXM stimulation. Panel B. EL mRNA after DXM treatment in macrophages. Panel C. LPL protein in Western blotting (the representative of two individual blots). Statistical comparisons between control and DXM treatment (DXM-LPL) are indicated as: *** p<0.001.
Figure 2-9. The EL overexpression in macrophages. Monocytes were transduced by lentivirus containing EL cDNA for 2 days, followed by PMA stimulation for 3 days. Panel A. EL mRNA (n=4), total phospholipase activity (n=4), and trioleinase activity (n=4) levels; Panel B. LPL mRNA in EL-overexpressing macrophages. Panel C. EL protein in Western blotting (The representative of 3 individual blots). Statistical comparisons between control and EL overexpression (EL-GOF) in macrophages are indicated as: *** p<0.001.
2.4.4 Lipases Promote Apo AI-mediated Cholesterol Efflux

In this study, apoAI was used to induce the cholesterol efflux in macrophages radiolabeled with $^3$H-cholesterol. Before the study, the kinetics of cholesterol efflux by apoAI was performed, showing that apoAI mediated cholesterol was saturated after 8–16 hours, even though the total effluxed cholesterol still increased with prolonged incubation (Appendix 6). The continuous increase in non-apoAI mediated cholesterol efflux could be most likely due to free diffusion, other mediators like bovine serum albumin in the medium could be cholesterol acceptors. Therefore, a four-hour incubation period with apoAI was chosen to evaluate the lipase effect on cholesterol efflux.

When macrophage LPL and EL were suppressed by lentivirus, apoAI-mediated cholesterol efflux was moderately but statistically significantly decreased by 18% to 20%, respectively (Figure 2-11A). This finding was reproduced in lipase-overexpressing macrophages, where apoAI-mediated cholesterol efflux was increased by almost 2 fold in DXM-treated macrophages,
and this effect was eliminated when DXM-induced LPL upregulation was abolished by LPL shRNA (Figure 2-11B). ApoAI mediated cholesterol efflux increased by ~1.5 fold in EL-overexpressing macrophages (Figure 2-11C). The stimulatory effect of EL on apoAI-mediated cholesterol efflux was also observed in FLP-IN 293 cells, with 2.3 fold increase in FLP-IN 293 EL cells compared with the control cells (Figure 2-11D).

![ApoAI-mediated Cholesterol Efflux in THP-1 Macrophages](image)

**Figure 2-11**: The effect of lipase suppression and overexpression on apoAI-mediated cholesterol efflux in macrophages and FLP-IN 293 cells. Cholesterol efflux was induced by 10μg/ml apoAI for 4 hours. Panel A. apoAI-mediated cholesterol efflux in lipase suppressed macrophages (n=10); Panel B. apoAI-mediated cholesterol efflux in DXM treated macrophages (n>6); Panel C and D. apoAI-mediated cholesterol efflux in EL-overexpressing macrophages and FLP-IN 293 cells (n>6). Statistical comparisons between lipase suppression (LPL-LOF or EL-LOF), overexpression (DXM-LPL, EL-GOF, or 293 EL) and controls are indicated as: *** p<0.001

The above findings were also verified in macrophages treated with exogenous lipases. When macrophages were treated with exogenous bovine LPL (bLPL), there was a dose-dependent increase in apoAI-mediated cholesterol efflux, with a 51% increase at the concentration of 5μg/ml (Figure 2-12A) In parallel, the addition of exogenous EL into macrophages dose-
dependently increased apoAI-mediated cholesterol efflux with an increase of 25% at a concentration of 0.5ug protein/ml (Figure 2-12B).

Figure 2-12. The effect of exogenous lipases on apoAI-mediated cholesterol efflux in macrophages. Cholesterol efflux was induced by 10ug/ml apoAI in the presence of either bovine LPL or EL in macrophages. Panel A. The dose-dependent increase of apoAI-mediated cholesterol efflux by bovine LPL (bLPL) (n=4); Panel B. The dose-dependent increase of apoAI-mediated cholesterol efflux by exogenous human EL (n=4). Statistical comparisons between control (0ug/ml) and exogenous lipase are indicated as: * p<0.05, ** p<0.01, *** p<0.001.

2.4.5 The Differential Role of Catalytic and Non-Catalytic Functions of Lipase in Cholesterol Efflux

In order to understand the role of catalytic and non-catalytic functions of lipases, we used THL and heparin alone or in combination to eliminate the catalytic and non-catalytic function or both. THL was shown to completely inhibit the LPL and EL activity as analyzed by trioleinase assay.

In DXM-treated macrophages, LPL-related, apoAI-mediated cholesterol efflux was significantly reduced by 61% after the addition of THL, and 60% when LPL was removed from cell surface by heparin treatment. The elimination of both catalytic and non-catalytic functions of LPL by combined treatment of THL and heparin almost abolished the LPL effect on apoAI-mediated cholesterol efflux (Figure 2-13A). In EL-overexpressing macrophages, the same pattern was also observed, with 59% and 29% decreases in EL-dependent, apoAI-mediated cholesterol efflux after THL and heparin treatment, respectively. With the inhibition of catalytic and non-catalytic functions of EL, 93% of the EL effect on apoAI-mediated cholesterol efflux was removed (Figure 2-13B). The same experiment was also repeated in FLP-IN 293 cells, catalytic
and non-catalytic functions of EL played a similar role in apoAI-mediated cholesterol efflux as in macrophages (Figure 2-13C).

Figure 2-13: The role of catalytic and non-catalytic functions of lipases in apoAI-mediated cholesterol efflux. Lipase-related,apoAI-mediated cholesterol efflux (clear bars of DXM-LPL, EL-GOF, and 293 EL) is expressed as 100%, the apoAI-mediated cholesterol efflux after the treatment of either THL, heparin alone or in combination (THL+Heparin) was expressed as percentage of lipase-related efflux. Panel A. ApoAI-mediated cholesterol efflux in DXM treated macrophages (n>6); Panel B. ApoAI-mediated cholesterol efflux in EL-overexpressing macrophages (n=6); Panel C. ApoAI-mediated cholesterol efflux in EL-overexpressing FLP-IN 293 cells (n=6). Statistical comparisons between treatment and non-treatment in lipase-related cholesterol efflux are indicated as: * p<0.05, ** p<0.01, *** p<0.001.

2.4.6 The Effect of Lipases on Cholesterol Efflux Is Independent of ABCA1
ApoAI mediated cholesterol efflux is largely through ABCA1 transporter, thus we investigated whether the expression of lipases would alter the ABCA1 expression. In lipase-suppressed macrophages, the ABCA1 mRNA and protein were not significantly changed compared to the control (Figure 2-14A). The increase of apoAI-mediated cholesterol efflux by EL is not accompanied with a parallel increase in ABCA1 expression as the ABCA1 mRNA and protein levels were not altered in EL-overexpressing macrophages or in FLP-IN 293 cells (Figure 2-14
B and C). However, ABCA1 levels in DXM-treated macrophages were considerably decreased by 62% in mRNA and 80% in protein, respectively (Figure 2-14B). Taking the increased cholesterol efflux after DXM treatment into consideration, it can be deduced that the enhanced apoAI-mediated cholesterol efflux by LPL in macrophages was independent of ABCA1.

Figure 2-14. ABCA1 expression after lipase suppression and overexpression in macrophages and FLP-IN 293 cells. Panel A. ABCA1 mRNA (n≥7) and protein (n=3) levels in lipase suppressed macrophages (LPL-LOF or EL-LOF); Panel B. ABCA1 mRNA (n≥4) and protein (n=2) levels in DXM-treated (DXM-LPL) and EL-overexpressing (EL-GOF) macrophages; Panel C. ABCA1 mRNA (n=6) and protein (n=2) levels in EL-overexpressing FLP-IN 293 cells (293 EL). Statistical comparisons between control and lipase suppression (LPL-LOF or EL-LOF) or overexpression (DXM-LPL, EL-GOF, or 293 EL) are indicated as: ** p<0.01.
2.4.7 Lipases Increase ApoAI Binding via Bridging Function

Since the stimulatory effect of lipases on apoAI-mediated cholesterol efflux was independent of ABCA1 expression in macrophages and 293 cells, we thereafter looked into the effect of LPL and EL on apoAI binding. With the suppression of LPL and EL, the apoAI binding was reduced by 32% and 34% respectively (Figure 2-15A). Compared to the control, the increased apoAI binding (empty part of clear bar) was observed in macrophages after the lipase overexpression, with 1.4- and 1.2- fold increases in DXM-treated and EL-overexpressing macrophages, respectively (Figure 2-15 B and C). The increased apoAI binding with

![Figure 2-15. Apo AI binding in macrophages and FLP-IN 293 cells after lipase suppression or overexpression.](image)

Panel A. apoAI binding in lipase-suppressed cells (LPL-LOF, EL-LOF) (n≥4); Panel B. apoAI binding in DXM treated macrophages (DXM-LPL, n=4); Panel C. apoAI binding in EL-overexpressing macrophages (EL-GOF) (n=4). Panel D. apoAI binding in EL-overexpressing FLP-IN 293 cells (293 EL, n=4). Statistical comparisons between the control (shaded bar) and lipase suppression (LPL-LOF or EL-LOF, clear bar) or overexpression (DXM-LPL, EL-GOF, or 293 EL, clear bar) in the absence or presence of treatments are indicated as: * p<0.05, ** p<0.01, *** p<0.001; Statistical comparisons between with and without treatment in lipase-related apoAI binding (empty part of clear bar) are indicated as: †† p<0.01, ††† p<0.001.
macrophages by either LPL or EL was not abolished after the catalytic inhibition of each lipase by THL; by contrast, heparin treatment eliminated 79% and 80% of lipase-related apoAI binding (empty part of clear bar) in macrophages in LPL- and EL-overexpressing macrophages, respectively (Figure 2-15 B and C). A similar relationship was also seen in FLP-IN 293 cells, EL increased apoAI binding by 34%, which was inhibited by heparin (40%) but not THL treatment (Figure 2-15D).

2.4.8 Lipases Change Phospholipid Composition in Macrophages

The fact that the catalytic inhibition of lipases decreased apoAI-mediated cholesterol efflux raised a question whether lipases could modify the membrane lipid composition and thus influence cholesterol efflux. Therefore, cell lipids were extracted from both lipase-suppressed and overexpressing macrophages and FLP-IN 293 cells for the analysis of phospholipid composition.

With the suppression of LPL and EL by shRNA lentivirus in macrophages, the intracellular total cholesterol including cholesteryl ester and free cholesterol was decreased by ~40%. A significant decrease in triglyceride level was detected only in EL suppressed macrophages. The major phospholipid component, phosphatidylcholine (PC), was increased in both LPL- and EL-suppressed macrophages where lysophosphatidylcholine (LPC), the metabolite of phosphatidylcholine, was significantly decreased (Figure 2-16 A and B). The levels of phosphatidylinositol (PI) and phosphatidylethanolamine (PE) were also modestly increased in LPL- and EL-suppressed macrophages, respectively (Figure 2-16 A and B).

When LPL expression was upregulated by DXM treatment, the level of PC was decreased by 35%, with a corresponding 23% increase of LPC. Similarly, 10% decrease in PC and 26% increase in LPC were observed in EL-overexpressing macrophages. Consistently, PE and sphingomyelin (SPH) were reduced in both LPL- and EL-overexpressing macrophages (Figure 2-17 A and B). EL overexpression increased intracellular cholesterol content by ~10%. However, the cholesterol level in DXM-treated macrophages was surprisingly decreased, mostly due to a 49% reduction in free cholesterol (Figure 2-17 A and B). This change could be explained by the drastic increase in cholesterol efflux after DXM treatment.
Figures 2-16. Lipid composition in lipase-suppressed macrophages. Panel A. Lipid composition in LPL suppressed macrophages (n=3); Panel B. Lipid composition in EL-suppressed macrophages (n=3). (LPC: lysophosphatidylcholine, PC: phosphatidylcholine, TC: total cholesterol, CE: cholesteryl ester, FC: free cholesterol, TG: triglycerides. FAA: free fatty acids, CL: cardiolipin, PE: phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine, SPH: sphingomyelin. Statistical comparisons between the control and lipase suppression (LPL-LOF or EL-LOF) are indicated as: * p<0.05, ** p<0.01, *** p<0.001.

The EL overexpression in FLP-IN 293 cells markedly increased cholesteryl ester and triglyceride levels by 4- and 1.7-fold, respectively. In parallel to the findings in lipase-overexpressing macrophages, a 14% reduction in PC and a corresponding 31% increase in LPC were detected in FLP-IN 293 EL cells as well (Figure 2-17C).
Figure 2-17. Lipid composition in lipase-overexpressing macrophages and FLP-IN 293 cells. Panel A. Lipid composition in DXM-treated macrophages (n=2); Panel B. Lipid composition in EL-overexpressing macrophages (n=3); Panel C. Lipid composition in EL-overexpressing FLP-IN 293 cells (n=3). (LPC: lysophosphatidylcholine, PC: phosphatidylcholine, TC: total cholesterol, CE: cholesteryl ester, FC: free cholesterol, TG: triglycerides, FAA: free fatty acids, CL: cardiolipin, PE: phosphatidylethanolamine, PI: phosphatidylinositol, PS: phosphatidylserine, SPH: sphingomyelin) Statistical comparisons between control and lipase overexpression (DXM-LPL, EL-GOF, or 293 EL) are indicated as: * p<0.05, ** p<0.01, *** p<0.001.)
2.4.9 Lysophosphatidylcholine Stimulates ApoAI-mediated Cholesterol Efflux

The effect of LPC on apoAI-mediated cholesterol efflux was also investigated in macrophages. With the increase in LPC concentration, apoAI-mediated cholesterol efflux was increased in a dose-dependent manner, with an 80% increase at the concentration of 10uM. (Figure 2-18).

Figure 2-18. Lysophosphatidylcholine (lyso-PC) effect on apoAI-mediated cholesterol efflux. Cholesterol efflux was induced by 10ug/ml apoAI in macrophages at the various concentrations of lysophosphatidylcholine (n=3). Statistical comparisons between no lyso-PC and lyso-PC treatments are indicated as: ** p<0.01.
2.5 Discussion

Lentivirus appears to be promising and advantageous in gene therapeutical modalities. The integration of HIV virus into the host genome does not necessitate cell division as successful transductions of differentiated cells and non-dividing cells (growth-arrested fibroblasts or non-dividing neuron, dendritic cells, and rat cardiac myocytes) have been reported. In our experiment, we utilized the lentivirus to either suppress or increase lipase expression in monocytes/macrophages, achieving 100% transduction efficiency at MOI of 20 which was also confirmed by EGFP expression under the fluoroscope. Positive EGFP expression was still detected in ~60% monocytes two weeks after the lentivirus transduction. Theoretically, a stable lentivirus-transduced THP-1 cell line could be established by fluorescence-activated cell-sorting. Lentiviral transduction introduced shRNA and EL cDNA into cells efficiently, with more than 75% suppression of target lipase and >100-fold increase of EL mRNA. Taken together, these results support that lentivirus can be used as a potential therapeutical tool to deliver either shRNAs or transgenes into monocytes/macrophages in clinical endeavors for disease treatment.

In the present study, two complementary strategies of loss-of-function and gain-of-function were utilized in order to elucidate the lipase role in cholesterol efflux more convincingly. Accumulating evidence supports that LPL and EL can be proatherogenic, so we initially hypothesized that the expression of LPL and EL would impair the cholesterol efflux in macrophages. To our surprise, the lipase suppression decreased cholesterol efflux to a moderate extent. Moreover, in the GOF study where LPL and EL were added exogenously or expressed endogenously, an increase in apoAI-mediated cholesterol was observed consistently. Therefore, the stimulatory effect of both LPL and EL on apoAI-mediated cholesterol efflux was supported by both LOF and GOF strategies.

The roles of the catalytic and non-catalytic functions of lipases appear to be additive since the inhibition of each function alone only partially decreased lipase-related, apoAI-mediated cholesterol efflux, and this was totally abolished after the removal of both catalytic and non-catalytic functions. No synergistic relationship between catalytic and non-catalytic functions in apoAI-mediated cholesterol efflux was found. In contrast to their facilitating role in lipid accumulation, the expression of LPL and EL promotes cholesterol efflux, adding to the complexity of the role of both lipases in the development of atherosclerosis. However, it
remains unclear whether lipase expression in macrophages in vivo can influence intracellular cholesterol concentration through this mechanism in an effort to maintain cholesterol homeostasis.

Gauster et.al have reported that EL reduced cholesterol efflux in Cos-7 cells where HDL was modified by EL and then used as the cholesterol recipient. Compared to our study, a different cell line and cholesterol acceptor were used. THP-1 derived macrophages was also investigated in that study, however, no apparent effect of EL-modified HDL on cholesterol efflux was detected in macrophages. HDL has been proven to interact with cell surface SR-BI and ABCG transporters during cholesterol efflux, whereas, apoAI induces cholesterol efflux mainly via the ABCA1 transporter, which may also contribute to the explanation of the divergence between previous and current studies. The phospholipid content in HDL is an important determinant for HDL and cell surface interaction, and EL has been shown to be capable of hydrolyzing phospholipids. Thus, it was proposed that the depletion of phospholipids in HDL by EL compromised the HDL affinity for the cell membrane, leading to decreased cholesterol efflux. The relative role of phospholipids in apoAI-mediated cholesterol efflux is not well characterized. In one study, it was reported that the association of apoAI with phospholipids reduced its ability to interact with ABCA1. Here, we believe that the overexpression of lipases, especially EL, would deplete phospholipids from and convert lipidated apoAI into lipid-free apoAI, enhancing cholesterol efflux (illustrated as ① in figure 2-19).

In the present study, lipases stimulated apoAI-mediated cholesterol efflux by increasing apoAI docking on the cell surface which was dependent on the non-catalytic function of the lipases. Both LPL and EL bear a lipid-binding domain in C-terminus, and interact with apoB100. EL and LPL also interact with HDL for the selective uptake of cholesterol, suggesting that lipases may have the intrinsic ability to interact with apoAI. The increased apoAI docking on cells by lipases makes the former much easier to accept cholesterol from membrane due to the spatial proximity.

The increased apoAI docking by lipases may also increase the interaction between ABCA1 and apoAI, especially when apoAI is anchored in the vicinity of ABCA1. This speculation was suggested by the following observations. First, in the lipase suppressed macrophages, decreased
apoAI-mediated cholesterol efflux was not accompanied with a parallel change in ABCA1 expression. Second, the increased cholesterol efflux after EL overexpression was independent of ABCA1 level. Herein, we speculate that lipases increase the interaction of ABCA1 and apoAI through binding to and providing apoAI to the ABCA1 transporter (illustrated as © in figure 2-19).

Although apoAI mainly interacts with ABCA1 to induce cholesterol efflux, the cholesterol outflow towards apoAI through a non-ABCA1 transporter pathway could also exist for lipase-related cholesterol efflux. In DXM-treated macrophages, there was a marked reduction of ABCA1, however, a significant increase in apoAI-mediated cholesterol efflux was still observed. This dissociation of ABCA1 expression with lipase-enhanced, apoAI-mediated cholesterol efflux indicates an alternative pathway for apoAI-induced cholesterol efflux. In support of this, a considerable portion of apoAI-mediated cholesterol efflux was preserved in ABCA1-/- mouse peritoneal macrophages. Similarly, an interaction of apoAI with membranes rather than proteins was suggested by diffusion parameters of membrane-associated apoAI. This evidence implies that apoAI can be docked by lipases independent of ABCA1, thus, increasing cholesterol efflux by facilitating free diffusion (illustrated as ® in figure 2-19).

The modification of cellular phospholipid composition by lipase expression may also contribute to the change in apoAI-mediated cholesterol efflux (illustrated as © in figure 2-19). Phospholipids are proven to be preferred substrates for EL, and the lipolytic action of LPL on phospholipids has been documented as well, and LPL deficient patients have increased phosphatidylcholine content on red blood cell membrane. In this study, the relative levels of two major phospholipid components, phosphatidylcholine and sphingomyelin, were decreased with the overexpression of LPL and EL. Membrane cholesterol is classified into a fast pool and a slow pool, the former is located in the outer leaflet of cellular membrane rich in phosphatidylcholine, the latter is situated in sphingomyelin-rich domains like caveolae as well as the inner leaflet. Phosphatidylcholine and sphingomyelin present in the plasma membrane are critical to maintain the two kinetic pools of cholesterol. The treatment of membrane with sphingomyelinase or phospholipase C dramatically increases cholesterol efflux from the fast pool via free diffusion.
In the fast pool, the hydrophobic interaction between cholesterol and phosphatidylcholine has been quantitatively measured, the decreased phosphatidylcholine content in the membrane after lipase overexpression may loosen the force to entrap cholesterol, accelerating the cholesterol desorption from the fast pool. The slow pool of cholesterol, which is stabilized by a relatively strong force of hydrogen bonding between sphingomyelin and cholesterol, can be also influenced as the sphingomyelin level was inversely related to lipase expression. The degradation of sphingomyelin significantly increases cholesterol desorption in fibroblasts, astrocytes, and CHO cells. As such, the reduction of sphingomyelin by lipase expression leads to the liberation of cholesterol from the slow pool. Furthermore, the removal of membrane phospholipids can also cause phosphatidylserine exofacial flipping during which an outward flow of cholesterol was driven concurrently. Other phospholipids like phosphatidylethanolamine and phosphatidylserine, the levels of which were changed with the modification of lipase expression, may impose some modulatory effects on membrane cholesterol stability as well. Throughout this process, apoAI docked on the cell surface by lipases becomes an immediate acceptor to receive cholesterol released from both fast and slow pools.

The production of lysophosphatidylcholine (LPC) in macrophages and transfected FLP-IN 293 cells was positively correlated with lipase expression. A dose-dependent increase in apoAI-mediated cholesterol efflux by LPC was demonstrated in macrophages in this study, which is consistent with previous reports. In addition, paraoxonase 1 was found to increase cholesterol efflux, and this effect was ascribed to the increase of LPC production by paraoxonase action. The released LPC may incorporate into and microsolubilize apoAI to facilitate the acceptance of cholesterol (illustrated as \( \mathbf{1} \) in figure 2-19). The metabolite of sphingomyelin, ceramide, was also reported to stimulate apoAI-mediated cholesterol efflux through ABCA1 pathway. The decreased level of sphingomyelin as found after lipase overexpression in this study may suggest that the production of ceramide may increase, and then enhance apoAI-mediated cholesterol efflux.

In summary, lipoprotein and endothelial lipases in macrophages promote the apoAI-mediated cholesterol efflux through enhancing apoAI binding and modulating phospholipid composition of the membrane. We propose a model where the potential mechanisms of lipase effect on
apoAI-mediated cholesterol efflux are illustrated (figure 2-19). Briefly, phospholipid hydrolysis by lipases converts pre-β HDL into lipid-free apoAI, which is more efficient in inducing cholesterol efflux through the ABCA1 transporter. Moreover, lipases enhance the binding of apoAI to facilitate the cholesterol outflow by either active transport via ABCA1 or free diffusion. The perturbation of membrane phospholipid composition by lipases may liberate the cholesterol to apoAI, and this process can be accelerated by the simultaneous release of lysophosphatidylcholine.

Figure 2-19. Proposed mechanisms of lipase-enhanced apoAI-mediated cholesterol efflux in macrophages. In this model, lipases (LPL and EL) convert pre-β HDL into lipid-free apoAI which can accept cholesterol from ABCA1 transporter more efficiently (indicated as ①). Lipases also enhanced apoAI binding, providing the spatial proximity between apoAI and ABCA1 (indicated as ②) or membrane cholesterol (indicated as ③) to facilitate the cholesterol outflow. The alteration of phospholipid composition by lipase hydrolysis drives the cholesterol release more readily (indicated as ④), the concurrent release of lysophosphatidylcholine also accelerates cholesterol release (indicated as ⑤).
2.6 References:


Chapter 3. Endothelial Lipase Expression Enhances the Binding and Uptake of Native and Oxidized Low Density Lipoprotein in Human Macrophages: A Mechanism Requiring Heparan Sulfate Proteoglycans

3.1 Introduction and Rationale

One striking feature of atherosclerosis is the formation of lipid-laden foam cells, of which most are derived from macrophages. Lipids in foam cells are mostly from circulating lipoproteins, particularly low density lipoprotein (LDL) and its modified forms such as oxidized or aggregated LDL. Macrophages express several lipoprotein receptors, including low density lipoprotein receptor (LDLR), low density lipoprotein receptor-related protein (LRP), and several scavenger receptors like SRA and CD36. These receptors are intimately involved in the lipoprotein modification and subsequent internalization by macrophages, promoting the formation of macrophage-derived foam cells.1–6

The role of EL in atherosclerosis remains unclear, and the results obtained from animal studies are not consistent. Ishida et al. reported the EL knockout in apoE-deficient mice lessened the aortic atherosclerosis by ~70% on both regular and chow diets.7 By contrast, this relationship was not established in another study where both apoE-deficient and LDLR-deficient mice were bred with EL knockout mice.8 The development of atherosclerotic lesions in EL-knockout animals did not differ from their controls. However, several clinical studies suggest that EL could be potentially pro-atherogenic. EL was increased in a population of moderately obese men, and also correlated to the inflammatory score calculated on the basis of C-reactive protein, interleukin-6, and secretory phospholipase A2 concentrations.9 The positive correlation of EL with metabolic syndrome factors and coronary calcification score was observed in 858 healthy participants with a family history of premature coronary heart disease.10 However, the proceeding experiments (Chapter 1) in this thesis indicate that macrophage-derived EL displayed an anti-atherogenic trait by promoting cholesterol efflux.

b A version of this chapter has been submitted for publication. Qiu, G. and Hill, J.S. Endothelial lipase expression enhances the binding and uptake of native and oxidized low density lipoprotein in human macrophages.
EL has been regarded to be a genetic regulator of plasma high density lipoprotein (HDL),\textsuperscript{11-13} and animal studies also show that EL can modulate plasma levels of apoB-containing lipoproteins.\textsuperscript{11-15} EL expression in transfected Chinese Hamster Ovary (CHO) cells resulted in a marked increase of LDL binding and uptake.\textsuperscript{16} However, the effect of EL on LDL metabolism in macrophages thus far has not been investigated.
3.2 Hypotheses and Specific Aims

3.2.1 Hypotheses
The expression of EL in THP-1 derived macrophages will promote the binding and uptake of both native and oxidized LDL. EL-mediated LDL metabolism will be dependent on cell surface HSPGs, whereas the catalytic activity of EL will not play a critical role. Furthermore, the interaction between EL and lipoprotein receptors (LDLR, LRP, and CD36) may mediate part of LDL metabolism.

3.2.2 Specific Aims:
1. To generate EL suppressed or overexpressing macrophages
2. To measure the binding and association of native and oxidized LDL in macrophages
   a. DiI labeling of LDL
   b. The oxidation of LDL and quantitation of oxidation degree of oxLDL
   c. The binding and association of native and oxLDL with cells.
3. To differentiate the roles of the catalytic and non-catalytic functions of EL in LDL metabolism in macrophages
   a. LDL and oxLDL binding and association after the inhibition of catalytic activity by THL
   b. LDL and oxLDL binding and association after the elimination of cell surface HSPGs by heparinase I.
4. To investigate the role of lipoprotein receptors in EL-mediated LDL metabolism
   a. The blockage of lipoprotein receptors by LDLR antibody, receptor associated protein (RAP), CD36 antibody
   b. LDL and oxLDL binding and association after the blockage of lipoprotein receptors.
3.3 Materials and Methods:

3.3.1 The suppression and over-expression in macrophages by lentivirus

The methods have been described in previous chapter.

3.3.2 DiI labeling and oxidation of LDL

For 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) labeling, native LDL (Biomedical Technologies INC., MA, USA) was incubated with DiI (final concentration of 300µg/ml) at 37°C overnight. Thereafter, both DiI-labeled and unlabeled LDLs were oxidized as following: LDL was incubated with 5 µM CuSO₄ for 2 hours or overnight at 37°C for mildly and extensively oxidized LDL (oxLDL), respectively. EDTA was added to final concentration of 1mM to terminate the reaction before purification. Subsequently, LDL was re-isolated into PBS using a PD-10 desalting column pre-equilibrated with PBS (GE Healthcare). The protein concentration of the LDL preparations was measured using a BCA protein assay kit (Pierce). Thiobarbituric acid reacting substances (TBARS) assay (ZeptoMetrix Co., NY, USA) was used to evaluate the oxidation extent of LDL. The amount of TBARS was determined by comparison to a standard of malondialdehyde (MDA) equivalents. A LDL preparation with a TBAR value of 20~30 nmole/mg protein of MDA equivalents was classified as mildly oxidized LDL whereas TBAR values greater than 50 nmole/mg protein of MDA equivalents was classified as extensively oxidized LDL.

3.3.3 LDL binding and association assay

THP-1 monocytes (5×10⁵) were transduced with lentivirus at MOI of 20 for 2 days, and then were differentiated into macrophages by 100nM PMA stimulation for 2 days. Thereafter, macrophages were starved in RPMI 1640 medium containing lipoprotein deficient serum (LPDS) for 24 hours. Subsequently, macrophages were treated with 10µg/ml DiI-LDL (native, mildly oxidized, and extensively oxidized LDLs), and incubated at 4°C or 37°C for the binding and association assays, respectively. For background binding/uptake analysis, 20-fold excess of non-Dil-labeled LDL was added into culture in competition with DiI-LDL. After 4 hour incubation, cells were washed twice with 0.2% BSA-PBS and PBS, and then lysed in RIPA buffer. Aliquots of cell lysates were measured for fluorescence at the excitation and emission wavelengths of 520nm and 580nm. Standard curves were made using serially-diluted DiI-LDL.
3.3.4 Cell treatments in LDL binding and association assay
Tetrahydrolipstatin (50µg/ml) and heparinase I (2.5 units/ml) were added to culture medium during the binding and uptake assays to inhibit catalytic and noncatalytic activities (bridging function) of EL, respectively. LDL receptor monoclonal antibody (15µg/ml, Fitzgerald), CD36 monoclonal antibody (10µg/ml, Novus Biochemicals), and Receptor Associated Protein (RAP, Calbiochem) which blocks LRP at the concentration of 4µg/ml were added one hour prior to the addition of Dil-LDL.
3.4 Results:

3.4.1 EL suppression reduces the binding and association of native and oxidized LDL
The binding and uptake of native and oxidized LDL were analyzed after EL suppression. The uptake of LDL is reported to be dependent on its degree of oxidation, so we modified LDL into mildly and extensively oxidized LDLs by incubating native LDL with copper sulfate for different times. Our experiments confirmed that the binding and association of LDL in macrophages is a function of oxidation extent, the higher oxidation rendered the higher binding and association of LDL. The average LDL binding to macrophages increased from ~300ng/mg cellular protein for native LDL to ~750ng/mg cellular protein for mildly oxidized LDL and ~1200ng/mg cellular protein for extensively oxidized LDL (Figure 3-1A, 3-1C, and 3-1E). Meanwhile, the amount of cell-associated LDL at 37°C, of which most was internalized by cells, increased from ~1ug/mg cellular protein for native LDL to ~1.5ug/mg cellular protein and ~2.0ug/mg cellular protein for mildly and extensively oxidized LDLs (Figure 3-1B, 3-1D, and 3-1F).

EL suppression was associated with significant reduction in binding and cell association of native LDL of 52.5% and 33%, respectively (Figure 3-1A and 3-1B). Similarly, EL suppression was also associated with significant decreases of 43.2% and 12.4% for the binding and association of mildly oxidized LDL (Figure 3-1C and 3-1D). Furthermore, the binding of extensively oxidized LDL was significantly reduced by 36% after the EL suppression (Figure 3-1E), but no significant difference was observed for the cell association of extensively oxLDL after EL suppression (Figure 3-1F).

3.4.2 Overexpression of EL increases the binding and association of native and oxidized LDL
We further examined the role of EL in the LDL binding and association in EL-overexpressing macrophages. Strikingly, EL over-expression increased native LDL binding and association by 2.96 and 2.12 -fold, respectively (Figure 3-2A and 3-2B). EL also stimulated the binding and association of both mildly and extensively oxidized LDL, with 1.7- and 1.4-fold increases for mildly oxidized LDL, and 1.6- and 1.5--fold increases for extensively oxidized LDL, respectively (Figure 3-2 C~F).
3.4.3 EL-mediated LDL binding and association is independent of catalytic function

To determine what extent the catalytic activity of EL may have on the observed effects of LDL binding and cell association, we performed experiments in the presence and absence of
tетрациклин (THL), a specific lipase inhibitor through covalently binding to serine residue in catalytic site. In our experiments, THL treatment diminished the basal binding and association of native, mildly and extensively oxidized LDL in the control cells (Figure 3-3 A-E), this effect could be due to the inhibition of endogenous lipases such as LPL, of which catalytic activity is known to increase the removal of LDL. After subtracting the basal LDL binding or association, we did not observe any difference in EL-mediated LDL binding (empty part of clear bar) for EL-overexpressing THP-1 macrophages after EL catalytic inhibition by THL (Figure 3-
3A). Whereas, EL-mediated native LDL association (empty part of clear bar) was decreased by ~40% in the presence of THL (Figure 3-3B). The catalytic function seems to be less critical in the metabolism of oxidized LDL, neither EL-mediated binding nor association (empty part of clear bar) of both mildly and extensively oxLDL was affected in the presence of THL (Figure 3-3 C~F).

Figure 3-3. Investigation of the role of EL catalytic activity on EL-mediated LDL/oxLDL binding and association in THP-1 derived macrophages. EL expression in THP-1 derived macrophages was upregulated by EL cDNA-containing lentivirus; the binding and association of native and oxLDL were analyzed in the absence (No THL) and presence (+THL) of 50ug/ml tetrahydrolipstatin. Panel A and B: native LDL binding and cell association; Panel C and D: mildly oxLDL binding and cell association; Panel E and F: extensively oxLDL binding and cell association. Control: macrophages transduced with empty lentivirus; EL-GOF: EL-overexpressing macrophages by lentivirus transduction. Statistical comparisons between the absence and presence of THL in controls are indicated as: ** p<0.01, *** p<0.001; Statistical comparisons between control and EL-GOF are indicated as: †† p<0.01, ††† p<0.001; Statistical comparisons between the absence and presence of THL in EL mediated binding or cell association of LDL/oxLDL (empty part of clear bar) are indicated as: ††† p<0.001, n=4.
3.4.4 Heparan sulfate proteoglycans are required for EL-mediated LDL binding and association

EL has a non-catalytic or "bridging" function, so we used heparinase I to eliminate the binding ability of EL to HSPGs, and then did LDL binding and association analyses. Compared to the

Figure 3-4. Investigation of the role of HSPG on EL-mediated LDL/oxLDL binding and association in THP-1 derived macrophages. EL expression in THP-1 derived macrophages was upregulated by EL cDNA-containing lentivirus, the binding and association of native and oxLDL were analyzed in the absence (No heparinase) and presence (+heparinase) of 2.5units/ml heparinase I. Panel A and B: native LDL binding and cell association; Panel C and D: mildly oxLDL binding and cell association; Panel E and F: extensively oxLDL binding and cell association. Control: macrophages transduced with empty lentivirus; EL-GOF: EL-overexpressing macrophages by lentivirus transduction. Statistical comparisons between the absence and presence of heparinase in controls are indicated as: ** p<0.01, *** p<0.001; Statistical comparisons between control and EL-GOF are indicated as: † p<0.05, †† p<0.01, ††† p<0.001; Statistical comparisons between the absence and presence of heparinase in EL mediated binding or cell association of LDL/oxLDL (empty part of clear bar) are indicated as: ‡ p<0.05, §§§ p<0.001, n=4.
heparinase untreated macrophages, the heparinase I treatment reduced the basal binding of both mildly and extensively oxidized LDL (49% and 60%, respectively, Figure 3-4 C~F), but not native LDL (Figure 3-4 A~B) in the control conditions. The basal association of native LDL and extensively oxLDL was also significantly decreased in the presence of heparinase (49% and 15%, respectively). Because HSPGs have been proven to be able to stimulate LDL turnover by macrophages,\textsuperscript{22-24} the decreased LDL turnover could be ascribed to the removal of HSPGs from cell. The presence of heparinase I almost completely eliminated the enhancement of LDL/oxLDL binding mediated by EL (empty part of clear bar). In the presence of heparinase I, only 9% and 5% of EL-mediated native LDL binding and association was retained in macrophages (Figure 3-4A and 3-4B). A reduction of 89% and 88% in EL-mediated mildly oxLDL binding and association was observed in the presence of heparinase I (Figure 3-4C and 3-4D). Similarly, the heparinase treatment caused a 91% and 71% reduction in EL-mediated binding and association of extensively oxidized LDL (Figure 3-4E and 3-4F).

3.4.5 The role of lipoprotein receptors in EL-mediated LDL binding and association

Macrophages express a variety of lipoprotein receptors responsible for the metabolism of native and oxidized LDL, among which LDLR, LRP, and CD36 are highly expressed and most important.\textsuperscript{1,3,6,25} To investigate the potential role of specific cell surface receptors in the ability of EL to mediate LDL/oxLDL binding and cell association, specific monoclonal LDLR and CD36 antibodies were pre-incubated with macrophages to block the subsequent binding of LDL and oxLDL. RAP, a multidomain protein containing two independent LRP binding sites, was used to competitively inhibit LDL binding to LRP. The blockage of receptors by specific antibodies significantly reduced the basal levels of native LDL and oxLDL binding and association in control conditions (Figure 3-5A~F). In the EL-overexpressing cells, EL-mediated native LDL binding and association (empty part of clear bar) were diminished by 24% and 54% respectively by LDLR antibody but not RAP (Figure 3-5 A and B). With the blockage of CD36 by its antibody, no noticeable effects on EL-mediated binding and association (empty part of clear bar) of both mildly and extensively oxidized LDL were observed (Figure 3-5 C and D).
Figure 3-5. Investigation of the role of LDLR, LRP, and CD36 on EL-mediated LDL/oxLDL binding and association in THP-1 derived macrophages. EL expression in THP-1 derived macrophages was upregulated by EL cDNA-containing lentivirus, the binding and association of native and oxLDL were analyzed in the absence (-) and presence (+) of blocking agents (LFLR-Ab, CD36-Ab, or RAP). Panel A and B: native LDL binding and cell association; Panel C and D: mildly oxLDL binding and cell association; Panel E and F: extensively oxLDL binding and cell association. Control: macrophages transduced with empty lentivirus; EL-GOF: EL-overexpressing macrophages by lentivirus transduction. Statistical comparisons between the absence and presence of blocking agents in controls are indicated as: ** p<0.01, *** p<0.001; Statistical comparisons between control and EL-GOF are indicated as: †† p<0.01, ††† p<0.001; Statistical comparisons between the absence and presence of blocking agents in EL mediated binding or cell association of LDL/oxLDL (empty part of clear bar) are indicated as: ‡ p<0.05, ‡‡ p<0.01, n=4.
3.5 Discussion

Although the influence of EL activity on the metabolism of circulating lipoproteins has been described, there is little knowledge about its tissue-specific function in macrophages. In the present study, we have used loss-of-function and gain-of-function approaches and demonstrated that macrophage-derived EL can mediate the binding and cell association of native LDL and oxLDL.

This study demonstrated that the EL expression in THP-1 derived macrophages significantly increased the binding and association of both native and oxidized LDL. A similar effect of EL was also observed in transfected Chinese Hamster Ovary (CHO) cells, where the expression of EL markedly enhanced LDL binding by 3-fold; furthermore, endogenous EL expression in COS cells by adenovirus resulted in the increased LDL internalization and degradation. These findings suggest that EL could promote foam cell formation if overexpressed in macrophages. Upregulation of EL expression has been found in human atherosclerotic lesions, and its existence is colocalized with macrophages in the atherosclerotic lesions. The role of EL in LDL metabolism in macrophages is similar to its next of kin, LPL, which promotes the formation of macrophage-derived foam cells and accelerates the development of atherosclerosis. By promoting lipid accumulation in macrophages, EL may also accelerate the development of atherosclerosis.

As demonstrated in this study, EL enhanced LDL metabolism in macrophages, however, how EL interacts with LDL is still not known. The knowledge of EL structure is mostly drawn from other members of the lipase gene family. The C-terminal domain of lipases is relatively hydrophobic, making it possible to interact with hydrophobic lipids. In LPL, the lipid binding domain has been located to a cluster of tryptophan residues (Trp390-Trp393-Trp394) in the C-terminus of LPL. Scanning of EL amino acid sequence also reveals the conservation of a tryptophan cluster in C-terminal region at 398, 406, and 410. However, their role in lipid binding thus far has not been verified.

We also observed that the catalytic activity of EL appears to influence the uptake of native LDL but not binding in macrophages. One possible explanation for this result is that the lipolytic modification of native LDL by EL occurs only after LDL binding takes place, and this
modification may cause the decrease in size and the rearrangement of surface molecules on LDL, which make this remodeled LDL more accessible for binding and consequent internalization in macrophages. The role of the catalytic function of EL in the metabolism of apoB-containing lipoproteins was also demonstrated in mouse models. The transgenic expression of human EL significantly reduced the concentrations of circulating apoB-containing lipoproteins, while catalytically inactive EL raised them. By contrast, the catalytic activity of EL does not assume a critical role in the metabolism of both mildly and extensively oxidized LDL as neither the binding nor the association of mildly and extensively oxidized LDL was considerably influenced after EL inactivation by THL. Consistent with previous reports, the oxidative modification of LDL markedly increases LDL binding for cells when compared to native LDL. This study disclosed that the role of EL in LDL metabolism became quantitatively less significant with extensive oxidation. Furthermore, the catalytic activity of EL is not as critical as its bridging function in the metabolism of apoB-containing lipoproteins in cell models. On the whole, the role of catalytic activity of EL in the metabolism of oxidized LDL appears minimal.

EL is capable of binding to cell surface HSPGs due to the conservation of alkaline amino acid residue clusters in its C-terminus. Like LPL, this heparin-binding function of EL is crucial for lipid binding and uptake in this study, because the displacement of EL from cell surface by treating cells with heparinase almost abolished the effects of EL on LDL/oxLDL binding and subsequent catabolism by EL in macrophages. Fuki et al. have shown that the EL-dependent increase of LDL binding to CHO cells was completely removed by heparin or sodium chlorate treatment which prevents EL binding to HSPGs. Furthermore, there was almost no effect of EL on LDL binding in HSPG-deficient CHO cells. This evidence is consistent with our findings from macrophages. The candidate sites for heparin binding were proposed to be Arg327-Lys329-Arg330-Lys333, Arg312-Lys313-Arg315, Gly184-Arg188, and Lys352-Arg450-Lys452-Lys459. Site-directed mutagenesis could be an useful tool to verify this.

The fact that oxidized LDL metabolism in macrophages is independent of scavenger receptor CD36, and a large portion of native LDL uptake is devoid of LDLR involvement, suggests the existence of an alternative pathway for EL-mediated LDL metabolism. Lipases can be internalized via HSPGs and recycled. For example, LPL which was initially bound to the cell surface was internalized along with HSPGs, and the treatment of the cells with heparinase or
heparin markedly reduced the amount of intracellular LPL internalized. Therefore, a lipoprotein receptor-independent but heparinase-sensitive metabolizing route was proposed for LPL bound LDL. Due to the structural and functional similarity between EL and LPL, it is speculated that a non-receptor dependent, HSPG-sensitive pathway may also exist for EL-mediated lipoprotein metabolism (as illustrated in figure 3-6).

Figure 3-6. Proposed pathways for EL-mediated LDL binding and association in THP-1 derived macrophages. The EL expression promotes the binding and association of both native and oxidized LDL in human macrophages. For LDL uptake, both the receptor and non-receptor dependent pathway exists. The receptor-dependent pathway is found for the EL mediated native LDL uptake, where EL binds to LDL and then renders LDL towards LDL receptor (LDLR) for internalization. The non-receptor pathway involves EL-HSPG interaction, and is important for the uptake of both native and oxidized LDL.

LDLR is involved in EL-dependent binding and association of native LDL. In these experiments, the blockage of LDLR by a monoclonal antibody partially reduced the EL-mediated native LDL catabolism by macrophages. The same relationship between LDLR and LPL-mediated lipid uptake was also reported. LPL increased the uptake of LDL by 2 -fold in wild-type (LDLR+/+) aortic endothelial and HepG2 cells, but there was virtually no increase of native LDL uptake in LDLR-deficient cells. Unlike LPL, which can directly interact with LRP and collectively contribute to the lipoprotein removal, EL stimulated increase of native LDL.
metabolism does not require the existence of LRP since the LRP blockage by RAP did not cause significant change in native LDL binding or association.

LPL has the ability to directly interact with several lipoprotein receptors such as LDLR, however, EL seemingly does not interact with LDLR when mediating native LDL metabolism in macrophages. If there was a direct binding between EL and LDLR, heparinase treatment would not be able to completely remove the effect of EL on native LDL metabolism. Our results have shown EL binding to HSPGs is a prerequisite for subsequent LDL binding and internalization via LDLR. Therefore, a two-step theory is hypothesized to explain the LDLR-associated but also EL-dependent LDL metabolism in macrophages; first, native LDL is bound to cell surface via the EL-HSPG interaction, and then EL provides LDL to LDLR for subsequent binding and internalization via this receptor (as illustrated in Figure 3-6).

In summary, macrophage-derived EL expression enhances the binding and association of both native and oxLDL in human THP-1 macrophages, an effect dependent on the presence of cell surface HSPGs. In addition, EL-enhanced native LDL metabolism involves LDLR, and neither LRP nor CD36 is found to be implicated in EL-mediated LDL/oxLDL metabolism.
3.6 References:


36. Mulder M, Lombardi P, Jansen H, van Berkel TJ, Frants RR, Havekes LM. Heparan sulphate proteoglycans are involved in the lipoprotein lipase-mediated enhancement of


Chapter 4. Suppression of endothelial lipase or lipoprotein lipase expression in THP-1 macrophages attenuates pro-inflammatory cytokine secretion

4.1 Introduction and rationale

It is well recognized that a variety of proinflammatory cytokines are expressed in activated macrophages including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1β (IL-1β), and monocyte chemoattractant protein-1 (MCP-1). These cytokines have been shown to promote lesion progression by either aggravating endothelial dysfunction, recruiting additional inflammatory cells, or stimulating smooth muscle proliferation.

The expression of LPL and EL have been associated with macrophages within human atherosclerotic lesions. Although the specific role of these lipases in atherosclerosis likely depends on their tissue localization, accumulating evidence supports a proatherogenic role of macrophage-derived LPL, and the strong association of plasma EL with inflammation, obesity, metabolic syndrome, and premature coronary heart disease highly suggests a proatherogenic role of EL.

The proatherogenic role of LPL and EL in macrophages has been largely ascribed to their non-catalytic or bridging function by which LPL stimulates the LDL binding and internalization. Similarly, endogenously produced EL enhances the binding and uptake of both native and oxidized LDL in macrophages in the present study and these in transfected Chinese hamster ovary cells. In addition, the role of EL and LPL as adhesion molecules to facilitate the binding of monocytes onto the surface of endothelial cells was also observed.

Nevertheless, there is little information on the effect of endogenous lipase expression on the secretion of proinflammatory cytokines in human macrophages. LPL expression in mouse macrophages and adipocytes is dampened by interferon-γ, TNF-α, lipopolysaccharide, IL-11, IL-1, and IL-6. Regarding EL, IL-1β and TNF-α have been shown to stimulate its expression in endothelial cells. However, very few studies have addressed how lipase

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expression may affect cytokine expression. Exogenous LPL has been shown to induce TNF-α by increasing TNF-α mRNA transcription and stability.\textsuperscript{19,20} To our knowledge, there are no reports on the influence of EL expression on cytokine expression.
4.2 Hypotheses and Specific Aims

4.2.1 Hypotheses
The expression of endothelial lipase and lipoprotein lipase is intimately related to the pro-inflammatory response in macrophages; therefore, the suppression of endothelial lipase and lipoprotein lipase will attenuate the expression of atherosclerosis-related genes and decrease the production of proinflammatory cytokines and chemokines (IL-1, IL-6, IL-8, TNF-alpha, MCP-1) in macrophages. The decreased proinflammatory response will be associated with decreased levels of lipolytic products and lipid accumulation in macrophages.

4.2.2 Specific Aims
1. To generate LPL and EL suppressed macrophages
2. To assess the effect of oxidized LDL on the production of proinflammatory cytokines and chemokines in macrophages
3. To measure the levels of proinflammatory cytokines and chemokines in lipase suppressed macrophages
4. To evaluate the expression of atherosclerosis-related genes in lipase suppressed macrophages by cRNA microarray
5. To measure the effect of lipase suppression on the production of proinflammatory cytokines and chemokines in oxidized LDL treated macrophages
6. To analyze lipid composition in lipase suppressed macrophages
4.3 Materials and Methods

4.3.1 Lentiviral Production, Macrophage Transduction, Lipase Quantitation by real-time qRT-PCR and Western Blot, and HPLC Assay of Phospholipid Composition. The detailed methods are described in chapter 2.

4.3.2 LDL Oxidation and Cell Treatment
Native LDL (Biomedical Technologies INC., MA, USA) was oxidized by incubating 1mg/ml LDL with 5 μM CuSO₄ for various times to generate mildly and extensively oxidized LDL (oxLDL) as described previously. The oxidative extent was assessed by TBARS assay (ZeptoMetrix Co., NY, USA). A LDL preparation with a TBAR value of 20–30 nmole/mg protein of MDA equivalents was classified as mildly oxLDL whereas TBAR values greater than 50 nmole/mg protein of MDA equivalents was classified as extensively oxLDL. THP-1 monocytes (5×10⁵ cells) were seeded into a 12-well plate and then transduced by lentivirus at multiplicity of infection of 20 on the same day. Phorbol 12-myristate 13-acetate (PMA) was added to the cells to a final concentration of 100 nM 2 days after lentiviral transduction to stimulate the differentiation of the monocytes into macrophages. Subsequently, medium was collected for cytokine analysis every 24 hours for 3 consecutive days. Oxidized LDL (50 μg/ml) was added to the macrophage culture 24 hours prior to PMA stimulation.

4.3.3 Cytokine ELISA
The quantitation of secreted proinflammatory cytokines was performed by ELISA assays (BioSource). IL-1β, IL-6, IL-8, MCP-1, and TNF-α were analyzed from aliquots of conditioned medium from THP-1 macrophages according to the manufacturer’s instructions. Briefly, aliquots of 100 μL were added to a pre-coated microplate containing 100 μL of dilution buffer and incubated for 3 hours at room temperature. Wells were washed 6 times before 100 μL of biotinylated anti-target solution was added for 45 min. Microplates were washed again before incubation with 100 μl Streptavidin-HRP working solution at room temperature for 45 minutes. A stabilized chromogen solution (100 μL) was added and incubated in the dark for 30 min after 6 wash steps. Absorbance was read at 450nm after adding 100 μL stop solution.
4.3.4 Microarray

An atherosclerosis-specific microarray (SuperArray) was used to evaluate the gene expression following lipase suppression. Total RNA was extracted from three individual wells with same treatment and pooled for cDNA synthesis. Total RNA (3 μg) was mixed with Truelabeling primer and cDNA synthesis enzyme mix in cDNA synthesis buffer and incubated at 42°C for 50 minutes. The synthesized cDNA was transcribed into cRNA at 37°C for 4 hours after the addition of an amplification master mix (biotin-16-UTP, amplification enzyme mix, and amplification buffer). Synthesized cRNA was then purified by adding lysis & binding buffer in combination with a spin column. Bound cRNA was eluted and 10 μg of biotin-labeled cRNA in 0.75ml of pre-warmed GEAhyb hybridization solution was added into a hybridization tube containing array membrane which was prehybridized with GEAhyb hybridization solution at 60°C for 2 hours with vigorous agitation. Hybridization was performed at 60°C overnight with gentle agitation. Thereafter, the array membrane was washed twice with 2X SSC, 1% SDS for first wash, and 0.1X SSC, 0.5% SDS for second wash at 60°C, and blocked using GEAblocking solution Q before the alkaline phosphatase-conjugated streptavidin was added. After 4 washes of 1X buffer F and one time rinsing of Buffer G, membrane was developed using CDP-Star substrate, and photographed by ChemiGenius 2 chemiluminescence system. The expression of genes was analyzed by GEArray® Expression Analysis software, each gene is represented by 4 spots on the microarray membrane.
4.4 Results

4.4.1 Cytokine Expression Secondary to Lipase Suppression
The suppression of LPL and EL has been successfully achieved as described in chapter 1. Following suppression of either LPL or EL, the concentration of five proinflammatory cytokines was monitored in THP-1 macrophages over three days (the complete data are listed in Appendices 6–8). Under control conditions there were marked and steady increases in IL-6, MCP-1 and TNF-α during the observed time period (Appendix 7). However, the level of IL-1β and IL-8 is relatively stable throughout the observation period; only modest differences were observed for IL-1β and IL-8.

The suppression of either LPL or EL dramatically changed the production of proinflammatory cytokines (Appendix 7). Significant decreases were observed for IL-1β for all time points following LPL suppression, with a decrease of 72% at day 1, 41% at day 2, and 33% at day 3 (P<0.05 in all cases). Similarly, IL-1β was significantly decreased by 77% and 67% at 24 and 48 hours for EL suppression (P<0.05 in all cases). The similar and significant decreases in cytokine levels following lipase suppression were also observed for IL-6, MCP-1, and TNF-α, with a maximum of 73% and 78% decrease in IL-6, 67% and 73% decrease in MCP-1, and 60% and 66% decrease in TNF-α for LPL and EL suppression, respectively (Figure 4-1, and Appendix 7). However, the lipase suppression has little effect on IL-8 production, there are modest (9%~31%) but consistent decreases in IL-8 under EL-suppressed conditions, and LPL suppression had no effect on IL-8 level except a mild decrease of 30% at 24 hours. Overall, the suppression of LPL and EL significantly decreased the production of IL-1β, IL-6, MCP-1, and TNF-α in THP-1 derived macrophages.

4.4.2 Cytokine Expression Secondary to Treatment with Oxidized LDL
Oxidized LDL exerts various effects on cellular biology, no consensus has been reached with regard to the oxidized LDL effect on cytokine production, and contradictory results have been published from different labs. Here, we also looked at oxidized LDL effect on cytokine expression in macrophages. Compared to control conditions, the treatment of THP-1 macrophages with mildly and extensively oxidized LDL was associated with lower cytokine
Figure 4-1. Cytokine expression in THP-1 macrophages following LPL or EL gene suppression. THP-1 monocytes were transduced with lentivirus containing either a scrambled shRNA sequence (control) or targeted sequences for LPL (LPL suppression) or EL (EL suppression). Macrophages were stimulated by PMA in the absence of oxidized; conditioned medium was collected for 3 days after PMA and analyzed for cytokine measurement by ELISA. Panel A, IL-1β; Panel B, IL-6; Panel C, IL-8; Panel D, MCP-1; Panel E, TNF-α. Lipase suppressed cells were compared with control conditions for each time point (Data are represented by mean ± SEM, n=3, Statistical comparisons are indicated as: * p<0.05, ** p<0.01)

levels of IL-1β, IL-6, MCP-1, and TNF-α with decreases of 28% and 23%, 38% and 62%, 21% and 25%, and 34% and 66% observed at 48 hours, and 38% and 33%, 60% and 57%, 9% and 29%, 37% and 58% at 72 hours, respectively (Figure 4-2). A similar trend in of IL-1β, IL-6, and TNF-α was also observed for the 24 hour time point where no significant change of MCP-1 was observed (Figure 4-2). Throughout the experiment period, the levels of IL-8 in oxidized LDL
treated macrophages were not significantly different from the controls except the 24 hour time point where a modest decrease of 32% in macrophages treated with extensively oxidized LDL. In general, the higher oxidation degree of LDL gave rise to greater inhibition over the production of proinflammatory cytokines when compared to the lower oxidation degree.

4.4.3 Lipase suppression superimposes the inhibitory effect on oxidized LDL treatment in cytokine expression

We further tested if the lipase suppression can superimpose further inhibition on proinflammatory cytokine production in oxidized LDL treated macrophages. When experiments

![Graphs showing cytokine levels](image)

Figure 4-2. The effect of oxLDL on the production of proinflammatory cytokines in THP-1 macrophages. OxLDL was added into cell culture 24 hours before PMA stimulation, conditioned medium was collected every 24 hours after PMA for 3 days and measured for cytokine levels by ELISA. Oxidized cells were compared to the controls at each time point (Data are represented as mean ± SEM, n=3, Statistical comparisons are indicated as: * p<0.05, ** p<0.01, *** p<0.001)
were performed in the presence of various oxidized forms of LDL, lipase suppression further lessened the production of the proinflammatory cytokines. There were significant decreases for all five cytokines measured for at least one time point for LPL and EL suppressed macrophages incubated with mildly oxidized LDL (Appendices 8 and 9). At the time point of 48 hours, there was a further reduction of 44% and 61%, 70% and 61%, 52% and 37%, and 57% and 43% at mildly oxidized LDL treatment, 66% and 70%, 9% an 47%, 53% and 33%, and 8% and 33% at extensively oxidized LDL treatment, in IL-1β, IL-6, MCP-1, and TNF-α after LPL and EL suppression, respectively (Figure 4-3). Similar findings were also observed at 24 and 72 hours.

Figure 4-3. The additive effect of lipase suppression and oxidized LDL treatment on proinflammatory cytokine production (A representative of 48 hours). The monocytes were transduced with shRNA-containing lentivirus prior to oxidized LDL treatment. Following the PMA stimulation, conditioned medium was analyzed by ELISA every 24 hours for 3 consecutive days. The cells with both lipase suppression and oxidized LDL treatment were compared to those with oxidized LDL treatment alone. (Data are represented as mean ± SEM, n=3, Statistical comparisons are indicated as: * p<0.05, ** p<0.01)
4.4.4 The effect of oxidized LDL on lipase expression in macrophages

To determine if oxLDL treatment influenced lipase expression, LPL and EL mRNA was quantified throughout the 3 day PMA stimulation period following the treatment of oxLDL. Both mildly and extensively oxidized LDL consistently and significantly decreased EL mRNA expression by 45%~50% throughout monocyte differentiation (Figure 4-4B), whereas LPL mRNA level did not appear to be affected by either type of oxidized LDL (Figure 4-4A).

![Figure 4-4. The effect of oxLDL on lipase expression in THP-1 macrophages. OxLDL was added into cell culture 24 hours before PMA stimulation, and mRNA was extracted everyday after PMA stimulation for 3 consecutive days. Panel A: Macrophage LPL expression after oxLDL treatment; Panel B: Macrophage EL expression after oxLDL treatment; Panel C: Macrophage EL expression at day 2 with lentivirus transduction and oxidized LDL treatment. (Data are represented as mean ± SEM, n=4, Statistical comparisons of oxLDL, EL suppression, and oxLDL+EL-LOF with the control are indicated as: *** p<0.001, Statistical comparisons between EL suppression and oxLDL+EL-LOF are indicated as: † p<0.05.)]

Thereafter, we examined whether oxidized LDL causes the further reduction of EL expression after lentiviral transduction. Because the oxidation degree seemed not to differ in terms of their effects on EL expression, the monocytes/macrophages transduced by EL-shRNA lentivirus were incubated with mildly oxidized LDL for this experiment. Strikingly, EL-shRNA incurred a
reduction of 94% in EL mRNA. When treated with oxidized LDL in lentivirus-transduced macrophages, a further 1% decrease in EL mRNA was noticed (Figure 4-4C).

4.4.5 Lipid Composition after Lipase Suppression

Lipases are closely involved in lipid metabolism, we therefore investigated the cellular lipid composition secondary to lipase suppression. The lipid composition of lipase-suppressed THP-1 macrophages was significantly altered (Figure 4-5). Following 3 days of differentiation by PMA treatment, relative decreases in total cholesterol by 39% and 38%, cholesteryl esters by 38% and 40%, unesterified free cholesterol by 40% and 36%, and triglycerides by 15% and 28% were observed in LPL and EL suppressed macrophages, respectively. Those results indicated that the lipid uptake by macrophages was significantly depressed after the lipase suppression.

Various phospholipids on cell membrane can be the substrates of lipases, so we looked into the relative level of several important phospholipids and their metabolites after the lipase suppression. With LPL and EL suppression, the relative lysophosphatidylcholine (LPC) concentration was decreased by 15% and 20% respectively. Correspondingly, the relative level of phosphatidylcholine was increased by 29% and 39% for LPL and EL suppression respectively. Other phospholipids including phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) also tended to increase after LPL and EL suppression.

Figure 4-5. The effect of LPL or EL suppression on lipid composition in THP-1 macrophages. Cell lipids were extracted from lipase-suppressed and control cells following 3 days of PMA stimulation and analyzed by HPLC. Results are presented as the ratio of each lipid mass to total lipid mass (mean ± SEM, n=3). Total cholesterol (TC), cholesteryl ester (CE), free cholesterol (FC), triglycerides (TG), Free fatty acid (FFA), Cardiolipin (CL), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylserine (PS), Phosphatidylcholine (PC), and Lysophosphatidylcholine (LPC). (* p<0.05, ** p<0.01)
4.4.6 Microarray of Atherosclerosis Pathway Specific Genes

Microarray analysis of 110 atherosclerosis-specific genes was carried out on EL and LPL suppressed macrophages. The suppression of either LPL or EL in macrophages changed the gene expression (>1.0 or <-1 in fold change, or absent) of approximately 50 of the 110 probed genes contained within the microarray (Appendix 10). The top 10 upregulated and downregulated genes are listed in Table 4-1. Analysis of the microarray data indicated that several pro-inflammatory cytokines including IL1-β, IL-6, IL-8, and TNF-α were downregulated by 1.84, 2.47, 4.35, 3.72 -fold respectively for LPL suppression and by 0.96, 1.89, 1.15, 0.91 -fold respectively for EL suppression.

Expression of many of the genes involved in cell adhesion and extracellular matrix including CCL-2, CCL-20, CCL5, ICAM-1, fibronectin, integrin α2, β5, and β7 were diminished by varying degrees (from 0.84- to 5.06-fold), whereas the expression of integrin α5, αX, and β2 increased to varying extents (from 0.67- to 3.26 -fold). Analysis of the microarray data also indicated marked decreases in the expression of growth factors and anti-apoptotic genes (M-CSF, VEGF, BCL-2).

Interestingly, some genes were upregulated after the lipase suppression. TGF-β1, one of modulatory proteins to mitigate the inflammatory response, was upregulated by 3.11- and 1.43 -fold in both LPL and EL suppressed macrophages, respectively. Also, LDLR, SRA, and SR-BI were increased in LPL suppressed macrophages (3.17-, 0.44-, and 1.30 -fold, respectively) and EL suppressed cells (2.72-, 1.92-, and 1.44 -fold, respectively). In addition, CD36 was also upregulated in the presence of EL suppression (2.11 fold).

The expression of nuclear receptors and transcription factors was also altered in LPL and EL suppressed cells as seen with 2.35- and 1.56-fold decreases for PPAR-γ, and 7.08- and 7.01-fold increases for NF-κB.
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Annotation: Genes which are expressed in control but absent in lipase suppressed cells are not listed in the table.
4.5 Discussion

LPL and EL have been shown to have significant effects on lipid metabolism and to influence the development of lesions in mouse models of atherosclerosis. In the present study, we used a lentiviral-mediated RNA interference approach to investigate the effect of LPL and EL suppression in cytokine secretion and cellular lipid composition. To differing extents IL-1β, IL-6, IL-8, MCP-1, and TNF-α were found to be decreased in the presence of endogenous lipase suppression. Lipase suppression was also accompanied by decreases in the concentration of cellular cholesterol, cholesteryl ester, triglycerides and lyso-PC.

The present study indicated that lipase suppression caused the decreased expression of proinflammatory cytokines such as IL-1β, IL-6, MCP-1 and TNF-α, as well as a decreased production of lyso-PC. Decreased expression of pro-inflammatory cytokines in macrophages could be ascribed to diminished lyso-PC production secondary to lipase suppression. LPL and EL are capable of hydrolyzing phospholipids although EL has been shown to more readily hydrolyze phospholipids contained within synthetic substrates. The incubation of artificial phospholipid emulsion with LPL generated hydrolytic products with preference for lipolysis at the 1-acyl ester bond of phosphatidylcholine. Furthermore, phosphatidylcholine content on cell membranes was increased in LPL-deficient patients because of decreased hydrolysis of phospholipids by LPL. The hydrolysis of phosphatidylcholine liberates lyso-PC, which possesses numerous biological activities. For example, lyso-PC has been shown to induce the expression of MCP-1, IL-8 and RANTES by human vascular endothelial cells and smooth muscle cells, and promote the chemotaxis of monocytes, neutrophils, and lymphocytes. Lyso-PC promotes proinflammatory changes in monocytes through stimulating arachidonic acid release. IL-1β expression was also augmented in macrophages by lyso-PC which acts on G2A, a receptor for lyso-PC. Thus, it is possible that lipase suppression results in decreased hydrolysis of phospholipids and subsequent lyso-PC production, which results in decreased proinflammatory cytokine expression.

It is also possible that a reduction in the non-catalytic function of these lipases influences cytokine regulation. Previously, it has been demonstrated that lipases are capable of facilitating lipid uptake and accumulation by lipase-expressing target cells including macrophages.
observed in the present study, the relative amount of cholesterol and triglycerides were
decreased in lipase suppressed macrophages. Moreover, studies from our laboratory have
indicated decreased binding of native LDL and oxLDL following lipase suppression. We
speculate that intracellular lipids may be depleted following lipase suppression due to a reduced
uptake of extracellular lipid. Intracellular cholesterol is utilized for a multiplicity of cellular
functions, including the maintenance of cell membrane integrity, hormonal production, cytokine
production, and intracellular signaling molecules. Furthermore, intracellular lipids can influence
cytokines at the gene expression level. Lipid loading increases oxidative stress and, as a result,
IL-1β is rapidly induced in foam cells.\(^{30}\) Meanwhile, free cholesterol-loaded macrophages are
considered to be an abundant source of TNF-α, IL-6, and IL-8.\(^{31,32}\) Thus, a decreased
concentration of intracellular lipids following lipase suppression may also mediate decreases in
cytokine expression.

Oxidized lipoproteins have been well recognized as proatherogenic, due to their enhanced
receptor-mediated endocytosis by target cells, mostly macrophages in atherosclerotic lesion. In
our study, both mildly and extensively oxidized LDL consistently decreased IL-1β, IL-6, MCP-1,
and TNF-α throughout 24 to 72 hours after PMA stimulation. However, the role of oxidized
lipoproteins on cytokine expression is not consistent among several studies. Increased
expression of cytokines including IL-1β, IL-6, IL-10 and 12, TNF-α and -β, interferon gamma
(IFN-γ) was observed after treatment with oxLDL challenge.\(^{33,34}\) However, the treatment with
either mildly or extensively oxidized lipoproteins for 48 hours consistently decreases
proinflammatory cytokine expression in macrophages in the present study. Kim and their
colleagues have demonstrated that the production of proinflammatory cytokines including
inducible NOS and NO, MCP-1 and TNF-α, was suppressed in an oxidation-dependent manner,
when microglia, one of the differentiated microphages in brain, were treated with either 2,2-(E-
azobis(2-amidimopropane) dihydrochloride or copper sulfate prepared oxLDL.\(^{35}\) In addition, the
LPS-stimulated expression of IFN-γ, IL-12, IL-1beta, IL-6, were repressed when mouse
macrophages were preincubated or coincubated with extensively oxLDL.\(^{36-38}\)

The discrepancy of the effect of oxLDL on cytokine expression may be related to the extent of
oxidation of lipoproteins as well as the amount and treatment duration. In macrophages, the
stimulatory effects were only observed in mildly and moderately oxLDL with short time
incubations.\textsuperscript{39} Minimally oxLDL may elicit a proliferatory action on macrophages after short-time (4-6 hours) treatment which is diminished after 24 hours, whereas incubation with extensively oxLDL consistently suppressed cytokine expression regardless of the duration of the incubation period.\textsuperscript{40,41} Thus, prolonged exposure (>24 hours) of macrophages regardless of oxidation status of LDL is associated with an inhibitory effect on cytokines. Lipid loading may lessen the responsiveness of macrophages towards inflammatory stimuli, as cholesterol-laden macrophages have a depressed inducibility of TNF expression.\textsuperscript{42} Furthermore, incubation with oxLDL elicits oxidative stress, mitochondrial dysfunction, and lysosomal destabilization, and then promotes apoptosis in macrophages.\textsuperscript{43-45} As a consequence of cell apoptosis, the expression of most of genes including proinflammatory response genes are downregulated.

Both lipase suppression and oxidized LDL treatment decreased the production of selected proinflammatory cytokines in this study, their effects are additive as a greater inhibition on cytokine levels were observed. Also observed from the microarray data in the present study, lipase suppression appears to favor a decreased proliferative state as mitogenic and antiapoptotic genes were downregulated, which may further deteriorated the apoptotic process in oxidized LDL treated cells, therefore further depress the proinflammatory response. Moreover, the oxidized LDL treatment further decreased EL expression in macrophages transduced by shRNA lentivirus, which could magnify their inhibitory effects on proinflammatory cytokine production when combined.

Microarray analysis also indicated decreased expression levels of proinflammatory cytokines consistent with the measured cytokine concentrations. Although IL-8 protein levels did not appear to be appreciably altered by targeted LPL suppression, marked decreases in IL-8 mRNA levels were observed. It is possible that further regulation at post-translational or catabolic stages may have affected this relationship. In addition, we found that some key nuclear factors such as PPAR\textgamma and NF-\kappaB were markedly changed. It is already known that hydrolytic products of lipids and intracellular lipid contents are the key regulator of PPARs and NF-\kappaB. Coincubation of LPL with LDL or VLDL dramatically elevates PPAR\alpha activity in correspondence to free fatty acid release, as well, transgenic mice overexpressing LPL had increased peroxisome proliferation.\textsuperscript{46} In addition, marked PPAR\alpha activation is observed after LPL is administrated or systemically released by heparin in vivo.\textsuperscript{46} Endothelial lipase provides an alternative pathway for the FFA production which is further incorporated into cells and then
activates PPARα. Released arachidonic or linoleic acid can be the agonist for PPAR/RXR heterodimer in macrophages. Moreover, the cholesterol accumulation in cells elicits its conversion to oxysterol, the latter is a potent endogenous activator for LXR, the activation of LXR can also interact with PPAR and RXR to regulate gene expression. Since lipase suppression would be consistent with decreased free fatty acid liberation and intracellular lipid accumulation, decreased expression of PPARs would be expected. NF-κB activation could be associated with the decreased concentration of hydrolytic lipid products and PPAR inactivity. Consistent with this hypothesis are results indicating that linoleic acid released by lipases is capable of inhibiting NF-κB in macrophages and, as a result of a negative transcriptional regulation of NF-κB, PPAR activation abrogates NF-κB activation in macrophages.

Strikingly, microarray analysis of gene expression also unveiled the upregulated expression of lipoprotein receptors (SRA, LDLR) and decreased expression of mitogenic and anti-apoptotic genes such as a variety of growth factors and Bcl-2. It is well documented that a source of exogenous lipid is required to maintain cell growth and proliferation. Generation of the lipid second messenger phosphatidylinositol 3,4,5 triphosphate from phospholipids as well as ceramide derived from sphingomyelin triggers the progression of the cell cycle. In response to a decrease in intracellular lipids from lipase suppression, lipoprotein receptor expression may be upregulated, whereas NF-κB may be stimulated to increase the expression of lipoprotein receptors. Also, a lack of lipids may alter cell proliferation and trigger apoptosis.

In the present study, oxLDL treatment suppressed EL expression in macrophages, and thus the reduced cytokine concentrations may in part be mediated by decreased EL expression. The discrepancy of the reported effects of oxLDL on cytokine expression may be related to the extent of oxidation of lipoproteins as well as the amount and treatment duration.

In summary, we have observed that the suppression of LPL or EL decreased the expression of proinflammatory cytokines in human macrophages and reduced intracellular lipid concentration. Lipase suppression also altered several genes associated with atherosclerotic pathways revealing their multifaceted role in the development of atherosclerosis.
4.6 References:


Chapter 5. Atorvastatin Decreases Lipoprotein Lipase and Endothelial Lipase Expression in Human THP-1 Macrophages

5.1 Introduction and Rationale

Hydroxymethylglutaryl-CoA Reductase inhibitors (satins) effectively reduce cholesterol and apoB-containing lipoprotein levels by blocking the endogenous cholesterol synthesis through competitive inhibition of Hydroxymethylglutaryl-CoA Reductase (HMG-CoA) reductase and are extensively used in the treatment of hypercholesterolemia. Beyond their lipid-lowering effect, statins are also credited with pleiotropic bioactivities, including the improvement of endothelial dysfunction, alleviation of inflammation and oxidative stress, and stabilization of the atherosclerotic plaque. As a consequence, statins have been proven to be able to regress or at least retard the development of atherosclerosis, and significantly decrease the incidence of atherosclerosis-related cardiovascular events.

The non-lipid-lowering effects of statins are often attributed to their ability to influence signaling pathways, among which Rho proteins, liver X receptor (LXR), and nuclear factor kappa B (NF-κB) are most studied. Rho proteins, which are the small G proteins, play an important role in many aspects of cellular biology, require the prenylation before translocating to the cytoplasmic membrane to exert their effects. The intermediates for cholesterol synthesis, such as farnesyl pyrophosphates and geranyl pyrophosphates, can supply isoprenoids to prenylate the Rho proteins, so that the inhibition of cholesterol intermediates by statin administration will change the status of Rho activation accordingly. The oxysterols, one of the cholesterol derivatives, are the natural ligands for LXR, therefore, the level of oxysterols will changes in response to the cholesterol synthesis inhibition by statins. By far, the relationship between these signaling molecules and lipase expression has not been fully explored in macrophages.

As mentioned before, LPL and EL are intimately involved in the development of atherosclerosis, and in-vivo and in-vitro studies suggest that the macrophage expression of

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\textsuperscript{d} A version of this chapter has been submitted for publication. Qiu, G. and Hill, J.S. Atorvastatin Decreases Lipoprotein Lipase and Endothelial Lipase Expression in Human THP-1 Macrophages.
LPL and EL is proatherogenic.\textsuperscript{7-15} In views of anti-atherogenic character of statins and the importance of macrophages in atherosclerosis, there could be a potential link between statin treatment and lipase expression in macrophages, in other words, it is interesting to assess whether statin's anti-atherogenic effect is partially mediated by the modulation of lipase expression in macrophages.

Unfortunately, there is a relative paucity of knowledge regarding statin influence on lipase expression in macrophages. In clinical studies, the systemic administration of high-dose simvastatin (80mg daily) was accompanied with 49\% increase in preheparin plasma lipase activity and a 21\% increase in postheparin LPL activity.\textsuperscript{16} Moreover, atorvastatin and pravastatin elevated pre-heparin LPL mass in type II diabetics with hypercholesterolemia, and the magnitude of LPL increase by simvastatin in rabbit was even greater, a 72\% increase in LPL activity.\textsuperscript{17,18} Due to their ubiquitous distribution and enormous reserve, adipocytes may be the major source for the increase in plasma LPL following statin treatment. Consistent with this hypothesis, increased LPL expression and activity were reported in 3T3-L1 preadipocytes when treated with pitavastatin, simvastatin, and atorvastatin.\textsuperscript{19,20} However, neither the expression of LPL or EL has been explored in human macrophages following statin treatment.
5.2 Hypotheses and Specific Aims

5.2.1 hypotheses
The treatment of macrophages with atorvastatin will modulate the expression of lipoprotein lipase and endothelial lipase. Atorvastatin will also alter the intracellular signaling pathways (Rho proteins, LXR, and NF-κB) in macrophages. The altered signaling pathways could be related to the change of lipase expression secondary to atorvastatin treatment.

5.2.2 Specific Aims
1. To profile LPL and EL expression in macrophages after atorvastatin treatment
2. To examine the levels of intracellular signaling molecules (Rho, LXR, and NF-κB) after atorvastatin treatment
3. To investigate Rho effects on LPL and EL expression in the presence and absence of atorvastatin treatment
4. To investigate LXR effects on LPL and EL expression in the presence and absence of atorvastatin treatment
5. To investigate NF-κB effects on LPL and EL expression in macrophages
6. To explore the relationship of Rho and LXR with NF-κB.
5.3 Materials and Methods

5.3.1 Reagents
Purified atorvastatin was provided by Pfizer, mevalonate (MEV, M4667), farnesylpyrophosphate (FPP, F6892), and lysophosphatidic acid (LPA, L7260) were purchased from Sigma Aldrich to activate Rho protein. Clostridium botulinum exoenzyme C3 (Exo-C3, G-130) from BioMol was used to inhibit Rho protein. T01901317 (575310, CalBiochem) and 22(R)-hydroxycholesterol (HO-Chol, 89355, Cayman Chemical) were used as synthetic and natural activators of LXR, respectively. Cell membrane permeable SN50 was obtained from Calbiochem for NF-κB inhibition.

5.3.2 Cell Culture and Treatment
THP-1 monocytes (American Type Culture Collection) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% antibiotic-antimycotic (Invitrogen), 1 mM sodium pyruvate, and 1.5% sodium bicarbonate at 37°C, 95% air and 5% CO2, and used within 20 passages. Phorbol 12-myristate 13-acetate (PMA, P8139, Sigma Aldrich) was added at a final concentration of 100 nM for 48 hours to differentiate THP-1 monocytes into macrophages. Atorvastatin was then added to cultured macrophages at various concentrations. Macrophages were treated with Rho activators (200 μM of MEV, 20 μg/ml of FPP, 2.5 μM of LPA), Rho inhibitor (5 units/ml of Exo-C3), LXR activators (1 μM of T0901317 and 2.5 μM of HO-Chol), or NF-κB inhibitor SN50 (18μM) in the absence and presence of atorvastatin.

5.3.3 Rho Pull-Down Assay
A Rho activation assay kit (17-294, Upstate) was used to perform a Rho pull-down assay. Briefly, 2×10^7 macrophage cells under different treatment conditions were lysed in Mg^2+ lysis/wash (MLB) buffer on ice for 15 minutes after two washes of ice-cold tris-buffered saline. Cell lysates were then collected after removing cell debris by centrifugation. A GTPase protein binding domain/agarose slurry (30 μL) was added to cell lysates to bind Rho protein, and incubated for 45 minutes at 4°C with gentle agitation. Rho-bound agarose beads were pelleted by brief centrifugation (10 second, 14,000 ×g, 4°C), and washed with 1 ml of MLB 3 times, and
then resuspended in 40 μL of 2× Laemmli reducing sample buffer plus 2 μL of 1M dithiothreitol, and then boiled for 5 minutes prior to gel electrophoresis and Western blotting.

5.3.4 Immunoprecipitation of Liver X Receptor
THP-1 macrophages (5 × 10⁶) were lysed in 200 μL of RIPA lysis buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich). For liver X receptor (LXR) immunoprecipitation, cell lysates were co-incubated with μMACS Protein A MicroBeads (130-071-001, Miltenyi Biotec) and rabbit anti-LXR polyclonal antibody (NB 400-157, Novus Biologicals) on ice for 30 minutes, and then loaded onto μMACS columns on a magnet platform. LXR-bound microbeads were retained in columns. After three wash steps of RIPA buffer and one wash step of low-salt buffer (20mM Tris-HCl, pH 7.5), LXR was eluted in 50 μL of preheated 2 × Laemmli reducing sample buffer.

5.3.5 Nuclear Extraction and NF-κB ELISA
THP-1 monocytes (1×10⁷ cells) were cultured and treated as mentioned above, after 2 times wash with PBS, 0.5ml hypotonic buffer A (10mM HEPES, pH 7.9, 10mM KCl, 0.1 mM EDTA, 1% protease inhibitor cocktail (Sigma), 1mM DTT, 0.4% IGEPAL) was added into culture dish, and incubated for 10 minutes at room temperature, and then cells were disrupted by pipetting, and scraped into microtubes. Nuclei were pelleted at 4°C at 14,000 rpm for 3 minutes, and disrupted in 50ul buffer B (20mM HEPES, pH 7.9, 0.4M NaCl, 1mM EDTA, 10% glycerol, 1% protease inhibitor cocktail, 1mM DTT) at 4°C for 2 hours with vigorous shaking. Supernatants were collected after centrifugation at 4°C, 14,000 rpm for 5 minutes. NF-κB p50/p65 transcription factor colorimetric assay (SGT510, Chemicon) was used for the measurement of active NF-κB in nuclear extracts following the manufacturer’s instructions. TNF-α treated Hela whole cell extract and negative oligonucleotide capture probe were used for positive and negative controls respectively.

5.3.6 Trioleinase assay, RNA Extraction and Real-Time qRT-PCR, and Western Blot
The methods are described in chapter 2.
5.4 Results

5.4.1 Atorvastatin decreases LPL and EL mRNA and protein level in THP-1 macrophages

THP-1 derived macrophages were treated under variable concentrations of atorvastatin in a range from 2 μM to 40 μM. LPL activity as represented by the proportion of salt-sensitive and apoCII dependent activity displayed a dose-dependent decrease with an increase in atorvastatin concentration with a reduction of 55% at 10uM, 76% at 20uM, and 82% at 40uM (Figure 5-1A).

![Atorvastatin effect on LPL activity](image)

Figure 5-1. Lipase downregulation in THP-1 macrophages after atorvastatin treatment. THP-1 monocytes (5x10^5 cells) differentiated by 48 hour stimulation of PMA were treated with atorvastatin at various concentrations for 24 hours. Cell lysates and heparin-challenged culture medium were analyzed by Western blot and trioleinase activity assay. Panel A. Atorvastatin dose-dependently decreased trioleinase activity (n=4). Panel B. LPL and EL mRNA in macrophages at 20uM atorvastatin (n>8). Panel C. A dose-dependent decrease in protein mass for LPL and EL on Western blot (Representative of 3 independent blots). Statistical comparisons between statin-treated and untreated macrophages are indicated as: *** p<0.001.

An in-well phospholipase activity was performed to measure the total phospholipase activity following atorvastatin treatment which was decreased by 56% at 20uM of atorvastatin (data not shown). LPL mRNA was decreased by 56% and EL mRNA by 63% at the concentration of 20uM of atorvastatin (Figure 5-1B). The LPL Western blot was consistent with the trioleinase activity results exhibiting a decreased LPL mass in a dose-dependent behavior, with decreases of 23% at 10uM, 50% at 20uM, and 78% at 40uM (Figure 5-1C). Similarly, Western blot of EL
protein showed a dose-dependent decrease of EL mass, with a reduction of 54% at 10μM, 73% at 20μM, and 84% at 40μM (Figure 5-1C). Because the activity and mass of LPL and EL were not substantially reduced beyond 20μM and no cytotoxicity was observed at this dose (data not shown), an atorvastatin concentration of 20μM was used in subsequent experiments.

5.4.2 Atorvastatin decreases Rho, LXR and NF-κB activity

In an attempt to determine the underlying mechanism by which atorvastatin suppresses LPL and EL expression in macrophages, we explored the intracellular signaling pathway of small G protein Rho, liver X receptor (LXR), and nuclear factor κB (NF-κB). Activated Rho protein and LXR levels were dose-dependently decreased by atorvastatin where a 20 μM concentration was associated with >80% and >60% reduction, respectively (Figure 5-2A and 5-2B). Measurement of NF-κB indicated that 20 μM atorvastatin decreased NF-κB activity by >80% (Figure 5-2C).

![Figure 5-2](image)

Figure 5-2. The levels of Rho protein, LXR-α, and NF-κB in macrophages after atorvastatin treatment. Cell lysates and nuclear extracts were collected as described in text. Panel A. Rho protein on Western blotting (representative of 3 experiments). Panel B. LXR-α on Western blotting (representative of 2 experiments). Panel C. ELISA assay of NF-κB in nuclear extracts (n=3). Statistical comparison between statin-untreated and treated macrophages is indicated as: *** p<0.001.
5.4.3 Rho inactivation by atorvastatin does not mediate a decrease in EL or LPL expression in THP-1 macrophages

In order to assess whether decreased Rho activation was responsible for the observed reduction in LPL and EL expression following atorvastatin treatment, we used Rho activators (MEV, FPP, and LPA) in an attempt to recover LPL and EL levels. In the presence of atorvastatin, Rho activation by MEV partially salvaged LPL mRNA and activity although LPL protein was not significantly affected as assessed by Western blotting. FPP did not increase LPL mRNA, activity or protein mass, and LPA even further decreased LPL mRNA, activity and mass (Figure 5-3 A–C). To further assess the relationship of Rho with LPL expression, we treated macrophages with Rho activators and a Rho inhibitor (exoenzyme C3) in the absence of atorvastatin. Interestingly, Rho activation by MEV and FPP stimulation did not noticeably change LPL mRNA. However, LPA stimulation was associated with 35% decrease of LPL mRNA (Figure 5-3D). In parallel with mRNA expression, LPL mass in Western blot was not altered appreciably although LPA decreased LPL mass (Figure 5-3F). In addition, exoenzyme C3 did not change LPL mRNA or protein levels (Figure 5-3 D and F). Although statistically significant, the reduction of trioleinase activity by either Rho stimulation or inhibition after mevalonate, FPP, LPA, and exoenzyme C3 was modest (12%-19%), with a maximal reduction associated with LPA treatment (Figure 5-3E). The discrepancy between trioleinase activity and LPL mRNA and protein levels may be attributed to the lack of complete specificity of the trioleinase activity assay for LPL. Based on these observations, Rho activation, particularly stimulation by LPA, tended to decrease LPL expression and a causative relationship between Rho inactivation and LPL suppression after atorvastatin treatment could not be established.

As far as EL expression was concerned, MEV treatment partially rescued EL mRNA expression in the presence of atorvastatin (58% of control in comparison to 37% of control in atorvastatin, figure 4A), but no significant differences were observed in Western blot analysis (Figure 5-4B). FPP stimulation did not elevate EL mRNA and protein levels and LPA even further depressed EL mRNA and protein levels (figure 5-4 A and B). In the absence of atorvastatin, Rho activation by MEV, FPP, and LPA consistently decreased EL mRNA and protein levels whereas Rho inhibition by exoenzyme C3 did not change either EL mRNA or protein levels (figure 5-4 C and D). These results suggest that atorvastatin induced Rho inhibition was not responsible for the observed reduction in EL expression.
Figure 5-3. The effects of Rho activators and inhibitor on LPL expression in macrophages in the presence or absence of atorvastatin. LPL mRNA, trioleinase activity, and protein were evaluated after 24-hour treatment of macrophages with either Rho activators (MEV, FPP, and LPA) or inhibitor exoenzyme C3 in the presence (solid bars) or absence (clear bars) of 20uM atorvastatin. Panel A–C: LPL mRNA (n=8), trioleinase activity (n > or = 4), and Western blot (representative of 3 independent experiments) respectively after Rho activator treatment in the presence of 20uM atorvastatin. Panel D–F: LPL mRNA (n=8 for Rho activators and n=4 for exoenzyme C3), trioleinase activity (n > or = 4), and Western blot (representative of 3 independent experiments) respectively after the treatment of Rho activators and inhibitor in the absence of atorvastatin. (Statistical comparisons between statin and statin plus additional treatments are indicated as: **p<0.01, and ***p<0.001 for panel A and B; Statistical comparisons between the control (no statin treatment) and treatments are indicated as: * p<0.05, **p<0.01, and ***p<0.001 for panel D and E)
Figure 5-4. The effects of Rho activators and inhibitor on EL expression in macrophages in the presence or absence of atorvastatin. EL mRNA and protein were evaluated after 24-hour treatment of macrophages with either Rho activators (MEV, FPP, and LPA) or inhibitor exoenzyme C3 in the presence (solid bars) or absence (clear bars) of 20uM atorvastatin. Panel A and C: EL mRNA by real-time qRT-PCR after Rho activation or inhibition in the presence or absence of 20uM atorvastatin respectively (n=6). Panel B and D: Western blot of EL protein after Rho activation or inhibition in the presence or absence of 20uM atorvastatin respectively (representative of 3 independent Western blots). (Statistical comparisons between statin and statin plus additional treatments are indicated as: * p<0.05 and **p<0.01 for panel A; Statistical comparisons between the control (no statin treatment) and treatments are indicated as: ***p<0.001 for panel B)

5.4.4 LXR inhibition by atorvastatin mediates LPL, but not EL suppression
To investigate the potential role of LXR in LPL and EL expression we applied LXR agonists T0901317 and 22(S)-hydroxycholesterol to THP-1 macrophages in the presence and absence of atorvastatin. Strikingly, the inhibition of LPL mRNA, activity, and protein by atorvastatin was completely rescued by the synthetic LXR agonist T0901317 (130%, 119%, and 110% of control), and partially recovered by the natural agonist 22(S)-hydroxycholesterol (84%, 99%, and 90% of control) (Figure 5-5 A~C). Moreover, LXR activation by both natural and synthetic substrates (T0901317 and 22(R)-hydroxycholesterol) significantly increased LPL mRNA (47% and 19%), activity (44% and 34%), and mass (35% and 25%) in the absence of atorvastatin
These observations strongly support that atorvastatin-induced LPL suppression is mediated by LXR suppression.

By contrast, LXR activation was not able to rescue the suppressive effects of atorvastatin on EL expression. Treatment with the natural substrate 22R-hydroxycholesterol amplified the suppressive effect of atorvastatin, further depressing EL mRNA and protein to 2% and <10% of control levels, respectively (Figure 5-6 A and B). The synthetic substrate T0901317 did not have any appreciable effects in the presence of atorvastatin. In the absence of atorvastatin, LXR activation by T0901317 and 22R-hydroxycholesterol suppressed EL mRNA expression by 55% and 92%, respectively and EL protein by 54% and 50%, respectively (Figure 5-6 A and C).
Figure 5-6. The effects of LXR agonists on EL expression in macrophages in the presence or absence of atorvastatin. EL mRNA and protein were evaluated after 24-hour treatment of macrophages with LXR agonists (T0901317 and OH-Chol) in the presence (solid bars) or absence (clear bars) of 20uM atorvastatin. Panel A: EL mRNA after LXR activation in the absence and presence of 20uM atorvastatin (n=6). Panel B and C: EL Western blot after LXR activation in the absence and presence of 20uM atorvastatin respectively (a representative of 3 independent blots). (Statistical comparisons between the control and treatments are indicated as: ***p<0.001; Statistical comparisons between statin and statin plus treatments are indicated as: ††† p<0.001)

5.4.5 EL suppression by atorvastatin is mediated by NF-κB inhibition

To further address the mechanism by which atorvastatin reduces EL expression, we then explored the role of NF-κB. When macrophages were treated with SN50, a peptide inhibitor of NF-κB which inhibited NF-κB level by ~45% in our experiments, EL mRNA expression was reduced by 29%, with a corresponding 30% decrease in protein levels as observed in Western blots (Figure 5-7A and B). In view of the inhibitory effect of atorvastatin on NF-κB, we postulate that atorvastatin may mediate EL suppression through decreased NF-κB activity. NF-κB inhibition did not cause any noticeable changes in LPL mRNA (Figure 5-7C). In addition, the effects of Rho and LXR activation on NF-κB were also assessed. NF-κB activity was inhibited to a varying degree from 37% to 55% upon Rho activation by MEV, FPP, and LPA, whereas Rho inhibition by exoenzyme C3 did not change the NF-κB level (Figure 5-7D). Furthermore, LXR activation by T0901317 and 22(R)-hydroxycholesterol exerted the same effect as Rho activation on NF-κB activity with a 42% and 77% decrease, respectively (Figure 5-7D). These results may explain in part the previous observations that Rho and LXR activators decrease EL expression.
Figure 5-7. The effect of SN50 on EL expression and the effects of Rho and LXR activators on NF-κB activity in macrophages. PMA-differentiated macrophages \( (5 \times 10^5 \text{ cells}) \) were treated with 18uM SN50 for 48 hours, and then EL was evaluated by real-time qRT-PCR for mRNA (Panel A. \( n=4 \)) and Western blot for protein (Panel B. \( n=2 \)). Panel C: NF-κB levels after Rho and LXR activations (n=3). (Statistical comparisons between control and treatments are indicated as: *** \( p<0.001 \))
5.5 Discussion

The non-lipid lowering effects of statins have been studied in numerous cell models for a wide range of biologic functions. However, the effect of statins on lipase expression in macrophages has not been addressed. We demonstrate in the present study that LPL and EL expression in human macrophages were markedly reduced by atorvastatin.

As statins are competitive inhibitors of HMG-CoA reductase, the metabolism of a variety of downstream cholesterol intermediates is affected. In particular, diphosphates such as FPP and geranyl pyrophosphate have been shown to mediate the prenylation and subsequent activation of Rho proteins. Similarly, since cholesterol is a precursor to oxysterol, statins also decrease this known endogenous activator of LXR. To determine if either of these transcription factors were responsible for the observed suppression of LPL and EL in THP-1 macrophages we applied a series of known activators and inhibitors of these pathways.

We observed that activation of Rho proteins did not rescue the suppressive effects on lipase suppression induced by atorvastatin treatment and, in some cases, caused a further depression in lipase expression. However, mevalonate treatment was able to partially salvage LPL mRNA levels in the presence of atorvastatin. This observation may be explained by the relative position of mevalonate compared to other downstream Rho activators in the biosynthetic pathway which may be more specific for Rho activation. Furthermore, Rho inhibition by exoenzyme C3 did not disturb LPL or EL expression, suggesting that Rho is not the mediator for atorvastatin action on lipase expression in macrophages. It is possible that the suppressive effects of Rho activators on lipase expression may be mediated through the transcription factor Oct-1 which interacts with the common cis-acting octamer element 5'-ATTTGCAT-3' on target genes. The binding site for Oct-1 has been identified and characterized at position -46 base pairs in the proximal human LPL promoter. Once phosphorylated on the homeodomain, its DNA binding activity could be inhibited in vivo and in vitro. Patients with T->C substitution mutation in this region have low plasma LPL activity due to an inability of Oct-1 binding. Furthermore, adipocytes, when treated with TNF-α, have a reduced expression of LPL related to the dissociation of Oct-1-like molecule from the Oct-1 consensus sequence on the LPL gene. Rho activation may lead to the phosphorylation of Oct-1 and retard its translocation from the cytosol to the nucleus.
subsequently decreasing LPL transcription. The octamer sequence for Oct-1 binding is also found within the EL gene, but its functional relationship has not been investigated.

LXR activation using synthetic and natural agonists increased LPL mRNA and activity in the absence of atorvastatin and rescued LPL suppression in the presence of atorvastatin. Consistent with this observation, Zhang et al. reported that a functional DR4 LXR response element is present in the intron region between exons 1 and 2 in the LPL gene and that gene expression could be modified by oxysterol. Furthermore, mice fed a diet containing high cholesterol or an LXR-selective agonist exhibited a significant increase in LPL expression in liver and macrophages whereas a defective response of LPL expression was observed in LXR-deficient mice. Taken together, it appears that atorvastatin may mediate the suppression of LPL by decreasing LXR activation.

By contrast, LXR activation was associated with a decrease in EL expression in the presence or absence of atorvastatin. It has been shown previously that the synthetic LXR agonist T0901317 suppressed EL expression in human endothelial cells, while 22(R)-hydroxycholesterol had no effect. Moreover, another natural activator of LXR, 24(S)-hydroxycholesterol, resulted in EL down-regulation at mRNA and protein levels in brain capillary endothelial cells. An LXR response element has not been identified within the EL gene so the mechanism of the influence of LXR on gene expression remains unclear. In addition, the PPARγ agonist pioglitazone also reduces EL expression. Due to cross-talk between LXR and PPARs, the effect of EL inhibition by LXR agonists could be mediated by subsequent PPARγ activation.

Since LXR did not appear to be responsible for EL suppression following atorvastatin treatment, we then investigated NF-κB, another transcription factor associated with EL expression. It has been reported that binding sites for NF-κB were present in the 5' region of −1250 bps of the EL promoter and co-transfection experiments using a luciferase reporter gene with the EL promoter in NIH-3T3 cells demonstrated direct regulation by NF-κB. In the present study, we demonstrated in THP-1 macrophages that atorvastatin dramatically inactivates NF-κB. The inhibitory effect of statins on NF-κB has been observed with lovastatin and simvastatin in human vascular endothelial and smooth muscle cells. In the present study, inhibition of NF-κB by SN50 resulted in decreased EL mRNA and protein levels. Consistent with this observation, NF-κB activation by IL-1β and TNF-α was shown to induce the expression of EL.

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mRNA and protein in HUVEC cells whereas the NF-κB inhibitor SN50 attenuated this response. Further investigation revealed that the treatment of both Rho and LXR activators led to the marked reduction of NF-κB level, perhaps explaining in part why Rho and LXR activation caused EL suppression. By contrast, NF-κB appears to be less critical in the regulation of LPL as NF-κB inhibition by SN50 did not cause any change of LPL expression and a NF-κB binding site has not been identified in the promoter area of the LPL gene.

The data in the present study is summarized by indicating intracellular signaling pathways responsible for the regulation of lipase expression in THP-1 macrophages (Figure 5-8). It appears that the expression of LPL and EL are influenced by LXR and NF-κB, respectively whereas NF-κB is negatively regulated by Rho and LXR. Atorvastatin treatment decreases LPL and EL expression in THP-1 macrophages by reducing the formation of oxysterol (LXR ligand) and inhibiting NF-κB, respectively. The ability of atorvastatin to decrease LPL and EL expression in THP-1 macrophages may be an additional mechanism that offers protection from the development of atherosclerosis.
Figure 5-8. Schematic illustration of signaling pathways involved in LPL and EL expression in THP-1 macrophages. The LPL gene contains an LXR response element within the promoter region such that it is positively regulated by LXR. Statins inhibit cholesterol synthesis and decrease oxysterol production, and thus decrease LXR activation and subsequent LPL expression. A consensus sequence for NF-κB binding is located upstream to the EL gene and positively regulates EL expression and thus NF-κB suppression by statins leads to EL downregulation. Rho and LXR activators may also inhibit EL expression by inhibiting NF-κB.
5.6 References:


Chapter 6. General Discussion and Conclusion

6.1 Summary

The formation of macrophage-derived foam cells is due to an imbalance between lipid uptake and efflux, this is characteristic of atherosclerosis, during which a variety of proinflammatory cytokines are also implicated. LPL and EL have been found to be expressed in atherosclerotic lesions, especially in macrophages, but their roles in macrophage functions have not been fully investigated. In the present thesis, I have described the investigation of the effects of LPL and EL on cholesterol efflux, lipoprotein binding and uptake, and proinflammatory cytokine expression in macrophages, providing additional knowledge of the roles of these lipases in human macrophage function.

The expression of LPL and EL in macrophages promoted apoAI-mediated cholesterol efflux, to which both catalytic and non-catalytic (bridging function) activities of lipases contribute differentially (illustrated as 1 in figure 6-1). More specifically, the bridging function of EL was associated with an increase in cell surface apo A-I binding whereas the catalytic activity appeared to influence membrane lipid composition. Both effects possibly contribute to increased cholesterol efflux. Just as evidence has been reported describing an interaction between lipases (LPL and EL) and apoB, a direct interaction between lipases and apoAI may also occur at the macrophage cell surface. Although LPL is reported to prefer triglycerides as substrates, our experiments suggest that LPL possesses sufficient phospholipase activity to alter membrane lipid composition and consequent cholesterol release, and so does EL.

In contrast to the stimulatory effect on cholesterol efflux, I demonstrated for the first time that EL can mediate lipoprotein catabolism by macrophages (illustrated as 2 in figure 6-1). Native and oxidized LDL were bound to and internalized by macrophages at a significantly higher level when EL was overexpressed and at a lower level when EL expression was suppressed. The systemic expression of EL reduces apoB-containing lipoproteins in animal models; whereas in vitro, its facilitating role in LDL metabolism was only studied in CHO cells where an increased binding and internalization of native LDL secondary to EL expression was observed. The facilitating role of EL in LDL metabolism requires its bridging function as HSPG removal by heparinase completely abolished the EL-mediated effect on LDL/oxLDL binding/uptake by macrophages, consistent with a previous report in transfected CHO cells. In addition, the
The catalytic activity of EL seems to play some role in native LDL metabolism as a partial reduction of LDL uptake was detected after catalytic inhibition. Neither LRP nor CD36 appeared to be involved in lipase-facilitated LDL/oxLDL metabolism whereas the blockage of LDLR led to a moderate reduction of native LDL binding and uptake.

There is little if any information on the relationship between macrophage lipase expression and proinflammatory cytokine secretion. A positive association of LPL with TNF-α and PDGF production was reported in two studies. In the present thesis, I have described a positive correlation between lipase expression and proinflammatory cytokine secretion (illustrated as 3 in figure 6-1). When LPL and EL were suppressed, the levels of IL-1β, IL-6, MCP-1, and TNF-α were significantly reduced consistent with a reduced proinflammatory response as revealed by microarray results of atherosclerosis-specific genes. The attenuated proinflammatory response of macrophages following lipase suppression could be associated with a decreased production of lysophosphatidylcholine since it stimulates the production of a collection of proinflammatory cytokines. The maintenance of normal cellular functionality necessitates the optimization of cellular lipid levels. For example, cholesterol-loaded macrophages are considered to be an abundant source of TNF-α, IL-6, and IL-8, and, thus, decreased cellular lipid content secondary to lipase suppression may cause the decreased cytokine secretion by macrophages.

Although many non-lipid lowering effects of statins have been revealed in various cell models, and oral administration of statins can increase plasma LPL levels to promote lipoprotein clearance. The lipase regulation by statins and their relationship with signaling pathways in macrophages have not yet been studied. In the present thesis, I have described a dose-dependent inhibition of both LPL and EL by atorvastatin (illustrated as 4 in figure 6-1). Atorvastatin decreased Rho protein level, but neither LPL nor EL inhibition by atorvastatin was related to Rho inhibition since Rho activation reduced both LPL and EL expression. The levels of LXR and NF-κB were diminished by atorvastatin and were associated with LPL and EL suppression, respectively. This observation is opposite to the reported adipocyte response to statin treatments, the latter was suggested to be responsible for the increase of plasma LPL following statin regimens. These data reported in the macrophage provide new information on the non-lipid lowering effects of statins.
Figure 6-1. The Lipase Regulation by Atorvastatin and Their Effects on Cholesterol Efflux, Lipoprotein Binding/Uptake, and Proinflammatory Cytokine Expression in Macrophages
6.2 Which is the dominant effect of macrophage-derived lipases, pro-atherogenic or anti-atherogenic?

The stimulatory effect of lipases on cholesterol efflux confers an antiatherogenic ability on macrophages, however, the increased LDL/oxLDL binding and uptake by EL as evidenced in this study, combined with the well-established role of LPL in facilitating LDL/oxLDL metabolism in macrophages imply proatherogenic qualities. Moreover, the positive relationship between lipase expression and proinflammatory cytokine production supports a proatherogenic role as well. Therefore, the overall role of LPL and EL in macrophages and atherogenesis needs further study.

If lipases stimulate foam cell formation, it may indicate that the observed effects on lipoprotein metabolism may quantitatively be more relevant than any effects on cholesterol efflux in regard to the evaluation of their atherogenic potential. As for macrophage-derived LPL, many studies conducted in vitro and in vivo have provided evidence of its proatherogenic role. In vitro, the exogenous addition of LPL markedly increased intracellular cholesterol levels in mouse J774 macrophages. Also, macrophages isolated from growth-hormone deficient patients had increased expression of LPL, and internalized more proatherogenic lipoproteins and transformed into foam cells more readily. Furthermore, in both C57BL/6 and LDL-/- mice, the wild-type (LPL+/+) macrophages caused a more complex atherosclerotic lesion with higher content of macrophage-derived foam cells when compared to LPL knockout macrophages. Despite its potential to enhance cholesterol efflux, these studies indicate that the net effect of macrophage LPL is enhanced lipid accumulation.

EL is highly expressed by macrophages in atherosclerotic lesions; however, its relationship with foam cell formation thus far remains unclear. In this study, EL overexpression appears to stimulate lipid accumulation in macrophages as the content of total cholesterol and triglycerides increased significantly, suggesting the net effect of EL is to increase lipid deposition. Nevertheless, this in-vitro implication must be confirmed by in vivo studies in which macrophage EL is over-expressed.
6.3 **Future Direction**

A large quantity of information regarding lipase effects on macrophage biology has been generated throughout the present research endeavors. However, there are various uncertainties and speculations which will guide our future research endeavors.

1) Further studies of the structure-function relationships of EL should provide mechanistic insights into its specific functional roles. Several critical structures in other lipases are conserved in EL; however, their roles have not been characterized.

   a. Lipoprotein binding domain. Compared to LPL whose lipoprotein binding domain has been mapped to a cluster of hydrophobic tryptophan residues (Trp390-Trp393-Trp394) in the C-terminus, several tryptophan residues in the EL C-terminal domain have been identified at positions 398, 406, and 410. Their roles in the interaction of EL with lipoproteins including HDL and LDL should be characterized. Site-directed mutagenesis followed by in vitro binding/uptake or in vivo lipoprotein metabolism assays could be useful tools.

   b. Heparin binding domain. Several heparin-binding domains have been proposed but not confirmed yet. They are Arg327-Lys329-Arg330-Lys333, Arg312-Lys313-Arg315, Gly184-Arg188, and Lys352-Arg450-Lys452-Lys459. A similar site-directed mutagenesis strategy as described above could be chosen.

   c. Apolipoprotein binding domain. The direct interaction between EL and apolipoproteins (apoAI, apoB) has not been documented. Therefore, the protein fragments of EL could be used on protein microarray/chip to identify the responsible domain(s) for the binding of apoAI and apoB. Similarly, the domain(s) in apoAI or apoB responsible for EL binding could also be identified. Subsequently, the functional effects of mutated domains could be assessed in cell or animal models.

2) With the elucidation of structure-function relationships, cell models with the overexpression of enzymatically inactive or heparin binding-defective EL could be generated to further confirm the findings described in this thesis. The generation of either enzymatically inactive or heparin binding-defective enzyme could more clearly define the catalytic and non-catalytic roles of EL.
3) Ideally, results obtained from vitro models should be tested in an in vivo setting. Thus, the in vivo study of animal models with macrophage EL knockout or overexpression will be critical in evaluating the atherogenic potential of macrophage-derived EL. In these models, a wild-type, enzymatically inactive, or heparin binding defective EL could be introduced into macrophages by bone marrow reconstitution via either fetal liver cell grafting or bone marrow transplantation. Through this strategy, the effect of macrophage EL and its relative catalytic and non-catalytic roles in lipid metabolism and atherosclerotic lesion development could be assessed.
6.4 Reference


Appendices

Appendix 1. Sequencing results of pSHAG-scramble-shRNA
Appendix 2. Sequencing results of pSHAG-LPL-shRNA
Appendix 3. Sequencing results of pSHAG-EL-shRNA
Appendix 4. Sequencing results of pWPI-EL
Appendix 5. Sequencing results of pcDNA5/FRT-EL
Appendix 6. Time-course curve of apoAI-mediated cholesterol efflux in macrophages
Appendix 7. Cytokine expression in lipase-suppressed macrophages
Appendix 8. Cytokine expression in mildly-oxLDL treated macrophages with or without lipase suppression
Appendix 9. Cytokine expression in extensively-oxLDL treated macrophages with or without lipase suppression
Appendix 10. Microarray of atherosclerosis-related genes in lipase suppressed macrophages
Appendix 4. DNA sequence of pWPI vector containing EL cDNA sequence
THP-1 macrophages prelabeled with $^3$H-cholesterol were incubated with 10µg/ml apoAI for various times. Percentage of medium $^3$H-cholesterol to total $^3$H-cholesterol (medium+cellular) was calculated to represent cholesterol efflux rate.
Appendix 7. Cytokine expression in THP-1 derived macrophages with LPL and EL suppression in the absence of oxidized LDL treatment

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Time (Hours after PMA)</th>
<th>Control</th>
<th>LPL Suppression</th>
<th>EL Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β (pg/ml)</td>
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<td>270±30</td>
<td>75.3±0.6*</td>
<td>62.0±0.4*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>300±40</td>
<td>178±4*</td>
<td>100±20*</td>
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<td></td>
<td>72</td>
<td>240±20</td>
<td>160±20*</td>
<td>140±50</td>
</tr>
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<td>670±20†</td>
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</tr>
<tr>
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<td>48</td>
<td>972±4</td>
<td>950±20</td>
<td>868±8†</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>1020±30</td>
<td>960±10</td>
<td>930±20*</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
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<td>70±10</td>
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<td>48</td>
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<td>117±4*</td>
<td>79±8*</td>
</tr>
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<td></td>
<td>72</td>
<td>1100±100</td>
<td>360±60*</td>
<td>300±20*</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
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<td>75±4*</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>350±40</td>
<td>210±20*</td>
<td>120±20*</td>
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<tr>
<td></td>
<td>72</td>
<td>430±50</td>
<td>170±20*</td>
<td>150±20*</td>
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*Comparison of control with lipase suppressed condition, P<0.05
†Comparison of control with lipase suppressed condition, P<0.01
Appendix 8. Cytokine expression in THP-1 derived macrophages after lipase suppression in the presence of mildly oxidized LDL treatment (oxLDL was added 24 hours before PMA).

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Time (Hours after PMA)</th>
<th>Control</th>
<th>LPL Suppression</th>
<th>EL Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β (pg/ml)</td>
<td>24</td>
<td>180±10</td>
<td>140±30</td>
<td>49±5†</td>
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<td></td>
<td>48</td>
<td>216±5</td>
<td>120±4†</td>
<td>80±20*</td>
</tr>
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<td>72</td>
<td>150±40</td>
<td>130±10</td>
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</tr>
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<td>980±10</td>
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<td></td>
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<td>TNF-α (pg/ml)</td>
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<td>72</td>
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<td>130±20*</td>
<td>66±4*</td>
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*Comparison of control with lipase suppressed condition, P<0.05
†Comparison of control with lipase suppressed condition, P<0.01
Appendix 9. Cytokine expression in THP-1 derived macrophages after lipase suppression in the presence of extensively oxidized LDL treatment. (oxLDL was added 24 hours before PMA).

<table>
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<th>Control (pg/ml)</th>
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<th>EL Suppression (pg/ml)</th>
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<tr>
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<td></td>
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<td>72</td>
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*Comparison of control with lipase suppressed condition, P<0.05
†Comparison of control with lipase suppressed condition, P<0.01
Appendix 10. The microarray data of atherosclerosis-specific pathway in lipase-suppressed macrophages

<table>
<thead>
<tr>
<th>Position</th>
<th>Gene Name</th>
<th>Function of Gene</th>
<th>LPL Suppression Fold Change</th>
<th>EL Suppression Fold Change</th>
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</thead>
<tbody>
<tr>
<td>1</td>
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<td>Production of angiotensin II</td>
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<td>Absent</td>
</tr>
<tr>
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<td>Adipose differentiation-related protein</td>
<td>Lipid particle</td>
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<tr>
<td>3</td>
<td>BCL1-associated X protein</td>
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<td>Absent</td>
</tr>
<tr>
<td>4</td>
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<td>Regulation of cell growth, anti-apoptosis</td>
<td>Absent</td>
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<tr>
<td>5</td>
<td>BCL2-related protein A1</td>
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</tr>
<tr>
<td>6</td>
<td>BCL2-like 1</td>
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<tr>
<td>7</td>
<td>BH3 interaction domain/BID</td>
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<td>2.47</td>
</tr>
<tr>
<td>8</td>
<td>Chemokine (C-C motif) ligand 2/CCL2</td>
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<td>Absent</td>
</tr>
<tr>
<td>9</td>
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Annotation: Genes indicated “absent” are expressed in control macrophages, but not detected (signal intensity less than blank control) in lipase suppressed macrophages.