Fatty acids liberated from lipoproteins by lipoprotein lipase negatively influences cholesterol efflux from THP-1 macrophages

by

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Abstract

Lipoprotein lipase (LPL) is an extracellular lipase that primarily hydrolyses triglycerides within circulating lipoproteins. Macrophage LPL contributes to atherogenesis, but the mechanisms behind it are poorly understood. I hypothesized that the free fatty acid (FFA) component of the products of lipoprotein hydrolysis generated by LPL promotes atherogenesis by inhibiting the cholesterol efflux ability by macrophages. To test my hypothesis, THP-1 macrophages were incubated overnight with lipoprotein hydrolysis products generated by LPL. Results showed that the hydrolysis products negatively modulated the transcripts encoding nuclear receptors, cholesterol transporters, and enzymes involved in FFA synthesis. A mixture of only purified FFA that matches those liberated by LPL yielded comparable results to those for lipoprotein hydrolysis products. Furthermore, the FFA mixture significantly attenuated apolipoprotein A-I-mediated cholesterol efflux. Overall, these data show that lipoprotein hydrolysis products generated by LPL may promote atherogenesis by inhibiting cholesterol efflux, which partially explains the pro-atherogenic role of macrophage LPL.

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List of Abbreviations

A/A	Antibiotic/antimycotic
ABCA1	Adenosine triphosphate-binding cassette transporter, sub-family A, member 1
ABCG1	Adenosine triphosphate-binding cassette transporter, sub-family G, member 1
ACAT	Acyl-coenzyme A:cholesterol acyltransferase
ACCA	Acetyl-coenzyme A carboxylase A
ANOVA	Analysis of variance
Apo	Apolipoprotein
Asn	Asparagine
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CM	Chylomicron
DMEM	Dulbecco's Modified Eagle Medium
DR4	Direct repeat 4
EL	Endothelial lipase

ESI-MS	Electrospray ionization-mass spectrometry
FAF-BSA	Fatty acid free bovine serum albumin
FASN	Fatty acid synthase
FFA	Free fatty acid
GC-MS	Gas chromatography-mass spectrometry
Gly	Glycine
GPIHBP1	Glycosylphosphatidylinositol anchored high-density lipoprotein binding protein 1
HDL	High-density lipoprotein
HL	Hepatic lipase
HSPG	Heparan sulfate proteoglycan
IDL	Intermediate-density lipoprotein
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low-density lipoprotein
LCT	Long-chain triglyceride
LPL	Lipoprotein lipase
LXR-α	Liver X receptor alpha
LXRE	Liver X receptor response element
MCT	Medium-chain triglyceride
mRNA	Messenger RNA

nSREBP	Nuclear sterol regulatory element binding protein
ox-LDL	Oxidized low-density lipoprotein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PKC	Protein kinase C
PL	Phospholipid
PMA	Phorbol 12-myristate-13-acetate
PPAR-α	Peroxisome proliferator activated receptor alpha
PPAR-γ	Peroxisome proliferator activated receptor gamma
PPRE	Peroxisome proliferator response element
PtdCho	Phosphatidylcholine
RCT	Reverse cholesterol transport
RXR	Retinoid X receptor
RPMI	Roswell Park Memorial Institute
SCD-1	Stearoyl-coenzyme A desaturase-1
SD	Standard deviation
Ser	Serine
Sp	Specificity protein
SR-BI	Scavenger receptor class B member 1
SREBP	Sterol regulatory element binding protein

TG	Triglyceride
THL	Tetrahydrolipstatin
TNF-α	Tumour necrosis factor alpha
UTR	Untranslated region
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cell

Chapter 1: Introduction

1.1 Lipoproteins

Lipoproteins are a complex assembly consisting of lipid and protein, allowing lipids to be transported within the circulation for delivery to different tissues (1). Lipoproteins are segregated into five major classes, categorized by density. Each class has a distinct lipid and protein composition, and each plays specific roles in lipid metabolism. These five classes of lipoproteins, in order of increasing density, are chylomicrons (CM), very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) (1). Lipoproteins share the same general structure, including a hydrophobic core and a surface monolayer with embedded apolipoproteins (apo), which are required for solubilizing the lipid and for receptor recognition (1). Specifically, the hydrophobic core consists of triglycerides (TG) and cholesteryl esters (CE), while the surface monolayer consists of amphipathic lipids, including phospholipids (PL) and cholesterol (1).

In the intestine, the dietary ingested TG and PL are hydrolysed by pancreatic lipase, generating free fatty acids (FFA). The generated FFAs subsequently enter the intestinal absorptive cells and are subsequently reassembled into TG and/or PL, which are then further packaged into CM along with cholesterol and CE (1). Following this, the newly synthesized CM is secreted into the lacteals and eventually enters the vascular circulation.

Within the circulation, the CMs are captured by lipoprotein lipase (LPL) in different tissues, and the hydrolysis of the TG components subsequently follows. As a result, the CM becomes a smaller and denser particle, that is known as a CM remnant. The CM remnant can be rapidly taken up by the liver, where its protein and lipid cargo can be used for intracellular functions, including the synthesis of VLDL (Figure 1) (2, 3).

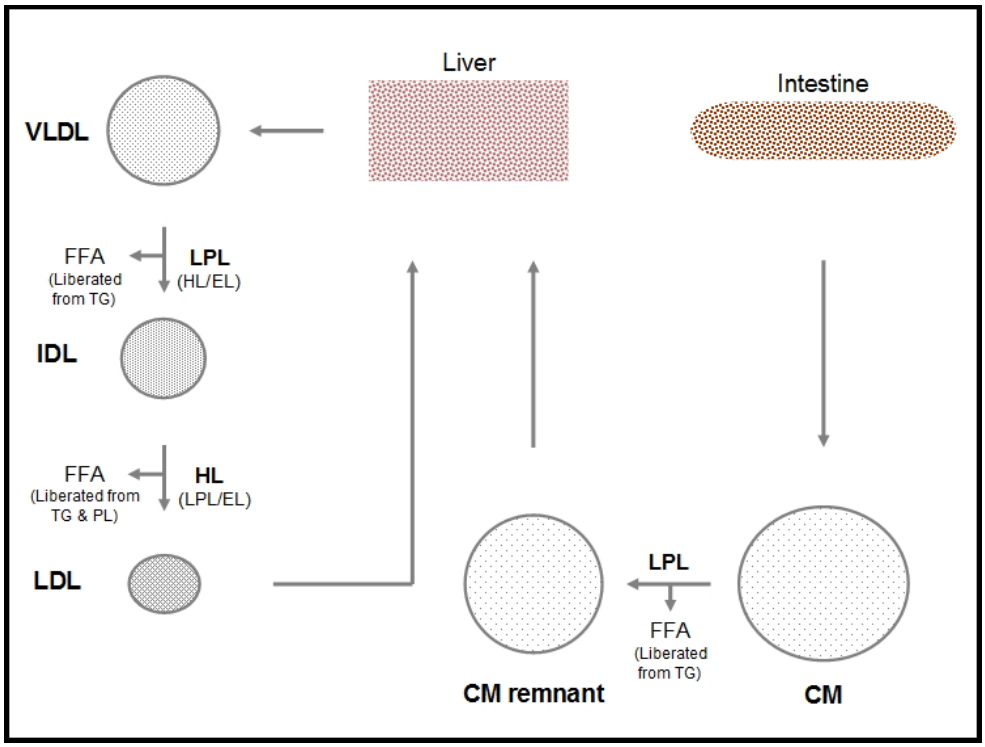
Similarly to CM, newly synthesized VLDL is secreted from the liver into the plasma, and is hydrolysed by LPL within the circulation. The hydrolysis of its TG component leads to an increase of lipoprotein density, thus generating IDL. IDL continues to travel through the circulation, and the TG and PL components are further hydrolysed by both hepatic lipase (HL) and LPL. The hydrolysis of the IDL TG and PL result in the IDL to become a cholesterol-rich lipoprotein, which is termed LDL (4). LDL functions in the delivery of cholesterol to cells. When a cell requires cholesterol for constructing its membrane or for synthesizing steroid hormones, circulating LDL will be recognized and endocytosed by the cellular LDL receptor. In addition, the excess LDL in the bloodstream will be eliminated by the liver via LDL receptor-mediated endocytosis to avoid high circulating levels of LDL (Figure 1) (1).

Different from the other 4 classes of lipoproteins, which function in delivering lipid to the peripheral cells, HDL is responsible for transporting excess cellular cholesterol to the liver for excretion into bile by the process of reverse cholesterol transport (RCT) (5, 6). Briefly, within the circulation, HDL precursors (a complex of

Figure 1: The metabolism of chylomicrons and VLDL

Chylomicrons (CM) are produced in the intestine and are ultimately delivered into the bloodstream. The circulating CM triglycerides (TG) are hydrolysed at different tissues by lipoprotein lipase (LPL); the hydrolysis liberates free fatty acids (FFA) and generates a cholesterol rich CM remnant. The CM remnant can be rapidly taken up by the liver, where its protein and lipid cargo can be used for various purposes, including the synthesis of very low-density lipoprotein (VLDL). Once synthesized by liver, VLDL is secreted into the circulation, where its TG and phospholipids (PL) are hydrolysed by LPL, hepatic lipase (HL), and endothelial lipase (EL). These actions increase the density of the VLDL into an intermediate-density lipoprotein (IDL) and low density lipoprotein (LDL). Lastly, excess circulating LDL can be removed by many tissues, including the liver.

Figure 1


Liver
Intestine
VLDL
FFA
(Liberated
from TG)
LPL
(HL/EL)
IDL
FFA
(Liberated from
TG & PL)
HL
(LPL/EL)
LDL
LPL
FFA
(Liberated
from TG)
CM remnant
CM

apoA-I and PL) continually obtain cholesterol that is effluxed from the peripheral cells to form a mature HDL particle, which is eventually eliminated from the circulation by the liver (Figure 2) (for more details see *section 1.4*).

1.2 Lipoprotein lipase and the *sn*-1 lipase subfamily

1.2.1 Overview of the *sn*-1 lipase subfamily

Lipases are water-soluble enzymes that function to liberate FFA from lipids by hydrolysing ester bonds. The *sn*-1 lipase subfamily specifically functions to hydrolyse the ester bond at the *sn*-1 position of circulating TG and PL (Figure 3). This subfamily includes LPL, HL and endothelial lipase (EL), which are thought to share structural homology. In addition to their catalytic function, LPL, HL, and EL also exhibit a non-catalytic function by binding to cell surface proteoglycans, which acts to facilitate the uptake of lipoproteins by cells by bringing the lipoproteins in close proximity to cell surface receptors. The non-catalytic function of these lipases is thought to contribute to lipoprotein metabolism independently of the catalytic function (7-11).

Previous studies have implicated LPL, HL, and EL in the modulation of atherosclerosis by both their catalytic and non-catalytic functions. LPL appears to exhibit an anti-atherogenic function; however, its expression in macrophages is tied with an increased risk of atherosclerosis (for more details see *section 1.3.2*) (12-17). The role of HL in atherosclerosis is unclear: hepatic HL has been suggested to play an

Figure 2: Reverse cholesterol transport

Excess non-hepatic cellular cholesterol and phospholipid is transferred to extracellular apolipoprotein A-I (apoA-I) by the cholesterol transporter adenosine triphosphate-binding cassette transporter, sub-family A, member 1 (ABCA1) to form nascent HDL, which can continue to accept cellular cholesterol via the cholesterol transporters adenosine triphosphate-binding cassette transporter, sub-family G, member 1 (ABCG1) and scavenger receptor class B member 1 (SR-BI). The newly acquired cholesterol can be esterified to cholesteryl esters (CE) by lecithin:cholesterol acyltransferase (LCAT). Through this process, the nascent HDL becomes a large mature HDL particle. In humans, the cholesteryl ester transfer protein (CETP) can move some of the HDL CE to triglyceride (TG)-rich lipoprotein particles in exchange for TG, thus allowing some of the CE to be removed from the circulation via very low-density lipoprotein (VLDL) or low-density lipoprotein (LDL) receptors. While on the way back to the liver, HDL is hydrolysed by hepatic lipase (HL) and endothelial lipase (EL), leading to the liberation of CE from HDL at the liver. As a result, the released CE will be taken up by the liver via the dual-directional cholesterol transporter SR-BI, while the liberated apoA-I is available to restart the RCT process.

Figure 2

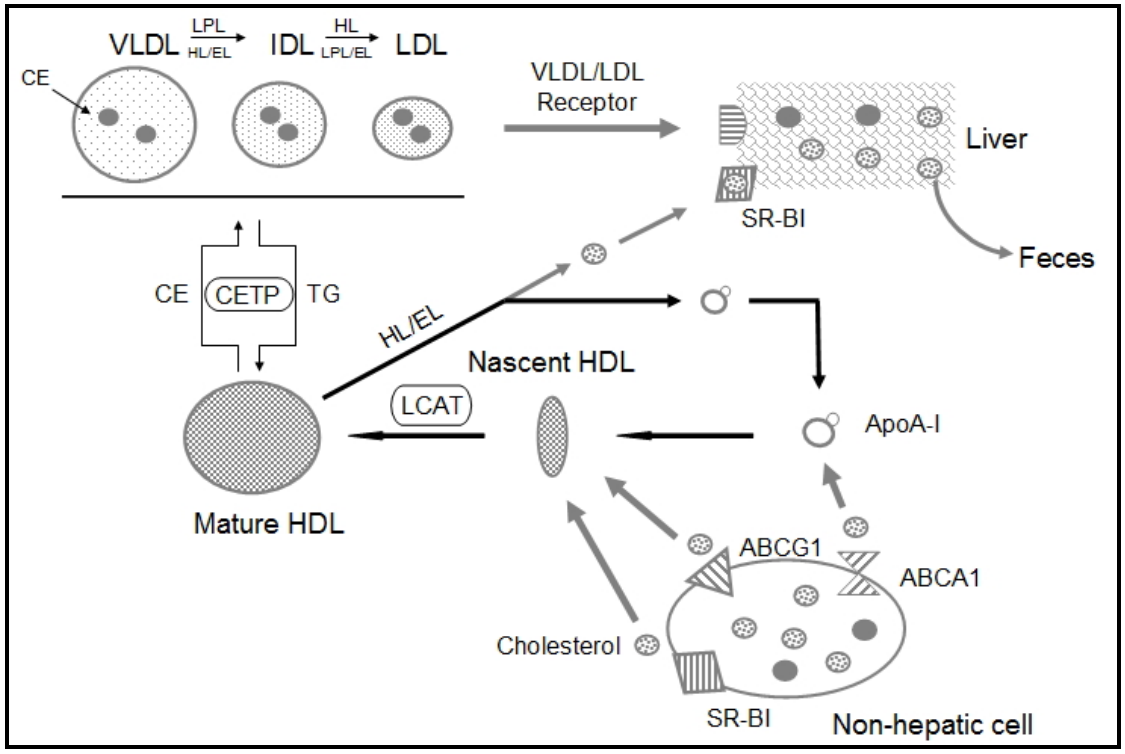
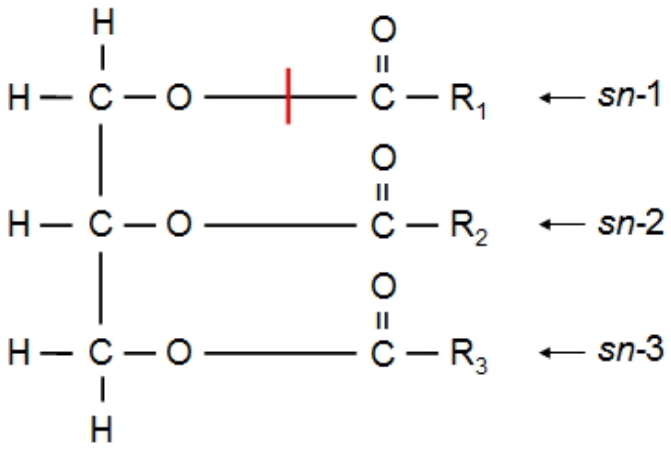

VLDL
LPL
HL/EL
IDL
HL
LPL/EL
LDL
CE
VLDL/LDL
Receptor
Liver
SR-BI
Feces
CE
CETP
TG
HL/EL
Nascent HDL
LCAT
ApoA-I
Mature HDL
ABCG1
ABCA1
Cholesterol
SR-BI
Non-hepatic cell

Figure 3: Triglyceride and phospholipid structure and the site of action of *sn*-1 lipase subfamily

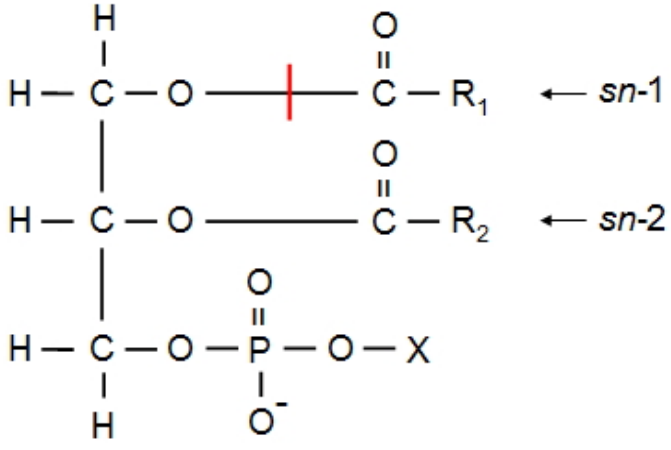
A: The structure of triglyceride. B: The structure of phospholipid. The red vertical line where shown indicates the site of hydrolysis by *sn*-1 lipase subfamily members.

Figure 3

A


H
O
H — C — O — C — R1
sn-1
O
H — C — O — C — R2
sn-2
O
H — C — O — C — R3
sn-3
H

B


H
O
H — C — O — C — R1
sn-1
O
H — C — O — C — R2
sn-2
O
H — C — O — P — O — X
O-
H

anti-atherogenic role by facilitating the elimination of pro-atherogenic TG-rich lipoproteins from the circulation (18-23); however, macrophage HL has been shown to increase aortic lesion areas, which suggests a pro-atherogenic role (23). Experimental results from EL knockout and overexpression studies suggest that EL is more likely to exhibit a pro-atherogenic property. Specifically, i) the transgenic expression of human EL in mice leads to a reduced level of circulating HDL, due to a high HDL catabolism rate in the liver and kidneys (24-27); ii) the expression level for EL positively correlates with the development of atherosclerosis (28, 29); and iii) EL-knockout mice have an increased HDL level and decreased atherosclerotic lesion area (27, 29-31). However, the exact role of EL in atherogenesis is currently uncertain.

1.2.2 The tissue expression profile and physiological action sites of the *sn*-1 lipase subfamily members

LPL, HL, and EL are commonly expressed in monocyte-derived macrophages and in hepatocytes (26, 32-36). Additionally, LPL is specifically expressed in the heart, skeletal muscle, adipose tissue, spleen, mammary glands, and lung (36-38). HL activity has been isolated from adrenal tissue and ovaries (39-42). The expression of EL has been identified in placenta, thyroid, lung, and kidney (26, 34).

Non-macrophage LPL and HL are first synthesized by parenchymal cells, where they are then translocated to the luminal surface of the capillary endothelium to bind with

cell surface proteoglycans (37, 38, 43-50). Here, they are exposed to the bloodstream to access the lipoproteins within the circulation. Non-macrophage EL is synthesized by arterial/venous endothelial cells and binds directly to the luminal surface of the capillary endothelium (26, 34).

1.2.3 The structure of the *sn*-1 lipase subfamily

LPL, HL, and EL exhibit at least a 40% amino acid sequence identity with each other (7). Furthermore, multiple conserved amino acid sequences have been identified, which include a catalytic triad of serine (Ser), aspartic acid (Asp), and histidine (His), and the lipase consensus sequence glycine (Gly)-x-Ser-x-Gly (where x represents any amino acid) (51, 52). Although the crystal structures for LPL, HL, and EL have not been elucidated, a putative model of the tertiary structures for LPL and HL were previously reported based on the three-dimensional structure of the related lipase pancreatic lipase (53, 54). The putative tertiary structures of LPL and HL include two major structural domains: an amino-terminal globular domain, and a carboxyl-terminal β-barrel domain. The amino-terminal globular domain has been identified to contain the Ser-Asp-His catalytic triad and a 'lid' domain, which are both important for the catalytic activity of the lipase; the catalytic triad is covered by the lid domain. As a result, the substrate only has access to the catalytic site when the lid domain moves away from the catalytic triad. Thus, the lid domain plays a partial role in determining the substrate specificity of the lipase (55, 56).

1.2.4 The substrate preference of the *sn*-1 lipase subfamily and its role in lipoprotein metabolism

LPL, HL, and EL exhibit different relative affinities to diverse lipid substrates. Specifically, LPL shows a high TG lipase activity and low phospholipase activity, whereas EL exhibits a dominant preference for PL; HL has an equal substrate preference for TG and PL (7, 57, 58). Due to the differences in substrate specificities, LPL, HL, and EL differ widely in their roles within lipoprotein metabolism. Specifically, LPL primarily hydrolyses TG rich lipoproteins, including CM, VLDL, and LDL; HL hydrolyses all classes of lipoproteins, while EL is mainly responsible for catalyzing the hydrolysis of HDL (35, 57, 59, 60).

1.2.5 The synthesis, processing, and secretion of LPL

Similar to any other secretory protein, the gene for LPL is first transcribed to messenger RNA (mRNA) in the nucleus. The mRNA is then further transported to the free cytosolic ribosome, where a signal sequence is synthesized. The signal sequence functions to assist the transport of the mRNA-ribosome complex to the rough endoplasmic reticulum (ER). Once the mRNA-ribosome complex binds to the rough ER, the signal sequence is removed and the translation of LPL mRNA is restored, while the newly synthesized polypeptide enters the ER lumen (61).

Within the ER, LPL is *N*-glycosylated with a high mannose content (rendering it

sensitive to endoglycosidase H cleavage) at asparagines 43 and 359 of full-length LPL (61-64). Next, glucose residues are trimmed from the glycosylation chains by glucosidase; this step is deemed to be critical for LPL function, as it induces partial catalytic activity (65-67). In addition, the existence of LPL activity in the ER suggests that the trimming of glucose residues allows for the dimerization of LPL (61, 66, 68-70). Lastly, the partially active LPL is transferred to the Golgi apparatus, where the *N*-linked oligosaccharide chains are further processed into more complex branched chains that become insensitive to endoglycosidase H cleavage (61). The fully processed LPL in the *trans*-Golgi network is then delivered to secretory vesicles. However, not all of the newly synthesized LPL will be secreted, as some is delivered to lysosomes for degradation (37, 61). A yet to be identified sorting process is believed to exist in the *trans*-Golgi network, which is responsible for the degradation of excess LPL (37, 61).

After being secreted from the parenchymal cell, LPL will immediately bind to the surface of the parenchymal cell through highly negatively charged, membrane bound chains of heparan sulfate proteoglycans (HSPG). Afterward, the LPL is displaced from the parenchymal cell surface. It crosses the interstitial space, and is finally transported to its physiological action site (37, 61). To date, few pathways explaining how LPL crosses the endothelial monolayer to reach the circulation have been identified. Nonspecific transport of LPL is achieved by either cellular vesicular transit or paracellular routes,

while specific transport has been identified that needs the assistance of both HSPG and VLDL receptors (60, 71-74). Furthermore, Davies *et al.* (75) have recently identified that glycosylphosphatidylinositol anchored high-density lipoprotein binding protein 1 (GPIHBP1) functions to transport LPL across endothelial cells, and a deficiency of GPIHBP1 in mice results in an incorrect LPL localization.

1.2.6 The Regulation of LPL

1.2.6.1 Transcriptional regulation

The transcriptional regulation of LPL is achieved through the interaction between transcription factors and *cis*-acting sequences, which are present in the regulatory regions of the LPL gene (37, 61). Previous studies have reported multiple mechanisms underlying the transcriptional regulation of LPL by metabolites and certain drugs. Firstly, in liver, adipose tissue, and macrophages, the transcript of LPL has been shown to be modulated through the binding of the peroxisome proliferator activated receptor (PPAR)-α, PPAR-γ, and 9-*cis* retinoic acid receptor to the peroxisome proliferator response element (PPRE) in response to fibrates, fatty acids, glucose, and thiazolidinediones (76-79). Secondly, in adipocytes, sterols induce modulation of LPL gene transcripts; this is partially mediated through the sterol regulatory element binding protein (SREBP) (80). Thirdly, Zhang *et al.* (81) previously showed that a high cholesterol diet could result in a selective expression of LPL in the liver and macrophages via the binding of liver X receptor/retinoid X receptor

(LXR/RXR) to its response element, which is located between exon 1 and 2 of the LPL gene. In addition, Hughes and colleagues (82) have shown that interferon-γ acts to decrease the DNA binding activity of the transcription factor specificity protein (Sp) Sp1, as well as the stability of Sp3. This further results in a decreased binding of Sp1 and Sp3 to the LPL promoter, and eventually leads to suppression of the transcript for LPL. Lastly, Tanuma *et al.* (83) showed that the transcripts of LPL could be silenced by the binding of an unknown LPL silencer protein (with a mass between 54 and 63 kDa) to the silencing element, which is located in the promoter at positions -225 to -81.

1.2.6.2 Translational regulation

Some mechanisms have been identified for the translational regulation of LPL. Yukht *et al.* (84) found that catecholamine can inhibit LPL synthesis through an interaction between a *trans*-acting factor (likely a protein) and the proximal 3' untranslated region (UTR) binding site of the LPL mRNA that lies between nucleotides 1599 and 1638. Kern and co-workers (85) found that LPL synthesis is enhanced in hypothyroid rat adipocytes versus wild type adipocytes due to the reduced activity of a translation-inhibitory factor, which acts to inhibit the translation of LPL by binding to the same proximal 3' UTR binding site of LPL mRNA as stated above . Ranganathan *et al.* (86) previously showed that epinephrine could inhibit LPL translation in adipocytes due to the binding of a *trans*-acting binding protein (of about 30 kDa) to another 3' UTR binding site within the LPL mRNA that resides between nucleotides 1601 and 1650 . Insulin also regulates the

translation of LPL. In insulin deficient rats, a reduced translation of LPL in adipose tissue was identified as a result of the binding of an unknown cytoplasmic protein to the 3' UTR binding site of the LPL mRNA between nucleotides 1834 and 1980 (87). Notably, in addition to modulating LPL expression at translational level, Semenkovich *et al.* (88) showed that insulin functions to increase the level of LPL mRNA by enhancing the LPL mRNA stability in adipocytes.

1.2.6.3 Post-translational regulation

The post-translational regulation of LPL includes the modulation of the degradation, processing, secretion, and translocation of LPL protein. Evidence exists to suggest that fasting may prevent either the elimination of glucose from LPL *N*-linked oligosaccharides, or the transfer of LPL from the ER to the *cis*-Golgi network, thus, resulting in a decrease of LPL activity (89). Specifically, although the mRNA level and synthesis rate of LPL in adipose tissue was increased by 2-fold in fasting mice, the LPL mass was unchanged and the LPL activity was decreased by 50% versus fed mice (89). Consistent with this observation, the LPL within adipose tissues of fasting mice was found to be 65% as an endoglycosidase H-sensitive (high mannose glycosylated ER) form and 35% as an endoglycosidase H-insensitive (low mannose Golgi-derived) mature from, while the refeeding of mice led to a LPL profile of 35% high mannose ER form and 65% Golgi-derived form (89). On the other hand, kinetic analysis indicated that fasting did not affect the degradation rate of ER-derived LPL, but markedly enhanced (by 3.5-fold) the

fractional catabolic rate of mature LPL in the secretory compartment of Golgi (89). Furthermore, fasting yielded a similar effect on LPL activity in heart, without affecting the LPL mRNA level and synthesis rate (89). Additionally, lipase maturation factor 1, a newly identified ER-transmembrane protein, has been shown to regulate LPL at the post-transcriptional level by affecting the dimerization of inactive LPL monomers (which is a crucial step in activating LPL), and the stabilization of active LPL homodimers (90, 91).

1.2.6.4 Signalling transduction mechanisms involved in the regulation of LPL

To date, multiple intracellular signalling transduction pathways have been identified which function to regulate the expression of LPL. Firstly, Tengku-Muhammad *et al.* (92) previously showed that lipopolysaccharide and cytokines function to regulate the expression of macrophage LPL by tyrosine kinases in the presence or absence of phosphatidylinositol-3-kinase activity. Secondly, in macrophages, the activation of protein kinase C (PKC) by agents including glucose, homocysteine, platelet-derived growth factor, and reactive oxygen intermediates, has been shown to be involved in stimulating LPL expression and secretion (76, 93-95). Specifically, Beauchamp *et al.* (76) showed that the treatment of macrophages with the PKC inhibitor Calphostin C abolishes the homocysteine-induced stimulation of LPL transcription and translation; similarly, the abolishment of homocysteine-induced stimulation of LPL expression by D-glucose has also been reported by Sartippour *et al.* (93). Renier *et al.* (95) showed that inhibiting PKC

activity within macrophages by Calphostin C completely inhibited reactive oxygen species-induced LPL secretion. Inaba *et al.* (94) have also shown that blocking PKC using staurosporine significantly suppressed platelet-derived growth factor (BB homodimer)-induced LPL activity in macrophages. Thirdly, Kraemer *et al.* (96) showed that in adipocytes, the insulin-stimulated activity of LPL can be restored in the presence of wortmannin by inhibiting the activity of phosphatidylinositol-3-kinase. They also found that similar to wortmannin, rapamycin acts to abolish the insulin-induced activation of LPL activity in adipocytes by indirectly inhibiting the activity of p70S6 kinase via the direct inhibition of the mammalian target of rapamycin protein (96, 97). Lastly, cyclic adenosine monophosphate (cAMP) exhibits species specificity in modulating the LPL synthesis. Studies using multiple human and rat tissues plus cell models (including the human adrenocortical carcinoma cell line-NCI-H295, rat brown adipose tissue, human THP-1 cells, and rat mesenchymal heart cells), showed that cAMP exhibits a positive correlation with LPL transcripts, while studies using mouse cell models (including J774 macrophage and 3T3-F442A adipocyte) found that the secretion and transcription of LPL was negatively regulated by cAMP (98-103). However, Gardette *et al.* (104) found that the treatment of human monocyte-derived macrophages with cAMP significantly attenuated the secretion of LPL, while Peinado-Onsurbe *et al.* (105) observed that an increase of cellular cAMP positively regulated the LPL activity in the neonatal mouse hepatoma cell line BWTG3.

1.2.7 Activity and substrate specificity of LPL

LPL exhibits a catalytic function, as well as a non-catalytic bridging function that is independent of catalysis. The catalytic function is the most well studied and is deemed to be the most important. LPL mainly acts to catalyze the hydrolysis of fatty acyl chains from TG within circulating lipoproteins. *In vivo*, the circulating TG is derived from two pathways: one is from diet by intestinal absorption (secreted as CM), and the other is endogenous synthesis by the liver (secreted as VLDL) (43, 106-108). Through the catalysis by LPL, the TG component of CM is removed, thus forming a cholesterol enriched particle known as a CM remnant; the CM remnants are removed from the circulation by the liver through LDL receptor family members (106, 109). In contrast, within the circulation, following a series of attachment/detachment events in different tissues, the liver derived VLDL are converted to IDL and further to LDL, in part through the catalysis by LPL, thus enabling the lipid to be distributed to tissues within circulation (106).

An *in vitro* study conducted by Deckelbaum and co-workers (110) showed that compared to long-chain TG (LCT) (with fatty acid chains containing 16 to 18 carbons), LPL displayed a marked preference for the hydrolysis of medium-chain TG (MCT) (with fatty acid chains containing 8 to 10 carbons). This is probably because MCT has a higher solubility and the increased mobility at the emulsion-water interface versus LCT (110).

The preferential hydrolysis of TGs by LPL is also relative to the degree of saturation of fatty acyl chains bound to the glycerol backbone (111). In general, TGs containing medium saturated or long unsaturated fatty acyl chains were shown to be better substrates for LPL versus TGs with long-chain saturated fatty acyl chains (111). Furthermore, due to the effects of steric hindrance, the first 8 carbons of the fatty acyl chains were shown to be critical for the ability of LPL to liberate the fatty acyl chains from TG. Specifically, LPL was shown to exhibit the highest enzyme affinity to the fatty acyl chain with 8 carbons, and it exhibited an advanced enzyme affinity to TG with fatty acyl chains that have a *cis*-double bond at C_9 position (111). Additionally, it has been shown that the liberation of saturated and monounsaturated C_{16}-$_{18}$ FFA from TG by LPL is faster than those for the C_{20} fatty acids arachidonic acid and eicosapentaenoic acid (112, 113). LPL hydrolyses the C_{22} docosahexaenoic acid ester bond from TG at a rate between the hydrolysis rate of C_{16}-$_{18}$ acid ester bonds and C_{20} acid ester bonds (112, 113). Moreover, the same trend was observed in the hydrolysis of fatty acid ester bond from PL by LPL (112).

The non-catalytic bridging function of LPL normally refers to its ability to bind to lipoproteins and cells simultaneously, which allows for facilitating the uptake and degradation of lipoproteins (9, 60, 114). Previous *in vitro* and *in vivo* studies showed that the cell binding sites of LPL are the sulfated proteoglycans of HSPG, plus cell surface

proteins, including LDL receptor related protein, LDL receptor, VLDL receptor, gp330, apoE receptor, and GPIHBP1 (9, 15, 60, 114-116). Mamputu *et al.* (117) also previously showed that a bridging action by LPL occurs between cells, as they found that LPL could bind simultaneously to both monocyte surface HSPG and to the cells of the arterial endothelium.

The functions of LPL can regulate other processes, including functioning to trigger the expression of tumour necrosis factor alpha (TNF-α), suppressing the secretion of apoE, and activating endothelial NAD(P)H oxidase (118-120). Specifically, Renier *et al.* (118) previously observed that the incubation of macrophages with LPL increased the expression of TNF-α by promoting the transcription of TNF-α via nuclear factor kappa-light-chain-enhancer of activated B cells, and an increased stability of the TNF-α mRNA. Lucas *et al.* (120) previously showed that either directly incubating macrophages in the presence of LPL or pre-binding LPL to macrophage cell surfaces led to a decreased secretion of apoE, likely through a mechanism associated with the LDL receptor. Esenabhalu *et al.* (119) have observed that the expression of human LPL in mouse aortic smooth muscle cells resulted in a deposition of FFA in the aorta, which further acts to activate the endothelial NAD(P)H through a PKC-dependent mechanism. Furthermore, Mamputu and colleagues (121) also showed that LPL promotes the proliferation of vascular smooth muscle cells (VSMC) through the co-operation between its catalytic and

non-catalytic function: they observed that the LPL induced proliferation of VSMC was abolished by inactivating LPL, and by releasing LPL from the cell surface using heparinase.

1.3 Atherosclerosis

1.3.1 Overview of atherosclerosis

Atherosclerosis refers to the thickening of the artery wall, which results from the formation of atheromatous plaques in the sub-endothelial space. The atherosclerotic plaque consists of lipid-laden foam cells, proliferating smooth muscle cells, and multiple extracellular materials, including collagen, sulfated glycosaminoglycans, fibrin, and cholesterol (122, 123). Atherosclerosis is initiated when the excess circulating plasma LDL invades the sub-endothelial space, where the LDL is susceptible to oxidation (124, 125). Once the LDL is oxidized, it will entice the blood circulating monocytes to migrate into the sub-endothelial space, where they further differentiate into macrophages that can take up the oxidized LDL (ox-LDL) (125). The continuing uptake of ox-LDL slowly turns the macrophages into lipid-laden foam cells. Concomitantly, with the accumulation of foam cells, a fatty streak is formed. This is considered as the early state of atherogenesis (125, 126).

In the development of atherosclerosis, the smooth muscle cells migrate into the sub-endothelial space from the medial layer of the artery wall. Similar to macrophages,

these smooth muscle cells subsequently transform into foam cells by taking up ox-LDL. More importantly, under the activation of ox-LDL, the smooth muscle cells generate several extracellular matrix proteins, which induce the fatty streak formation and cause the foam cells to form a fibrous cap (122, 123, 127). Meanwhile, the interactions between cells and the cytokines within the sub-endothelial space act on triggering the apoptosis of macrophages and smooth muscle cells, resulting in the accumulation of extracellular cholesterol and the formation of the necrotic core, which is characteristic of advanced atherosclerotic plaques (122, 123). *In vivo*, advanced atherosclerotic plaques mainly lead to the ischemic symptoms of atherosclerosis by narrowing the vessel lumen. However, the neovascularization together with the matrix metalloproteinases, which are secreted by macrophages and smooth muscle cells, act on weakening the atherosclerotic plaque (128, 129). Thus, in lesions with a thin fibrous cap and a large necrotic core, the plaque is prone to rupture. This leads to the thrombosis of the vessel that eventually can cause an acute cardiovascular event, such as stroke or myocardial infarction (130, 131).

1.3.2 The pro-atherogenic effects of macrophage LPL

The pro-atherogenic property of macrophage LPL was first reported by Zilversmit (132), based on an observation in cholesterol fed rabbits that the arterial LPL content positively correlated with the amount of aortic cholesterol and the rate of plasma CE influx to the artery. Following this report, evidence from *in vivo* studies further confirmed a link

with LPL expression and atherogenesis. This included: i) the existence of LPL mRNA and protein in atherosclerotic lesion macrophages (133); ii) an enhanced peritoneal macrophage LPL mRNA and protein expression level in an atherosclerosis-susceptible mouse strain (134); and iii) elevated plasma cholesterol in apoE-null mice positively related with the accumulation of CE in atherosclerotic lesion macrophages and the expression of LPL (135).

LPL clearly influences lesion development *in vivo*. Babaev and colleagues (136) have reported that the injection of homozygous LPL deficient fetal hepatic cells in irradiated C57BL/6 mice results in a significant decrease of atherosclerotic lesion size versus mice injected with wild type hepatic cells. They also showed that the injection of homozygous LPL deficient fetal hepatic cells in irradiated male LDLR-null mice fed an atherogenic diet for 8 weeks significantly reduced the proximal aortic lesion area (by 33%) and *en face* aortic lesion size (by 38%) at the end of 8 weeks, versus mice injected with wild type hepatic cells. In addition, at the end of 19 weeks, the *en face* aortic lesion size in the mice injected with fetal homozygous LPL-deficient hepatic cells was reduced by 69%, versus mice injected with wild type hepatic cells. (137). Notably, the post-heparin plasma LPL activities and plasma lipid profiles were unchanged in these studies, which suggested that the attenuated atherosclerosis in these mouse models is a result of lowered LPL expression levels in the atherosclerotic lesion (136, 137). On the other hand, a study conducted by Wilson and colleagues (138) showed that the macrophage-specific

overexpression of human LPL in male apoE null mice resulted in a markedly increased aortic lesion size by 51% versus control. Furthermore, recent studies showed that LPL promotes the deposition of lipid in atherosclerotic-susceptible sites. Localized human LPL transgene expression in both the balloon-injured carotid arteries of rabbits and mouse carotid arteries result in a marked lipid accumulation (139, 140), which suggested that the locally liberated hydrolysis products of lipids by LPL might augment atherogenic lesion progression.

The pro-atherogenic activity of macrophage LPL has also been reported from *in vitro* studies, as LPL could result in an accumulation of lipid within macrophages that might further trigger the macrophages to transform into foam cells (7, 136-145). In THP-1 macrophages, LPL was shown to increase the binding and uptake of mildly-oxidized lipoproteins, and stimulated the uptake and degradation of LDL independently of the LDL-receptor (144, 145). Similarly, in J774 mouse macrophages, LPL has been shown to increase the accumulation of VLDL-derived CE and TG (143). Lastly, the secretion of interleukin (IL)-1β, IL-6, monocyte chemoattractant protein-1, and TNF-α (which are pro-inflammatory cytokines) by THP-1 macrophages were markedly attenuated when LPL expression was suppressed, while the incubation of VLDL and LPL with macrophages would significantly activate the expression of TNF-α, suggesting that the lipid hydrolysis products generated by LPL might be responsible for the ability of LPL to modulate pro-inflammatory cytokines and adhesion molecules (141, 142).

Taken together, given the potent pro-atherogenic activity of macrophage LPL, a further understanding of the mechanisms behind these pro-atherogenic activities may allow for macrophage LPL to be a potential therapeutic target for the prevention and treatment of atherosclerosis.

1.4 Reverse cholesterol transport

1.4.1 Overview of reverse cholesterol transport

RCT is an atherosclerotic protective process that drives the excess cholesterol from peripheral tissues back to the liver for excretion (146, 147). RCT requires the transfer of cholesterol from cells to HDL (Figure 2).

ApoA-I, the major protein component of HDL, is mainly synthesized in the liver and intestine, or is derived from lipoproteins. *In vivo*, newly synthesized apoA-I is secreted into circulation as a lipid-poor particle, which functions as a cholesterol acceptor (146-148). Adenosine triphosphate-binding cassette transporter, sub-family A, member 1 (ABCA1) is a cholesterol efflux transporter that is embedded in the cell membrane. ABCA1 functions to remove (or efflux) excess cholesterol plus PL from non-hepatic tissues to the cholesterol acceptor apoA-I when it travels through the circulation (146). The loading of cholesterol and PL leads to the transformation of apoA-I to form what is known as nascent HDL (or preβ-HDL), which can continue to take up cholesterol that is effluxed from peripheral cells by the cholesterol transporters adenosine

triphosphate-binding cassette transporter, sub-family G, member 1 (ABCG1) and scavenger receptor class B member 1 (SR-BI) (149).

Lecithin:cholesterol acyltransferase (LCAT) is mainly synthesized in the liver and is activated by apoA-I after it binds to HDL; LCAT plays an important role in promoting the ability of HDL to accept cholesterol from the peripheral cells (150, 151). Specifically, the cholesterol that is accumulated in the nascent HDL (located on the surface of the particle) is esterified to a CE by LCAT, and the CE is subsequently moved to the hydrophobic core of the HDL particle, thus providing HDL with more room to accept additional cholesterol from peripheral tissues (149). Furthermore, the continued loading of cholesterol progressively increases the size of the HDL particles, leading to the transformation of the discoid nascent HDL into spherical mature HDL.

In humans, the cholesteryl ester transfer protein (CETP) plays a role in HDL metabolism. CETP is a hydrophobic glycoprotein that is secreted into the circulation after being synthesized by the liver and adipose tissue (152). CETP acts by transferring CE from HDL and exchanges CE for TG from TG-rich lipoprotein particles. This modulation by CETP allows for some of the HDL CE to be taken up by the liver via VLDL and/or LDL receptors, while generating TG rich/CE depleted HDL particles whose PL can be hydrolysed more effectively by HL and EL, thus generating smaller and more dense HDL particles (153).

When the HDL is at the liver, the TG and PL components within the particle are readily hydrolysed by HL and EL, leading to the alteration of lipid composition of the particle that in turn promotes the liberation of the CE and apoA-I from HDL (146). As a result, the released CE will be taken up by the liver via the dual-direction cholesterol transporter SR-BI, while the liberated apoA-I and PL are now available to restart the RCT process (149).

Lastly, once CE is transported into the liver, it is converted back to free cholesterol that can subsequently be eliminated from the body through bile secretion, thus completing the RCT process (146).

1.4.2 Cholesterol efflux

Cholesterol efflux, the initial step of the RCT process, refers to the removal of excess cholesterol from non-liver tissues by apoA-I and HDL (154). The function of cholesterol efflux in maintaining cell cholesterol homeostasis is a critical process that can inhibit the progression of atherosclerosis (146, 147).

To date, multiple mechanisms of cholesterol efflux have been identified, which includes aqueous diffusion and transporter meditated efflux. (154, 155). Specifically, removal of cholesterol by aqueous diffusion commonly occurs with all cell types, but with a very limited efficiency (155). Thus, the ability of cells to efflux cholesterol is mainly dependent on cholesterol transporters (155). The transporters that are involved in

cholesterol efflux include ABCA1, ABCG1, and SR-BI (154).

ABCA1 is highly expressed in liver and tissue macrophages (154). ABCA1 functions by promoting the transfer of peripheral cholesterol and PL onto lipid-poor apoA-I, which is responsible for initiating the formation of nascent HDL particles (154, 156). ABCG1 functions to efflux cholesterol to PL-containing acceptors, which include PL-only small unilamellar vesicles, nascent HDL, and mature HDL (157). The ability of ABCG1 to export cholesterol to PL-containing acceptors is thought to facilitate the ABCA1-induced formation of preβ-HDL from apoA-I, which suggests a co-operative relation between these two cholesterol transporters in cholesterol efflux (154, 157). Furthermore, the markedly increased expression of ABCG1 in ABCA1-deficient macrophages suggests a compensatory role for ABCG1 in cholesterol efflux (158).

Unlike ABCA1 and ABCG1, the flux of cholesterol mediated by SR-BI is bidirectional. It has been shown that SR-BI not only exhibits an ability to efflux cholesterol from macrophages to circulating lipoproteins, it also functions to influx cholesterol from circulating lipoproteins to the liver and steroidogenic tissues (that include the adrenals, ovaries, and testis) (154, 159, 160).

The expression level of these cholesterol transporters has become a critical factor for determining the ability to export cholesterol from arterial macrophages and preventing atherogenesis.

1.4.3 Modulation of cholesterol efflux by nuclear receptors in macrophages

LXR-α is a member of the nuclear receptor family that acts on modulating the transcription of genes involved in cholesterol efflux, transport and excretion (161, 162). LXR-α serves as a cholesterol sensor that is activated by oxysterols and intermediates of the cholesterol biosynthetic pathway (161). LXR-α acts on inducing the transcripts of its target genes by binding to LXR response elements (LXRE) after forming heterodimers with RXR (163-165).

PPAR-α and PPAR-γ are ligand-activated transcription factors that can also influence cholesterol efflux. PPAR-α is highly expressed in the liver, muscle, kidney, and heart, while PPAR-γ is mainly expressed in the intestine, mammary gland, and adipose tissue (166-170). The endogenous ligands for PPARs include fatty acids and eicosanoids (166). Once activated, PPARs will first heterodimerize with RXR, and following this they activate the transcripts of target genes by binding to the PPREs that are located in the promoter regions of the target genes (171, 172). Notably, one of the target genes of PPARs includes *NR1H3*, which encodes for LXR-α, since the PPRE has been identified and located on its promoter region (173, 174). Additionally, PPARs were also shown to function to suppress gene transcripts by interfering with signalling pathways (reviewed in (175)). Both PPAR-α and PPAR-γ have been implicated as critical modulators of cholesterol efflux within macrophages by regulating the expression of the cholesterol

transporters ABCA1, ABCG1, and SR-BI through either a LXR-α dependent or independent pathway (Figure 4) (176-179).

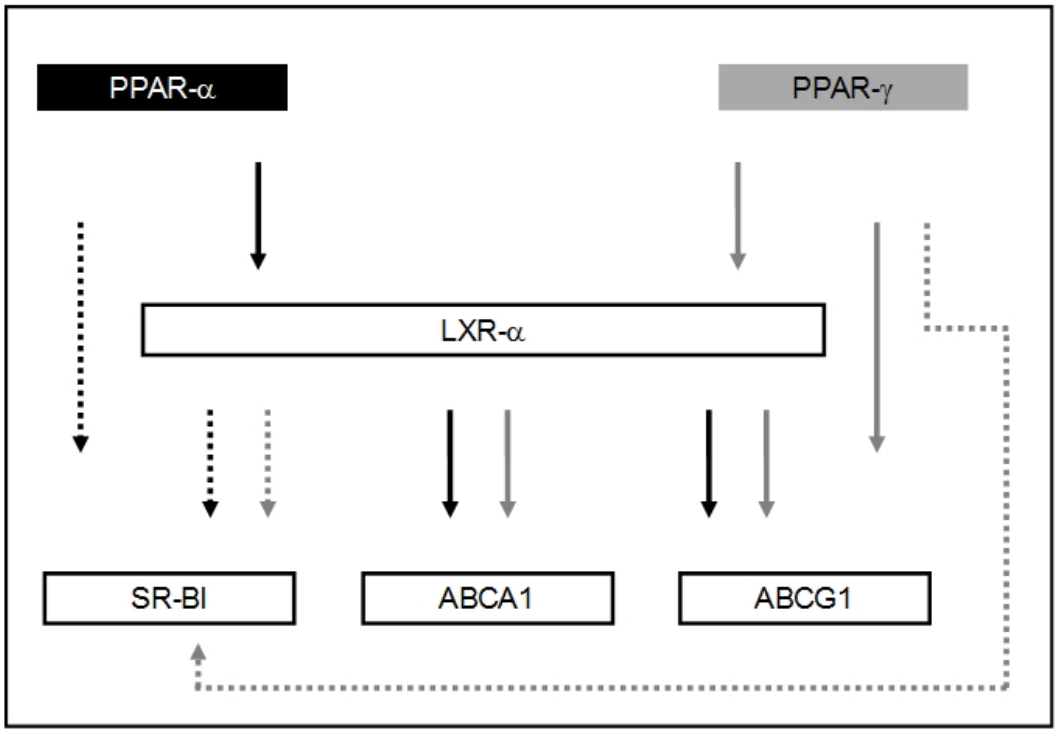
Evidence has shown that the activation of either PPAR-α or PPAR-γ significantly increases the expression of LXR-α and ABCA1, and subsequently elevates ABCA1 and apoA-I mediated cholesterol efflux (179). Furthermore, the identification of an LXRE on the ABCA1 promoter indicated that ABCA1 is a direct target for LXR-α (180-182). Thus, both PPAR-α and PPAR-γ exhibit the ability to regulate the expression of ABCA1 via the transcriptional cascades in the PPAR-α and LXR-α pathways (Figure 4).

The transcriptional cascades in the PPAR-γ and LXR-α pathway were also shown to be responsible for regulating the expression of ABCG1. Specifically, four LXREs were identified and characterized within the intron downstream of exon 2 (178). The expression of ABCG1 could be induced by PPAR-γ, which is associated with an increased expression of LXR-α, and the disruption of PPAR-γ results in lowered expression of ABCG1 and reduced cholesterol efflux (183). On the other hand, it has been reported that the activation of PPAR-γ could also activate the expression of ABCG1 in the absence of LXR-α (177). Thus, PPAR-γ could act on modulating ABCG1 in either a LXR dependent or independent pathway (Figure 4). Additionally, the agonists of either PPAR-α or PPAR-γ have been reported to significantly induce SR-BI mediated cholesterol efflux, which suggests that PPAR-α and PPAR-γ have an ability to modulate

Figure 4: The pathway of the transcriptional regulation of cholesterol transporters by PPARs

PPAR-α functions to indirectly modulate the transcription for genes encoding ABCA1 and ABCG1 via LXR-α (black arrows); PPAR-γ functions to indirectly modulate the transcription for genes encoding ABCA1 and ABCG1 via LXR-α, and to directly modulate the transcription for the gene encoding ABCG1 (gray arrows). Both PPAR-α and PPAR-γ have an ability to modulate the expression of SR-BI, however whether this modulation requires the activity of LXR-α is uncertain (black and gray broken arrows).

Figure 4



the expression of SR-BI. However, whether the PPARs regulation of SR-BI requires the activity of LXR-α is uncertain (Figure 4) (176).

1.5 Intracellular FFA synthesis and its regulation

In addition to regulating cholesterol efflux at a transcriptional level, LXR-α also functions to modulate intracellular lipid metabolism by regulating the expression of LPL (as described in *section 1.2.6.1*) and enzymes involved in FFA synthesis (184). Specifically, LXR-α was shown to indirectly modulate the transcripts for genes encoding enzymes involved in FFA synthesis via the transcriptional cascades in the LXR-α and SREBP-1c pathways (184-186). Furthermore, LXR-α has also been shown to directly promote the transcription of the gene encoding fatty acid synthase (FASN) (184, 186).

SREBP-1c, a downstream target of LXR-α, is a member of the basic helix-loop-helix leucine zipper transcription factor family. The activation of target genes by SREBP-1c is achieved through its amino-teminal domain, namely nuclear SREBP (nSREBP) (187). The nSREBP enters the nucleus after it is cleaved from the SREBP-1c by both site-1 protease and site-2 protease. Once inside the nucleus, nSREBP will bind to sterol response elements, which are found in the promoter regions of its target genes (187). The downstream target genes of SREBP-1c that are involved in the intracellular FFA synthesis include the genes encoding acetyl-CoA carboxylase A (ACCA), FASN, and stearoyl-CoA desaturase-1 (SCD-1) (188-191).

ACCA is a biotin-dependent enzyme, which exhibits a biotin carboxylase and a carboxyl transferase activity (192, 193). Within the FFA synthesis process, ACCA functions by catalyzing the carboxylation of acetyl-CoA to generate malonyl-CoA, which is a substrate for FASN (193). FASN is a multifunctional enzyme, which consists of two independent multifunctional polypeptides (194). FASN is responsible for catalyzing the synthesis of the saturated FFA palmitate and myristate (194). Notably, in addition to being indirectly modulated by LXR-α via SREBP-1c, a direct mechanism for the regulation of FASN by LXR has been identified by Joseph and colleagues (186). They found that when SREBP processing was suppressed, the LXR-induced activation of FASN expression was reduced, but not abolished, and FASN was activated by LXR agonists without altering the expression level of SREBP-1 (186). Moreover, LXREs were indeed identified and characterized within the *FASN* promoter region (186).

SCD-1 is a key lipogenic enzyme, which functions by catalyzing the desaturation of stearic acid to synthesize the mono-unsaturated fatty acid oleic acid (195). The synthesis rate of oleic acid by SCD-1 was shown to be strongly related to the storage and the transport of cholesterol, since oleic acid is the preferred fatty acid for the esterification of cholesterol (196).

1.6 Hypothesis and Objectives

1.6.1 Hypothesis

I hypothesize that the hydrolysis products of plasma total lipoproteins generated by LPL will significantly suppress the transcripts of genes associated with cholesterol efflux and lipogenesis. I also hypothesize that the FFA component of the hydrolysis products is responsible for influencing the expression of genes related to cholesterol efflux and lipogenesis.

1.6.2 Objectives

The objectives of this research are: i) to investigate the influence of the hydrolysis products of total lipoproteins generated by LPL and of the FFA, liberated from total lipoproteins by LPL, on the levels of gene transcripts associated with cholesterol efflux and lipid synthesis within THP-1 human macrophages; and ii) to assess the ability of macrophages treated with FFA that match the ratios to those liberated from total lipoproteins by LPL to efflux cholesterol from macrophages.

1.7 Significance

Macrophage LPL exhibits a positive correlation with atherogenesis; however, the mechanisms underlying any pro-atherogenic property of macrophage LPL remain poorly understood. By studying the pro-atherogenic property of macrophage LPL, the results of

my experiments will offer some insight as to whether macrophage LPL promotes atherogenesis by attenuating the ability of cells to efflux cholesterol. The results from this study will hopefully allow for future studies to investigate whether partial inhibition or elimination of macrophage LPL activity can positively modulate cholesterol efflux. If the inhibition of macrophage LPL activity is successful in activating cholesterol efflux, this could make macrophage LPL a potential therapeutic target in atherosclerosis.

Chapter 2: Materials and Methods

2.1 Cell culture and reagents

2.1.1 Cell lines and maintenance

2.1.1.1 Human embryonic kidney 293T cell line

The human embryonic kidney cell line HEK-293T was donated by Dr. Sherri Christian (Department of Biochemistry, Memorial University of Newfoundland). Cells were maintained in a complete growth medium of Dulbecco's Modified Eagle Medium (DMEM)/High Glucose (which contained 4 mM L-glutamine, 4.5 g/L glucose and 110 mg/L sodium pyruvate) (HyClone, South Logan, UT, USA), 10% v/v fetal bovine serum (FBS) (Hyclone), and 1% v/v of an antibiotic/antimycotic (A/A) stock solution (containing 10,000 U/mL penicillin G, 10,000 μg/mL streptomycin, and 25 μg/mL amphotericin B) (HyClone). The cells were incubated in T75 cell culture flasks (BD Biosciences, Mississauga, ON, Canada) at 37°C, with an atmosphere of 95% air and 5%$CO_{2(g)}$. The cells were sub-cultured every 2 to 3 days with a 1:10 sub-cultivation ratio. Briefly, cell culture medium was discarded and the cells were washed briefly using 5 mL plain DMEM. To detach the cells from the cell culture flasks, the cells were rinsed with 2.5 mL of 0.25% w/v trypsin (HyClone), and then placed at 37°C for 2 minutes. Next, 10 mL fresh complete DMEM medium was used to re-suspend the cells. Lastly, 1 mL of mixed cells

was removed and added into a new T75 flask that contained 14 mL fresh complete DMEM medium.

2.1.1.2 THP-1 cell line

The human monocytic leukemia cell line THP-1 was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in a complete growth medium of Roswell Park Memorial Institute (RPMI)-1640 medium (which contained 25 mM HEPES and 0.3 mg/L L-glutamine) (HyClone), 10% v/v FBS, and 1% v/v A/A. The cells were incubated in T75 cell culture flasks at 37°C, with an atmosphere of 95% air and 5%$CO_{2(g)}$. The cells were sub-cultured every 3 to 4 days when the concentration reached 8×10^5 cells/mL by removing 3 mL of media (containing non-adherent cells) to a new T75 flask containing 12 mL fresh complete RPMI.

2.1.2 HEK-293T cell transfection

HEK-293T cells at 60-80% confluency in 100-mm dishes (BD Falcon) were transfected using Lipofectamine™ (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. To reach a confluency of 60-80% at the time of transfection, 18 to 24 hours before the transfection, one confluent T75 flask was split 1:3 into a 100-mm plate. Cells in 100-mm plates were transfected using Lipofectamine™ with no cDNA (Non), 5.85 μg of pcDNA3 (Invitrogen) containing the cDNA for human LPL [GenBank:NM_000237] (a gift from Dr. Daniel J. Rader, University of Pennsylvania,

Philadelphia, PA, USA), or 5.85 μg empty pcDNA3 mammalian expression vector (empty vector control transfection). At 24 hours post-transfection, the culture media were discarded, and the cells were washed once with 5 mL plain DMEM media. Then, 5 mL DMEM media containing 10 U/mL heparin (Organon, Toronto, ON, Canada) and 1% v/v A/A was added to each dish; heparin was used to release LPL from cell surfaces. Cells were then incubated for another 23.5 hours at 37°C with 5% $CO_{2(g)}$. At 47.5 hours post-transfection, 1 mL DMEM media containing 100 U/ml heparin and 1% v/v A/A was added to each dish to raise the overall heparin concentration to 25 U/mL, and the cells were incubated at 37°C with 5% $CO_{2(g)}$ for additional 30 minutes (end at 48 hours after transfection). At 48 hours post-transfection, the conditioned medium in each dish was harvested and transferred to individual 15 mL centrifuge tubes. In order to remove the cell debris, the conditioned medium was centrifuged at 1,050 rpm for 7 minutes at 4°C. The supernatant was collected and divided into 200 μL and 500 μL aliquots, and stored at -80°C until needed. LPL hydrolytic activity was evaluated as described in *section 2.3.3*.

2.1.3 THP-1 cell differentiation

The differentiation of THP-1 monocyte cells into macrophage cells was performed as previously described (197). One confluent T75 flask was equally divided into two 15 mL centrifuge tubes, and followed by centrifuging at 750 rpm for 7 minutes at room temperature. The supernatant (culture medium) was discarded, and the cells were

resuspended in complete RPMI medium with addition of 100 nM phorbol 12-myristate-13-acetate (PMA) (Sigma, St. Louis, MO, USA) at a concentration of 3.86 × 10^5 cells/mL. Next, 9.65 × 10^5 cells were added to each well of a 6-well plate, and cultured at 37°C with 5% $CO_{2(g)}$ for 48 hours. After 48 hours, the adherent cells in each well were washed 3 times with 2 mL RPMI-1640 media containing 0.2% w/v fatty acid free bovine serum albumin (FAF-BSA) (Sigma), then cultured for 24 hours with RPMI containing 1% v/v A/A, 0.2% w/v FAF-BSA and 100 nM PMA (2.5 mL per well) at 37°C with 5% $CO_{2(g)}$. After 24 hours, the THP-1 macrophage cells were ready to be treated with hydrolysis products or purified FFA mixture.

2.2 Analysis of LPL expression

2.2.1 Quantification of LPL mRNA

The quantification of LPL mRNA was performed exactly as previously described (197). Briefly, total RNA from transfected HEK-293T cells was extracted using the RNeasy Mini Kit (Qiagen, Toronto, ON, Canada); the mRNA was stored at -80ºC and cDNA was generated using the iScript RT Supermix cDNA synthesis kit (which contains oligo(dT) and random primers) (Bio-Rad). Real-time PCR analyses with the cDNA samples were performed using SYBR-Green and primers (Integrated DNA Technologies, Toronto, ON, Canada) against LPL and β-actin (for details see *section 2.7*).

2.2.2 Immunoblot analysis

To denature the LPL protein in conditioned medium, the samples (40 μL) were mixed with an equal volume of a 2X treatment buffer containing 0.125 M Tris-HCl (pH 6.8), 20% v/v glycerol, 5% v/v β-mercaptoethanol, 4% w/v SDS, and 0.01% v/v bromophenol blue. Samples were incubated at 100°C for 7 minutes. Whole cell lysates in 100 mm dishes were prepared by scraping cells into 500 μL of 2X treatment buffer, and samples were incubated at 100°C for 7 minutes. Denatured media and cell samples were then loaded (20 μL per well) and separated by SDS-PAGE (stacking gel: 5%, separating gels: 10%) for approximately 60 minutes at 200 VDC; the running buffer for gel electrophoresis consisted of 25 mM Tris, 192 mM glycine, and 0.1% w/v SDS (pH 8.3) (Bio-Rad). Proteins were subsequently transferred to nitrocellulose membranes using a wet electroblotting system (at 350 mA, at 4°C, for 2 hours) (Bio-Rad); the buffer used for the transfer was 48 mM Tris base, 39 mM glycine, 1.3 mM SDS, and 20% v/v methanol. Once the samples were transferred to the membrane, the membrane was then incubated for 2 hours with a blocking solution (5% w/v non-fat dry milk (Bio-Rad), 0.05% v/v Tween-20 (Sigma Aldrich), and 0.05% w/v NaN_3 (Fisher Scientific, Toronto, ON, Canada) in phosphate-buffered saline (PBS) (3.8 mM NaH_2PO_4, 16.2 mM Na_2HPO_4, 0.15 M NaCl) at room temperature on a platform shaker. Next, the membranes were incubated for 16 to 18 hours with a 1:1,000 dilution (diluted by PBS containing 5% w/v non-fat dry milk,

0.05% v/v Tween-20, and 0.05% w/v NaN_3) of an anti-human LPL polyclonal antibody (#sc-32885, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature on a platform shaker. After incubation, the membranes were washed 4 times with PBS, and incubated for 2 hours with a 1:1,000 dilution (diluted by PBS containing 5% w/v non-fat dry milk and 0.05% v/v Tween-20) of a horseradish peroxidase-conjugated anti-rabbit IgG (#SA1-200, Pierce Biotechnology, Rockford, IL, USA) at room temperature on a platform shaker. After 2 hours, the membranes were washed 4 times with PBS, and developed using the ECL™ Prime chemiluminescent reagent (GE Healthcare, Baie d'Ufre, QC, Canada). Chemiluminescence was subsequently detected on an ImageQuant 4000 gel imager (GE Healthcare).

2.3 Lipoprotein isolation, analysis and hydrolysis

2.3.1 Isolation of human plasma total lipoproteins by KBr density gradient ultracentrifugation

The total lipoprotein fraction (d<1.21 g/mL), including VLDL, LDL, IDL, and HDL, was isolated by density gradient ultracentrifugation as previously described (198). Briefly, a total of 80 mL of human blood was collected from two normolipidemic donors fasted overnight (Human Investigation Committee approval number 11-109); immediately upon collection, ethylenediaminetetraacetic acid (EDTA) was added from a 0.5 M stock (pH 7.4) to reach a final concentration of 2 mM. To isolate the plasma, the blood was

centrifuged at 2,800 rpm for 15 minutes at 4°C, and then the plasma (supernatant) was collected and pooled. Next, the density of the plasma was adjusted to 1.21 g/mL using a d=1.35 g/mL KBr solution (in 154 mM NaCl and 2 mM EDTA, pH 7.4). Subsequently, the plasma with the density of 1.21 g/mL was ultracentrifuged at 4°C in a 70.1Ti rotor (Beckman, Mississauga, ON, Canada) at 50,000 rpm for 44 hours. After ultracentrifugation, the top ¼ of the supernatant (d<1.21 g/mL) was collected. The total lipoproteins were dialyzed in a cellulose tubular membrane (with a molecular weight cut off of 3,500) against PBS for 24 hours at 4°C; the PBS was changed every 6 hours. After the dialysis, the lipoproteins were collected and stored under $N_{2(g)}$ at 4°C.

2.3.2 Quantification of phospholipid in isolated human plasma total lipoproteins

The PL concentration of the isolated plasma total lipoproteins was measured using a commercial kit (Phospholipids C - Wako Diagnostics, Richmond, VA, USA), according to manufacturer's instructions. Briefly, in triplicate, a 5 μL aliquot of the isolated lipoproteins was mixed with 15 μL PBS, and subsequently the diluted lipoproteins were mixed with 200 μL of the kit's color reagent solution in a well within a 96-well plate. The mixture was incubated at 37°C for 5 minutes, then the absorbance of the sample was measured at 600 nm wavelength on a Synergy fluorescent plate reader (Bio-Tek, Winooski, VT, USA). Samples were compared to a standard curve, prepared from the PL standard stock (at 300 mg/dL) within the kit. To prepare the standard curve, the stock solution was

diluted to 5 mg/dL, 10 mg/dL, 25 mg/dL, 50 mg/dL, 100 mg/dL, 150 mg/dL using PBS. To a separate well for each dilution, 20 uL of the diluted standard solutions or PBS (as a reagent blank) were mixed with 200 μL color reagent and assessed as above.

2.3.3 Lipoprotein hydrolysis by LPL

PBS or heparinized media without (no cDNA transfection or mock transfection) or with LPL from the transfected HEK-293T cells (as prepared and described in *section 2.1.2*) were gently mixed with an equal volume of isolated plasma total lipoproteins (as prepared and described in *section 2.3.1*, with a PL concentration of 3.1 mM (as measured and described in *section 2.3.2*)). FAF-BSA was added to each mixture to reach a final concentration of 0.2% w/v, then these mixtures were incubated for 4 hours at 37°C. The FFA present in each sample after 4 hours was evaluated using a commercial kit (HR Series NEFA-HR 2) (Wako), according to manufacturer's instructions. Briefly, in triplicate, a 4 μL aliquot of medium from each mixture was added to individual wells in a 96-well microplate. Mixtures were incubated with kit reagents to generate a color change corresponding to FFA content, and samples were measured at 560 nm wavelength on a Synergy fluorescent plate reader. A standard curve was created using 0+4, 0.5+3.5, 1+3, 1.5+2.5, 2+2, 2.5+1.5, 3+1 and 4+0 μL of standard solution (1 mM oleic acid) + water in separate wells. Data were expressed as the amount of FFA (nmol) generated per μL of heparinized media (or PBS) per 4 hours. No differences were observed for LPL hydrolytic

activity from experiment to experiment.

2.4 Mass spectrometry analyses

Tandem electrospray ionization-mass spectrometry (ESI-MS) and gas chromatography-mass spectrometry (GC-MS) analyses of lipoprotein hydrolysis products were performed by Dr. Robert Brown (Department of Biochemistry, Memorial University of Newfoundland) at the laboratory of Dr. David Ford at the Center for Cardiovascular Research at Saint Louis University (St. Louis, MO, USA). Data analyses were carried out by Yanbo Yang. Lipid extractions and analyses are described below.

2.4.1 Lipid extraction

Lipids from 10 μL lipoprotein-media mixtures (prepared as described in *section 2.3.3*, but with 3.9 mM stock total lipoproteins (by PL)) were extracted using the Bligh-Dyer method, exactly as previous described (199), in the presence of non-naturally occurring internal standards for each class of lipid assessed. Internal standard lipids that were added to each lipoprotein-media mixture include 0.5 μg triheptadecenoin (tri-17:1 TG) (Nu-Chek Prep, Elysian, MN, USA), 5 μg diarachidoyl phosphocholine (di-20:0 PtdCho) (Avanti Polar Lipids, Alabaster, AL, USA), 1.2 μg 1-heptadecanoyl lysophosphatidylcholine (17:0 lysoPtdCho) (Avanti Polar Lipids), and 1 μg arachidic acid (20:0 FFA) (Nu-Chek Prep). Extracted lipids were resuspended in 500 μL chloroform and stored at -20°C under $N_{2(g)}$ until needed.

2.4.2 Electrospray ionization-mass spectrometry analyses

ESI-MS analyses were carried out as previously described (200-202). Briefly, 200 μL methanol and 2 μL of 10 mM NaOH (in methanol) were mixed with 50 μL of lipid extract, and subsequently injected into a Thermo TSQ Ultra tandem ESI-MS system at a rate of 3.5 μL per minute; the electrospray needle voltage (with a capillary temperature of 270ºC) was set at 3,500 VDC in positive ion mode. Sodiated adducts of select PtdCho and lysoPtdCho species were quantified by scanning for the neutral loss of choline (*m/z* 59.1) using a collision energy of -28 eV, and sodiated adducts of TG were quantified by survey scanning for $[M+Na]^+$ between *m/z* 800 and 950. Isotopic contributions toward all collected ESI-MS data were corrected as previous described (200-202).

2.4.3 Gas chromatography-mass spectrometry analyses

The GC-MS analyses were performed as previously described (203), to quantify the individual species of FFA within lipoprotein-media mixtures. Briefly, the FFA were firstly esterified into pentafluorobenzyl esters, then quantified by selective ion monitoring.

2.5 Preparation of FFA mixture

2.5.1 Stock solution preparation

Briefly, myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), stearic

acid (18:0), oleic acid (18:1), linoleic acid (18:2), arachidonic acid (20:4), and docosahexaenoic acid (22:6) (all from Nu-Chek Prep) were dissolved in high performance liquid chromatography grade methanol to a concentration of 10 mg/mL. The prepared fatty acid stock solutions were sealed under $N_{2(g)}$, and stored at -20°C until needed.

2.5.2 Preparation of a purified FFA mixture that matches the amounts liberated from lipoproteins by LPL

A mixture of FFA that matches the amounts to those liberated from human plasma total lipoproteins by LPL was prepared as previously described (197). Briefly, to prepare a mixture of purified FFA that matches the amount of 250 μL total lipoprotein hydrolysis products generated by LPL, a certain amount (nmol) of each species of FFA (as shown in Table 1) was removed from its stock solution (as described and prepared in *section 2.5.1*) to a 1.5 mL Eppendorf tube. The methanol was evaporated using $N_{2(g)}$ at 35°C, and the fatty acids were resuspended in 10 μL dimethylsulfoxide. Next, with continuous vortexing, the prepared fatty acid mixture was added to 240 μL DMEM medium containing 1% A/A at a rate of 1 μL/minute.

Table 1: Components of the purified FFA mixture that matches the amount liberated from total lipoproteins by LPL

Species of FFA	Amount (nmol)
Myristic acid (14:0)	18.6
Palmitoleic acid (16:1)	23.7
Palmitic acid (16:0)	275
Linoleic acid (18:2)	70
Oleic acid (18:1)	241.8
Stearic acid (18:0)	45.4
Arachidonic acid (20:4)	0.9
Docosahexaenoic acid (22:6)	0.4

Listed are the nmol of each free fatty acid (FFA) present in 250 μL of the prepared purified FFA mixture.

2.6 Incubation of lipoprotein hydrolysis products and purified FFA mixture with THP-1 macrophages

2.6.1 Incubation of lipoprotein hydrolysis products with THP-1 macrophages

Differentiated THP-1 cells in 6-well plates (as prepared and described in *section 2.1.3*) were washed once with RPMI (2 mL/well). Next, 1 mL of RPMI containing 0.2% w/v FAF-BSA, 25 μg/mL tetrahydrolipstatin (THL) (Sigma) and 1% v/v A/A was added to each well. THL inhibits the lipolytic activity of any endogenous *sn*-1 lipases within the THP-1 macrophages (197). The cells were incubated at 37°C with 5% $CO_{2(g)}$ for 1 hour.

Meanwhile, the total lipoprotein hydrolysis products (as prepared and described in *section 2.3.3*) were diluted in a ratio of 1:4 and 1:8 separately using RPMI containing 0.2% w/v FAF-BSA, 25 μg/mL THL, 100 nM PMA and 1% v/v A/A to give a concentration of 0.68 mM and 0.34 mM, respectively (based on post-hydrolysed FFA). After 1 hour, 930 μL of diluted hydrolysis products was added to each well of cells, and cells were incubated for 18 hours at 37°C with 5% $CO_{2(g)}$. After 18 hours, the THP-1 macrophage RNA was isolated for analysis by real-time PCR.

2.6.2 Incubation of FFA mixture with THP-1 macrophages

The differentiated THP-1 cells (as prepared and described in *section 2.1.3*) were first washed once with RPMI-1640 media (2 mL/well). Next, 1 mL of RPMI containing 0.2% w/v FAF-BSA, 25 μg/mL THL and 1% v/v A/A was added to individual wells. The cells were then incubated for 1 hour at 37°C with 5% $CO_{2(g)}$. Meanwhile, the fatty acid containing medium (as prepared and described in *section 2.5.2*) was diluted in a ratio of 1:4 or 1:8, as described in *section 2.6.1*, to give a final FFA concentration of 0.68 mM or 0.34 mM. After 1 hour, 930 μL of diluted purified FFA mixture was added to each well of cells; cells were incubated for 18 hours at 37°C with 5% $CO_{2(g)}$. After 18 hours, the THP-1 macrophage cells were ready for real-time PCR analysis (as described in *section 2.7*) or Oil red O staining. For staining, the cells were washed twice with PBS and then were incubated with Oil red O dye (0.36% in 60% isopropanol) (Millipore, Toronto, ON, Canada) for 15 minutes on a rocker at room temperature. After 15 minutes, Oil-red O dye was removed, and the stained THP-1 macrophages were examined by using a light microscope (40x objective, bright field), and images were captured using a digital camera.

2.7 Real-time PCR analysis

2.7.1 RNA extraction

Total RNA from the THP-1 macrophage cells was extracted using the RNeasy Mini Kit (Qiagen, Toronto, ON, Canada) according to manufacturer's instructions. The

isolated RNA was stored at -80°C until needed.

2.7.2 cDNA synthesis

The cDNA was synthesized from the isolated total RNA (as described and prepared in *section 2.7.1*) using iScript RT Supermix (Bio-Rad, Mississauga, Ontario, Canada) according to manufacturer's instructions. Briefly, for each reaction, 1 μg of total RNA was reverse transcribed with the iScript reverse transcriptase in a total volume of 20 μL, using the following reaction conditions: 5 minutes at 25°C, followed by 30 minutes at 42°C, followed by 5 minutes at 85°C. The synthesized cDNA was collected and stored at -20°C until needed.

2.7.3 SYBR Green real-time PCR assay

Quantitative real-time PCR was performed using the iQ SYBR Green Supermix kit (Bio-Rad) and primers against β-actin, LXR-α, PPAR-α, PPAR-γ, ABCA1, ABCG1, SR-BI, ACCA, FASN, SCD-1, SREBP-1c, and LPL (Integrated DNA Technologies) on a Mastercycler ep realplex (Eppendorf, Mississauga, ON, Canada) real-time PCR system. The final reaction mixture (25 μL) contained 75 ng of cDNA or non template control (in 3 μL), 1.25 μL of 500 nM forward primer, 1.25 μL of 500 nM reverse primer, 7 μL of RNase-free water (provide in kit), and 12.5 μL iQ SYBR Green Supermix (provided in kit). The sequences of primers are shown in Table 2. The PCR was carried out using the following program: 1 cycle of 95^{o}C for 3 minutes; 40 cycles of 95^{o}C for 15 seconds,

Table 2: Real-time PCR primer sequences used to quantify the mRNA level of reference and target genes

Gene	Forward Primer	Reverse Primer
ACTB	5′- ACC TTC TAC AAT GAG CTG CG -3′	5′- CCT GGA TAG CAA CGT ACA TGG -3′
NC_000007.13	Template: 349 368	Template: 537 517
NR1H3	5′- TCC TTT TTC TGA CCG GCT TC -3′	5′- GAT GAA TTC CAC TTG CAG CC -3′
XM_005252718.1	Template: 444 463	Template: 591 572
PPARA	5′- GCT ATC ATT ACG GAG TCC ACG -3′	5′- TCG CAC TTG TCA TAC ACC AG -3′
XM_005261658.1	Template: 460 480	Template: 547 528
PPARG	5′- GAG CCC AAG TTT GAG TTT GC -3′	5′- GCA GGT TGT CTT GAA TGT CTT C -3′
NM_138711.3	Template: 1367 1386	Template: 1514 1493
ABCA1	5′- AAC GAG ACT AAC CAG GCA ATC -3′	5′- ACA CAA TAC CAG CCC AGA AC -3′
XM_005251780.1	Template: 1778 1798	Template: 1925 1906
ABCG1	5′- GAG GGA TTT GGG TCT GAA CTG -3′	5′- CTG TTC TGA TCA CCG TAC TCG -3′
XM_005261209.1	Template: 1108 1128	Template: 1197 1177
SCARB1	5′- ATC CTC ACT TCC TCA ACG C -3′	5′- TTC ACA GAG CAG TTC ATG GG -3′
XM_005253637.1	Template: 979 997	Template: 1108 1089
ACACA	5′- TCG CTT TGG GGG AAA TAA AGT G -3′	5′- ACC ACC TAC GGA TAG ACC GC -3′
XM_005257267.1	Template: 417 438	Template: 508 489
FASN	5′- ACA GGG ACA ACC TGG AGT TCT -3′	5′- CTG TGG TCC CAC TTG ATG AGT -3′
NM_004104.4	Template: 2483 2503	Template: 2633 2613
SCD	5′- CTC CAC TGC TGG ACA TGA GA -3′	5′- AAT GAG TGA AGG GGC ACA AC -3′
NM_005063.4	Template: 2768 2787	Template: 3018 2999
LPL	5′- ACA CTT GCC ACC TCA TTC C -3′	5′- ACC CAA CTC TCA TAC ATT CCT -3′
NM_000237.2	Template: 525 543	Template: 645 625
SREBF1	5′- GGA GGG GTA GGG CCA ACG GCC T -3′	5′- CAT GTC TTC GAA AGT GCA ATC C -3′
XM_005256773.1	Template: 35 56	Template: 114 93

The gene symbols (with corresponding national center for biotechnology information reference accession number) encode the following proteins: *ACTB* encodes β-actin, *NR1H3* encodes LXR-α, *PPARA* encodes PPAR-α, *PPARG* encodes PPAR-γ, *ABCA1* encodes ABCA1, *ABCG1* encodes ABCG1, *SCARBI* encodes SR-BI, *ACACA* encodes ACCA, *FASN* encodes FASN, *SCD* encodes SCD-1, *LPL* encodes LPL, and *SREBF1* encodes SREBP-1c The efficiencies for each pair of primers are as shown in the Appendix under Supplemental Table 1.

56°C for 15 seconds, 72°C for 20 seconds; all reactions were performed in triplicate. The mRNA levels were normalized to β-actin, which was used as a house keeping gene.

2.7.4 Measurement of real-time amplification efficiencies of target and reference genes

The real-time PCR amplification efficiencies for each mRNA assessed were calculated according to the following equation (204):

$$E = 10^{[-1/slope]} \quad \text{(Equation 1)}$$

The slopes were calculated from the threshold cycle (Ct) value, which were measured as described in *section 2.7.3.*, versus input cDNA concentration (1 ng, 5 ng, 10 ng, 50 ng, and 100 ng). All reactions were performed in duplicate, and the data are shown in Appendix, Supplemental Table 1. The melting curve analysis showed that only one specific melting peak can be detected for each PCR product. This suggested that only one product has been amplified in each reaction (data not shown).

2.7.5 Calculation of gene expression

The relative expression ratio of a target gene was calculated according to a modified equation based on the delta delta C_t mathematical model (Equation 2) (204):

$$\text{Ratio} = (E_{target})^{\triangle CP_{target\ (control-sample)}} / (E_{ref})^{\triangle CP_{ref\ (control-sample)}}$$

(Equation 2)

Specifically, E_{target} and E_{ref} represent the amplification efficiency of the target genes and the reference gene β-actin, respectively, which were measured as described in *section 2.7.3*. ΔCP_{target} is the difference between the C_t value of the control and the experimental sample of the target gene transcript; ΔCP_{ref} is the difference between the C_t value of the control and the experimental sample of β-actin transcript. The relative transcriptional level of control samples was expressed as 100% ± standard deviation (SD).

2.8 Cholesterol efflux assay

2.8.1 Desalting of apolipoprotein A-I

Lyophilized apoA-I (Sigma) in 10 mM ammonium bicarbonate was resuspended in water and salt-exchanged for PBS using a PD-10 desalting column (GE Healthcare) equilibrated with PBS. The absorbance of the eluate was read at 280 nm (using PBS as blank), and the concentration of apoA-I was calculated using the Beer–Lambert law where the molar absorption coefficient of apoA-I equals 1.23 mL/ mg · cm (205).

2.8.2 Cholesterol efflux assay

Cholesterol efflux assays were performed as similarly described (206). Briefly, THP-1 cells were differentiated into macrophages by PMA in a 12-well plate (4.63×10^5 cells/well) as described in *section 2.1.3*. After 48 hours incubation, media were replaced with 500 µl RPMI-1640 containing 1% v/v FBS, 1% v/v A/A and 1 µCi/mL [^{3}H]cholesterol (PerkinElmer, Waltham, MA, USA); cells were cultured for 24 hours at 37°C with 5% $CO_{2(g)}$ to load cells with labelled cholesterol. After 24 hours, the cells in each well were washed twice using 750 µL media containing 0.2% w/v FAF-BSA, 100 nM PMA, and 1% v/v A/A. Following washes, the cells were cultured with 480 µL RPMI containing 0.2% w/v FAF-BSA, 100 nM PMA, and 1% v/v A/A for 5 hours at 37°C with 5% $CO_{2(g)}$. After 5 hours, the culture media were replaced with 480 µL RPMI containing 0.2% w/v FAF-BSA, 100 nM PMA, 1% v/v A/A, and 25 µg/mL THL; cells were then cultured for 1 hour at 37°C with 5% $CO_{2(g)}$. After 1 hour, the culture media were replaced with RPMI containing 0.2% w/v FAF-BSA, 100 nM PMA, 1% v/v A/A, and 25 µg/mL THL ± FFA mixture (0.68 mM) (as described and prepared in *section 2.6.2*); cells were then cultured for 18 hours at 37°C with 5% $CO_{2(g)}$. After 18 hours, cells were washed with 750 µL RPMI containing 0.2% w/v FAF-BSA, 100 nM PMA, and 1% v/v A/A. Following washes, the media were replaced with 446.4 µL RPMI containing 0.2% w/v FAF-BSA, 100 nM PMA, and 1% v/v A/A ± 25 µg/mL apoA-I; cells were then incubated for 6 hours

at 37°C with 5% $CO_{2(g)}$. After 6 hours, the medium in each well was collected into a labelled scintillation vial containing 553.6 μL ddH_2O. The cells in each well were lysed using 500 μL of 0.2 M NaOH for 30 minutes at room temperature; cell lysates were subsequently collected and placed into scintillation vials. Following this, additional 500 μL fresh 0.2 M NaOH was added to each well to resuspend and collect any cell lysis remnants. Both the media and cells were assessed by liquid scintillation counting for [^{3}H]cholesterol. The amount of [^{3}H]cholesterol effluxed was calculated as a percentage of [^{3}H]cholesterol effluxed into the medium per amount of total cell and medium [^{3}H]cholesterol. The background (BSA only data) was subtracted to obtain efflux data specific for apoA-I.

2.9 Statistical analyses

Where indicated in figure legends, one-way analysis of variation (ANOVA) with a Tukey's test for multiple comparisons, and two-way ANOVA with a Sidak multiple comparison test were performed on data using GraphPad Prism Version 5.0 (Trial) for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Where indicated in figure legends, Student's *t*-tests were performed on data using Microsoft Office Excel 2010 (Microsoft Canada Inc., Mississauga, ON, Canada). Error bars on the data are mean ± SD.

Chapter 3: Results

3.1 Generation of recombinant LPL

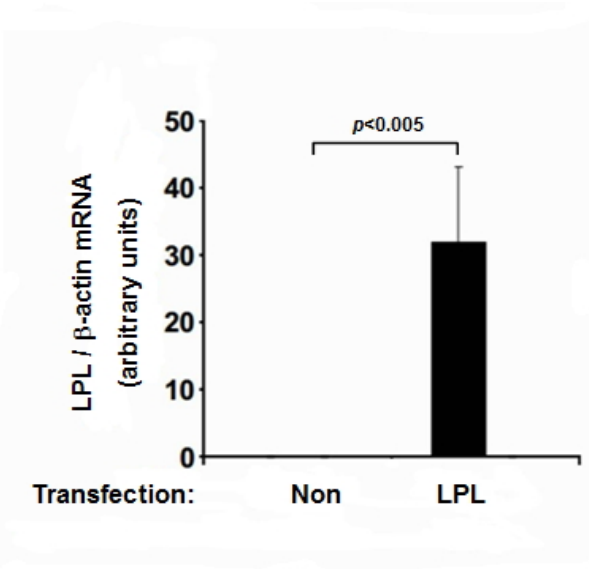
To generate the LPL that was used in the current study, a plasmid with the human LPL cDNA was transfected to HEK-293T cells as described in *section 2.1.2*. As shown in Figure 5A, real-time PCR analyses for LPL transcripts were markedly increased within HEK-293T cells expressing LPL. Furthermore, immunoblots for human LPL show that the transcript was actively translated in cells, and that it was released into media in the presence of heparin (Figure 5B). Triplicate assays to assess the FFA released from the hydrolysis of 1.05 mM total lipoproteins (by PL) from human plasma by conditioned media from HEK-293T cells expressing LPL showed that the quantity of FFA that was liberated was significantly greater than the amount liberated by media from mock transfected cells ($p<0.001$) (Figure 5C). No significant differences were observed in the quantity of FFA between hydrolysis reactions using either mock-transfected heparinized media or heparinized media from pcDNA3 transfected cells (Figure 5D). Taken together, these results show that the transfection was successful in producing recombinant human LPL, and the conditioned media from those cells transfected with human LPL possess considerable lipase activity.

Figure 5: Expression of LPL

A: Real-time PCR analyses of total RNA from transfected HEK-293T cells expressing no lipase (Non) or LPL to quantify LPL expression. Data represent the means ± SD from 3 independent experiments. *, $p<0.005$ (*t*-test). B: Immunoblot analyses for LPL in cells and heparinized media from transfected HEK-293T cells expressing no lipase (Non) or LPL. Data are representative of triplicate transfections. C: 1.05 mM total lipoproteins ($d<1.21$ g/mL) were hydrolysed over 4 hours at 37°C by heparinized media from transfected HEK-293T cells expressing no lipase (Non) or LPL (over 4 hours at 37°C). FFA released were quantified, and data represent the means ± SD from 3 independent experiments. Statistical analyses were performed using a *t*-test. D: 1.05 mM total lipoproteins ($d<1.21$ g/mL) were incubated for 4 hours at 37°C with heparinized media from transfected HEK-293T cells expressing no lipase (Non) or pcDNA3. FFA released were quantified, and data represent the means ± SD from 3 independent experiments.

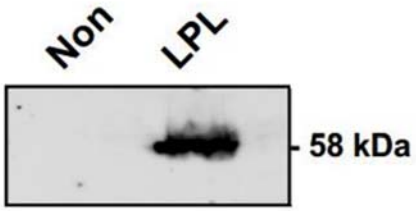
Figure 5

A



B

HEK-293T Cells:



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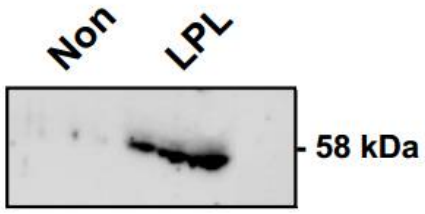
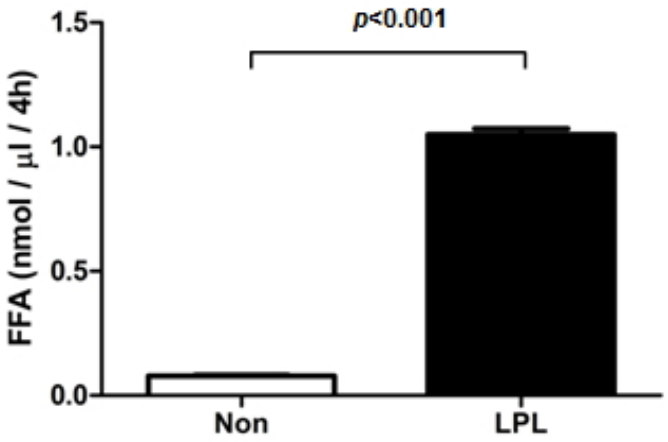
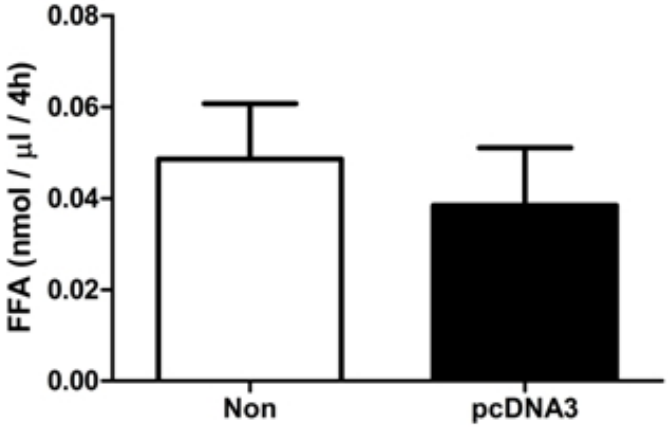


Figure 5 (cont.)

C



D



3.2 Lipidomic analysis of hydrolysis products generated by LPL from total lipoproteins

Shotgun lipidomic analyses on the lipoprotein hydrolysis products generated by conditioned media in the absence versus presence of LPL provide details for the individual molecular lipid species of the substrates and products. The substrates include molecular species of TG, PtdCho and lysoPtdCho, and the products include lysoPtdCho (derived from PtdCho) and FFA (derived from all substrates).

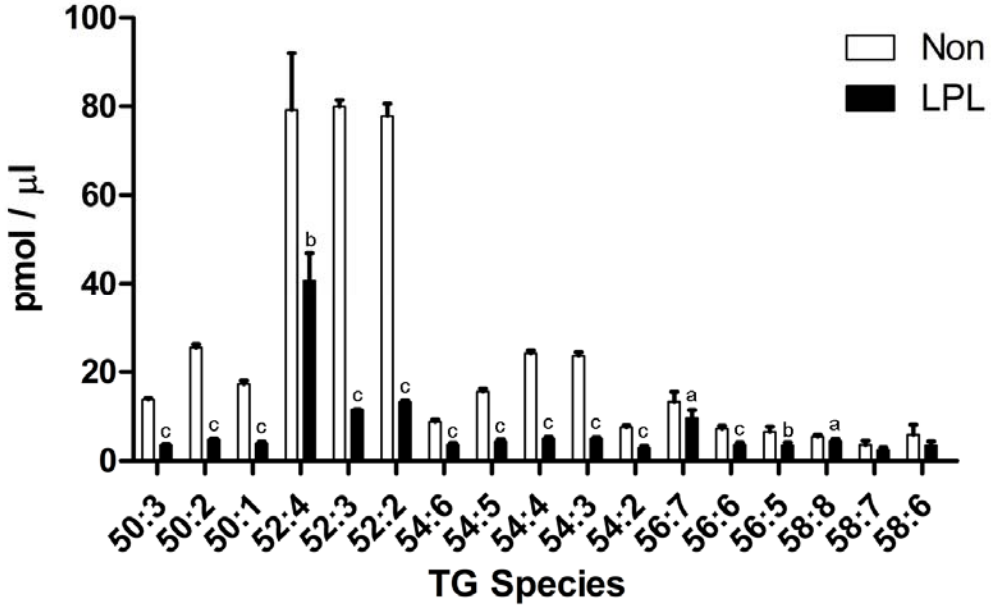
ESI-MS analyses of the hydrolysis reaction show that compared to mock transfected media incubations with total lipoproteins, 15 of the 17 species of TG that were assessed were significantly decreased following the hydrolysis of total lipoproteins by conditioned media containing LPL (Figure 6A). Furthermore, LPL significantly reduced the levels of 8 of the 10 PtdCho species assessed versus the PtdCho levels from mock transfected media incubations with total lipoproteins (Figure 7A). No significant differences for the levels of lysoPtdCho species were observed between the LPL and mock transfected media incubations (Figure 8A). Only modest differences were observed to the levels of individual species of TG (Figure 6B), PtdCho (Figure 7B), and lysoPtdCho (Figure 8B) between hydrolysis reactions in the presence of mock transfected media or PBS. Using GC-MS to measure FFA from hydrolysis reactions, results show that compared to mock transfected media incubations, all of the species of

Figure 6: Triglyceride species levels following total lipoprotein hydrolysis by heparinized media from LPL and no-lipase expressing HEK-293T cells

A: Lipids were extracted from total lipoprotein hydrolysis products from total lipoproteins (d<1.21 g/mL, 1.95 mM by PL) that were generated by heparinized media from HEK-293T cells expressing either LPL or no lipase (Non). Using triheptadecenoin as an internal control, the pmol triglyceride (TG) species per μL were quantified as described in *section 2.4.2*; a, $p<0.05$ versus Non; b, $p<0.01$ versus Non; c, $p<0.001$ versus Non (*t*-test). B: Lipids were extracted from total lipoprotein hydrolysis products from total lipoproteins (d<1.21 g/mL, 1.95 mM by PL) generated by heparinized media from mock transfected HEK-293T cells or PBS (vehicle control). The pmol TG species per μL were quantified as described above; a, $p<0.05$ versus PBS; b, $p<0.01$ versus PBS (*t*-test). All data are means ± SD from triplicate experiments.

A

Figure 6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80
60
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20
0
pmol / µl
Non
LPL
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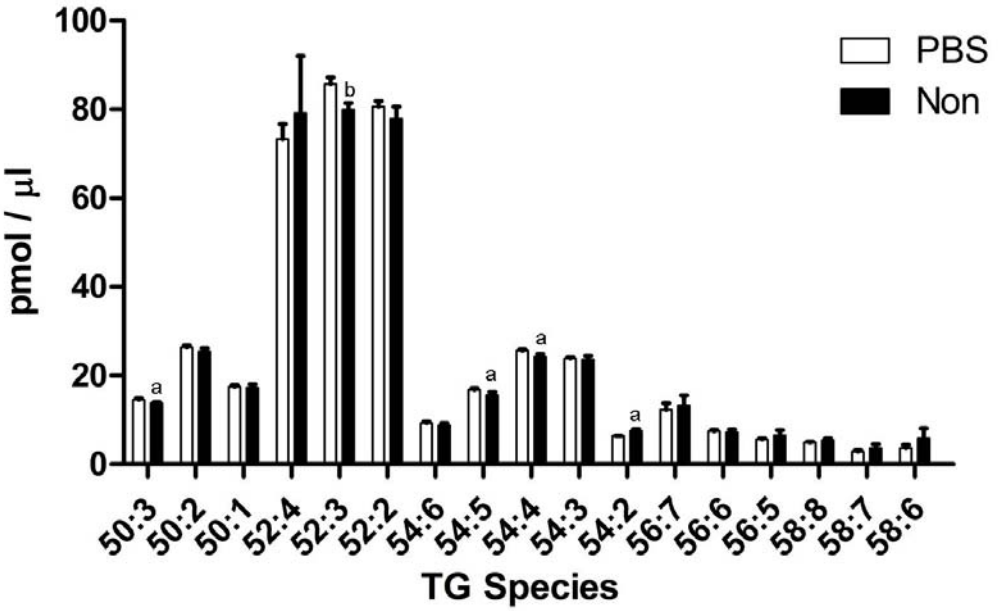
58:7
58:6
TG Species

B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60
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pmol / µl
PBS
Non
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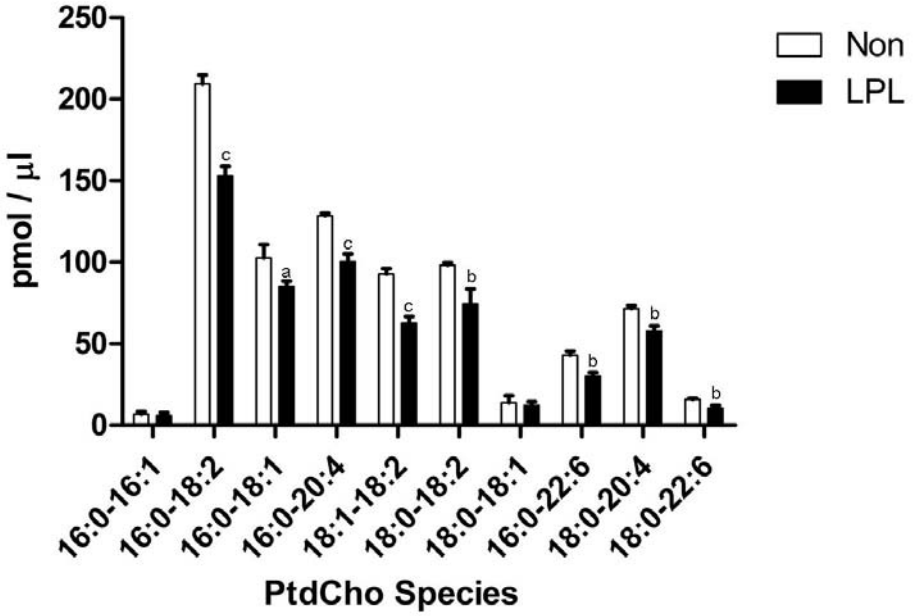
54:5
54:4
54:3
54:2
56:7
56:6
56:5
58:8
58:7
58:6
TG Species

Figure 7: Phosphatidylcholine species levels following total lipoprotein hydrolysis by heparinized media from LPL and no-lipase expressing HEK-293T cells

A: Lipids were extracted from total lipoprotein hydrolysis products from total lipoproteins (d<1.21 g/mL, 1.95 mM by PL) that were generated by heparinized media from HEK-293T cells expressing either LPL or no lipase (Non). Using diarachidoyl phosphocholine as an internal control, the pmol phosphatidylcholine (PtdCho) species per μL were quantified as described in *section 2.4.2*; a, $p<0.05$ versus Non; b, $p<0.01$ versus Non; c, $p<0.001$ versus Non (*t*-test). B: Lipids were extracted from total lipoprotein hydrolysis products from total lipoproteins (d<1.21 g/ml, 1.95 mM by PL) generated by heparinized media from mock transfected HEK-293T cells or PBS (vehicle control). The pmol PtdCho species per μL were quantified as described above; a, $p<0.05$ versus PBS; b, $p<0.01$ versus PBS (*t*-test). All data are means ± SD from triplicate experiments.

A

Figure 7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200
150
100
50
0
pmol / µl
Non
LPL
c
c
a
c
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b
b
b
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16:0-18:2
16:0-18:1
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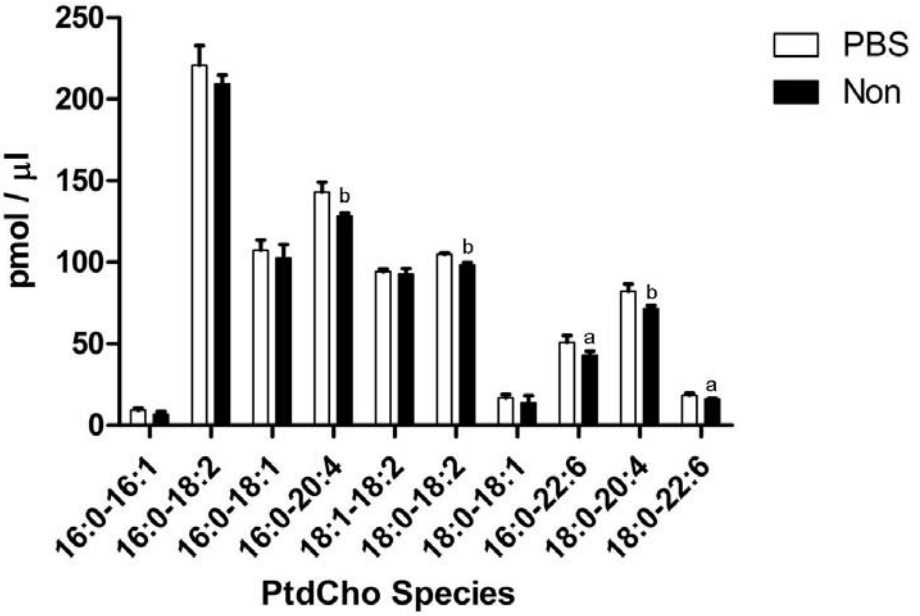
PtdCho Species

B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200
150
100
50
0
pmol / µl
PBS
Non
b
b
a
b
a
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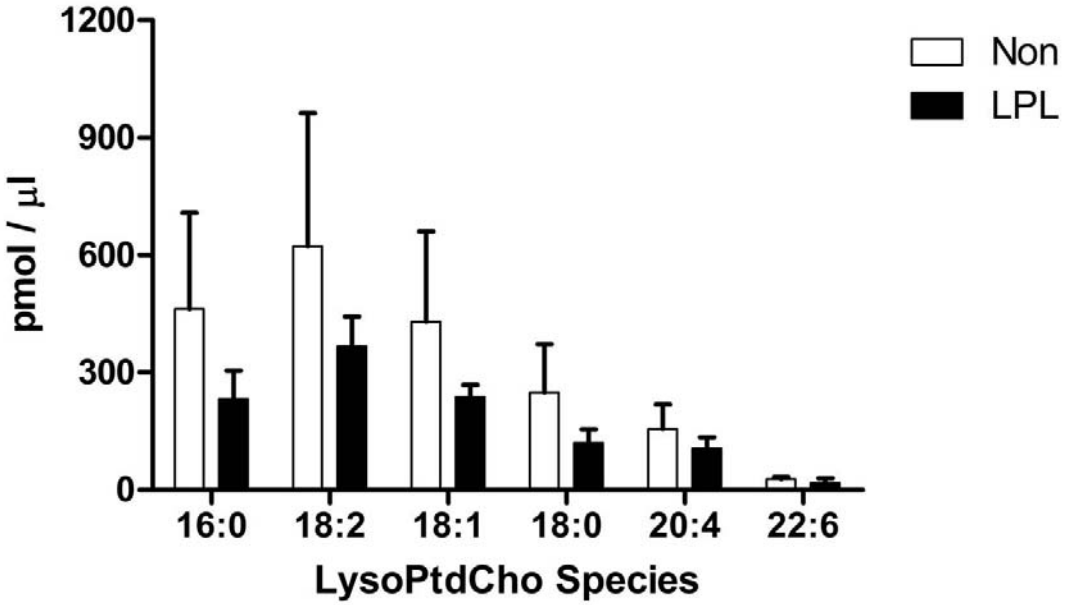
16:0-20:4
18:1-18:2
18:0-18:2
18:0-18:1
16:0-22:6
18:0-20:4
18:0-22:6
PtdCho Species

Figure 8: Lysophosphatidylcholine species levels following total lipoprotein hydrolysis by heparinized media from LPL and no-lipase expressing HEK-293T cells

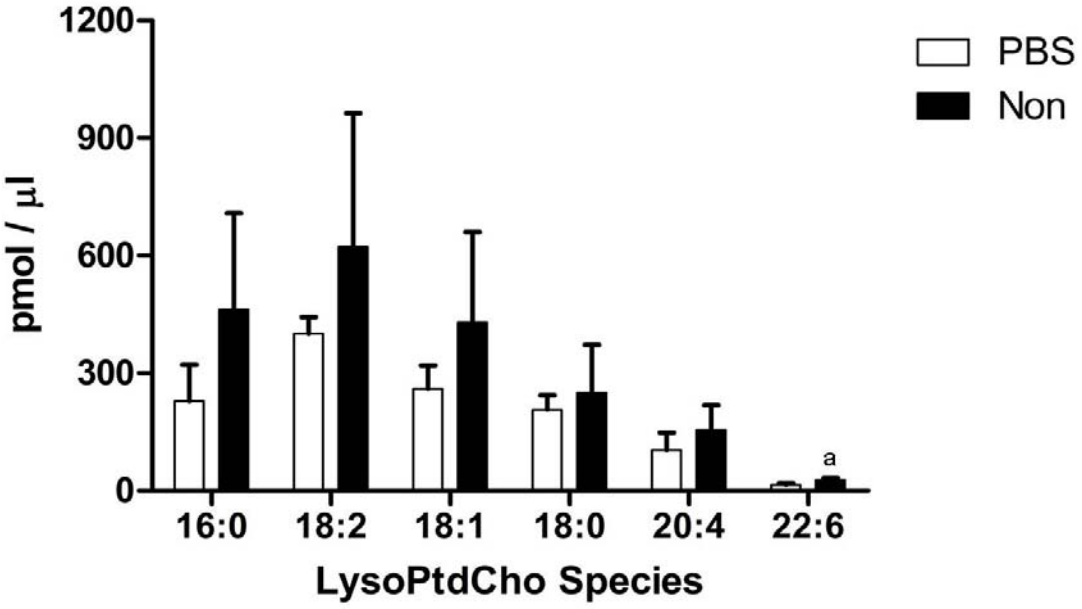
A: Lipids were extracted from total lipoprotein hydrolysis products from total lipoproteins (d<1.21 g/mL, 1.95 mM by PL) that were generated by heparinized media from HEK-293T cells expressing either LPL or no lipase (Non). Using 1-heptadecanoyl lysophosphatidylcholine (lysoPtdCho) as an internal control, the pmol lysoPtdCho species per μL were quantified as described in *section 2.4.2*. B: Lipids were extracted from total lipoprotein hydrolysis products from total lipoprotein (d<1.21 g/mL, 1.95 mM by PL) generated by heparinized media from mock transfected HEK-293T cells or PBS (vehicle control). The pmol lysoPtdCho species per μL were quantified as described above; a, $p<0.05$ versus PBS (*t*-test). All data are means ± SD from triplicate experiments.

A

Figure 8


1200
900
600
300
0
pmol / µl
Non
LPL
16:0
18:2
18:1
18:0
20:4
22:6
LysoPtdCho Species

B


1200
900
600
300
0
pmol / µl
PBS
Non
a
16:0
18:2
18:1
18:0
20:4
22:6
LysoPtdCho Species

FFA assessed were significantly increased after the hydrolysis of total lipoproteins by media containing LPL (Figure 9A). Compared to the hydrolysis reactions in the presence of PBS (vehicle control), 5 species of FFA were modestly increased in the hydrolysis reactions in the presence of mock transfected media (Figure 9B).

The concentration of the FFA liberated by LPL, as determined by GC-MS was 2.72 mM. This value was used to define the concentration of the total lipoprotein hydrolysis products and FFA that were added to THP-1 macrophages in subsequent studies: a dilution ratio of 1:4 (high concentration) (as described in *section 2.6.1*) is defined as 0.68 mM, and a dilution ratio of 1:8 (low concentration) (as described in *section 2.6.1*) is defined as 0.34 mM. These diluted products and FFA are representative of FFA concentrations found in the bloodstream of fasted persons *in vivo* (207).

3.3 The negative influences of the total lipoprotein hydrolysis products generated by LPL on gene transcripts within THP-1 macrophages

3.3.1 The mRNA levels for nuclear receptors LXR-α, PPAR-α, and PPAR-γ were significantly decreased by LPL lipoprotein hydrolysis products

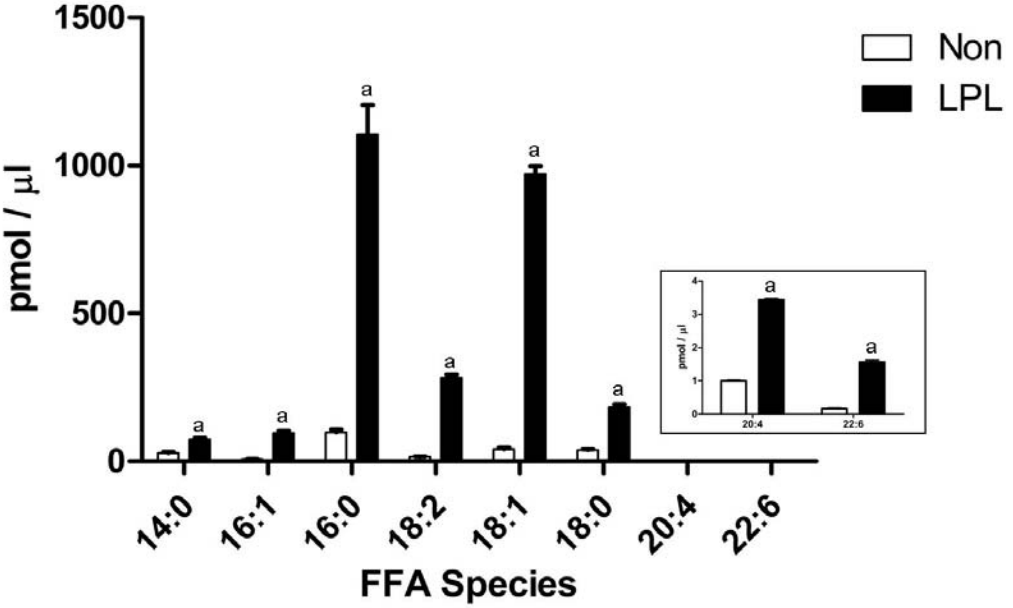
The incubation of THP-1 macrophages with lipoprotein hydrolysis products generated by LPL significantly reduced the mRNA levels of the lipid–activated nuclear receptors PPAR-α, PPAR-γ, and LXR-α (Figure 10). Both the low concentration (0.34

Figure 9: Free fatty acid species levels following total lipoprotein hydrolysis by heparinized media from LPL and no-lipase expressing HEK-293T cells

A: Lipids were extracted from total lipoprotein hydrolysis products from total lipoproteins (d<1.21 g/mL, 1.95 mM by PL) that were generated by heparinized media from HEK-293T cells expressing either LPL or no lipase (Non). Using arachidic acid as an internal control, the pmol free fatty acid (FFA) species per μL were quantified as described in *section 2.4.3*; a, $p<0.001$ versus Non (*t*-test). B: Lipids were extracted from total lipoprotein hydrolysis products from total lipoproteins (d<1.21 g/mL, 1.95 mM by PL) generated by heparinized media from mock transfected HEK-293T cells or PBS (vehicle control). The pmol FFA species per μL were quantified as described above; a, $p<0.05$ versus PBS; b, $p<0.01$ versus PBS, c, $p<0.001$ versus PBS (*t*-test). All data are means ± SD from triplicate experiments.

A

Figure 9


1500
1000
500
0
pmol / µl
Non
LPL
a
a
a
a
a
a
14:0
16:1
16:0
18:2
18:1
18:0
20:4
22:6
FFA Species
20:4
22:6

B

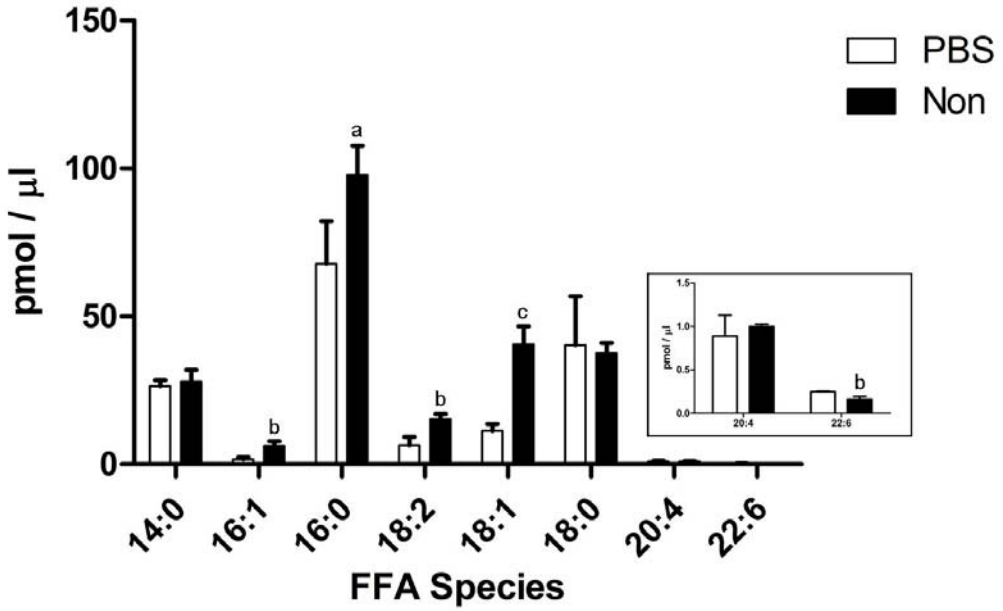
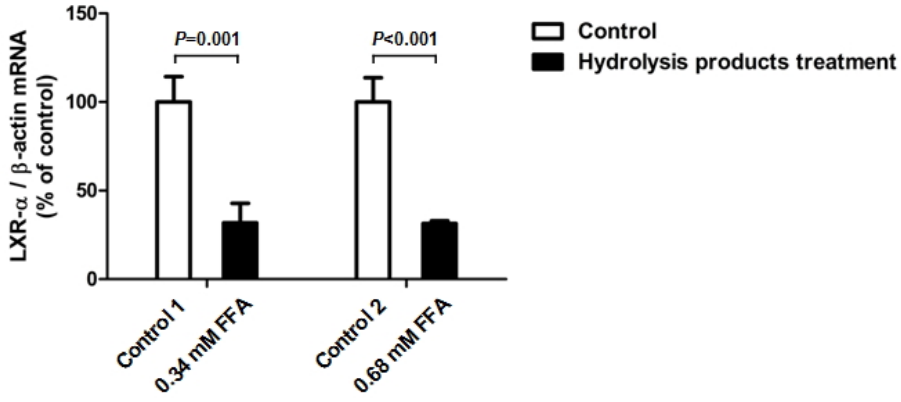

150
100
50
0
pmol / µl
PBS
Non
a
b
b
c
14:0
16:1
16:0
18:2
18:1
18:0
20:4
22:6
FFA Species
20:4
22:6
b

Figure 10: mRNA levels for nuclear receptors in macrophages incubated with lipoprotein hydrolysis products

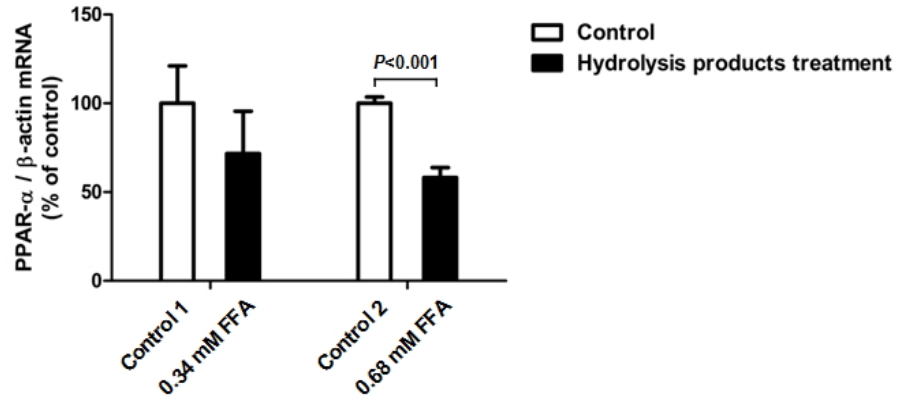
Differentiated THP-1 cells were treated in triplicate for 18 hours with 0.34 mM and 0.68 mM FFA (based on post-hydrolysis) of total lipoprotein hydrolysis products generated by LPL. A: The mRNA levels for LXR-α, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of LXR-α between the low concentration and high concentration hydrolysis products treatments. B: The mRNA levels for PPAR-α, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of PPAR-α between the low concentration and high concentration hydrolysis products treatments. C: The mRNA levels for PPAR-γ, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of PPAR-γ between the low concentration and high concentration hydrolysis products treatments. Control 1 and 2, represent the treatment of cells with total lipoprotein hydrolysis products generated by conditioned media with no lipase; respectively, they are the controls for the 0.34 mM and 0.68 mM FFA treatment. All data are means ± SD; *p* values, where written, indicate significance between control and treatment (*t*-test). Statistical analyses between hydrolysis products treatments were performed using a two-way ANOVA with a Sidak multiple comparison test.

A

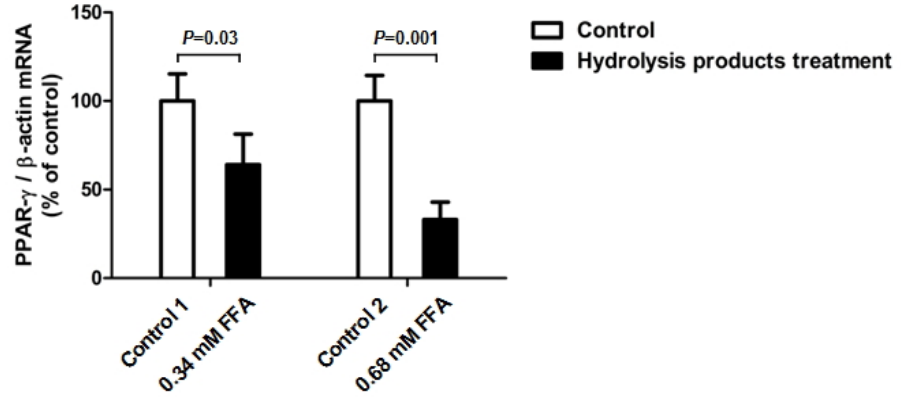
Figure 10



B



C



mM FFA based on post-hydrolysis) and the high concentration (0.68 mM FFA based on post-hydrolysis) of lipoprotein hydrolysis products significantly reduced the mRNA level of LXR-α (by 68% and 69%, respectively) (Figure 10A). The mRNA level of PPAR-α was markedly decreased by the high concentration of lipoprotein hydrolysis products by 42%, while the low concentration of lipoprotein hydrolysis products did not yield any significant effect on the transcript for PPAR-α (Figure 10B). Furthermore, the mRNA level of PPAR-γ was markedly decreased by low concentration and high concentration of LPL lipoprotein hydrolysis products by 36% and 67%, respectively (Figure 10C). No significant difference was observed for the mRNA levels of LXR-α, PPAR-α, and PPAR-γ between the low concentration and high concentration hydrolysis products treatments.

The observations of the suppressed transcription of genes encoding LXR-α and PPARs led us to speculate that the lipoprotein hydrolysis products generated by LPL may negatively modulate the genes that associate with cholesterol transport and lipogenesis. Thus, we further quantified the mRNA levels of multiple cholesterol transporters and enzymes involved in FFA synthesis in THP-1 macrophages that were incubated for 18 hours with lipoprotein hydrolysis products generated by LPL.

3.3.2 The mRNA levels for cholesterol transporters ABCA1, ABCG1, and SR-BI were significantly decreased by LPL lipoprotein hydrolysis products

The results from real-time PCR showed that the incubation of THP-1 macrophages with lipoprotein hydrolysis products generated by LPL significantly reduced the mRNA levels for ABCA1, ABCG1, and SR-BI (Figure 11). Both the low concentration (0.34 mM FFA) and high concentration (0.68 mM FFA) of lipoprotein hydrolysis products generated by LPL significantly decreased the mRNA levels for ABCA1 (by 48% and 24%, respectively) (Figure 11A) and ABCG1 (by 40% and 53%, respectively) (Figure 11B). The mRNA levels for SR-BI were significantly decreased only by the high concentration of LPL hydrolysis products (Figure 11C). A significant difference in the mRNA levels for ABCA1 was observed between the low concentration and high concentration treatment, while there was no significant difference in the mRNA levels for ABCG1 and SR-BI between the two treatments.

3.3.3 The mRNA levels for enzymes involved in FFA synthesis: ACCA, FASN, and SCD-1 were significantly decreased by LPL lipoprotein hydrolysis products

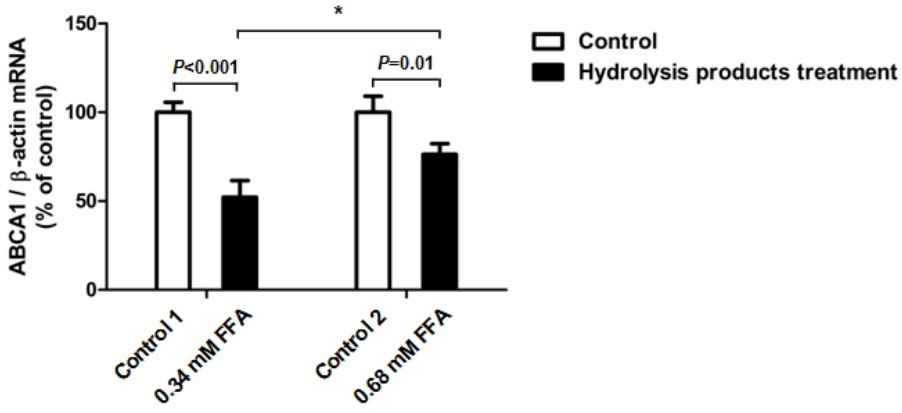
The transcriptional levels for the downstream target genes of LXR-α, including ACCA, FASN, and SCD-1, in THP-1 macrophages were assessed following incubation of the cells with lipoprotein hydrolysis products generated by LPL (Figure 12). The low concentration (0.34 mM FFA) and the high concentration (0.68 mM FFA) of lipoprotein

Figure 11: mRNA levels for cholesterol transporters in macrophages incubated with lipoprotein hydrolysis products

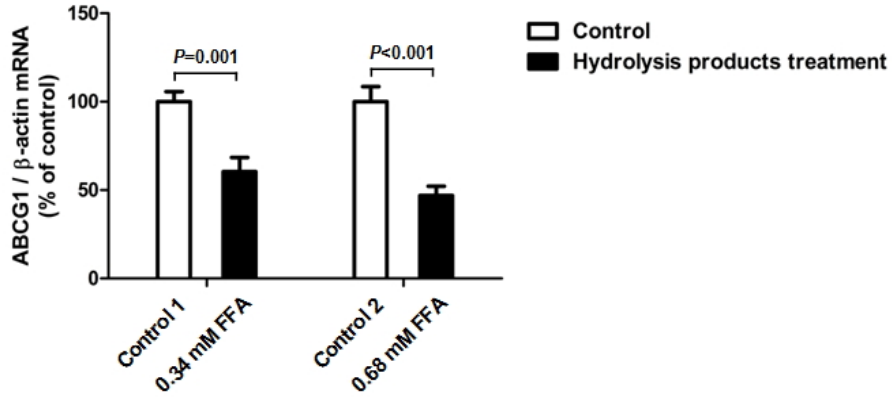
Differentiated THP-1 cells were treated in triplicate for 18 hours with 0.34 mM and 0.68 mM FFA (based on post-hydrolysis) of total lipoprotein hydrolysis products generated by LPL. A: The mRNA levels for ABCA1, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. A significant difference was observed for the mRNA levels of ABCA1 between the low concentration and high concentration hydrolysis products treatments (two-way ANOVA with a Sidak multiple comparison test. *, $p<0.01$). B: The mRNA levels for ABCG1, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of ABCG1 between the low concentration and high concentration hydrolysis products treatments (two-way ANOVA with a Sidak multiple comparison test). C: The mRNA levels for SR-BI, normalized to the mRNA levels of β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of SR-BI between the low concentration and high concentration hydrolysis products treatments (two-way ANOVA with a Sidak multiple comparison test). Control 1 and 2, represent the treatment of cells with total lipoprotein hydrolysis products generated by conditioned media with no lipase; respectively, they are the controls for the 0.34 mM and 0.68 mM FFA treatment. All data are means ± SD; *p* values, where written, indicate significance between control and treatment, based on Student's *t*-test.

A

Figure 11



B



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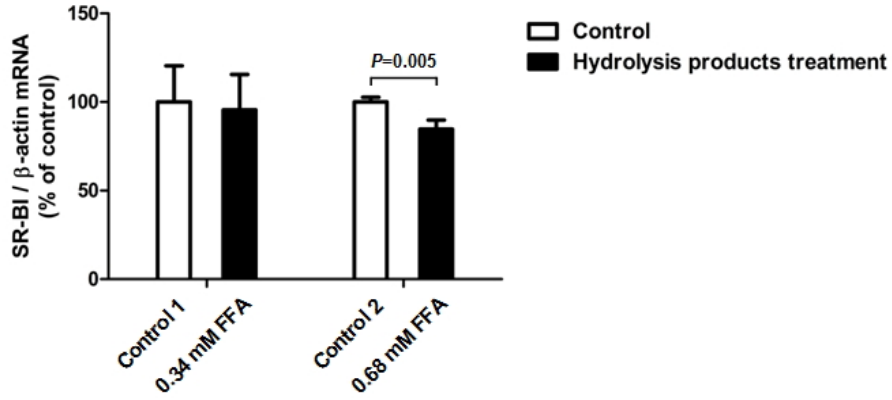
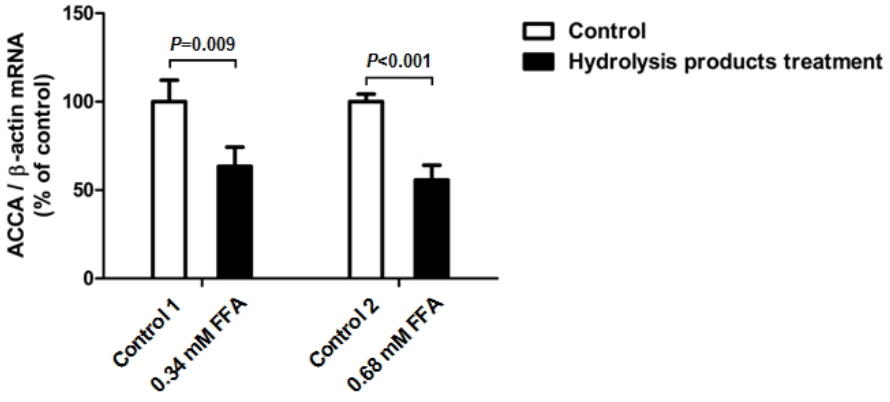


Figure 12: mRNA levels for enzymes involves in FFA synthesis in macrophages incubated with lipoprotein hydrolysis products

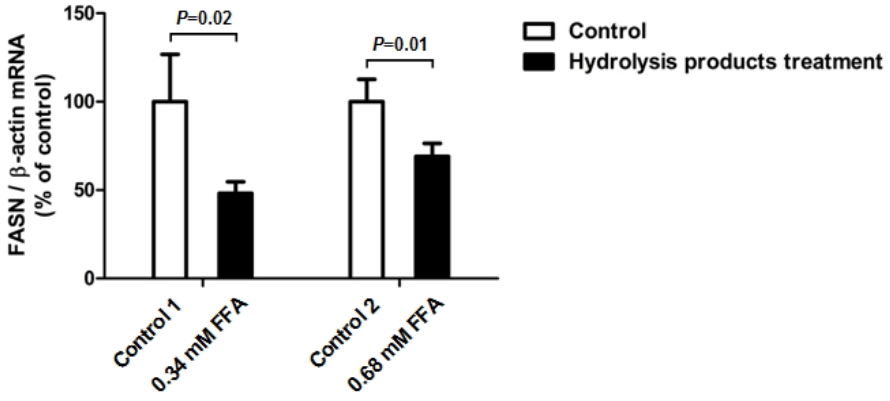
Differentiated THP-1 cells were treated in triplicate for 18 hours with 0.34 mM and 0.68 mM FFA (based on post-hydrolysis) of total lipoprotein hydrolysis products generated by LPL. A: The mRNA levels for ACCA, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of ACCA between the low concentration and high concentration hydrolysis products treatments (two-way ANOVA with a Sidak multiple comparison test). B: The mRNA levels for FASN, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of FASN between the low concentration and high concentration hydrolysis products treatments (two-way ANOVA with a Sidak multiple comparison test). C: The mRNA levels for SCD-1, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of SCD-1 between the low concentration and high concentration hydrolysis products treatments (two-way ANOVA with a Sidak multiple comparison test). Control 1 and 2, represent the treatment of cells with total lipoprotein hydrolysis products generated by conditioned media with no lipase; respectively, they are the controls for the 0.34 mM and 0.68 mM FFA treatment. All data are means ± SD; *p* values, where written, indicate significance between control and treatment, based on Student's *t*-test.

A

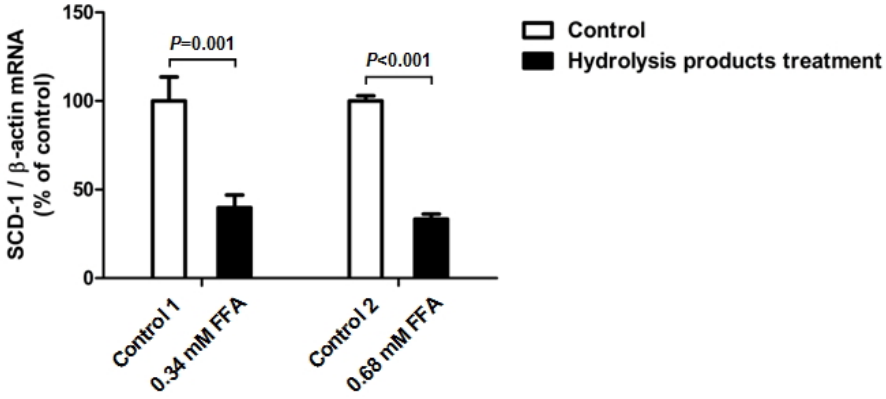
Figure 12



B



C



hydrolysis products generated by LPL reduced the mRNA levels for ACCA by 37% and 44%, respectively (Figure 12A), FASN by 52% and 31%, respectively (Figure 12B), and SCD-1 by 60% and 67%, respectively (Figure 12C). No significant differences were observed for the mRNA levels of ACCA, FASN, and SCD-1 between the low concentration and high concentration hydrolysis products treatments.

3.3.4 The mRNA levels for LPL were significantly decreased by LPL lipoprotein hydrolysis products

In order to find out whether or not a feedback mechanism of LPL exists in macrophages, we investigated the influence of lipoprotein hydrolysis products generated by LPL on the transcript for the gene encoding LPL. The real-time PCR results showed that both the low concentration (0.34 mM FFA) and high concentration (0.68 mM FFA) of lipoprotein hydrolysis products significantly reduced the mRNA level for LPL by 56% and 69%, respectively (Figure 13). No significant difference was observed between the low concentration and high concentration hydrolysis products treatments.

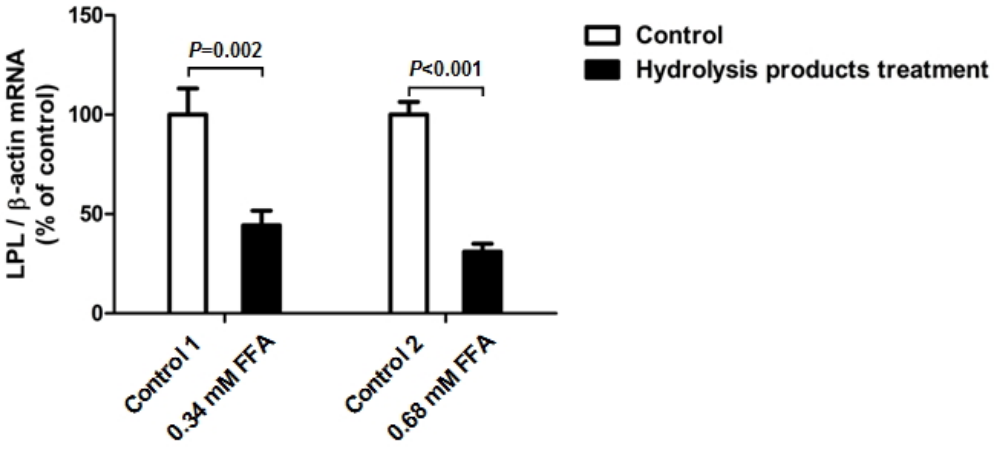
3.4 The pro-atherogenic influence of the purified FFA mixture on intracellular lipid homeostasis

In order to investigate the influence of the FFA liberated from total lipoproteins by LPL on intracellular lipid storage, a mixture of FFA that matched the concentrations of individual FFA species that were present in lipoprotein hydrolysis products generated

Figure 13: mRNA levels for LPL in macrophages incubated with lipoprotein hydrolysis products

Differentiated THP-1 cells were treated in triplicate for 18 hours with 0.34 mM and 0.68 mM FFA (based on post-hydrolysis) of total lipoprotein hydrolysis products generated by LPL. The mRNA levels for LPL, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of LPL between the low concentration and high concentration hydrolysis products treatments (two-way ANOVA with a Sidak multiple comparison test). Control 1 and 2, represent the treatment of cells with total lipoprotein hydrolysis products generated by conditioned media with no lipase; respectively, they are the controls for the 0.34 mM and 0.68 mM FFA treatment. All data are means ± SD; *p* values, where written, indicate significance between control and treatment, based on Student's *t*-test.

Figure 13


LPL / β-actin mRNA
(% of control)
150
100
50
0
P=0.002
P<0.001
Control 1
0.34 mM FFA
Control 2
0.68 mM FFA
Control
Hydrolysis products treatment

by LPL was prepared and incubated with differentiated THP-1 cells to assess its effect on lipid droplets. THP-1 macrophages were incubated overnight with low (0.34 mM) and high (0.68 mM) concentrations of the FFA mixture (in serum-free culture media containing PMA and 0.2% w/v FAF-BSA); cells were subsequently stained with Oil red O to stain the intracellular neutral lipids. Figure 14 shows that compared to cells treated with the vehicle control (of DMSO in media) (Figure 14A), Oil red O positive lipid droplets were clearly present in the THP-1 macrophages incubated with either a low concentration (0.34 mM) (Figure 14B) or a high concentration (0.68 mM) (Figure 14C) of the purified FFA mixture. Moreover, THP-1 macrophages incubated with the purified FFA mixture led to the development of foam cell-like macrophages: macrophages that were incubated with the high concentration of the purified FFA mixture (0.68 mM) presented with the most pronounced foam cell-like phenotype (Figure 14C), whereas macrophages incubated with the vehicle control (no FFA) did not exhibit a foam cell-like appearance (Figure 14A).

3.5 The negative influences of the purified FFA mixture on gene transcripts within human THP-1 macrophages

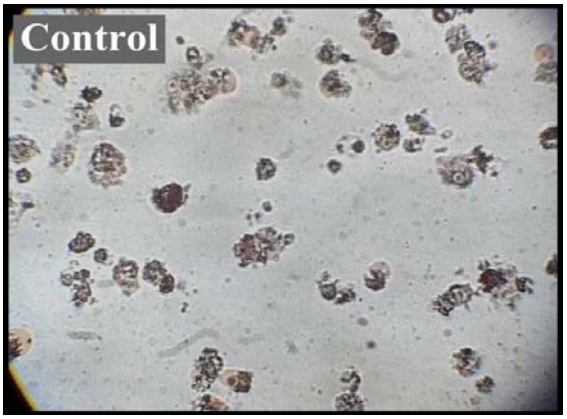
The results from quantitative real-time PCR analyses demonstrate that a purified FFA mixture, that matches the amounts to those added to cells from human plasma total lipoproteins hydrolysed by LPL, yield comparable results to those for lipoprotein

Figure 14: Oil red O Staining

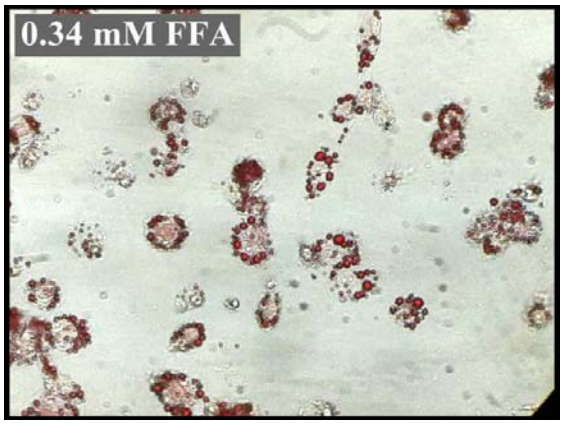
Differentiated THP-1 cells were treated for 18 hours with A: the vehicle control (of DMSO in media); B: with a low concentration (0.34 mM) of purified FFA mixture; and C: with a high concentration (0.68 mM) of purified FFA mixture. Cells were subsequently stained with Oil red O. Data are representative of triplicate experiments.

Figure 14

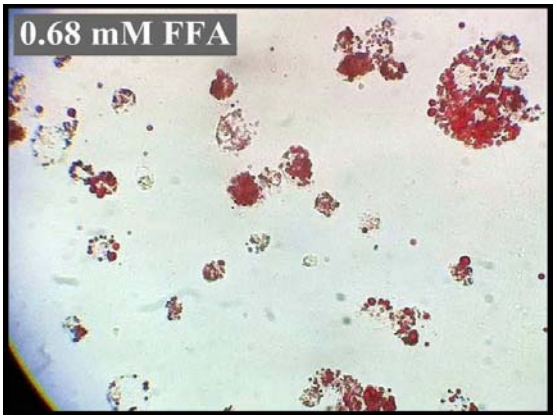
A



B



C



hydrolysis products generated by LPL on gene transcription.

3.5.1 The mRNA levels for nuclear receptors LXR-α, PPAR-α, and PPAR-γ were significantly decreased by the purified FFA mixture

The low concentration (0.34 mM) and the high concentration (0.68 mM) of the purified FFA mixture treatments of THP-1 macrophages for 18 hours significantly reduced the mRNA levels for LXR-α (by 74% and 33%, respectively) (Figure 15A) and PPAR-γ (by 47% and 28%, respectively) (Figure 15C). However, neither the low concentration (0.34 mM) nor the high concentration (0.68 mM) of the purified FFA mixture had a significant influence on the level of transcripts for gene encoding PPAR-α (Figure 15B). No significant differences were observed for the mRNA levels of LXR-α, PPAR-α, and PPAR-γ between the low and high concentrations of FFA treatments.

3.5.2 The mRNA levels for cholesterol transporters ABCA1, ABCG1, and SR-BI were significantly decreased by the purified FFA mixture

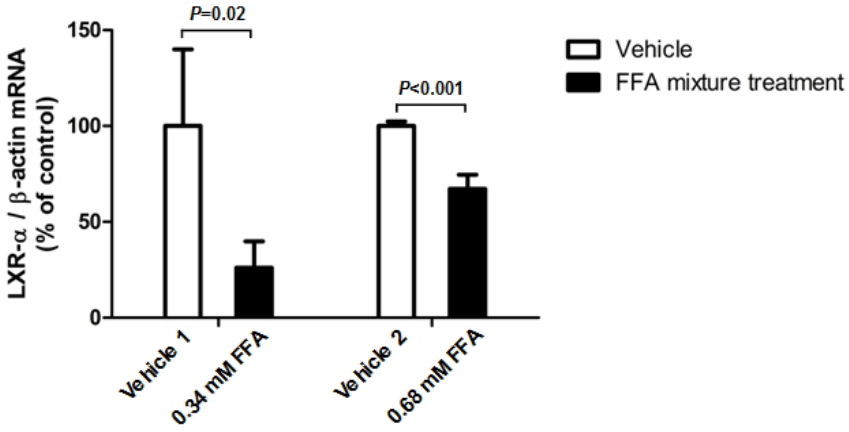
Both the low concentration (0.34 mM) and high concentration (0.68 mM) of the purified FFA mixture significantly reduced the mRNA levels for ABCA1 (by 70% and 27%, respectively), (Figure 16A) ABCG1 (by 83% and 67%, respectively) (Figure 16B), and SR-BI (by 42% and 20%, respectively) (Figure 16C). A significant difference was observed for the mRNA levels of ABCA1 between the low concentration and high concentration treatments, while there was no significant difference for the mRNA levels

Figure 15: mRNA levels for nuclear receptors in macrophages incubated with purified FFA mixture

Differentiated THP-1 cells were treated in triplicate for 18 hours with a mixture of purified FFA (that represents the species' of FFA liberated by LPL from total lipoproteins). A: The mRNA levels for LXR-α, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of LXR-α between the low concentration and high concentration FFA mixture treatments (two-way ANOVA with a Sidak multiple comparison test). B: The mRNA levels for PPAR-α, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of PPAR-α between the low concentration and high concentration FFA mixture treatments (two-way ANOVA with a Sidak multiple comparison test). C: The mRNA levels for PPAR-γ, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of PPAR-γ between the low concentration and high concentration FFA mixture treatments (two-way ANOVA with a Sidak multiple comparison test). Vehicle 1 is the control for 0.34 mM FFA, and Vehicle 2 is the control for 0.68 mM FFA. All data are means ± SD; *p* values, where written, indicate significance between control and treatment, based on Student's *t*-test.

A

Figure 15



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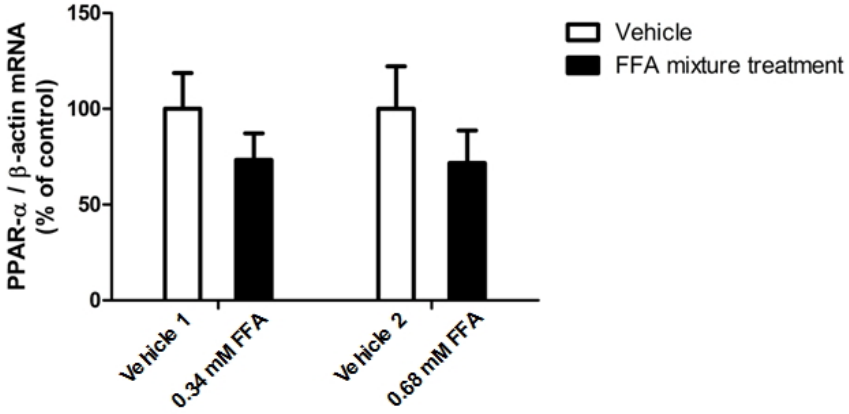


C

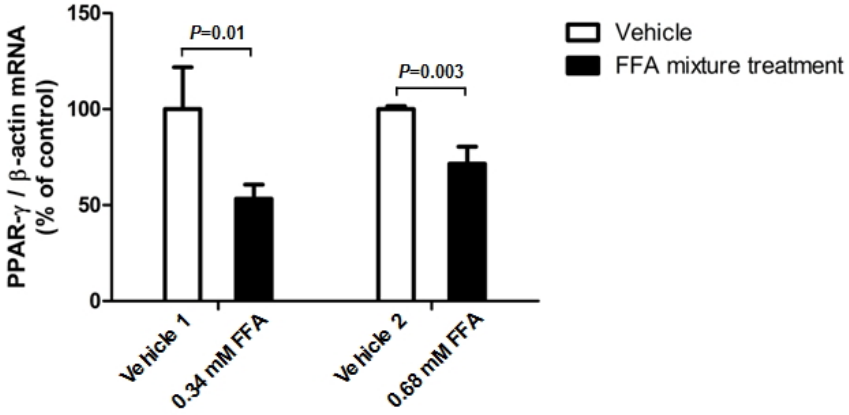
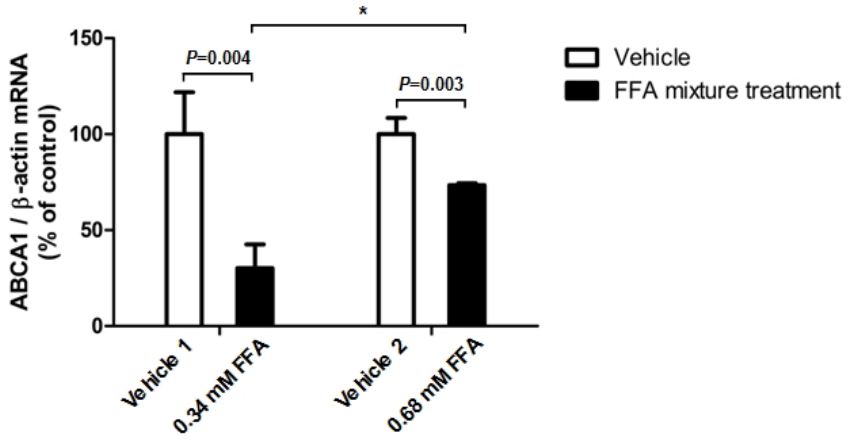


Figure 16: mRNA levels for cholesterol transporters in macrophages incubated with purified FFA mixture

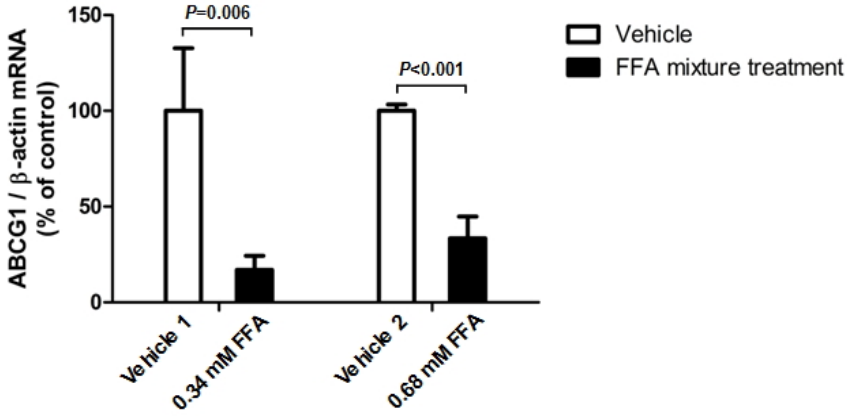
Differentiated THP-1 cells were treated in triplicate for 18 hours with a mixture of purified FFA (that represents the species' of FFA liberated by LPL from total lipoproteins). A: The mRNA levels for ABCA1, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. A significant difference was observed for the mRNA levels of ABCA1 between the low concentration and high concentration FFA mixture treatments (two-way ANOVA with a Sidak multiple comparison test. *, $p<0.01$). B: The mRNA levels for ABCG1, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of ABCG1 between the low concentration and high concentration FFA mixture treatments (two-way ANOVA with a Sidak multiple comparison test). C: The mRNA levels for SR-BI, normalized to the mRNA levels of β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of SR-BI between the low concentration and high concentration FFA mixture treatments (two-way ANOVA with a Sidak multiple comparison test). Vehicle 1 is the control for 0.34 mM FFA, and Vehicle 2 is the control for 0.68 mM FFA. All data are means ± SD; *p* values, where written, indicate significance between control and treatment, based on Student's *t*-test.

Figure 16

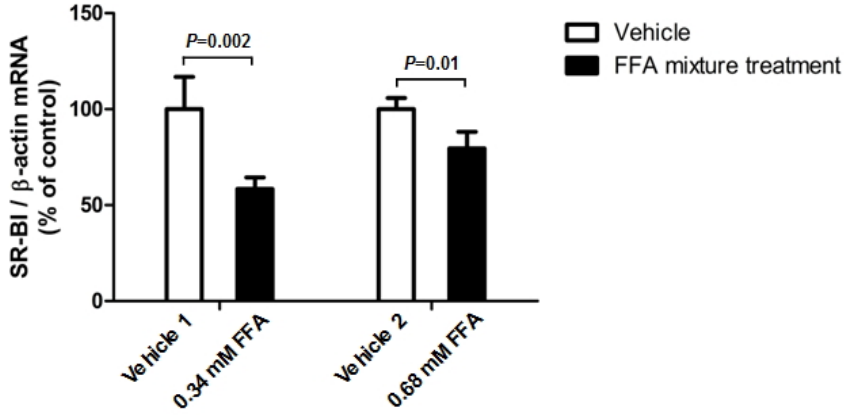
A



B



C



of ABCG1 and SR-BI between the two treatments.

3.5.3 The mRNA levels for enzymes involved in FFA synthesis were significantly decreased by the purified FFA mixture

As shown in Figure 17, both the low (0.34 mM) and high (0.68 mM) concentrations of the purified FFA mixture markedly decreased the mRNA levels for ACCA (by 57% and 34%, respectively) (Figure 17A), while only the low concentration of the purified FFA mixture significantly decreased the transcription of genes for FASN and SCD-1 by 49% (Figure 17B) and 79% (Figure 17C), respectively. No significant difference was observed for the mRNA levels of ACCA, FASN, and SCD-1 between the low concentration and high concentration FFA treatments.

3.5.4 The mRNA levels for SREBP-1c were significantly decreased by the purified FFA mixture

Both the low concentration (0.34 mM) and the high concentration (0.68 mM) of the purified FFA mixture significantly decreased the mRNA levels for SREBP-1c (by 75% and 85%, respectively) (Figure 18). No significant difference was observed for the mRNA levels of SREBP-1c between the low concentration and high concentration FFA treatments.

3.5.5 The mRNA levels for LPL were unchanged by the purified FFA mixture

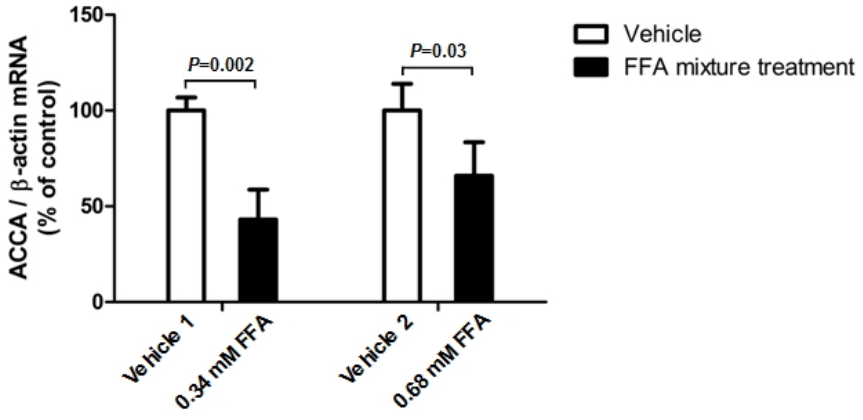
Unlike the observation on THP-1 macrophages incubated with lipoprotein

Figure 17: mRNA levels for enzymes involved in FFA synthesis in macrophages incubated with purified FFA mixture

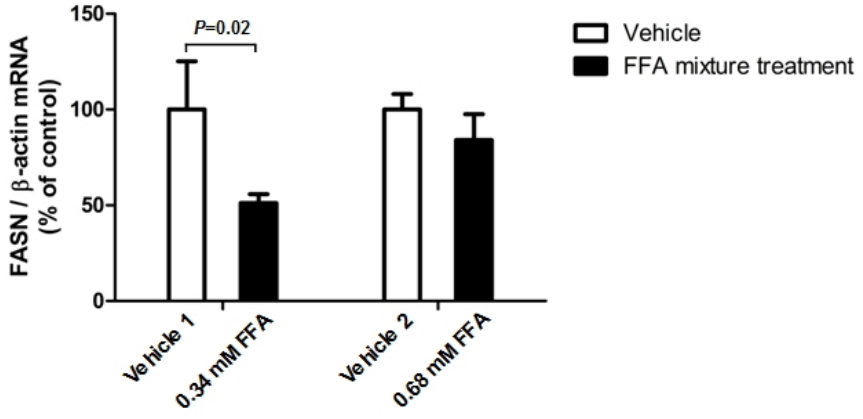
Differentiated THP-1 cells were treated in triplicate for 18 hours with a mixture of purified FFA (that represents the species' of FFA liberated by LPL from total lipoproteins). A: The mRNA levels for ACCA, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of ACCA between the low concentration and high concentration FFA mixture treatments (two-way ANOVA with a Sidak multiple comparison test). B: The mRNA levels for FASN, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of FASN between the low concentration and high concentration FFA mixture treatments (two-way ANOVA with a Sidak multiple comparison test). C: The mRNA levels for SCD-1, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of SCD-1 between the low concentration and high concentration FFA mixture treatments (two-way ANOVA with a Sidak multiple comparison test). Vehicle 1 is the control for 0.34 mM FFA, and Vehicle 2 is the control for 0.68 mM FFA. All data are means ± SD; *p* values, where written, indicate significance between control and treatment, based on Student's *t*-test.

A

Figure 17



B



C

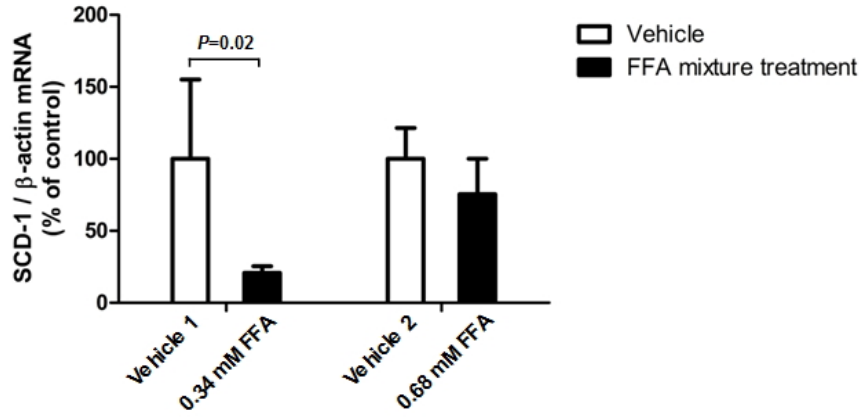
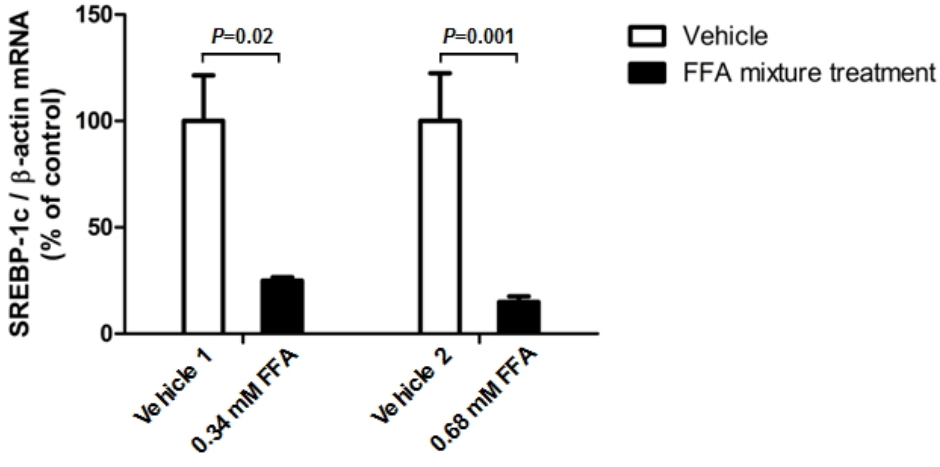


Figure 18: mRNA levels for SREBP-1c in macrophages incubated with purified FFA mixture

Differentiated THP-1 cells were treated in triplicate for 18 hours with a mixture of purified FFA (that represents the species' of FFA liberated by LPL from total lipoproteins). The mRNA levels for SREBP-1c, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. No significant difference was observed for the mRNA levels of SREBP-1c between the low concentration and high concentration FFA mixture treatments (two-way ANOVA with a Sidak multiple comparison test). Vehicle 1 is the control for 0.34 mM FFA, and Vehicle 2 is the control for 0.68 mM FFA. All data are means ± SD; *p* values indicate significance between control and treatment, based on Student's *t*-test.

Figure 18


SREBP-1c / β-actin mRNA
(% of control)
150
100
50
0
P=0.02
P=0.001
Vehicle
FFA mixture treatment
Vehicle 1
0.34 mM FFA
Vehicle 2
0.68 mM FFA

hydrolysis products generated by LPL, neither the low concentration (0.34 mM) nor the high concentration (0.68 mM) of the purified FFA mixture significantly influenced the mRNA levels for LPL versus vehicle controls (Figure 19). A significant difference was observed for the mRNA levels of LPL between the high concentration and low concentration FFA treatments.

3.6 The purified FFA mixture negatively influences apolipoprotein A-I mediated cholesterol efflux

We tested whether or not the reduced mRNA levels for cholesterol transporters in THP-1 macrophages treated with FFA would translate into an attenuated ability of macrophages to efflux cholesterol. Compared to vehicle control treated cells, the efflux of cholesterol to apoA-I from THP-1 macrophages labeled with [^{3}H]cholesterol was significantly reduced from 3.7% to 1.3% when pre-incubated with the 0.68 mM purified FFA mixture (Figure 20).

Figure 19: mRNA level for LPL in macrophages incubated with purified FFA mixture

Differentiated THP-1 cells were treated in triplicate for 18 hours with a mixture of purified FFA (that represents the species' of FFA liberated by LPL from total lipoproteins). The mRNA levels for LPL, normalized to the mRNA levels for β-actin, were quantified in triplicate by real-time PCR. A significant difference was observed for the mRNA levels of LPL between the low concentration and high concentration FFA mixture treatments (two-way ANOVA with a Sidak multiple comparison test. *, $p<0.05$). Vehicle 1 is the control for 0.34 mM FFA, and Vehicle 2 is the control for 0.68 mM FFA. All data are mean ± SD; *p* values, where written, indicate significance between control and treatment, based on Student's *t*-test.

Figure 19

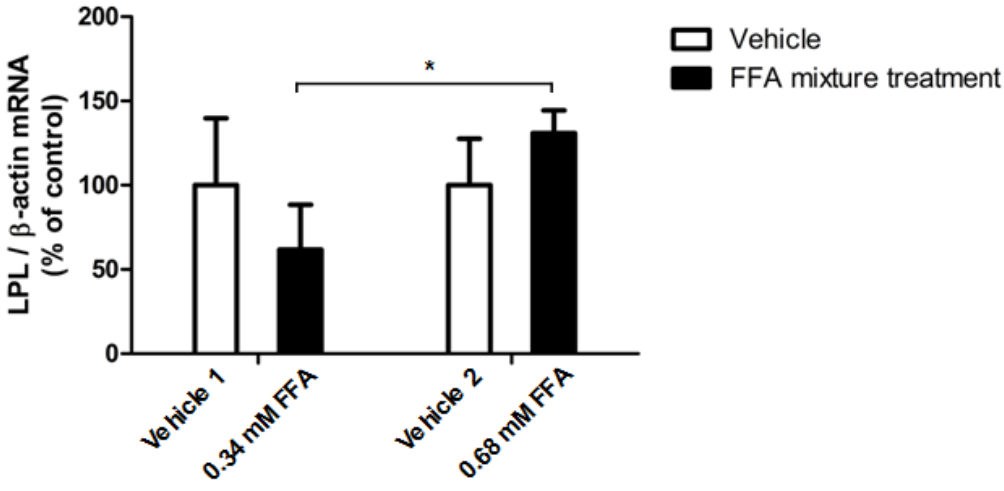
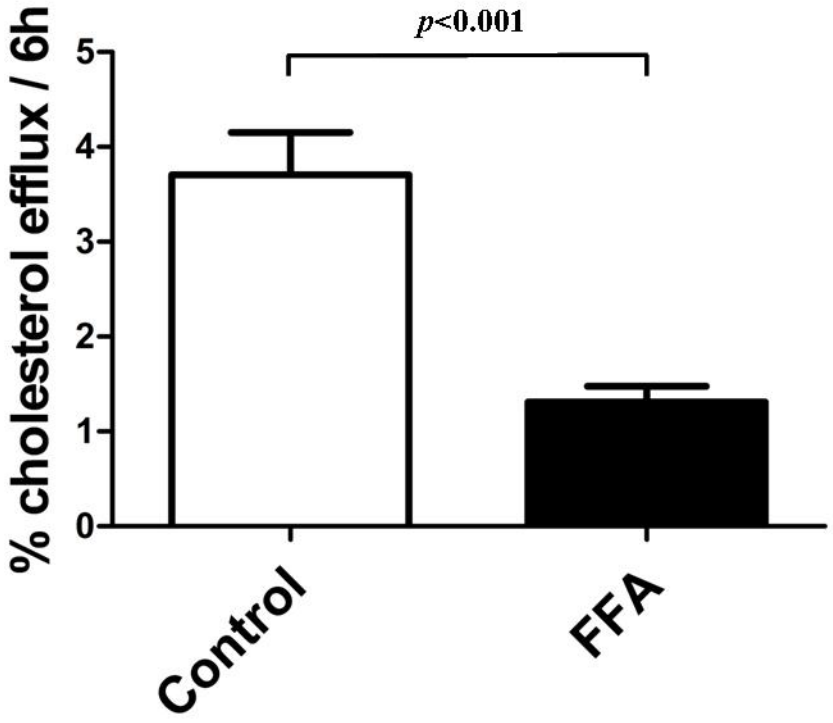

LPL / β-actin mRNA
(% of control)
200
150
100
50
0
*
Vehicle
FFA mixture treatment
Vehicle 1
0.34 mM FFA
Vehicle 2
0.68 mM FFA

Figure 20: The FFA component of total lipoprotein hydrolysis by LPL attenuated apoA-I mediated cholesterol efflux

Differentiated THP-1 cells labelled with [^{3}H]cholesterol were treated for 18 hours with a mixture of purified FFA (0.68 mM) (as described in *section 2.5.2*), then subsequently tested for cholesterol efflux ability to apoA-I over 6h. Efflux was calculated as a percentage of media [^{3}H]cholesterol per total cell and media [^{3}H]cholesterol. All data were corrected for control efflux experiments to 0.2% w/v FAF-BSA. Data are means ± SD; n=6. Statistical analyses were performed using a Student's t-test.

Figure 20



Chapter 4: Discussion

4.1 Discussion

4.1.1 Shotgun lipidomic analyses of lipoprotein hydrolysis products generated by LPL provided insight into the activities of LPL at the molecular level

In this study, LPL liberated from total lipoproteins all of the FFA species that were assessed by GC-MS. This was tied to significantly decreased levels of individual species of TG and PtdCho. While LPL preferentially hydrolyses TG, it does have a phospholipase A_1 activity (208). The significant hydrolysis of lipoprotein PtdCho in the current study by LPL suggests that once a maximal degree of hydrolysis of TG by LPL was reached, LPL likely would move toward hydrolysing PtdCho. In contrast, the lack of significant hydrolysis of lipoprotein lysoPtdCho suggested that LPL may not have an activity toward lysoPtdCho *in vitro*.

In the hydrolysis reactions with mock-transfected media, we unexpectedly observed that the levels for 5 species of FFA were increased versus the hydrolysis reaction in the presence of PBS. However, the comparable levels of individual species of TG and PtdCho between lipoproteins incubated with either mock-transfected media or PBS indicated that the mock-transfected media does not exhibit a significant LPL activity. Thus, I speculate that the significantly elevated levels for 5 species of FFA in the incubation of mock-transfected media with lipoproteins are derived from the HEK-293T

cells and not from the hydrolysis reaction.

4.1.2 The lipoprotein hydrolysis products generated by LPL negatively modulate the transcripts for genes encoding PPARs and LXR-α

In the current study, by using quantitative real time-PCR analyses, we discovered that the hydrolysis products generated by LPL from total lipoproteins significantly reduced the mRNA levels for the nuclear receptors PPAR-α, PPAR-γ, and LXR-α, while the FFA component of the hydrolysis products suppressed the transcripts for genes encoding LXR-α and PPAR-γ. The unchanged mRNA levels for PPAR-α in macrophages incubated with the purified FFA mixture suggests that other components from the LPL lipoprotein hydrolysis, such as diglycerides and liberated proteins, are likely responsible for the suppressive effects on PPAR-α. It is unlikely that lysoPtdCho contributed in this study, as their levels were not different following hydrolysis. The decreased mRNA levels for LXR-α in macrophages incubated with the purified FFA mixture might be in part a result of down-regulation by PPAR-γ, since the unsaturated FFA arachidonic acid blocks the ligand-dependent activation of LXR-α without influencing the basal expression of LXR-α (209).

On the other hand, given Inoue *et al.* (210) previously reported that individual species of FFA differently affect the mRNA level of PPAR-γ in human umbilical vein endothelial cells (i.e. oleic acid and eicosapentaenoic acid lead to an increase in PPAR-γ

mRNA levels, while arachidonic acid and linoelaidic acid lead to a decrease in PPAR-γ mRNA levels), I speculate that the net effects of the FFA mixture resulted in a decrease in PPAR-γ mRNA. Furthermore, some other mechanism may also exist to account for the decreased mRNA level of PPAR-γ in macrophages incubated with the lipoprotein hydrolysis products generated by LPL. Previous studies have reported that TNF-α acts to down regulate the transcript of the gene encoding PPAR-γ in adipocytes, and the expression levels of TNF-α are inversely related to LPL activity (211, 212). Thus, I speculate that other components from LPL lipoprotein hydrolysis products, but not FFA, may trigger a feed back regulation, such that the expression of TNF-α may be increased, thus leading to decrease in LPL mRNA plus a suppression of the transcription of the gene encoding PPAR-γ. The exact mechanism by which LPL lipoprotein hydrolysis products suppresses the transcripts of genes encoding PPARs and LXR-α merits further study, which may tie a pro-inflammatory response to hydrolysis products with the mechanism.

4.1.3 The suppressed transcripts of genes encoding nuclear receptors likely further result in lowered mRNA levels for cholesterol transporters

Given the identified roles of PPAR-α and PPAR-γ in directly or indirectly (through LXR-α) modulating the transcripts of genes encoding the cholesterol transporters ABCA1, ABCG1, and SR-BI (173, 176-178, 180, 213, 214), it was anticipated that the decrease of PPARs and LXR-α mRNA expression, caused by total

lipoprotein hydrolysis products generated by LPL, would contribute to a suppression of the transcripts of genes encoding ABCA1, ABCG1 and SR-BI. As expected, we found that the transcripts encoding these cholesterol transporters were indeed reduced. Notably, the low concentration FFA mixture was shown to significantly decrease the SR-BI mRNA level. While it would be expected that a similar result would be observed from the low concentration hydrolysis products treatment (since the low concentration of hydrolysis products has the exact same FFA profile as the low concentration FFA mixture), the low concentration of hydrolysis products did not have a significant effect on SR-BI mRNA level. Since a significantly suppressed transcript for SR-BI was observed in macrophages incubated with high concentration hydrolysis products, it is unlikely that other components from the hydrolysis products are offsetting the FFA induced reduction in SR-BI mRNA level. The exact explanation behind this discrepancy remains unknown.

Uehara *et al.* (215) previously showed that unsaturated FFA can markedly down-regulate the expression of ABCA1 without changing the expression of LXR-α. They further found that unsaturated FFA could suppress the expression of ABCG1 and inhibit the activity of ABCA1 and ABCG1 promoters by a mechanism that involves the binding of LXR/RXR to the direct repeat 4 (DR4) element (which is a positive regulatory element for LXR/RXR) (216). Similarly, Yoshikawa *et al.* (217) have clearly demonstrated that polyunsaturated FFA can suppress the promoter activity of SREBP-1c

by inhibiting the binding of LXR-α, which suggested that these species of FFA have the ability to modulate the target genes of LXR-α via post-transcriptional regulation. Taken together, given that the purified FFA mixture contains both polyunsaturated FFA and monounsaturated FFA (Table 1), I speculate that in addition to the suppression of transcripts for ABCA1, ABCG1, and SR-BI via the transcriptional cascade in the PPARs and LXR-α pathways (as described in *section 1.4.3*), the FFA may also act to reduce the mRNA levels for these cholesterol transporters by negatively modulating LXR-α at the post-translational level by inhibiting the binding of LXR to the promoters of ABCA1, ABCG1, and SR-BI, which contains a DR4 element. DR4 (or LXRE) exists within the promoters of ABCA1, ABCG1, and SR-BI, and they are located between residues -803 and +165, residues -1104 and +37, and residues -1200 to -937, respectively (215, 216, 218). However, the exact mechanism by which LPL lipoprotein hydrolysis products suppresses the transcripts of genes encoding cholesterol transporters remains to be determined and warrants further study.

Interestingly, although both the high and low concentration of hydrolysis products/FFA mixture reduced the ABCA1 mRNA level, results from the two-way ANOVA analysis indicated that the low concentration yielded a more potent effect than the high concentration. However, the mechanisms behind this are currently unknown.

4.1.4 The lowered mRNA levels for cholesterol transporters are tied to an attenuated ability for THP-1 macrophages to efflux cholesterol to apoA-I

Inducing the transcripts and/or expression of cholesterol transporters by the nuclear receptors PPARs and LXR was shown to significantly promote cholesterol efflux and RCT (181, 182, 219, 220). In contrast, lowering the expression or increasing the degradation of cholesterol transporters markedly attenuated cholesterol efflux and promotes atherogenesis (183, 221). Consistent with these previous observations, the current study shows that suppressing the transcripts for ABCA1, ABCG1, and SR-BI by the FFA component of hydrolysis products was tied to a reduction of the ability for THP-1 macrophages to efflux cholesterol to apoA-I.

Wang *et al.* (221) showed that unsaturated FFAs act to attenuate the ability of macrophages to efflux cholesterol to apoA-I by increasing the degradation of ABCA1 and decreasing the binding of apoA-I to ABCA1 at the cell surface. Thus, given that the purified FFA mixture contains unsaturated FFA, it is possible that in addition to the negative modulation on the transcripts of gene encoding ABCA1, the lipoprotein hydrolysis products generated by LPL might also function to reduce the ability for THP-1 macrophages to efflux cholesterol to apoA-I by inducing the degradation of ABCA1 and/or decreasing the binding of apoA-I to ABCA1/cell surface.

Notably, this study chose to carry out cholesterol efflux assays to evaluate the

influence of purified FFA on the ability of macrophages to efflux cholesterol. The effect of hydrolysis products was not evaluated because the lipoprotein remnants in the hydrolysis products could function as cholesterol receptors, which might give unreliable and irreproducible results for apoA-I mediated cholesterol efflux ratio.

In addition to a reduction to ABCA1 mRNA levels, ABCG1 mRNA levels were also decreased by both lipoprotein hydrolysis products generated by LPL, and the FFA mixture. It is likely that cholesterol efflux to small, mature HDL particles (such as HDL_3) would be reduced, which warrants testing in future studies.

4.1.5 The purified FFA mixture led to an accumulation of lipid within macrophages

Results from my study also show that suppressing the transcripts for ABCA1, ABCG1, and SR-BI by the FFA component of hydrolysis products was tied to an accumulation of intracellular lipids and foam cell-like formation. The FFA component of hydrolysis products induced lipid accumulation within macrophages, which is consistent with previous reports: i) macrophage and smooth muscle cell LPL can locally liberate hydrolysis products from lipoproteins and further facilitate the cellular uptake of lipids (119, 139, 140, 222); and ii) macrophages with high LPL expression levels and activity are more easily converted to foam cells, and the amounts of extracellular FFA localized with these cells are greater than that for macrophages with normal levels of LPL (223). In the current study, although unlikely, the lipid accumulation within macrophages that were

incubated with the purified FFA mixture may just simply be a result of increased storage of the lipid. However, it may also be part of an unknown effect of a FFA-induced negative modulation on LXR, as LXR deficiency in mice leads to an increase in foam cell formation from macrophages (224).

4.1.6 The lowered mRNA levels for enzymes involved in FFA synthesis are likely due to lowered LXR-α expression

The current study also shows that consistent with the suppressed transcript levels of LXR-α, the mRNA levels for the downstream target genes ACCA, SCD-1 and FASN were all reduced. Of these enzymes that are involved in FFA synthesis, FASN was shown to play a promotional role in atherosclerosis: Schneider *et al.* (225) observed that a deficiency of macrophage FASN in apoE-null mice acts to significantly attenuate atherogenesis by inducing the transcription of LXR-α, which will favour cholesterol efflux and attenuate cholesterol uptake by increasing the expression of ABCA1 and decreasing the expression of cluster of differentiation 36, respectively.

SCD-1 was shown to exhibit properties that are both pro- and anti-atherogenic. The pro-atherogenic properties include: i) SCD-1 activity is positively associated with plasma TG levels, which is deemed to be a significant independent risk factor of cardiovascular disease (226); and ii) SCD-1 and its products function to attenuate ABCA1 mediated cholesterol efflux by elevating the destabilization of ABCA1 (215, 221,

227-229). The anti-atherogenic property of SCD-1 represents its critical role in the homeostasis of the pro-atherogenic lipoprotein LDL (229-231). Specifically, a deficiency of SCD-1 in mice results in a significantly increased plasma LDL level, likely as a result of a decreased LDL clearance rate (230).

Taken together, due to the different properties of FASN (which exhibits a pro-atherogenic property), SCD-1 (which exhibits properties that are both pro- and anti-atherogenic), and ACCA (which does not seem to have a detrimental role in atherogenesis), the net effects of the suppressed transcripts of these enzymes, as found in the current study, on atherosclerosis remain to be investigated in future studies.

The mechanism by which lipoprotein hydrolysis products generated by LPL suppresses the transcripts of genes encoding ACCA, FASN and SCD-1 is not resolved. Knowing SREBP-1c functions to modulate lipid homeostasis in vertebrate cells (187), in part by directly targeting the transcripts encoding ACCA, FASN, and SCD-1 (188-191), and given that Hannah, *et al.* (232) previously demonstrated that unsaturated FFAs could significantly repress the transcript encoding SREBP-1c, it was expected that the purified FFA mixture would suppress the transcription of genes involved in FFA synthesis via a reduction of the mRNA levels for SREBP-1c. As expected, we found that the purified FFA mixture in the current study indeed dramatically reduced the mRNA level for SREBP-1c (Figure 18). Moreover, since SREBP-1c is a downstream target gene of

LXR-α, and given the current study shows that the purified FFA significantly suppresses the transcripts encoding LXR-α, I speculate that the FFA can be involved in regulating cellular FFA homeostasis, partially by acting on the identified transcriptional cascades in the LXR-α and SREBP-1c pathways (Figure 4) (184).

The formation of TG can protect cells from any toxic effects due to sudden increases of FFA influx (233). Though not tested in the current study, I speculate that the uptake of FFA from LPL mediated lipoprotein hydrolysis by macrophages would result in increased cellular TG synthesis. It has been shown that the activation of PPAR-α promotes β-oxidation (234). Given the observed decrease in PPAR-α mRNA within macrophages incubated with LPL lipoprotein hydrolysis products, I also speculate that β-oxidation might be suppressed in the macrophages, which will favour the formation of TG.

Esterification of cholesterol to cholesteryl esters by acyl-coenzyme A:cholesterol acyltransferase (ACAT) is critical for protecting macrophages from cholesterol toxicity (147). Given the observed attenuation of cholesterol efflux capacity in macrophages incubated with the purified FFA mixture, and knowing PPAR-γ acts to negatively modulate the expression of ACAT in macrophages (235, 236), it is anticipated that the macrophages incubated with the FFA mixture would protect themselves from the accumulated cholesterol and/or sudden increase of FFA influx by increasing the activity

of ACAT, which will promote intracellular esterification of cholesterol and the FFA.

4.1.7 The lowered mRNA levels for LPL by LPL lipoprotein hydrolysis products suggest the existence of a negative feedback loop for the regulation of LPL

Lipoprotein hydrolysis products generated by LPL markedly decreased the transcripts encoding LPL within THP-1 macrophages, which suggests the existence of a negative feedback loop for LPL regulation. This might be regarded as an anti-atherosclerosis mechanism, since the negative feedback regulation of LPL will decrease the synthesis of lipoprotein hydrolysis products, thus temporarily reducing the burden on the macrophages from the pro-atherogenic activities of these lipoprotein hydrolysis products. The suppression of transcripts encoding LPL may be due in part to the reduced levels of PPARs and LXR-α. This speculation is supported by the observation that LXR-α exhibits an ability to regulate the transcription of LPL by binding to the LXRE of the LPL promoter, as well as the observation that the activation of either PPAR-α or PPAR-γ in human monocyte derived macrophages significantly elevates the levels of LPL mRNA and the secretion of LPL protein (81, 237). In contrast, in the current study, the purified FFA mixture significantly reduced the mRNA levels for PPAR-γ, but no appreciable changes were observed at the transcriptional level for LPL in macrophages that were incubated with the purified FFA mixture. This result suggests that different from the regulatory effects of lipoprotein hydrolysis products on the

macrophage LPL transcripts (as stated above), other factors that are independent of PPARs may be involved in the regulatory effects of FFA on macrophage LPL transcripts, such as through the PKC/c-*fos* pathway (238-244). Michaud *et al.* (244) previously reported that i) different species of FFA yield different effects on the mRNA levels for LPL (i.e. the incubation of arachidonic acid and eicosapentaenoic acid with macrophages reduce the mRNA levels for LPL; the incubation of linoleic acid, palmitic acid, and stearic acid with macrophages increase the mRNA levels for LPL; the incubation of oleic acid with macrophages has no effect on mRNA levels for LPL); and ii) the incubation of macrophages with arachidonic acid and eicosapentaenoic yield parallel effects on reducing the mRNA levels for c-*fos* and LPL. Taken together, it is possible that the effects of each species of FFA in the purified FFA mixture in the current study on modulating the mRNA levels for LPL could cancel each other and result in no net effect, but other components within the lipoprotein hydrolysis products generated by LPL are likely responsible for the observed decrease in LPL mRNA in the THP-1 macrophages.

4.2 Overall conclusion

The current results establish that the lipoprotein hydrolysis products generated by LPL can act to suppress the transcript of genes for encoding nuclear receptors LXR-α and PPARs in cultured human THP-1 macrophages. This suppression appears to explain, at least partially, the ability of LPL lipoprotein hydrolysis products to lower the mRNA levels

for cholesterol transporters, the transcription of which has been shown to be regulated by PPARs and LXR-α (173, 176-178, 180, 213, 214). Moreover, the lowered transcripts for cholesterol transporters, which led to an attenuation of the THP-1 macrophages to efflux cholesterol to apoA-I, could contribute to the promotion of atherosclerosis development.

4.3 Direction of future study

Previous studies have shown that macrophage LPL exhibits a positive correlation with atherogenesis; however, the mechanism underlying any pro-atherogenic property of macrophage LPL remains poorly understood. Revealing the negative influences by the total lipoprotein hydrolysis products generated by LPL on cholesterol efflux provides new insight into the mechanism underlying the pro-atherogenic property of macrophage LPL. Since the hydrolysis products released from substrates with different lipid/lipoprotein profiles might have different effects on cholesterol efflux, future studies investigating the influence of LPL hydrolysis products liberated from total lipoproteins collected from dyslipidemic subjects on cholesterol efflux is necessary.

Moreover, given macrophage specific suppression of LPL using lentivirus-mediated RNA interference has been well established (141), future studies examining the impact of lipoprotein hydrolysis products on cholesterol efflux due to an RNA interference mediated reduction of endogenous macrophage LPL might reveal a potentially beneficial effect toward RCT. It is anticipated that the elimination of LPL will

facilitate cholesterol efflux by protecting the macrophages from taking up pro-atherogenic hydrolysis products. However, future study also is required to exclude other sources of hydrolysis products and their effects on cholesterol efflux. Firstly, LPL, HL, and EL may exhibit compensatory activities in hydrolysing lipoprotein lipids, thus it is possible that HL and EL might compensate for the eliminated LPL activity in macrophages. Secondly, while the hydrolysis products generated by cell-associated LPL likely will be directly taken up by local cells, it is possible that the lipoprotein hydrolysis products generated by non-macrophage LPL could be transported to macrophages through the bloodstream, thus further acting to promote atherosclerosis by negatively influencing the ability of macrophages to efflux cholesterol (119, 139, 140).

In conclusion, investigating the effects of inhibiting macrophage access to LPL hydrolysis products on cholesterol efflux *in vitro* and *in vivo* will help to accentuate the promise in using genetic approaches as a potential future therapy for preventing atherogenesis and cardiovascular disease.

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Appendix

Supplementary Table 1: Real-time amplification efficiencies of reference and target genes

Genes	Amplification Efficiency
ACTB	0.73
NR1H3	1.06
PPARA	1.25
PPARA	0.81
ABCA1	0.71
ABCG1	0.73
SCARB1	0.72
ACACA	0.92
FASN	0.63
SCD	0.83
LPL	1.08
SREBF1	1.03

The gene symbols (with corresponding national centre for biotechnology information reference accession number) encode the following proteins: *ACTB* (NC_000007.13) encodes β-actin, *NR1H3* (XM_005252718.1) encodes LXR-α, *PPARA* (XM_005261658.1) encodes PPAR-α, *PPARG* (NM_138711.3) encodes PPAR-γ, *ABCA1* (XM_005251780.1) encodes ABCA1, *ABCG1* (XM_005261209.1) encodes ABCG1, *SCARBI* (XM_005253637.1) encodes SR-BI, *ACACA* (XM_005257267.1) encodes ACCA, *FASN* (NM_004104.4) encodes FASN, *SCD* (NM_005063.4) encodes SCD-1, *LPL* (NM_000237.2) encodes LPL, and *SREBF1* (XM_005256773.1) encodes SREBP-1c. All primer pairs for the above generated only one product, as determined through melting curve analyses (data not shown).