



Characterization of oxysterols produced in macrophages and mechanisms of regulation

Yinan Chen

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**Characterization of oxysterols produced in
macrophages and mechanisms of regulation**

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ABSTRACT

Macrophages play a key role in atherosclerosis. After massive uptake of oxidized LDL (oxLDL), subendothelial macrophages are overloaded with cholesterol thereby leading to the formation of foam cells, which is one characteristic of atherogenesis. Oxysterols, the oxidation products of cholesterol, are one of major components of oxLDL; they are involved in the regulation of cholesterol homeostasis, induction of cellular oxidative stress and cytotoxicity.

Our works show that both LDL derived-cholesterol and cellular cholesterol can be strongly oxidized in human THP-1 and murine RAW 264,7 macrophages, especially during exposure of oxLDL. The major oxidative products are 7-ketocholesterol and 7 α / β -hydroxycholesterol.

Moreover, we demonstrate that both oxysterols derived from LDL cholesterol and cellular cholesterol can be exported to HDL, whereas not to apo-AI. Then, we studied the functionality of modified HDL and diabetic HDL on oxysterol efflux. A decrease of oxysterol efflux was observed with oxidized and glycoxidized HDL. Compared to the HDL of healthy controls, the HDL of diabetic subjects are less efficient to efflux oxysterols.

Taken together, the increased production of oxysterols in presence of oxLDL and their lower efflux by modified HDL support the detrimental role of these oxidative compounds in pathophysiological conditions like diabetes.

LIST OF SCIENTIFIC PUBLICATIONS

Published articles:

→ **Chen, Y.**, Arnal-Levron, M., Lagarde, M., Moulin, P., Luquain-Costaz, C., Delton, I. (2015)
THP1 macrophages oxidized cholesterol, generating 7-derivative oxysterols specifically released by HDL. *Steroids* 99, 212-218.

→ Arnal-Levron, M, **Chen, Y.**, Delton-Vandenbroucke, I, and Luquain-Costaz, C. (2013)
Bis(monoacylglycero)phosphate reduces oxysterol formation and apoptosis in macrophages exposed to oxidized LDL. *Biochemical Pharmacology* 86, 115-121.

Submitted article:

→ Arnal-Levron, M., **Chen, Y.**, Greimel, P., Gaget, K., Calevro, F., Riols, F., Bertrand-Michel, J., Hullin-Matsuda, F., Olkkonen, VM., Delton, I., Luquain-Costaz, C. (2015).
Bis(monoacylglycero)phosphate regulates OSBP-related protein 11 dependent sterol trafficking. *Submitted in Journal of Biological Chemistry.*

LIST OF SCIENTIFIC COMMUNICATIONS

Poster communications:

→ **Chen, Y .**, Arnal-Levron, M., Guichardant, M., Luquain-Costaz, C., Delton-Vandenbroucke, I. Formation intracellulaire d'oxystérols dans les macrophages THP-1 exposés aux LDL oxydées. *19ème journée de l'EDISS (Ecole Doctorale Interdisciplinaire Sciences Santé)*, 16 octobre 2014, Lyon

→ **Chen, Y .**, Arnal-Levron, M., Lagarde, M., Moulin, P., Hullin-Matsuda F., Luquain-Costaz, C., Delton, I. Oxidative modification of HDL alters its ability to efflux oxysterols from THP-1 macrophages. *16ème Journée scientifique de l'IMBL (Institut Multidisciplinaire de Biochimie des Lipides)*, le jeudi 26 Mai 2016, Lyon

→ **Chen, Y .**, Arnal-Levron, M., Lagarde, M., Moulin, P., Hullin-Matsuda F., Luquain-Costaz, C., Delton, I. Oxidative modification of HDL alters its ability to efflux oxysterols from THP-1 macrophages. *57th ICBL (International Conference on the Bioscience of Lipids)*, 4th-8th September, 2016, Chamonix.

→ **Chen, Y .**, Arnal-Levron, M., Lagarde, M., Moulin, P., Hullin-Matsuda F., Luquain-Costaz, C., Delton, I. Oxidative modification of HDL alters its ability to efflux oxysterols from THP-1 macrophages. *6th ENOR (European Network for Oxysterol Research)*, 29th-30th September, 2016, Paris.

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LIST OF ABBREVIATIONS

[³ H]	Tritium
7 α / β -HC	7 α / β -hydroxycholesterol
7-KC	7-ketocholesterol
24-HC	24-hydroxycholesterol
24(S),25-EC	24(S),25-epoxycholesterol
25-HC	25-hydroxycholesterol
27-HC	27-hydroxycholesterol
ABCA	ATP-binding cassette-type A
ABCG	ATP-binding cassette-type G
ACAT	acyl-CoA:cholesterol acyltransferase
Apo	apolipoprotein
BHT	butylated hydroxytoluene
BMP	bis(monoacylglycero)phosphate
CCL2	CC-chemokine ligand 2
CCL5	CC-chemokine ligand 5
CCR2	C-C chemokine receptor type 2
CD36	cluster of differentiation 36
CE	cholesterol ester
CETP	cholesteryl ester transfer protein
c-glyHDL	control of glycated HDL
c-glycoHDL	control of glycoxidized HDL
CHD	coronary heart disease
COP II	coat protein complex II
CVA	cerebrovascular accident
CXCL1	chemokine (C-X-C motif) ligand 1
diaHDL	diabetic HDLs

DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EDTA	ethylenediaminetetraacetic acid
Egr1	early growth response protein 1
ER	endoplasmic reticulum
ERC	endocytic recycling compartment
FBS	fetal bovine serum
FC	free cholesterol
GC-MS/MS	gas chromatography-tandem mass spectrometry
glyHDL	glycated HDL
glycoHDL	glycoxidized HDL
HDL	high density lipoproteins
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HMGCR	HMG-CoA reductase
h-oxLDL	highly oxidized LDL
ICAD	inhibitor of caspase-activated DNase
ICAM-1	intercellular adhesion molecule-1
IDL	intermediate density lipoproteins
IFN- γ	interferon gamma
IL8	interleukin-8
Insig	insulin induced gene
LCAT	lecithin-cholesterol acyltransferase
LDL	low density lipoproteins
LDLR	LDL receptors
LE	late endosomes
LOX-1	lectin-like oxidized LDL receptor-1
LPDS	lipoprotein deficient serum
LXR	liver X receptor
MCP1	monocyte chemoattractant protein 1

MCS	membrane contact site
M-CSF1	macrophage colony stimulating factor-1
MI	myocardial infarction
MLN64	metastatic lymph node 64
MMP-9	matrix metalloproteinase-9
m-oxLDL	minimally oxidized LDL
NADPH	nicotinamide adenine dinucleotide phosphate
nCEH	neutral cholesterol ester hydrolase
NF- κ B	nuclear factor kappa-light-chain enhancer of activated B cells
nLDL	native LDL
NOS	nitric oxide synthase
NPC1	Niemann-Pick type C1
NPC2	Niemann-Pick type C2
ORP	OSBP-related protein
OSBP	oxysterol binding protein
oxHDL	oxidized HDL
oxLDL	oxidized LDL
PAF-AH	platelet-activating factor acetyl hydrolase
PLTP	phospholipid transfer protein
PM	plasma membrane
PMA	phorbol-12-myristate-13-acetate
PON	paraoxonases
PPAR- γ	peroxisome proliferator-activated receptor- γ
Rab	Ras-related protein
ROS	reactive oxygen species
RXR	retinoid X receptor
S1P	site 1 protease
S2P	site 2 protease
SAA	serum amyloid A

SCAP	SREBP cleavage activating protein
SCP	sterol carrier protein
SMC	smooth muscle cell
SR-AI	scavenger receptor class A type I
SR-BI	scavenger receptor class B type I
SRE	sterol regulatory element
SREBP	sterol regulator element binding protein
SSD	sterol sensing domain
StAR	steroidogenic acute regulatory protein
START	StAR-related lipid transfer
TG	triglyceride
TIMP	tissue inhibitor of metalloproteinase
TLC	thin layer chromatography
TGF- β	transforming growth factor- β
TNF- α	tumor necrosis factor- α
TO901317	N-(2,2,2-trifluoro-ethyl)-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamid
U18666A	3- β -[2-(diethylamine)ethoxy]androst-5-en-17-one
UV	Ultra-Violet
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen 4
VLDL	very low density lipoprotein

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GENERAL INTRODUCTION

Atherosclerosis is a chronic inflammatory disease; it is defined by WHO as "a variable combination of changes of the intima of arteries (as distinguished from arterioles) consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes". After a long time asymptomatic process, atherosclerosis may be associated with multiple complications, including the coronary artery diseases, the carotid artery disease, and the peripheral artery disease. According to the report of WHO, ischemic heart disease and stroke, two complications or atherosclerosis, are the worldwide leading causes of death in 2015.

The initiation of atherosclerosis is occurred from the oxidation of low density lipoproteins (LDL) in narrow sub-endothelial space. The oxidized LDL are highly atherogenic since they can trigger the differentiation of monocytes into macrophages and induce a strong accumulation of cholesterol esters in subendothelial macrophages. The macrophages overloaded with cholesterol esters are called foam cells, which are the major components of fatty streaks(Aluganti Narasimhulu, Fernandez-Ruiz et al. 2016). The oxidized LDL contain the apolipoprotein B-100 with modified structure and various oxidation products of lipids among them oxysterols. Oxysterols are produced from cholesterol oxidation through enzymatic pathways (oxidative position mainly on the side chain of cholesterol), as well as non-enzymatic pathways (oxidative position mainly on the nucleus circles of cholesterol). Several oxysterols, such as 25- and 27-hydroxysterols, are beneficial; they can stimulate the efflux of cholesterol by binding to LXR. While other oxysterols, such as 7-ketocholesterol and 7 α / β -hydroxycholesterol, are deleterious as they induce oxidative stress and play an apoptotic role in cells (Alkazemi, Egeland et al. 2008). When the oxLDL are captured, oxysterols are also accumulated in macrophages. In advanced atherosclerotic plaques, 27-hydroxycholesterol is the major oxysterol component followed by 7-ketocholesterol, 7 β -hydroxycholesterol and 7 α -hydroxycholesterol(Brown and Jessup 1999).

However, we know little about the intracellular formation of oxysterols in macrophages. To investigate this question, we developed two complementary analytical approaches by radiolabeling and mass assay to both identify and quantify the cellular production of oxysterols in human and murine cell lines. Since macrophages acquire the bulk of cholesterol through LDL and oxLDL endocytosis, both cellular and LDL-derived cholesterol were considered in our studies. We also determined the intracellular oxidative site of cholesterol as being the late endosomal/lysosomal compartment. The excessive cholesterol in macrophages can be exported to HDL and apoA-I. Thus, the second aim of our work was to study the effects of HDL and apoA-I on oxysterol efflux.

Diabetes features with hyperglycemia and the high levels of blood sugar. The patients with type 2 diabetes have 2-3 fold elevated risk of atherosclerosis (Emerging Risk Factors 2010). Therefore, the third aim of our work is to evaluate the functionality of diabetic HDL on oxysterol efflux from macrophages. The HDL in diabetics undergo oxidation, glycation and glycoxidation. So we also prepared the modified HDL to analyze the impact of the different modifications of HDL on oxysterol efflux.

In this thesis, the first chapter is literature review, which contains two parts. The part I is about the metabolism of lipoproteins (mainly LDL and HDL), cholesterol and oxysterols. Part II is about atherosclerosis. In this part, we present some details of oxysterols, oxLDL and HDL in the progress of atherogenesis. Chapter 2 shows the general experimental work, including the materials and methods. Chapter 3 is composed of two articles. In these articles, we present the production of oxysterols in human THP-1 and murine RAW 264,7 macrophages and the role of HDL and apo A-I on oxysterol efflux. Chapter 4 refers to a study which is still in progress about the functionality of diaHDL and modified HDL. At last, chapter 5 presents the conclusion, discussion and perspectives of my work.

CHAPTER 1:

Literature review

I. Metabolism of lipoproteins, cholesterol and oxysterols

a. Lipoproteins

1. Generality of lipoproteins

Lipoproteins are complex particles made of proteins and fat whose major role is to transport lipids in the circulation for the delivery to all the tissues of the body.

After extraction of plasma, four major kinds of lipids are recovered: triacylglycerols (TG), phospholipids (PL), cholesterol, cholesterol esters (CE), and free fatty acids.

The classification of lipoproteins is based on their density: chylomicron (from intestine; the largest, with the lowest density due to high lipid/protein ratio), VLDL (very low density lipoprotein; from liver), IDL (intermediate density lipoprotein, created by the metabolism of VLDL), LDL (low density lipoprotein), HDL (high density lipoprotein). Different lipoproteins feature different lipid/protein ratios (Table 1). When the fatty acids of chylomicron are digested but the cholesterol remains, the particle is called the chylomicron remnant. It travels to the liver, where the cholesterol is metabolized. Triacylglycerol is the predominant lipid in chylomicrons and VLDL, whereas the cholesterol and the phospholipids are the major lipids in the LDL and HDL, respectively.

Table 1. Composition of the lipoproteins

Lipoprotein	Diameter (nm)	Density	Proteins (%)	Total lipids (%)	Composition			
					Percentages of total lipids			
					TG	PL	CE	FC
Chylomicrons	90-1000	<0,95	1-2	98-99	88	8	3	1
VLDL	30-90	0,95-1,006	7-10	90-93	56	20	15	8
IDL	25-30	1,006-1,019	11	89	29	26	34	9
LDL	20-25	1,019-1,063	21	79	13	28	48	10
HDL ₁	20-25	1,019-1,063	32	68	2	53	34	11
HDL ₂	10-20	1,063-1,125	33	67	16	43	31	10
HDL ₃	5-10	1,125-1,210	57	43	13	46	29	6
Pre β -HDL	<5	>1,21	70	30	...	83	...	17

2. Structure of lipoproteins

The general structure of a lipoprotein particle includes a non-polar lipid core consisting of triacylglycerols and cholesterol esters, a polar surface composed of the monolayer of phospholipid, unesterified cholesterol and specific apolipoproteins.

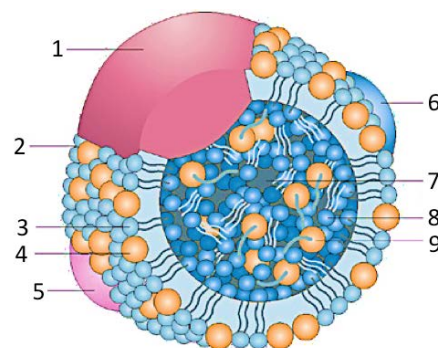


Figure 1. General structure of lipoprotein
(Wasan, Brocks et al. 2008)

1, 5, 6. Apolipoprotein. 2. Polar surface coat. 3. Phospholipid. 4. Unesterified cholesterol.
7. Non polar lipid core. 8. Triacylglycerol. 9. Cholesterol ester.

Distribution of apolipoproteins is a characteristic of lipoproteins (Table 2). Apolipoproteins can function as a part of lipoprotein structure to make the lipid molecules more stable, or as enzyme cofactors. They can also bind to specific cell surface receptors, which enable cells to take up the lipoproteins through receptor-mediated endocytosis. Some apolipoproteins are integral and cannot be dissociated, while some can be freely transferred to other lipoproteins (e.g. Apo A-IV).

Table 2. Characteristic of the apolipoproteins

Apolipoprotein	Lipoprotein	Functions
Apo A-I	HDL, Chylomicrons	Transporter of cholesterol. Activator of lecithin: cholesterol acyltransferase (LCAT). Ligand of ABCA1 and SR-BI.
Apo A-II	HDL, Chylomicrons	Inhibitor of LCAT. A modulator of reverse cholesterol transport.
Apo A-IV	Secreted with chylomicrons, but transferred to HDL	Associated with the formation of triacylglycerol-rich lipoproteins. Activator of LCAT.
Apo B-100	LDL, VLDL, IDL	Secretion of LDL by the liver. Ligand of LDL receptor.
Apo B-48	Chylomicrons, chylomicrons remnant	Secretion of chylomicrons in the intestine.
Apo C-I	VLDL, HDL, chylomicrons	Activator of LCAT. Inhibitor of CETP.
Apo C-II	VLDL, HDL, chylomicrons	Activator of LPL.
Apo C-III	VLDL, HDL, chylomicrons	Inhibitor of Apo C-II.
Apo D	Subfraction of HDL	Perhaps function as a lipid transfer protein.
Apo E	VLDL, HDL, chylomicrons, chylomicrons remnants	Ligand of chylomicrons remnants receptor in liver, and LDL receptor.

3. LDL

3.1 LDL and oxidized LDL

Low density lipoprotein (LDL) is the last remnant of VLDL and it plays an important role in the transport of cholesterol in human body. As shown in Table 1, the diameter of LDL particle is about 20-25nm; the protein and lipids represent 21% and 79% of total lipoprotein weight, respectively. It is estimated that an “average” LDL particle contains 700 molecules of phospholipids, 600 molecules of unesterified cholesterol, 1600 molecules of cholesterol esters, 180 molecules of TG and 1 molecule of apolipoprotein B-100. Besides lipids and protein,

various antioxidants, such as ubiquinol, α tocopherol, γ tocopherol, β carotene and lycopene, are present in this lipoprotein (Pirillo, Norata et al. 2013).

Apo B-100 is the unique apolipoprotein associated with LDL; it is composed of 4536 amino acid residues and is one of the largest monomeric proteins in human body with a molecular mass about 550 kDa. The structure of apo B-100 is usually divided into 5 domains as $\text{NH}_2\text{-}\beta\alpha 1\text{-}\beta 1\text{-}\alpha 2\text{-}\beta 2\text{-}\alpha 3\text{-COOH}$ (Magnolo, Noto et al. 2016). Because of the hydrophilic property of the $\text{NH}_2\text{-}\beta\alpha 1$ domain and several hydrophobic segments of the apolipoprotein, apo B-100 is able to distributed both at the surface (outside the particle) and in the lipid core (strong interactions with lipids), which stabilizes the structure of LDL.

Oxidized low density (oxLDL) has been studied for over 30 years. It is defined as “a particle derived from circulating LDL that may have peroxides or their degradation products generated within the LDL molecule or elsewhere in the body associated with the particle” (Parthasarathy, Raghavamenon et al. 2010). The oxidation of LDL can be catalyzed by metal cations (e.g. Cu^{2+} , Fe^{3+}) and several enzyme systems, including lipoxygenase, myeloperoxidase, xanthine oxidase, and NADPH oxidase (Delporte, Van Antwerpen et al. 2013). It leads to different extents of both lipids and protein oxidative alterations. However, not all the oxidation mechanisms are comparable and repeatable even under *in vitro* conditions. The oxidative products are various, including fatty acid oxidation products (e.g. malondialdehyde (MDA), 13-HPODE, 13-HODE), lipid derived products (e.g. lysophosphatidylcholine, oxysterols), protein carbonyls, modified amino acids (e.g. cysteine, cystine, histidine, lysine, arginine), and protein cross-links.

In human body, the circulating LDL is only minimally oxidized in blood because of the presence of antioxidants, such as vitamin and β carotene, and the efficient anti-oxidant enzymes (e.g. superoxide dismutase). Nevertheless, in the narrow sub-endothelial space, the level of antioxidants is much lower than that in blood; therefore, LDL is oxidized by the oxidants secreted by macrophages, endothelial cells and smooth muscle cells (Salvayre, Negre-Salvayre et al. 2016).

3.2 Capitation of LDL and oxidized LDL through receptor-mediated mechanisms

The LDL receptor (LDLR) is located on the plasma membrane (PM) and belongs to the family of LDL receptors, which internalize extracellular ligands for lysosomal degradation (Dieckmann, Dietrich et al. 2010). The members of the LDL receptors family can internalize VLDL and VLDL remnants after binding to apo E, and LDL after binding to apoB-100.

LDLR is synthesized in the endoplasmic reticulum (ER), then turns into its mature form in Golgi apparatus by glycosylation before being transported to PM, which involves Rab13 and Rab3b (Yamamoto, Nishimura et al. 2003, Nokes, Fields et al. 2008). Rab3b mediates the direct transport of LDLR to the cell surface, whereas Rab13 acts between the Golgi apparatus and the recycling endosome. ARH and Dab2 are the adaptors of LDLR. They interact with the NPxY motif of LDLR's cytoplasmic tail and recruit clathrin and other proteins involved in endocytosis (Wijers, Kuivenhoven et al. 2015). Depending on the cell type, LDLR either binds to ARH or binds to Dab2 and is internalized via clathrin-coated pits. Moreover, the LDLR is highly regulated by intracellular cellular cholesterol content (Chapter 1, page 42, Regulation of cellular cholesterol content).

In addition, apo B-100 plays a crucial role in the recognition of LDL by different membrane receptors. It has been shown that the modifications of apo B-100 can decrease the affinity between LDL and LDL receptor (Rutledge, Su et al. 2010). The modification caused by MDA, which changes 16% of the lysine residues of apo B-100, leads to an inability of recognition by LDLR, but generates the ligand for the scavenger receptors (SRs) (Haberland and Steinbrecher 1992).

Oxidized LDL is usually divided into two categories: the “minimally oxidized LDL” (m-oxLDL) and the “highly oxidized LDL” (h-oxLDL). The components of m-oxLDL are different from normal LDL; whereas it can still be recognized by LDLR, but not by most of the scavenger receptors (SRs). The h-oxLDL can be internalized by macrophages via multiple SRs, but cannot be recognized by LDLR.

Scavenger receptor class A type I/II (SR-AI/II) on macrophages was first discovered by Goldstein in 1979, and it was originally called the ‘acetyl LDL receptor’ (Goldstein, Ho et al.

1979, Krieger 1994). Scavenger receptors are cell-surface proteins. They can bind to senescent cells and modified lipoproteins, such as acetylated LDL (acLDL) and oxLDL. In contrast with LDLR, even though the uptake of modified LDL induces the elevated cholesterol content in macrophage, the expression of scavenger receptor is not regulated by the intracellular cholesterol quantity. In addition, since acetylated LDL is not a nature particle in human body, oxidized LDL was supposed be a physiological ligand for the SR-AI/II. Nevertheless, it has been shown that only 30% of the uptake of oxLDL is through the SR-AI/II, indicating that almost 70% of the oxidized LDL were internalized by macrophages attributed to other oxidized LDL receptors (Lougheed, Lum et al. 1997). Scavenger receptor class B type I (SR-BI) can also bind to oxLDL and acLDL; it is associated with caveolae on membrane, and is localized in macrophages, steroidogenic tissues and adipose tissues. However, the major function of SR-BI is considered as the HDL receptor, especially for the uptake of HDL cholesterol esters by liver and steroidogenic tissues.

Besides class A and class B scavenger receptors, there are a number of cell membrane receptors which can bind oxLDL with high affinity. A common characteristic of oxLDL receptors is that they are multiligand receptors. CD36 is another physiological receptor for oxLDL (Amézaga, Sanjurjo et al. 2014); it is sensitive only to oxidized phospholipids. Compared with normal subjects, the CD36-deficient human subjects show a 40% decreased rate of oxLDL uptake. Macrosialin, also called CD68, is reported to be able to recognize oxLDL without subsequent internalization (De Beer, Zhao et al. 2003). Lectin-like oxidized LDL receptor-1 (LOX-1), expressed in endothelial cells, macrophages and smooth muscle cells, shows a higher affinity towards the mildly-oxidized LDL (3-6h oxidation) than the extensively-oxidized LDL (12-24h oxidation). Unlike the SR-AI/II, LOX-1 cannot bind to acLDL, indicating that the mechanism of LOX-1 is different from the class A scavenger receptors (Pirillo, Norata et al. 2013).

3.3 The endocytic pathway of LDL

Macrophages internalize LDL by the endocytic pathway (Figure 2) (Brown and Goldstein 2013). At first, the LDL particles bind to the LDL receptors, which cluster in clathrin-coated pits at the cell surface. Then, the coated pits pinch off and the LDL-LDLR complexes are internalized into the clathrin-coated vesicles. After being uncoated, the endocytic vesicles are directed to the early endosomes, where the low pH causes a conformational change of the receptors allowing the release of LDL particles. Subsequently, the receptors are either directed back to the cell surface for recycle or degraded in lysosomes. Before degradation, LDL receptor can be approximately recycled 100 times. The LDL particles are delivered to the late endosomal/lysosomal compartment, in which the apolipoprotein and cholesterol esters are hydrolyzed and free cholesterol is released.

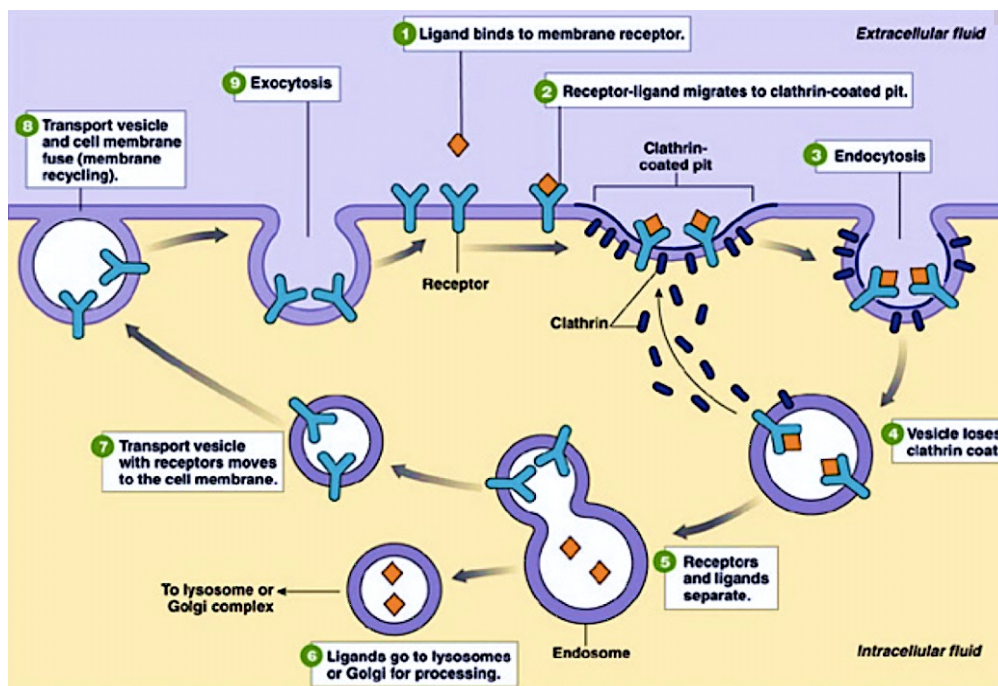


Figure 2. Endocytosis of LDL

(Pearson Education 2007)

4. HDL

High-density lipoproteins (HDL) are the small, dense, protein-rich lipoproteins with a mean size of 5-17 nm in diameter and density of 1.063-1.21 g/ml (Kontush and Chapman 2011). Compared with other lipoproteins, the particles of HDL have the highest protein/lipid ratio and the capability of carrying cholesterol away from the tissues to the liver for catabolism, lowering blood cholesterol levels. Because of the qualitative and quantitative differences in lipid and protein content, HDL particles are multi-shaped molecules with varying fluidity and charge. Based on their densities, HDL particles can be subclassified into HDL₂(1,063-1,125 g/ml) and HDL₃ (1,125-1,21 g/ml); and according to their sizes, they can also be separated by non-denaturing gel electrophoresis into 5 subclasses, from the largest to the smallest, as HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b} and HDL_{3c}. They can be quasi-spherical or discoid complexes composed largely of polar lipids and apolipoproteins. Apolipoprotein A-I (Apo A-I) is the most abundant protein of HDL; the other apolipoproteins, such as Apo A-II, apo A-IV, apo-C, apo E, and apo J, are found in lower proportion on HDL. Besides the various apolipoproteins, HDL contains multiple other proteins, which have enzymatic activity or the capacity to transfer lipids, including lecithin-cholesterol acyltransferase (LCAT), acetyl-PAF hydrolase, paraoxonase (PON), phospholipid transfer protein (PLTP) and cholesterol ester transfer protein (CETP). Due to the diversity of the proteins, HDL particles are highly heterogeneous.

4.1 The major protein components of HDL particles

Apolipoprotein A-I:

Apolipoprotein A-I (Apo A-I) is the major structural and functional protein of HDL. It can be found in almost all HDL particles and accounts for almost 70% of the total HDL protein (Schaefer, Santos et al. 2010). The main site for synthesis and secretion of Apo A-I is the liver. But it can also be secreted with chylomicrons from the intestinal enterocyte, and quickly

transferred to HDL in bloodstream. The major functions of Apo A-I are (1) interaction with cellular receptors for efflux of cholesterol and phospholipid; (2) activation of lecithin/cholesterol acyltransferase (LCAT); (3) athero-protective function of HDL. The C-terminal α -helix of Apo A-I plays a crucial role in the efflux of cholesterol; the relatively high hydrophobicity and lipid affinity of this segment are significant for the activity of Apo A-I (Lyssenko, Hata et al. 2012).

Apolipoprotein A-II:

Apolipoprotein A-II is the second major apolipoprotein of HDL. It accounts for about 15-20% of the total HDL protein and can be found in approximately half of HDL particles (Shimano 2009). Apo A-II is synthesized in liver and intestine; and it is more hydrophobic than Apo A-I. It exists as a homodimer composed of two identical polypeptide chains that are connected by a disulfide bridge at position 6. In addition, apo A-II can also form heterodimers with other cysteine-containing apolipoproteins. Apo A-II might be an antagonist for cellular cholesterol efflux; with a low Apo A-I level, the level of Apo A-II is not elevated as compensation, and the remaining Apo A-II is not able to protect against atherosclerosis.

Apolipoprotein E:

Compared with Apo A-I, the content of apolipoprotein E (Apo E) in HDL is much lower; but Apo E still plays a key role in HDL structure and activities (Lund-Katz and Phillips 2010). It contributes to the accumulation of cholesterol ester at the lipid core, thereby leading to the formation of the large, lipid-rich HDL. Apo E is synthesised in many tissues and cell types, such as liver, central nervous system and macrophages. It is mainly carried by triglyceride-containing lipoproteins as a ligand of apoB/apoE membrane receptors.

LCAT:

Lecithin:cholesterol acyltransferase (LCAT) is a plasma N-glycosylated protein which catalyzes the transfer of an sn-2 acyl group from phosphatidylcholine to the 3- β -hydroxyl group of cholesterol to form a cholesteryl ester (Figure 3) (Rousset, Vaisman et al. 2009). It forms almost all plasma cholesteryl ester in humans and plays a key role in promoting the transfer of excess cellular cholesterol from peripheral tissues to liver. LCAT has the tertiary structure, which is maintained by two disulfide bridges; it is mainly synthesized in liver, and its mRNA can also be found in brain and testis at appreciable levels. Apo A-I is the principal activator of LCAT. The oxidized LDL or phospholipid hydroperoxides can reduce the activity of LCAT (Rosenson, Brewer Jr et al. 2016). The main fatty acid transferred by LCAT is linoleic acid (Lin, Steiner et al. 2010).

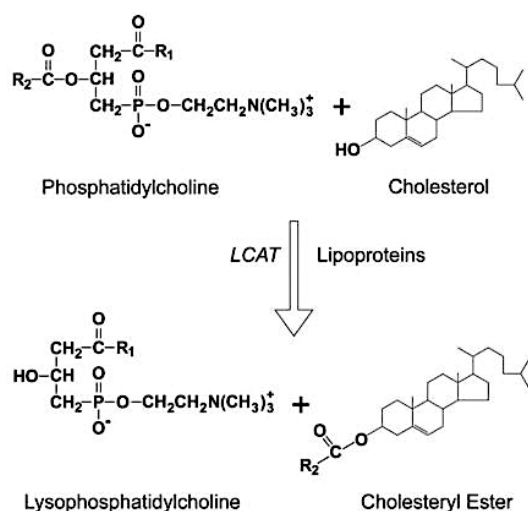


Figure 3. Reaction catalyzed by lecithin-cholesterol acyltransferase (LCAT)

PON:

Human paraoxonases (PON) are circulating calcium-dependent lactonases, which have three members PON1, PON2 and PON3 (Goswami, Tayal et al. 2009). Like LCAT, PON1 is a

N-glycosylated protein. It is largely secreted by liver (Mackness, Beltran-Debon et al. 2010) and most of PON1 is associated with HDL, preferentially in HDL₃, only small fractions are free or bound to VLDL and chylomicrons. It catalyzes the hydrolysis of organophosphate substrate paraoxone together with other organophosphate substrates and aromatic carboxylic acid esters. It can be fully activated by apo A-I. PON1 functions as an athero-protective protein by preventing the oxidation of LDL and HDL. The increase of free PON1 in plasma has been associated with diseases with increased oxidative stress (Rosenblat, Ward et al. 2012). Two calcium atoms are necessary for the stability of its structure; and the catalytic site is in the central tunnel of the enzyme. PON2 is the second member of the PON family. It is an intracellular enzyme expressed in many tissues, such as brain, liver, kidney and testis. This enzyme hydrolyses organophosphate substrates and aromatic carboxylic acid esters. The properties of PON3 are similar to those of PON1. It is also a N-glycosylated protein, which is synthesized in liver; it binds to HDL and needs the calcium for the structure stability. PON3 is a potent lactonase, and also a limited arylesterase.

Platelet-activating factor acetyl hydrolase (PAF-AH):

Platelet-activating factor acetyl hydrolase (PAF-AH), also called lipoprotein-associated phospholipase A₂ (LpPLA₂), is a calcium-independent, N-glycosylated enzyme. It stimulates the degradation of PAF by hydrolyzing the sn-2 ester bond to form the biologically inactive lyso-PAF (Mallat, Lambeau et al. 2010). The substrates of PAF-AH are the phospholipid with a short residue at the sn-2 position, so it is able to hydrolyze the proinflammatory oxidized short-chain phospholipids, but not the long-chain non-oxidized phospholipids. In HDL₂, the presence of PAF-AH is associated with the antioxidative function of this particle (Küçük, Küçük et al. 2015). In plasma, it is associated with the small, dense LDL and HDL particles (McIntyre, Prescott et al. 2009). Two hydrophobic clusters build a lipid-binding domain, which can ensure the association of PAF-AH with the lipoproteins; its active site is at the surface of lipoprotein that facilitates the access to the aqueous phase (Samanta and Bahnson 2008).

PLTP and CETP:

Phospholipid transfer protein (PLTP) is a member of the bactericidal permeability-increasing protein (BPI)/lipopolysaccharide (LPS)-binding protein (LBP)/Plunc superfamily. It can be found in liver and intestine and macrophages (Lee-Rueckert, Vikstedt et al. 2006). Circulating PLTP is predominantly associated with HDL particles. It stimulates the exchange of phospholipids between VLDL and HDL, and regulates the size of HDL to form larger and smaller particles (Ji, Wroblewski et al. 2014). The activities of PLTP are stimulated by lipoprotein lipase-induced lipolysis. In addition, it may play a key role in HDL athero-protective activities (Vaisar, Hutchins et al. 2015).

CETP is also a member of the BPI/LBP/Plunc superfamily. This transfer protein has multiple N-glycosylation sites. It is predominantly synthesized in liver and adipose tissue. CETP can transfer CE from HDL to Apo B-containing lipoproteins and stimulates a reverse transport of TG between these two kinds of lipoproteins. In addition, the hydrophobic tunnel of CETP, which is composed by cholesterol esters and amphiphilic phosphatidylcholine (PC) molecules, facilitates its neutral lipids transfer activity (Qiu, Mistry et al. 2007).

4.2 Complexity and heterogeneity of HDL particles

Metabolism of HDL is related with the modification of its composition and structure (Figure 4). The lipid/protein ratio of HDL is regulated by the action of several enzymes and the interactions between HDL and specific receptors (Rye and Barter 2014). After secretion by liver and intestine, Apo A-I associates phospholipids and free cholesterol to form lipid-poor discoidal HDL. Then, the enrichment of the lipid core with cholesterol ester by the enzyme LCAT generates the small spherical HDL₃, which can exchange phospholipid with VLDL by PLTP (Rye 2013). CETP further increases the size of HDL particles by exchanging CE and TG between HDL and VLDL/LDL. Subsequently, the cholesterol ester, triglycerides

and phospholipids are delivered to liver by the action of hepatic lipase and the interaction with SR-BI. Due to various factors, a large set of HDL particles is generated during the remodeling process. As it is shown in Figure 4, the factors include (A) several exchangeable apolipoproteins, such as Apo A-I, Apo A-II and Apo E, in HDL particle and their ability to dissociate and/or interchange between particles (Cavigiolio, Geier et al. 2010); (B) the constant conformational changes of these apolipoproteins (Curtiss, Bonnet et al. 2000); (C) more than 80 proteins associated with HDL that contains specific functions (Davidson, Silva et al. 2009); (D) different lipid compositions of HDL which activate different signaling pathways and play important roles for HDL functions (Kontush, Lhomme et al. 2013).

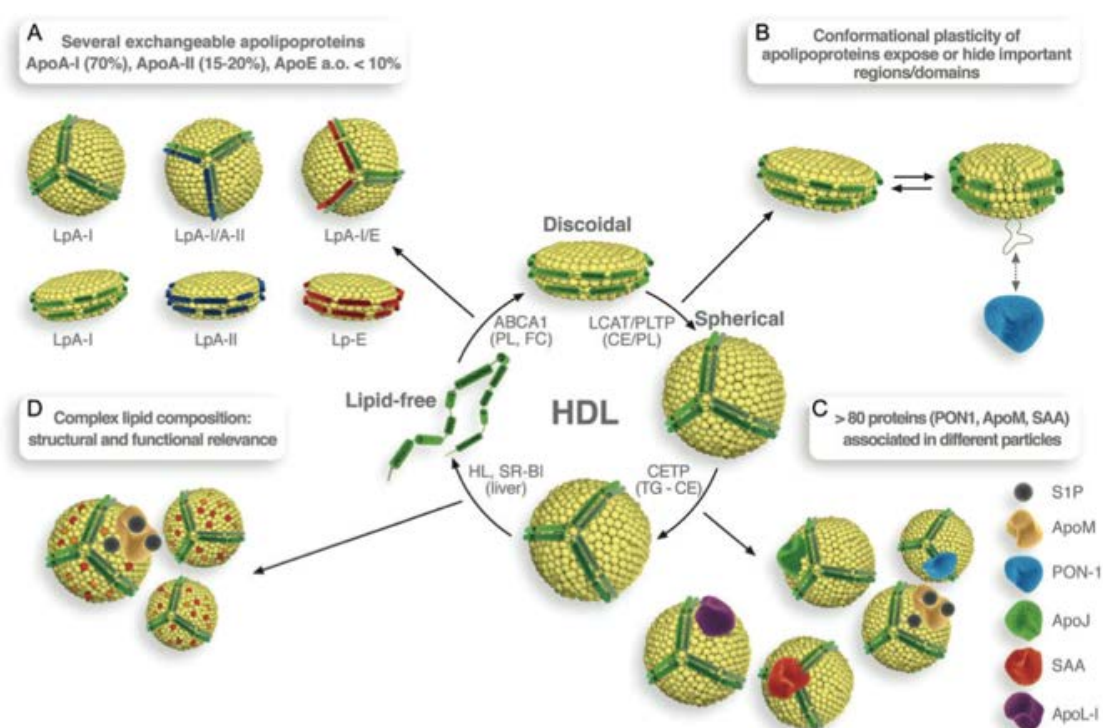


Figure 4. HDL particle complexity and heterogeneity
(Kratzer, Giral et al. 2014)

LpA-I: lipoprotein A-I; PL: phospholipids; FC: free cholesterol; LCAT: lecithin cholesterol acetyltransferase; CE: cholesterol ester; PLTP: phospholipid transfer protein; TG: triglyceride; HL: hormone sensitive lipase; PON-1: Paraoxonase 1; ApoJ: Clusterin; SAA: serum amyloid A; S1P: sphingosine 1 phosphate.

The complexity and heterogeneity of HDL indicate that the functions of HDL exist not only in lipid metabolism, but also in acute-phase response, innate immune response, complement activation, plaque stability and proteolysis inhibition (Heinecke 2009). Therefore, the whole HDL fraction represents “a collection of individualized species with distinct functionalities that happen to have similar physicochemical properties” (Shah, Tan et al. 2013).

4.3 HDL and type 2 diabetes

4.3.1 Generality of diabetes

Diabetes is defined by WHO as “a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces”. It is characterized by hyperglycemia, the raised level of blood sugar. There are three types of diabetes. Type 1 diabetes is characterized by deficient production of insulin and requires daily administration of insulin. Type 2 diabetes is caused by the ineffective use of insulin in human body (Assal and Groop 1999); and it is usually observed in adults, whereas recently it can be occurred more and more frequently in children. The symptoms of these two types of diabetes are various, such as losing weight, excretion of urine, thirst, constant hunger, vision changes and fatigue. Gestational diabetes is occurred during pregnancy. The expectant mothers suffered from this diabetes and their children are at increased risk of type 2 diabetes in the future.

4.3.2 Alteration of HDL in type 2 diabetes

In type 2 diabetes, multiple anti-atherosclerotic functions performed by HDL particles are impaired, such as efflux of cholesterol, antioxidative capacity, anti-inflammatory activity,

cytoprotective activity and protection on endothelium-dependent vasorelaxation (Rosenson, Brewer Jr et al. 2016). However, hyperglycemia alone is not sufficient to yield functionally deficient HDL and promote the development of atherogenesis (Kanter, Johansson et al. 2007). These functional deficiencies of HDL are associated with the dyslipidemia and the alteration of HDL structure. Dyslipidemia is an important characteristic of type 2 diabetes (Chapman, Ginsberg et al. 2011); it features decreased HDL cholesterol levels, increased HDL TG levels, elevated TG-rich lipoproteins (mainly VLDL) levels, and the dominance of small dense LDL. This abnormal lipid profile is associated with chronic low-grade inflammation and oxidative stress; therefore the dyslipidemia plays a proatherosclerotic role in type 2 diabetes. Moreover, it has been demonstrated that the cholesterol-lowering therapy is extremely efficient for preventing coronary heart disease (CHD) in patients with type 2 diabetes (Unit 2005, Trialists 2008). It is proposed that the treatment on lipoprotein metabolism is more effective for CHD than the correction of hyperglycemia (Ray, Seshasai et al. 2009).

Alteration of HDL lipids composition:

The enrichment of TG and the depletion of CE in HDL particles are caused by the elevated activity of CETP (Le Goff, Guerin et al. 2004). The CE/TG ratio of HDL core is a key factor for the cholesterol efflux capacity of HDL. With a low CE/TG ratio, there are conformational alterations of the C-terminal domains of apo A-I, which impacts the interaction between HDL and membrane receptors (Rosales, Patel et al. 2015). The enrichment of TG in HDL core can also decrease the conformational stability of apo A-I and makes HDL lose apo A-I easily. In blood, the HDL without apo A-I are cleared quickly. Moreover, it has been proposed that the structural alterations of apo A-I play an important role in the decreased antioxidative activity of HDL, since the changed domains of apo A-I may act as an acceptor of oxidized lipids (Rached, Chapman et al. 2015).

Besides CE and TG, the content of PL is another important factor for the efflux capacity of HDL (Schwendeman, Sviridov et al. 2015). In type 2 diabetes, the content of

sphingomyelin in HDL particles is elevated, which is accompanied by the reduction of phosphatidylcholine. The increased sphingomyelin/phosphatidylcholine ratio augments the rigidity of PL monolayer, thereby leading to an impairment of cholesterol efflux.

Apolipoproteins:

Apolipoprotein is one of the major factors to realize the HDL anti-atherogenic functions. However, in type 2 diabetes, the capacities of apolipoproteins can be significantly impacted by serum amyloid A (SAA), oxidative stress and glucose. SAA is an acute-phase protein; in type 2 diabetes, it can replace the apo A-I and other apolipoproteins on the surface of HDL₃ particles. The HDL with SAA is cleared rapidly from circulation (Han, Tang et al. 2016). Oxidative stress, caused by the increased levels of reactive oxygen, nitrogen and chlorine species in vessels, is an important feature of type 2 diabetes (Henriksen, Diamond-Stanic et al. 2011). With oxidative stress, the amino acids of apo A-I, including Met, Cys, Lys and Tyr, are selectively oxidized. In addition, the oxidation of apo A-I catalyzed by myeloperoxidase, especially the oxidation on the Tyr192 and Tyr166 residues, inhibits the cholesterol efflux dependent on transporter ABCA1, and impairs the lipid-binding capacity of apo A-I (Shao and Heinecke 2011). Glycation is another important modification for the functions of HDL apolipoproteins. In vitro, the non-enzymatic modification of apo A-I impairs its anti-inflammatory activity by decreasing its inhibition on CD11b expression (Hoang, Murphy et al. 2007). Furthermore, the accumulation of glycated apo A-I, apo A-II and other HDL proteins has been found in the body with hyperglycemia (Sun, Shen et al. 2013). It also has been shown that for the glycated HDL, the combination with anti-apo A-I was decreased because of the alteration of apo A-I structure (Igau, Castro et al. 1997). The protective effect of HDL on the endothelium-dependent vasorelaxation is also impaired by glycation (Brindisi, Duvillard et al. 2013) and oxidation (Perségol, Brindisi et al. 2015). Both oxidation and glycation can impact the capabilities of HDL and apo A-I, whereas these two modifications are carried out by unrelated mechanisms (Hermo, Mier et al. 2005). Moreover, it has been

shown that the glycoxidation of HDL leads to the formation of proapoptotic HDL and gives rise to the death of endothelial cells (Riwanto, Rohrer et al. 2013).

Enzymes:

The levels of HDL associated enzymes, such as PON1, PAF-AH, and LCAT are altered in type 2 diabetes, which may be caused by oxidation (Dullaart, Gruppen et al. 2016), glycation (Arora, Patra et al. 2016, Mogarekar, Dhabe et al. 2016) and the replacement by SAA (Rosenson, Brewer Jr et al. 2016). The concentrations and activity of PON1 in circulation are decreased in type 2 diabetes, leading to the increased severity of inflammation and oxidative stress. In glycated HDL, the activity of paraoxonase is reduced almost 65% (Hedrick, Thorpe et al. 2000). Moreover, compared with normal subjects, the activities of PAF-AH and PON1 in HDL₃ particles are reduced in patients with type 2 diabetes (Inagaki, Nakagawa-Toyama et al. 2012). PON 1 and LCAT can be glycated and oxidized in vitro, thereby impairing the protective function of HDL on the oxidation of LDL. The replacement of PON1, PAF-AH and LCAT by acute-phase protein SAA decreases the activities of these enzymes and gives rise to the proinflammatory effect of HDL (Han, Tang et al. 2016).

b. Cholesterol

1. Generality of cholesterol

Cholesterol was identified for the first time in its solid form in gallstones by François Poulletier de la Salle in 1769. In 1815, the French chemist Michel Eugène Chevreul named it as "cholesterine". Its empirical formula ($C_{27}H_{46}O$) was established in 1888 by Reinitzer. Its correct structure was made by Wieland and Dane, in 1932 (Vance and Van den Bosch 2000).

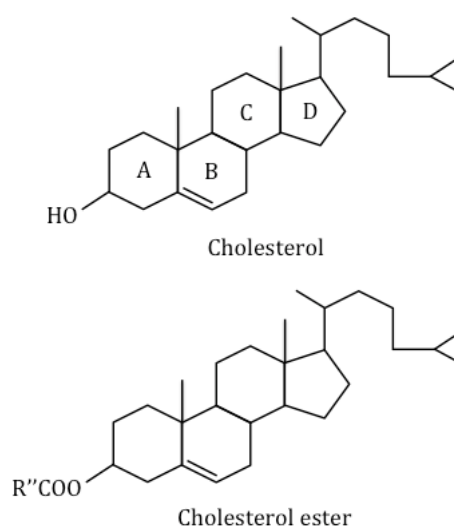


Figure 5. Structure of cholesterol and cholesterol ester

Cholesterol is the most common steroid in eukaryotes. Its structure is characterized by four “fused” rings: three six-member cyclohexane rings (marked A, B and C) and one five-member cyclopentane ring (the D ring); and there is a side chain of 8 carbon atoms at C-17 position (Figure 5). The carbon atoms of cholesterol are numbered from 1 to 27. There is a double bond between carbons 5 and 6 on the B ring, and a hydroxyl group on carbon 3 in β position (above the plane of the A ring).

In cells, cholesterol exists mainly in two forms: the unesterified form and the esterified form. Unesterified cholesterol, also called free cholesterol, is one of major constituents of cell membranes as well as a precursor for the synthesis of steroid hormones and bile acids, while the esterified cholesterol exists as a storage form in cells. Cholesterol is transported throughout body within lipoproteins, which can direct the lipids to certain tissues.

Mammalian cells obtain cholesterol by endocytosis of LDL or by de novo synthesis in the endoplasmic reticulum (ER). In human body, approximately half of cholesterol is produced by synthesis (~700 mg/day) and another half is provided by food. Approximately 10% of the total cholesterol is synthesized respectively by liver and intestines. Almost 9mg cholesterol per kg body weight is synthesized by peripheral tissues each day and has to be transported to liver for effective catabolism.

2. Intracellular distribution of cholesterol

Cholesterol is not uniformly distributed throughout cell. The uneven distribution of cholesterol is important for maintaining many biological functions of mammalian cells, including signal transduction and membrane traffic.

The plasma membrane (PM) is highly cholesterol-enriched, holding approximately 70% of total cellular cholesterol; and it has been estimated that cholesterol is 30-40% of the total lipid in PM. By contrast, the content of cholesterol in ER is very low, only about 0.5–1% of cellular cholesterol, even though this is the organelle where cholesterol is synthesized and the surface area of ER exceeds that of the PM in many cells. The content of cholesterol on the Golgi apparatus is intermediate between those of ER and PM. PM is sphingomyelin-rich, and that may be the reason of the high-level cholesterol in PM, since cholesterol has highest affinity for membranes enriched in sphingolipids and saturated phospholipids.

Cells with significant endocytosis may contain more intracellular cholesterol. The concentration of cholesterol also varies in different compartments of the endocytic pathway. The endocytic-recycling compartment (ERC), which is responsible for recycling membrane proteins and lipids, contains high-levels cholesterol. Contrary to the late endosomes/lysosomes, the early endosomes appears relatively rich in cholesterol.

Moreover, the inner and outer leaflets of the biological membranes also have different lipid compositions. A recent study shows that phosphatidylserine is a key factor for maintaining cholesterol in the cytosolic leaflet of the plasma membrane (Maekawa and Fairn 2015).

Finally, biological membranes have a greater lateral heterogeneity. “Lipid rafts” or “rafts” are microdomains enriched with cholesterol on biological membranes.

3. Microdomains rich in cholesterol

Many types of membrane microdomains coexist in cells. The plasma membrane is described as a mosaic of microdomains having different compositions in lipids and proteins

(Schmitz and Grandl 2008). Some microdomains contain very specific structures, such as the clathrin-coated pit or the caveolae-like invagination. Caveolae is one type of specialized, raftlike domain. It is associated with caveolins and has a flask shape with a diameter of about 60 nm. Lipid raft is another type of microdomains whose assemblage needs strong lipid-lipid and lipid-protein interactions. Both of these two kinds of microdomains are enriched with cholesterol, sphingolipids, glycosphingolipids and phospholipids containing saturated fatty acyl long chain. This particular lipid environment generates a region more ordered and compact (liquid-ordered phase or L_o) than the surrounding membranes (liquid-disordered phase or L_d), and attracts some proteins, in particular the proteins with a GPI anchor or palmitoyl groups, like the SRC-kinases. The high lateral mobility and the small size of L_o domains ensure the frequent encounter between molecules and the raft boundaries and facilitate individual molecules to leave the raftlike regions rapidly (Mishra and Joshi 2007). The plasma membrane is not the only one being composed of microdomains. The internal membranes, including ER, the Golgi apparatus and late endosomes, also comprise specific microdomains (Sobo, Chevallier et al. 2007).

These membrane microdomains are involved in some signaling pathways, endocytosis mechanisms and intracellular cholesterol transport. Indeed, most of the proteins playing a role of cholesterol carriers are associated with microdomains (Orlowski, Coméra et al. 2007). The caveolae plays an important role in intracellular cholesterol transport, particularly in the efflux of cholesterol to HDL and the cholesterol transport from ER to PM (Parton and del Pozo 2013). Caveolin contains the cholesterol binding sites. Other proteins involved in the transport of cholesterol are also associated with the microdomains rich in cholesterol. For example, SR-BI, the receptor involved in uptake of oxidized LDL by macrophages (Gillotte-Taylor, Boullier et al. 2001); and ABCG1, the transporter involved in the cholesterol efflux stimulated by HDL (Kobayashi, Takanezawa et al. 2006).

4. Biosynthesis of cholesterol

Mammalian cells contain all the enzymes required for de novo synthesis of cholesterol. The liver and intestine are the primary sites for the synthesis of cholesterol and the peripheral cells, including macrophages, can also synthesize some of their cholesterol. Cholesterol is synthesized in the endoplasmic reticulum (ER) from acetyl coenzyme A (acetyl-CoA) and through a series of about 30 successive enzymatic reactions (Figure 6).

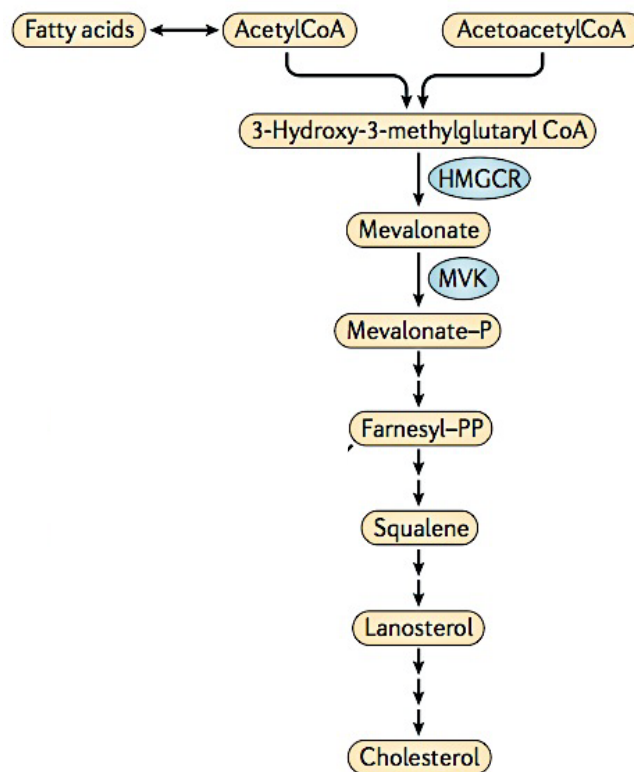


Figure 6. Biosynthesis of cholesterol
(Cyster, Dang et al. 2014)

3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase, officially abbreviated HMGCR), the rate-controlling enzyme in the cholesterol biosynthetic pathway, catalyzes the produce of mevalonate. Then, the farnesyl pyrophosphate and squalene are synthesized. Squalene possesses a structure that resembles the structure of the steroid nucleus,

the four fused rings. Under the reaction catalyzed by the squalene cyclase, lanosterol, the first sterol molecule in this biosynthetic pathway is produced. After several steps, including the loss of three methyl groups, the reduction of double bonds and the change of positions, cholesterol is formed from lanosterol (Berg, Tymoczko et al. 2002).

5. Regulation of cellular cholesterol content

SREBP (Sterol Regulatory Element Binding Protein), originally identified as basic helix-loop-helix (bHLH) leucine zipper transcription factors, are now established as the main regulator of the intracellular lipid synthesis (Goldstein, DeBose-Boyd et al. 2006). SREBP1 can activate expression of the genes involved in the biosynthesis and esterification of fatty acid; SREBP2 is an activator of the genes involved in intracellular cholesterol metabolism. SREBPs are synthesized and located in the membrane of ER in an inactive form, associated with SCAP (SREBP Cleavage Activating Protein) and Insig. The SCAP acts as the cholesterol sensors; sterol-sensing domain (SSD), a special area of SCAP, is able to assess the amount of cholesterol in ER (Radhakrishnan, Ikeda et al. 2007). Insig is the sensor of oxysterols, the oxidative products of cholesterol. Therefore, several oxysterols, such as 25-hydroxycholesterol and 24(S), 25-epoxycholesterol can also regulate cholesterol biosynthesis (Howe, Sharpe et al. 2016).

When the cellular cholesterol levels are depleted, there is a conformational change of the SSD that allows the SREBP-SCAP complex to dissociate from Insig; SCAP escorts SREBP in COPII vesicles to the Golgi apparatus, where the SREBPs are cleaved by the site 1 and site 2 proteases (S1P and S2P) (Ikonen 2008). The N-terminal active part of SREBP is cleaved and released by the two proteases and migrates to the nucleus of the cell. The SREBP transcription factor then binds to a specific DNA sequence, the Sterol Regulatory Element (SRE), and activates transcription of target genes, including the gene of HMG-CoA reductase and the gene of LDL receptor. Thus, when the amount of cholesterol decreases, SREBP can activate the synthesis of these two proteins that will respectively accelerate the synthesis of cholesterol and

increase the amount of cholesterol taken up through endocytosis of LDL (Figure 7). In addition, the enzyme HMG-CoA reductase can also bind to lanosterol, an intermediate in the cholesterol biosynthesis pathway, and then be degraded by proteasome.

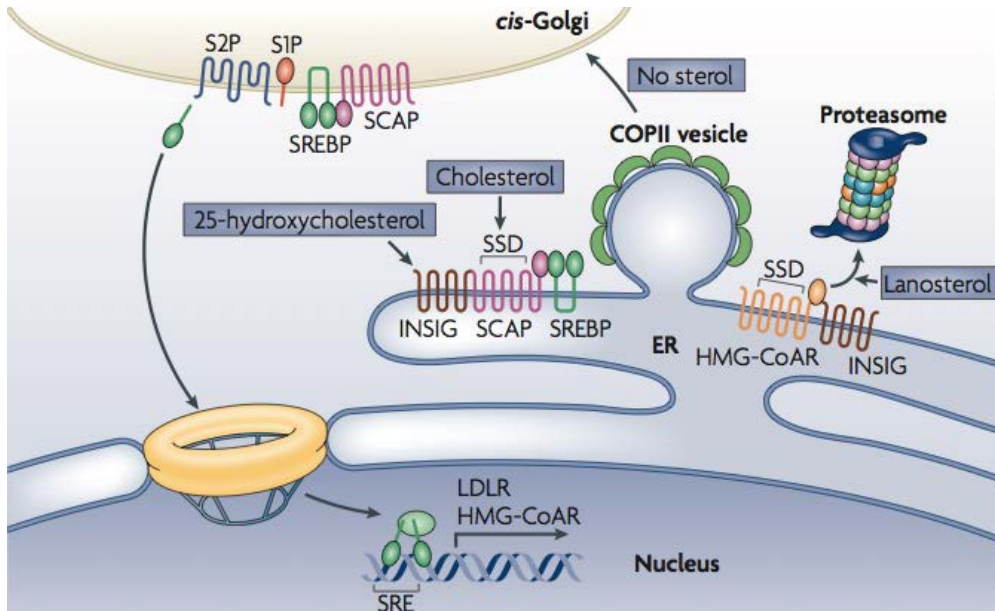


Figure 7. SREBP regulation of cholesterol metabolism
(Ikonen 2008)

However, when the amount of cholesterol in the ER is too high, Insig prevents the entry of SREBP-SCAP complex to COPII-coated vesicles and associates with HMG-CoA reductase to make it be degraded by the proteasome (Goldstein, DeBose-Boyd et al. 2006). Furthermore, when the cells acquire exogenous cholesterol via uptake of LDL, the cholesterol derived from LDL increases the activity of Acyl-CoA cholesterol acyltransferase (ACAT), which converts free cholesterol into cholesterol esters that are stored in cytosolic lipid droplets (Yang, Galea et al. 2012).

6. Cycle of hydrolysis and esterification of cholesterol

In cells, the hydroxide radical of cholesterol can be esterified with a fatty acid (primarily oleic acid) by the enzyme acyl-CoA: cholesterol acyltransferase (ACAT). In macrophages, ACAT is localized in ER where it catalyzes the esterification of cholesterol from PM, novo biosynthesis or endocytosis of LDL. The ACAT is regulated allosterically by cholesterol; therefore its activity is increased by large quantities of free cholesterol. The cholesterol esters produced by ACAT are stored with triglycerides in the core of cytosolic lipid droplets. These droplets are formed with a mechanism involving caveolin-1 protein. Exchanges may also occur between lipid droplets and ER via transport proteins (Prinz 2007).

Cholesterol esters in the lipid droplets can be hydrolyzed to release free cholesterol, which are then transported to ER or other compartments. The fatty acids released from lipid droplet are equilibrated instantaneously with the cytosolic pool buffered by specific binding proteins. The hydrolysis is catalyzed by the neutral cholesterol ester hydrolase (nCEH), an enzyme present in the cytoplasm. The lipid droplets, as the dynamic compartments of cholesterol, undergo a permanent cycle of hydrolysis and esterification. The control of this cycle is essential for the maintenance of cellular cholesterol homeostasis. Since the free cholesterol is more cytotoxic than the cholesterol esters, ACAT may serve as a protector in cells. However, when the amount of cholesterol reaches a certain threshold, the activity of ACAT is dramatically increased, which stimulates the development of macrophages into foam cells (Aluganti Narasimhulu, Fernandez-Ruiz et al. 2016). Only free cholesterol can participate in the cellular efflux, therefore, nCEH may also protect the cells by preventing or relenting the formation of foam cells. Both of these two enzymes can be protective, but perhaps at different stages of atherogenesis.

7. Intracellular transport of cholesterol

Intracellular cholesterol transport is important for maintaining cholesterol homeostasis, through multiple metabolic pathways including synthesis in ER, efflux to plasma lipoprotein,

uptake by LDL receptor, and regulation of content by SREBP and LXR transcription factors. A summary of the different transports is shown in Figure 8.

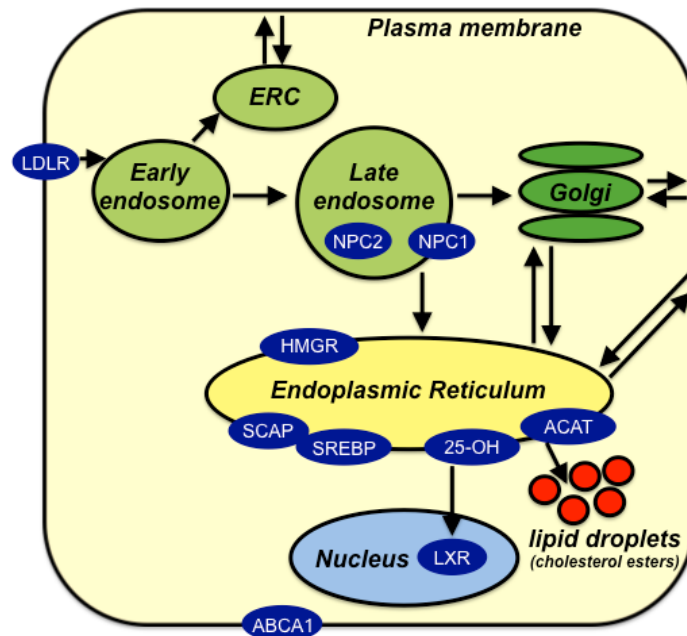


Figure 8. Intracellular cholesterol transport
(Soccio and Breslow 2004)

7.1 Different modes of intracellular transport of cholesterol

Cholesterol is a highly hydrophobic lipid; its passive diffusion in cytoplasm is very slow. Thus, the transport of cholesterol from one cellular compartment to another is mainly via (1) the vesicular transport, (2) the transport with carrier proteins, (3) transport across membrane contact sites. (Figure 9)

Cholesterol can be present in the membrane of transport vesicles or tubules, which carry membrane constituents among compartments. This transport process requires the intact cytoskeleton for the tracks of vesicles movement and the energy from ATP for the motor proteins, but does not need a change in the transversal distribution of cholesterol in the donor membrane. The amount of cholesterol in vesicles depends on its affinity for the microdomains.

When the vesicles are blocked, cholesterol can be shuttled through the nonvesicular transport, which is mediated by diffusible carrier proteins. These specific proteins contain hydrophobic cavities to bind cholesterol and transport it across the aqueous cytosol. There are many proteins that can function as the cholesterol carriers, such as the StAR (Steroidogenic Acute Regulatory) and START (StAR Lipid-Related Transfer domain), the NPC2 (Niemann-Pick Type C) which is involved in cholesterol transport from late endosomes, CPS-2 protein (Sterol Carrier protein 2) and caveolin. In this pathway, cholesterol must desorb from the cytoplasmic leaflet of the donor membrane, therefore the transbilayer distribution of cholesterol can affect this process (Ghosh 2012).

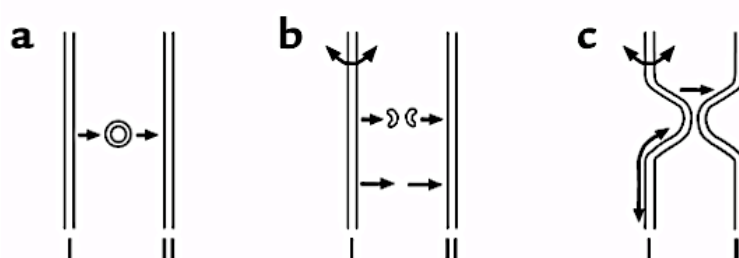


Figure 9. Basic mechanisms of cholesterol transport between two membranes
(Maxfield and Wüstner 2002)

(a) Vesicular transport (b) Diffusion through the cytoplasm either bound to a carrier protein (upper arrows) or by free diffusion (lower arrows) (c) Transport across membrane contacts.

Finally, the cell membrane contact sites (MCSs) are the regions where the membranes of two cellular compartments are bridged closely, typically less than 30nm (Helle, Kanfer et al. 2013). Therefore, the MCSs, particularly the site between RE and the other organelles, facilitate the transfer of cholesterol between the two membranes, either by diffusion or with the aid of transfer proteins (Holthuis and Menon 2014).

7.2 Transport of cholesterol from early endosome

After LDL particles are internalized by macrophages via LDL receptors and clathrin-coated pits, the vesicles containing LDL particles are fused with early endosomes (Elkin, Lakoduk et al. 2016). In the acidic condition of early endosome, the LDL receptors dissociate from LDL and localize to early endosomal tubular extensions, the bud of early endosome that subsequently fuse with the endocytic recycling compartment (ERC). Through this way, some free cholesterol on the early endosomal membrane can also be transferred to the ERC. Finally, the LDL receptors and lipids from ERC are transported to the plasma membrane.

7.3 Transport of cholesterol from late endosome/lysosome

The early endosomes proceed to late endosomes perhaps by a process involving vesicular transport or by the evolution of early to late endosomes. In late endosome/lysosome (LE/Ly), the LDL cholesterol esters are hydrolyzed by acid lipase to release free cholesterol. NPC1 (Niemann–Pick Type C1) and NPC2 proteins play essential roles for the transport of cholesterol from late endosomes. NPC2, localized inside the lumen of LE/Ly, is a soluble cholesterol-binding protein. It accepts and delivers the free cholesterol to NPC1, a transport protein localized on LE/Ly membrane. NPC1 protein binds free cholesterol with its N-terminal domain and inserts the cholesterol into the membrane of LE/Ly for export (Deffieu and Pfeffer 2011). Niemann–Pick type C is a lysosomal storage disease associated with mutations in NPC1 and NPC2 genes. The mutations of these two genes disrupt the transport system and result in the accumulation of cholesterol in lysosomes (Du and Yang 2013).

The free cholesterol derived from LDL exits LE/Ly membrane and reaches endoplasmic reticulum, plasma membrane and other organelles for its regulatory and structural roles. On plasma membrane, cholesterol can be transported to various extracellular acceptors or be returned to the endoplasmic reticulum, where it could be esterified by ACAT. Almost 70% of

the LDL cholesterol travels to ER through plasma membrane, but it can also be transported directly from LE to ER (Eden 2016). Both vesicular and non-vesicular pathway are involved in the endosomal transport of cholesterol. Rab GTPases regulate many steps of membrane traffic, including vesicle formation and vesicle movement. In addition, several proteins on LE with a cholesterol-binding site have been proposed to play a role in the non-vesicular transport of cholesterol from the LE, such as MLN64 (Martin, Kennedy et al. 2016) and ORP1 (Olkkonen 2015).

Finally, the internal membrane of late endosome contains a high proportion of bis(monoacylglycero)phosphate (BMP). BMP, a phospholipid that forms special microdomains on the LE membrane, plays a key role in the dynamic structure of late endosome and in the intracellular transport of cholesterol derived from LDL (Arnal-Levron, Chen et al. 2013, Erazo-Oliveras, Najjar et al. 2016).

7.4 Transport of cholesterol from endoplasmic reticulum

After synthesis in endoplasmic reticulum, cholesterol is rapidly transported to the plasma membrane. This process involves several pathways and proteins. In the first pathway, cholesterol is transported with vesicles and through Golgi apparatus (Wüstner and Solanko 2015). The second pathway requires the membrane contact sites (MCS), which is able to facilitate the transfer of cholesterol, between ER and other cellular compartments, including plasma membrane (Levine and Loewen 2006). In addition, several transport proteins, such as caveolin, SCP-2 and ORP2, play important roles in this intracellular cholesterol transport; but the exact roles of these proteins are still uncertain. Caveolin can bind cholesterol directly and may form a complex with chaperone proteins to transfer cholesterol from ER to PM, bypassing the Golgi apparatus (Sengupta 2012). The traffic of cholesterol between ER and PM is dynamic, since it has been estimated that the entire cholesterol-pool of PM shuttles to ER and back with a half time of 40 minutes (Lange, Strebel et al. 1993).

7.5 Transport of cholesterol from plasma membrane

The cholesterol in the plasma membrane is continually re-internalized in cells especially to the ER, where it could be esterified by ACAT and also participates in the transcriptional regulation controlled by SREBP. The damage on the cytoskeleton inhibits the transport of cholesterol from the plasma membrane to the endoplasmic reticulum, suggesting that the vesicular route is involved in this transport (Inder, Zheng et al. 2012). Furthermore, in the vesicular pathway between the PM and ER, cholesterol can be transferred through endosomes and Golgi apparatus. In addition, the cholesterol-rich microdomains play an important role in intracellular redistribution of cholesterol from the plasma membrane; and the non-vesicular mechanisms are also involved in this transport (Bharti 2014).

8. Efflux of cholesterol from macrophage

High-density lipoprotein (HDL) can capture the excess cholesterol from peripheral tissues, including macrophages, and subsequently deliver it to liver and steroidogenic organs, where cholesterol is used for the synthesis of lipoproteins, bile acids, vitamin D, and steroid hormones (Fisher, Feig et al. 2008). This process is called reverse cholesterol transport (RCT), which is initiated from the efflux of cholesterol from cells. This efflux is characterized by a transfer of free cholesterol from plasma membrane to different extracellular acceptors.

In macrophages, there are four pathways for exporting free cholesterol to an extracellular acceptor (Jessup and Kritharides 2008). The passive pathways include simple aqueous diffusion and facilitated diffusion mediated by SR-BI. The active processes are mediated by the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1, which are membrane lipid translocases.

8.1 Aqueous diffusion efflux pathway

Despite of its high hydrophobicity, cholesterol is sufficiently water-soluble to be released from cell membrane by aqueous diffusion mechanism to meet an acceptor in plasma (Phillips 2014). Aqueous diffusion, an unmediated bidirectional pathway that occurs in all cell types, involves desorption of cholesterol molecules from the donor membrane, diffusion of these molecules through the surrounding water layer, and incorporation of the cholesterol into a phospholipid-containing acceptor. With a constant donor concentration, the diffusion rate is dependent on the concentration and size of the acceptor particles. Since cholesterol in lipoproteins and membranes has high affinity with phospholipid, the free cholesterol/phospholipid ratios of donor and acceptor particles are also important for the rate and the net transfer of cholesterol.

The cholesterol aqueous diffusion occurs in the time scale of hours; therefore it is not an efficient pathway for cholesterol efflux. SR-BI can facilitate this process (Zanotti, Favari et al. 2012).

8.2 SR-BI efflux pathway

SR-BI is also involved in efflux of cholesterol by facilitating bidirectional transport between plasma membrane and HDL. Therefore, like the aqueous diffusion, the net transfer of cholesterol through SR-BI is dependent on the direction of the cholesterol gradient.

SR-BI, a homo-oligomeric glycoprotein, is a member of CD36 superfamily and shares almost 30% homologous sequence with the other proteins of the family. In plasma membrane, SR-BI is located in the caveolae and lipid rafts, the areas of the cell membrane that are rich in both cholesterol and sphingomyelin. The large extracellular domain (408 aa) of SR-BI, which is anchored in cell membrane with two N- and C-terminal transmembrane domains, plays a crucial role in mediating the bidirectional flux of free cholesterol, as it forms a nonpolar

channel that promotes the movement of lipid molecules between the various acceptors and the plasma membrane (Meyer, Graf et al. 2013).

SR-BI is expressed in many tissues and organs, including macrophages. It is also expressed in human atherosclerotic lesions (Kartt, Holme et al. 2014). However, it has been shown that its level of expression in macrophages derived from patients suffering atherosclerosis is the same as that in healthy subjects (Svensson, Englund et al. 2005). Moreover, some in vitro studies indicate that the accumulation of cholesterol in macrophages decreases the expression of SR-BI through LXR and SREBP and oxidized LDL also tend to decrease the expression of SR-BI (Yu, Cao et al. 2004).

It was suggested that SR-BI might play an anti-atherogenic role. In the mice deficient in SR-BI, the atherosclerotic lesions grow faster (Covey, Krieger et al. 2003). However, there are studies that show that SR-BI only plays a minor role in cholesterol efflux from macrophages (Wang, Collins et al. 2007).

With a low concentration of HDL, SR-BI accelerates the diffusion of cholesterol through a direct bond with the HDL particles. However, when the concentration of HDL is higher and the binding of SR-BI is saturated, the SR-BI-mediated efflux still presents a large dependence on HDL concentration, which means that SR-BI accelerates the diffusion of cholesterol regardless of its association with HDL particles but by simply changing the distribution and availability of cholesterol in the surrounding membranes (Phillips 2014).

The phospholipid component of acceptor is also crucial for the SR-BI-mediated efflux. It has been shown that there is no efflux of cholesterol from SR-BI to lipid-free apo A-I, even though the lipid-free apo A-I can bind to SR-BI (de la Llera-Moya, Rothblat et al. 1999). It has also been shown that the enrichment of HDL with PC increases the efflux of cholesterol through SR-BI, whereas the treatment of HDL with phospholipase A₂ (PLA₂) decreases the diffusion (Bostan, Uydu et al. 2013). Moreover, both PC and sphingomyelin on HDL particles can increase the net efflux of free cholesterol via SR-BI, but the mechanisms are different. With the enrichment of PC, the rate of efflux to HDL is increased, whereas with the enrichment of sphingomyelin, the rate of free cholesterol influx from acceptors is decreased.

8.3 ABCA1 and ABCG1 efflux pathway

It has been proved that the absence of ABCA1 and ABCG1 in macrophages accelerates the process of atherogenesis by accumulating a mass of cholesterol in cells. Therefore, the ABCA1 and ABCG1 play essential roles in the removal of excess cholesterol. (Out, Hoekstra et al. 2008)

ATP-binding cassette (ABC) transporters is a large family of proteins, which contain the common structural motifs and use ATP as an energy source to transfer various kinds of substrates, such as lipids, ions, and cytotoxins. ABCA1 is a member of the A subfamily of ABC proteins, which has two transmembrane domains (TMD) and two nucleotide-binding domains (NBD) (Sano, Ito et al. 2014). The molecule of ABCA1 contains 2 large extracellular loops and both of the loops are crucial for interaction with apo A-I. ABCG1 and ABCG4 are members of the ABCG subfamily, the half-type ABC proteins, which means that the ABCG proteins must partner with another ABCG protein, either by homo- or hetero-dimerization, in order to function. The ABCG proteins are composed of an N-terminal cytosolic NBD and a C-terminal TMD, which has 6 helices. ABCG1 is expressed widely but highest in macrophage-rich tissue such as spleen, lung, and brain. In membranes, ABCG1 can either serve as a homodimer or function as a heterodimer with ABCG4, but this heterodimerization still need to be proved (Kobayashi, Takanezawa et al. 2006). At the sub-cellular level, both ABCG1 and ABCG4 are thought to be located in intracellular vesicles as well as on the plasma membrane.

ABCA1 can stimulate a unidirectional transfer of free cholesterol and phospholipids from the plasma membrane to a lipid-poor apolipoprotein, such as apo A-I, apo A-II, and apo A-IV. The lipidation of apo A-I forms the nascent HDL. On the contrary, ABCG1 and ABCG4 can only transport lipid to HDL particles. Thus, it has been proposed that ABCA1 and ABCG1 may work sequentially. (Figure 10) At first, ABCA1 generates a HDL particle from apo A-I; then the HDL particle stimulates the efflux of cholesterol via ABCG1. ABCA1 and ABCG1 contribute together to the majority of cholesterol efflux from macrophages (Gelissen, Harris et al. 2006). In addition, ABCG1 stimulates the efflux of oxidized cholesterol (oxysterols),

especially the oxysterols with the oxidation at C-7 position, and protects cells from the deleterious effects of the oxidative products of cholesterol (Terasaka, Wang et al. 2007).

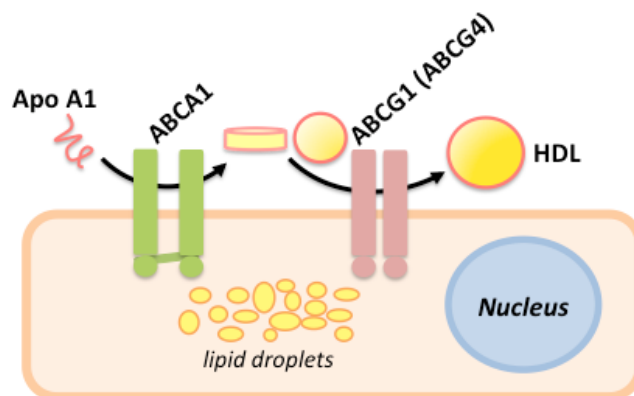


Figure 10. Efflux of cholesterol by ABCA1 and ABCG1

ABCA1 is distributed both in the plasma membrane (PM) and the late endocytic vesicles and it can shuttle between them rapidly. Its distribution to the PM is promoted by palmitoylation (Singaraja, Kang et al. 2009). It is suggested that the presence of ABCA1 on plasma membrane changes membrane morphology, which means that the quantity of PL and FC in the outer leaflet of the PM is increased (Landry, Denis et al. 2006). In addition, the interaction of apo A-I with ABCA1 and the surrounding lipid domains of PM are necessary for efflux. Apo A-I diffuses literally in the membrane and forms a productive complex with ABCA1 (Panagotopoulos, Witting et al. 2002).

The mechanism of cholesterol efflux through ABCA1 is still uncertain. Several mechanisms and models have been proposed, such as “two-step” model and “one-step” model (Phillips 2014, Jin, Freeman et al. 2015). For the “two-step” model (also called “sequential” model), the individual molecules of phospholipid are transferred before the cholesterol molecules to apolipoproteins. For the “one-step” model, phospholipid and cholesterol are mobilized simultaneously as discrete units, either small (microsolubilization) or large (macrosolubilization). Both of the two models are remained to be more proved.

Degradation of ABCA1 occurs rapidly after its transcription. Its cellular level is sensitive to the presence of apo A-I, since the interaction with apo A-I can protect it from the calpain-mediated proteolysis (Yokoyama, Arakawa et al. 2012). The genes of ABCA1 and ABCG1 are highly expressed when there is accumulation of cholesterol in cells. The expression of these genes is also regulated by the liver X receptor (LXR), and in some macrophages, by cAMP (Schmitz and Langmann 2005). Moreover, some studies indicate that the protein caveolin-1 is involved in cholesterol efflux stimulated by ABCA1 and ABCG1. However, these results remain controversial (Lin, Ma et al. 2007, Storey, Gallegos et al. 2007). Wang and the colleagues estimate that the function of caveolin-1 on cholesterol efflux is depending on cell types and the expression levels of caveolin-1 (Wang, Gu et al. 2014).

c. Oxysterols

Oxysterols are derived from oxidation of cholesterol by various pathways. Cells can gain oxysterols by intracellular oxidation of cholesterol or by the uptake of oxidized lipoproteins. Like cholesterol, most of the unesterified oxysterols in cells are localized in membranes. Besides of being the intermediates in cholesterol catabolism, oxysterols are bioactive lipids that play important roles in regulation of lipid metabolism, inflammation and act as toxic factors, which contribute to atherosclerosis. Many studies have shown that the content of oxysterols in macrophages is increased in the patients with diabetes, hypercholesterolemia and atherosclerosis (Alkazemi, Egeland et al. 2008).

1. Biological sources of oxysterols

Oxidation of cholesterol generates hydroxyl-, keto- or epoxy- groups at different positions of the cholesterol molecule. Generally, there are two main categories of oxysterols (Figure 11):

the oxidation on the sterol ring (mainly at the 7-position), such as 7-ketocholesterol (7-KC) and 7 β -hydroxycholesterol (7 β -HC); the oxidation on the side-chain, such as 25-hydroxycholesterol (25-HC) and 27-hydroxycholesterol (27-HC). Most of the ring-oxidized sterols are generated via the non-enzymatic pathway, and the side-chain-oxidized sterols are the products of enzymatic reaction. However, 25-HC and 7 α -HC are the exceptions, they can be formed by both enzymatic and non-enzymatic ways.

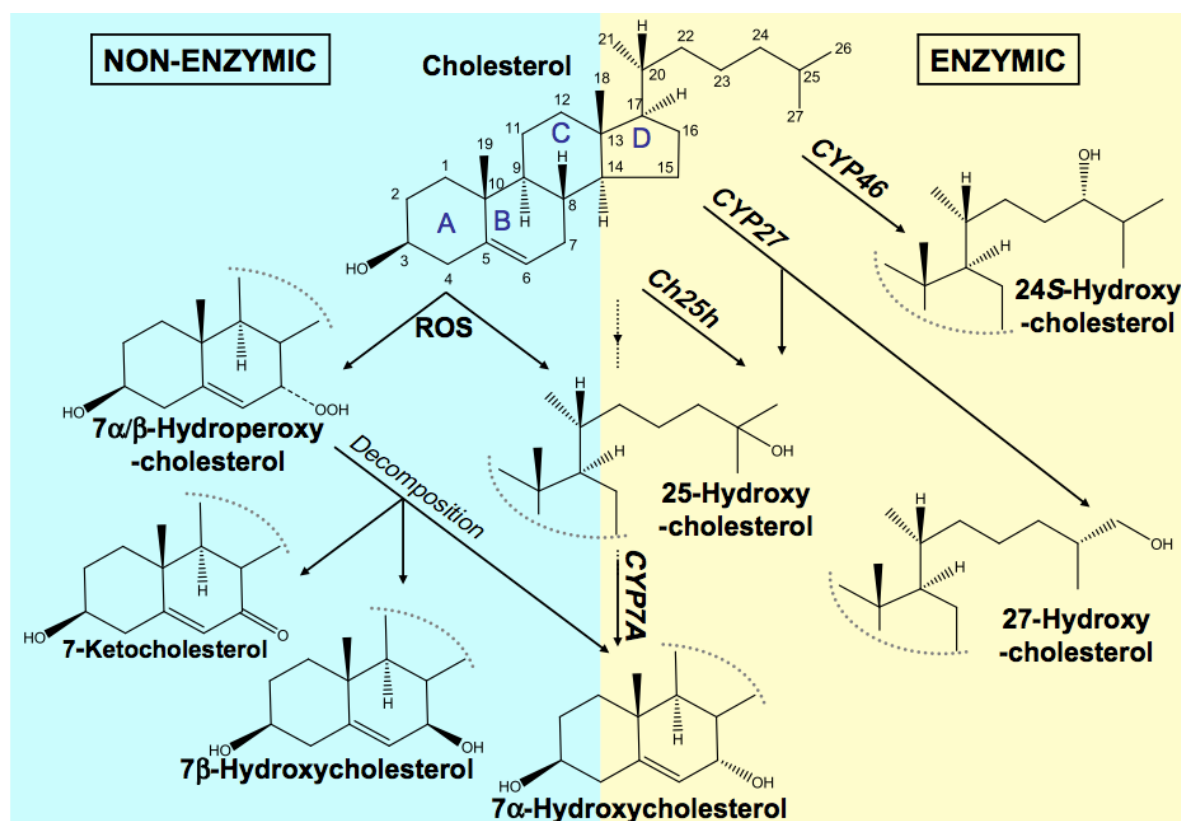


Figure 11. Structure of several major biological oxysterols
(Brown and Jessup 2009)

1.1 Formation from non-enzymic pathway

The non-enzymatic cholesterol oxidation (also called autoxidation of cholesterol) can be

triggered by trace amounts of metals (for example, Cu^{2+}) and reactive oxygen species (ROS) that include peroxides, superoxide, hydroxyl radical, and singlet oxygen. This oxidation pathway occurs in various model systems, such as aqueous emulsion, liposomes, monomolecular films spread on water or even the crystalline state. During the process of autoxidation, several radicals are able to oxidize cholesterol to form numerous stable or unstable intermediates and end products. The radical-triggered oxidation of cholesterol is generally classified into two types of reactions: the initiation and the propagation. Initiation is the first reaction, which is induced by lipid peroxidation process and is focused on a carbon with labile hydrogen. After the initial reaction, a reactive species is formed and can subsequently recruit other non-oxidized lipids to start a chain reaction called propagation phase. Copper is a very effective catalyst; its rate of oxidation is 60 times higher than that of iron (Halliwell and Gutteridge 2015).

The site preferred by non-enzymatic oxidation is the C-7, since the carbon-hydrogen bond here is relatively weak. Therefore, the main oxysterols generated by this mechanism are 7-KC and 7 α/β -HC. The dissociation energy of this C-H bond is 88 kcal/mol, little higher than the C-H bond of polyunsaturated fatty acid (75 kcal/mol) of phospholipids. However, in cell membranes, PUFAs are less prone to be oxidized than cholesterol, because of the abundance of cholesterol and the complex and fluid membrane environment (Iuliano 2011).

Finally, the level of oxysterols formed by cholesterol autoxidation is declined in animals that are fed the lipophilic antioxidants, such as probucol, butylated hydroxytoluene (BHT) and vitamin E (Shibata and Glass 2010).

1.2 Formation from enzymic pathway

Enzymic oxidation is able to induce the addition of a hydroxyl group to cholesterol side-chain and form the 24-, 25- and 27-hydroxycholesterols with specific enzymes.

The mitochondrial P450 enzyme 27-hydroxylase (CYP27A1) is expressed in many tissues, especially in liver and macrophages (Raza, Schommer et al. 2015). It catalyzes the formation of

27-hydroxycholesterol. This reaction is the first step in the oxidation of the side chain of sterol and is involved in the classical and alternative bile acid pathway. 27-hydroxylase can also produce 27-hydroxy-derivatives of cholesterol precursors, such as lanosterol, desmosterol and 7-dehydrocholesterol.

24-hydroxylase (CYP46A1), another P450 enzyme, is able to convert cholesterol into 24S-hydroxycholesterol (24-HC). CYP46A1 is located in ER and highly expressed in neural cells of the brain and retina (Bretillon, Diczfalusy et al. 2007). In brain, 24-HC can be transferred across the blood-brain barrier to maintain the cholesterol homeostasis.

Unlike 27-hydroxylase and 24-hydroxylase, 25-hydroxylase is not a P450 enzyme, but belongs to a smaller family of enzymes, which use di-iron as cofactors to convert cholesterol into 25-hydroxycholesterol (25-HC) (Diczfalusy 2013). 25-hydroxylase is expressed in most tissues at very low levels and is localized in the membranes of ER and Golgi apparatus. Its product, 25-HC, is an efficient suppressor of sterol synthesis in cells.

In addition, there are other oxysterols generated by enzymatic pathways. 7α -HC, produced by 7α -hydroxylase (CYP7A1), is found in the liver and functions as intermediate in the synthesis of bile acid. 24(S),25-epoxycholesterol (24(S),25-EC) is generated by shunt pathway during the biosynthesis of cholesterol (Nelson, Steckbeck et al. 1981).

1.3 Dietary sources of oxysterols

In cholesterol-rich foods, such as meat, eggs and dairy products, we can found relatively high levels of oxysterols. The content of oxysterols in food depends on the way of cooking and the length of storage periods. It has been shown that the oxysterols are absorbed in gut and then are transported by chylomicrons (Carpenter 2002). The absorption extent of dietary oxysterols is various in different systems, from 6% to 93% (Salejda and Krasnowska 2016), which means that the uptake, metabolism and elimination of these oxysterols are diverse *in vivo*.

2. Regulation of cellular cholesterol homeostasis by oxysterols

Several oxysterols generated enzymically from cholesterol are able to regulate the content of intracellular cholesterol through these mechanisms (Figure 12): 1. accelerate the degradation of HMG-CoA reductase; 2. inhibit the activation of SREBP; 3. increase cholesterol efflux by acting as activating the LXR; 4. increase the storage of cholesterol ester in cells.

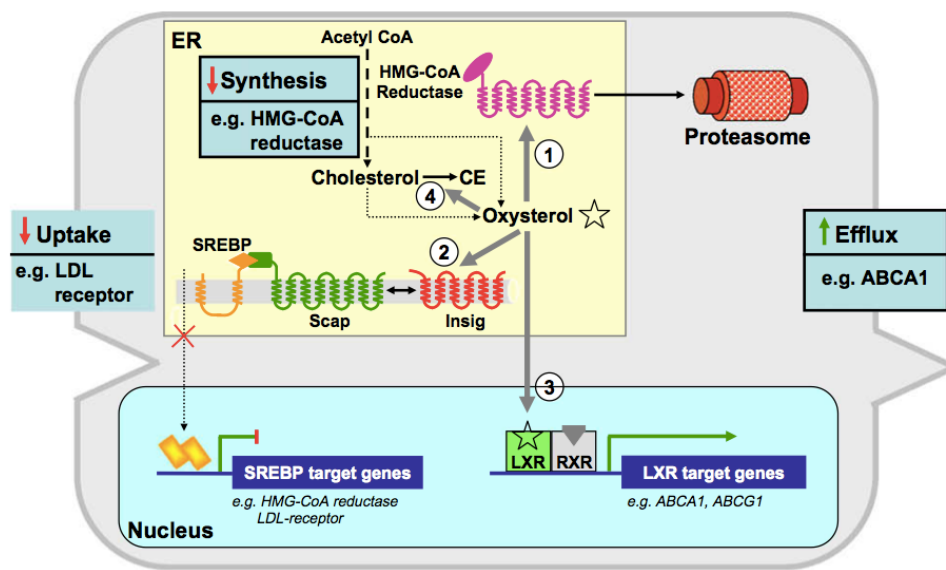


Figure 12. Functions of oxysterols for regulation of cholesterol homeostasis
(Brown and Jessup 2009)

2.1 LXR

The nuclear receptors LXR (Liver X Receptors) are transcription factors that bind both steroidal and non-steroidal ligands. Several oxysterols derived from enzymatic processing, such as 24(S),25-EC, 22(R)-HC, 24(S)-HC, and 27-HC, are potent ligands of LXRs. There are two members of the LXR subfamily, the LXR α and LXR β . LXR α is expressed mainly in liver and small intestine, whereas LXR β is widely expressed (Wójcicka, Jamroz-Wiśniewska et al. 2016).

In order to regulate the expression of genes, LXRs must form heterodimers with retinoid X receptors (RXRs) (Figure 13). RXRs are related members of the nuclear receptor superfamily; they can be regulated by 9-cis-retinoic acid and long-chain polyunsaturated fatty acids. Without the presence of its ligands, the LXR-RXR heterodimer binds to LXR response elements (LXREs) in the promoter region of target genes and remains inactive by binding the corepressors. When bound by its ligands, the heterodimer releases the corepressors and recruits coactivators, activating the functions of LXR (Vaisar, Hutchins et al. 2015).

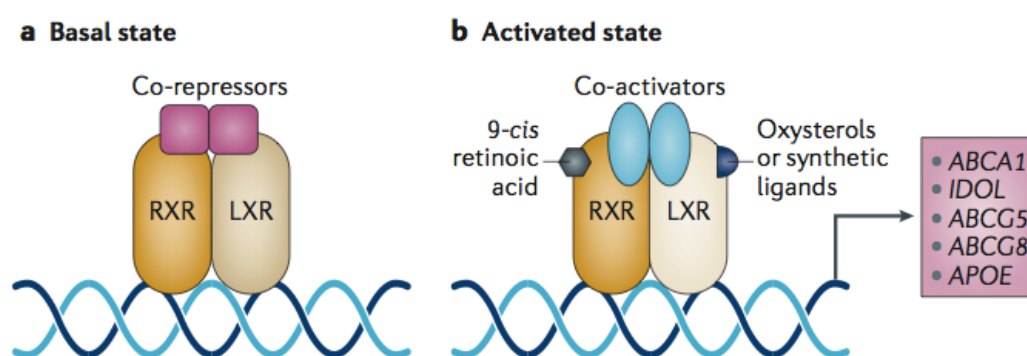


Figure 13. Action mechanisms of Liver X Receptors
(Hong and Tontonoz 2014)

The activation of LXRs can regulate a number of genes involved in cholesterol homeostasis, such as ABCA1, apolipoprotein E and SREBP1c. In macrophages, the uptake of oxLDL increases cellular concentrations of cholesterol and oxysterols. The activation of LXRs can decrease the expression of genes involved in cholesterol biosynthesis, particularly the gene of enzyme HMG-CoA reductase (Sharpe and Brown 2013), and can also stimulate cholesterol efflux by increasing the expression of ABCA1 (Hsieh, Kim et al. 2014). Therefore, LXRs may play an important role in reducing the development of atherosclerotic lesions (Joseph, McKilligin et al. 2002, Kappus, Murphy et al. 2014, Gui, Yao et al. 2016).

2.2 SREBP pathway

Insig, an oxysterol sensor, is another key regulator in ER membrane. It can inhibit the function of SREBP (Sterol Regulatory Element Binding Protein) by trapping and retaining the SREBP-SCAP complex at ER (Figure 12②). Thus, several oxysterols, such as 22(R)-, 25-HC and 24(S),25-EC, are able to attenuate cholesterol biosynthesis, expression and activity of LDL receptor in macrophage (Shao and Espenshade 2012).

2.3 OSBP and ORP

Oxysterols can also control the esterification of cholesterol and the activity of HMG-CoA through the oxysterol binding protein (OSBP) and the OSBP-related proteins (ORP) (Weber-Boyavt, Kentala et al. 2015). In human body, there are at least 12 OSBP/ORP proteins. They are characterized by an oxysterols-binding domain at C-terminal and a membrane interaction domain at N-terminal. OSBP is a cytosolic protein with natural ligands for various oxysterols, including 25-HC and 7-KC. The combination of 25-HC and OSBP induces a conformational change of the protein and promotes the translocation of the protein to Golgi apparatus. It has been shown that the overexpression of OSBP in CHO cells can decrease the activity of ACAT and increase the transcription of LDL receptor gene and HMG-CoA reductase gene, thereby stimulating the biosynthesis of cholesterol (Lagace, Byers et al. 1997).

OSBP, ORP1 and ORP2 are involved in vesicle transport of cholesterol, particularly the transport between ER and Golgi apparatus (Olkkonen 2015). ORP1L, a sterol-dependent regulator, localizes at the late endosome membrane and can regulate the positioning of late endosome (Rocha, Kuijl et al. 2009). ORP2 is located on cytoplasmic lipid droplets. The overexpression of ORP2 in cells can stimulate the transport of cholesterol from ER, decrease the esterification of cholesterol and increase the efflux of cholesterol to different extracellular acceptors (Laitinen, Lehto et al. 2002). The overexpression of ORP4 is able to disrupt the transport of cholesterol derived from endocytosed LDL (Cheng, JeBailey et al. 2002). The ORP8 expressed in THP1 cells is a negative regulator of the expression of ABCA1; this

suppressive effect involves the function of LXR in the ABCA1 promoter (Yan, Mäyränpää et al. 2008). These results suggest that several ORP proteins play an opposite role with LXR in the efflux of cholesterol. However, the precise mechanisms of the ORPs influence on cholesterol metabolism are still unknown. The different effects of ORPs and LXR might be caused by their different affinities and specificities for oxysterols (Shibata and Glass 2010).

3. Oxysterols as substrates of LCAT and ACAT

Cholesterol esters, the preferred form for circulation and intracellular storage of cholesterol in human body, are the products of plasma lecithin:cholesterol acyltransferase (LCAT) and cellular acyl-coenzyme A:cholesterol acyltransferase (ACAT). Like cholesterol, oxysterols can be substrates of these two enzymes to generate esters of oxysterols. As the major form of oxysterols in plasma and atherosclerotic lesions (Alkazemi, Egeland et al. 2008), the esterified oxysterols can facilitate the incorporation of oxysterols in lipoproteins and attenuate their toxicity in cells (Monier, Samadi et al. 2003).

It has been shown that when 27-HC is incubated with LCAT, it can form both mono- and di-esters with the 3 β - and 27-hydroxyl groups, whereas the incubation of other oxysterols, such as 25-HC, 7 α -HC and 7 β -HC, can only generate the 3 β -oleoyl monoesters (Szedlacsek, Wasowicz et al. 1995). In addition, the ester of 25-HC can also be transferred by cholesteryl ester transfer protein (CETP) between lipoproteins in plasma (García-González, Gutiérrez-Quintanar et al. 2014).

The esterification catalyzed by ACAT, can only generate the esters of several oxysterols, such as 7 α -HC and 5 α ,6 α -epoxycholesterol. The other oxysterols, including 7-KC, 7 β -HC and 5 β ,6 β -epoxycholesterol, are poorly esterified by this enzyme (Zhang, Yu et al. 2003).

4. Orientation of cholesterol and oxysterols in membrane

Cholesterol molecule is composed of a tetracyclic ring structure and a branched isooctyl side chain. The tetracyclic ring structure (sterol nucleus) is flat and contains a hydroxyl group at the position 3. As it shown in figure 14, in biological membrane, cholesterol molecular is roughly parallel with the phospholipid molecules forming a tight package with the saturated fatty acid chains of sphingolipids and glycerophospholipids; its hydrophobic side chain points to the inner part of the bilayer and its 3 β -hydroxyl group locates at the membrane-water interface with the phospholipid head groups. Because of its structural features, cholesterol is able to form the liquid ordered (Lo) phases in biological membrane.

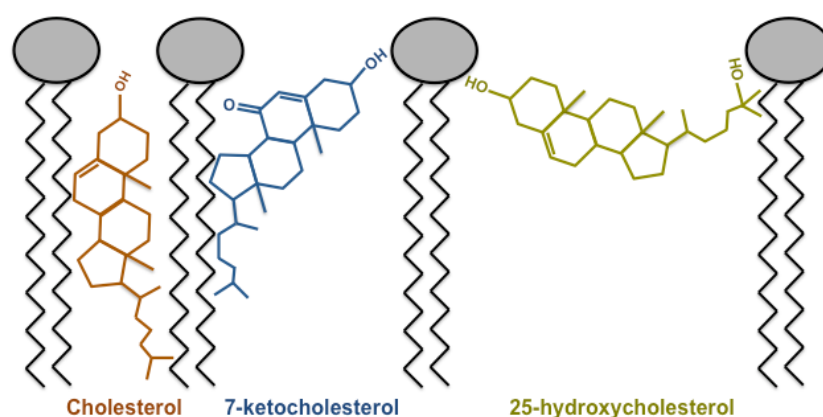


Figure 14. Orientation of cholesterol and selected oxysterols in membrane (Olkkonen and Hynynen 2009)

As the oxidative products of cholesterol, oxysterols can also participate in the constitution of membrane. Oxidation of cholesterol generates various oxygen substitutions in oxysterols molecules, such as hydroxyl-, carbonyl-, epoxy- and hydroperoxy-. These polar oxygen substitutions make sterol molecules much more hydrophilic. Therefore, oxysterols are able to move more rapidly between membranes than cholesterol. It has been shown that the rate of oxysterol transfer depends on the distance between the 3-hydroxyl group and the second oxidized function, which means that the oxysterols with an oxidation on the side chain

are transferred more quickly than the species with oxidation on the sterol nucleus (Meaney, Bodin et al. 2002).

In addition, depending on the different positions of the oxidized groups (usually the hydroxyl group), the orientation of oxysterols in membrane is different. (Figure 14) The oxysterols generated through enzymatic pathway containing an oxidized group on their side chains, for example 25-HC, have two polar ends on the molecule, preferring a vertical orientation with the phospholipid in the biological membrane. For the oxysterols oxidized on the tetracyclic ring structure, such as 7-KC and 7 β -HC, there are two hydrophilic groups on the same side of the molecule, which leads to a vertical orientation in the membrane and a decreased condensing of the membrane lipids compared to cholesterol (Phillips, Geng et al. 2001). Thus, the membrane lipid order and the package properties are altered by these polar oxidized groups, and the specific structure of the enzymic pathway oxysterols promotes the membrane permeability and cholesterol departure (Olsen, Schlesinger et al. 2012).

II. Atherosclerosis

a. Generality of atherosclerosis

Atherosclerosis is defined by World Health Organization (WHO) in 1957 as "a variable combination of changes of the intima of arteries (as distinguished from arterioles) consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes. " In other words, it is a chronic inflammatory disease caused by a disorder of lipid metabolism. Since there is differences in blood flow dynamics, the cell regions with low fluid shear stress - the sites of arterial branching or curvature - are much more susceptible (Wentzel, Chatzizisis et al. 2012). Therefore, the abdominal aorta, coronary arteries and carotid bifurcations are the predilection vessels for the formation of atherosclerotic plaques.

The initial event of atherosclerotic plaques can be observed very early in life, in young children, or even infants. Therefore, atherosclerosis is an evolutionary disease with an asymptomatic process for a long time and can be revealed by one of its complications, such as coronary heart disease (CHD), myocardial infarction (MI) and cerebrovascular accident (CVA). According to the data of WHO, ischaemic heart disease and stroke, two complications or atherosclerosis, are the leading causes of death in 2015 in the world; and they are also estimated to be the leading causes of mortality in 2030. The MONICA ("monitoring trends and determinants in cardiovascular disease") project showed that the frequency of cardiovascular disease is very high in North America and Europe, whereas it is much lower in Asia, the Mediterranean areas and the third world.

The risk factors for atherosclerosis are multiple; they can be classified as genetic and environmental factors. The genetic factors include the elevated levels of LDL/VLDL, the reduced levels of HDL, the elevated blood pressure, family history, diabetes and obesity, gender (male), systemic inflammation and metabolic syndrome. The environmental factors are high-fat diet, smoking, lack of exercise and infectious agents.

b. Classification of atherosclerotic lesions

Many groups have contributed to the classification of atherosclerotic lesions by analyzing different arteries (e.g. aorta, myocardium, coronary arteries) in different age groups. Stary and his colleagues reviewed numerous researches in this field and summarized three articles as the richest resources for microscopic descriptions of atherosclerosis (Stary, Blankenhorn et al. 1992, Stary, Chandler et al. 1994, Stary, Chandler et al. 1995). Then, in 2000, a histological classification of lesions was proposed by Stary; that is the American Heart Association classification, types I-VIII (Stary 2000). In this classification, the numbers are not consecutive. For example, the type IV lesion can directly develop to a much more advanced lesion bypassing the type VI lesion.

Later, Virmani introduced a much simpler classification (Figure 15) (Virmani, Kolodgie et al. 2000). The primary lesion types of this classification are xanthoma, pathological intimal thickening, fibroatheroma and fibrocalcific plaque. A patient with severe condition usually has different types of lesion at different areas of arteries.

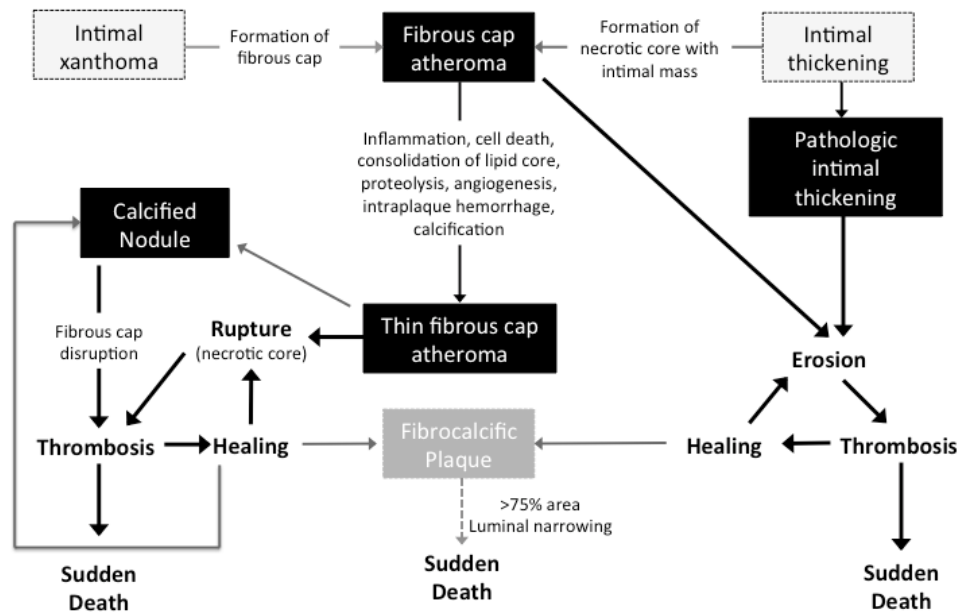


Figure 15. Simplified scheme for classifying atherosclerotic lesions modified from the current AHA recommendations

c. Atherogenesis

The developmental process of atherosclerotic plaques, also called atherogenesis, depends on a number of mechanisms. The appearance of the plaque at the early stage of development is the result of the accumulation of LDL in the sub-endothelial space where the lipoprotein particles undergo modifications (mainly oxidation) and massive endocytosis by macrophages thereby leading to intracellular accumulation of cholesteryl esters. The macrophages with superfluous intracellular cholesterol esters are called foam cells and deposit in the arterial

walls. This series of events is crucial in the early stage of atherogenesis, and is also critical in the whole progression of atherosclerosis, since it is a continuous process in the formation of the plaques.

1. Structure of a normal artery

Arteries consist of three morphologically distinct layers (Figure 16); they are the intima, the media and the adventitia.

The intima is the thinnest (exaggerated in Figure 16) and the innermost layer, where the atherosclerotic plaques develop. On the luminal side, it is bounded by the endothelium, a monolayer of endothelial cells. The endothelium is a selectively permeable barrier between blood and tissues and is also able to generate the molecules that can regulate thrombosis, inflammation, and vascular remodeling. In the sub-endothelial space, there are immune cells (macrophages and T cells), extracellular connective tissue matrix (mainly proteoglycans and collagen), and the elastin lamina on the peripheral side.

The media is the middle layer and is composed primarily of smooth muscle cells (SMCs), which ensure a permanent remodeling of the vessels in response to the physiological changes in body (Campbell and Campbell 2012). In addition, SMCs are able to produce and secrete the collagen, elastin and proteoglycans for the formation of fibrous matrix in the sub-endothelial space. Besides SMCs, the media also contains collagen and elastin; and its thickness is various according to the type of artery.

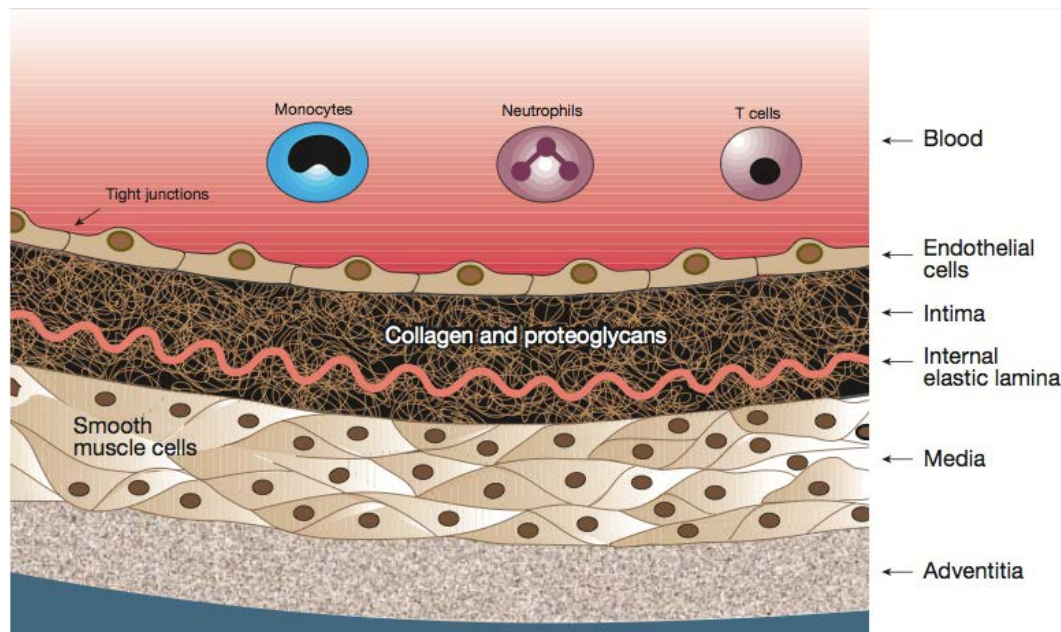


Figure16. Structure of a normal artery
(Lusis 2000)

The adventitia is composed of connective tissues. As the outer layer, it ensures the anchorage of artery to its environment. It is very rich in collagen and elastic fibers; and it contains interspersed fibroblasts, SMCs and adipocytes.

2. Lesion initiation

After a high-cholesterol and high-fat diet, the level of circulating LDL is augmented in blood. LDL cross passively the endothelium of intima and are accumulated in the sub-endothelial space at the predilection sites of the artery walls. (Figure 17) It has been proposed that the retention of LDL depends on the interactions between apo B-100 and the proteoglycans of sub-endothelial matrix (Usman, Ribatti et al. 2015). Subsequently, the LDL accumulated in the vessel wall undergo modifications, such as oxidation, lipolysis, proteolysis and aggregation. Among them, oxidation of LDL is the most important reaction and it plays a crucial role in formation of early lesions. The early oxidation of LDL can be triggered by the oxidative products of vascular cells (e.g., hydroperoxyeicosatetraenoic acid, HPETE) and can

only produce the minimally oxidized LDL (m-oxLDL), which cannot be recognized by the macrophage scavenger receptors but exert pro-inflammation activity. HPETE is the product of 12/15 lipoxygenase (12-LO). Lipoxygenases are critical sources of ROS for LDL oxidation. It has been shown that the inhibition of 12/15 lipoxygenase *in vivo* and *in vitro* can decrease atherosclerosis (Sukhanov, Snarski et al. 2015).

HDL exhibits strongly protective activity against atherosclerosis. Besides the role in efflux of excessive cholesterol from peripheral tissues, HDL contains antioxidant compounds like paraoxonase (PON1) and vitamin E.

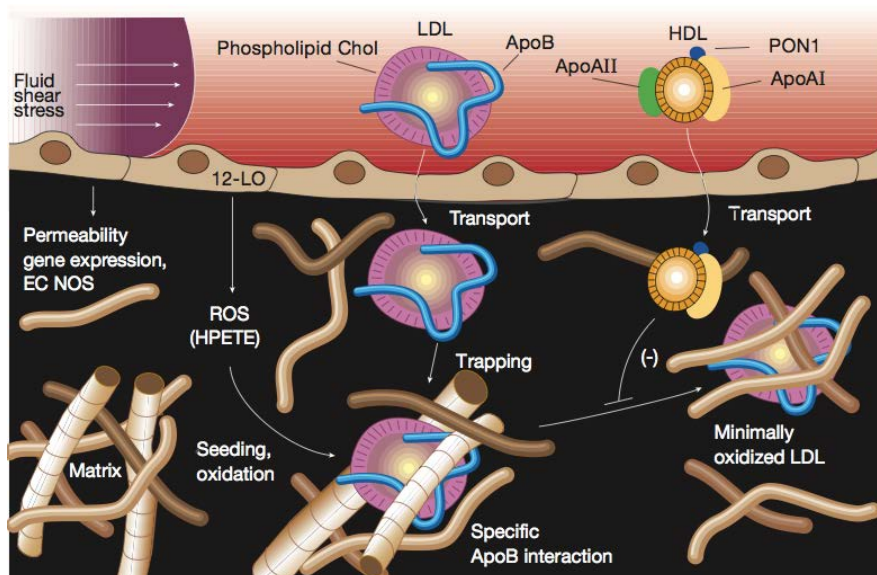


Figure 17. Initiation of atherosclerosis
(Lusis 2000)

3. Inflammation

The m-oxLDL plays an important role in the process of atherogenesis through its pro-inflammatory activity. After the m-oxLDL are generated, they stimulate the endothelial cells (ECs) and SMCs to secrete various pro-inflammatory molecules, such as adhesion molecules (P-selectin, E-selectin, ICAM-1, PCAM-1 and VCAM-1), chemotactic proteins

(MCP-1, CCL5 and CXCL1), and growth factors (M-CSF). Nitric oxide (NO) is a strong vasodilator that has many anti-atherogenic properties. Oxidized LDL can decrease the production of NO and augment blood pressure (Knowles, Reddick et al. 2000).

Subsequently, the leukocytes (monocytes and lymphocytes), but not neutrophils, are recruited by the pro-inflammatory molecules to the endothelium surface. The traverse of the leukocytes through ECs monolayer is regulated by adhesion molecules and chemoattractant. At first, the leukocytes rotate along the endothelium under the mediation of adhesion molecules (P-selectin, E-selectin and ICAM) which are able to bind the carbohydrate ligands on the leukocytes membranes. Then, the integrin VLA-4 on the monocytes and T cells interacts with the VCAM-1 (vascular cell adhesion molecule-1) on the ECs leading to the firm adhesion of leukocytes to endothelium. In addition, the chemotactic protein MCP-1 (monocyte chemoattractant protein-1) and its receptor CCR2 (C-C chemokine receptor type 2) play a key role in promoting atherogenesis, since in the mice lack of the two factors, the atherosclerotic plaques are dramatically reduced (Poupel and Combadière 2009).

In the sub-endothelial space, the monocytes proliferate and differentiate into macrophages by the activity of cytokine M-CSF (macrophage colony-stimulating factor). Macrophages are large mononuclear cells (20-50 microns in diameter) with a relatively large and eccentric core, a highly developed Golgi system and many cytoplasmic granules. They are also able to express a large number of membrane receptors. The atherosclerotic lesions are remarkably reduced *in vivo* with the silence of M-CSF, indicating that macrophages play a necessary and central role in atherosclerosis (Fenyo and Gafencu 2013).

Finally, hyperglycemia can also exacerbate inflammation by generating the advanced glycosylation endproducts (AGEs) which can interact with the RAGE (receptor for AGE) on the surface of endothelium (Hanssen, Wouters et al. 2014).

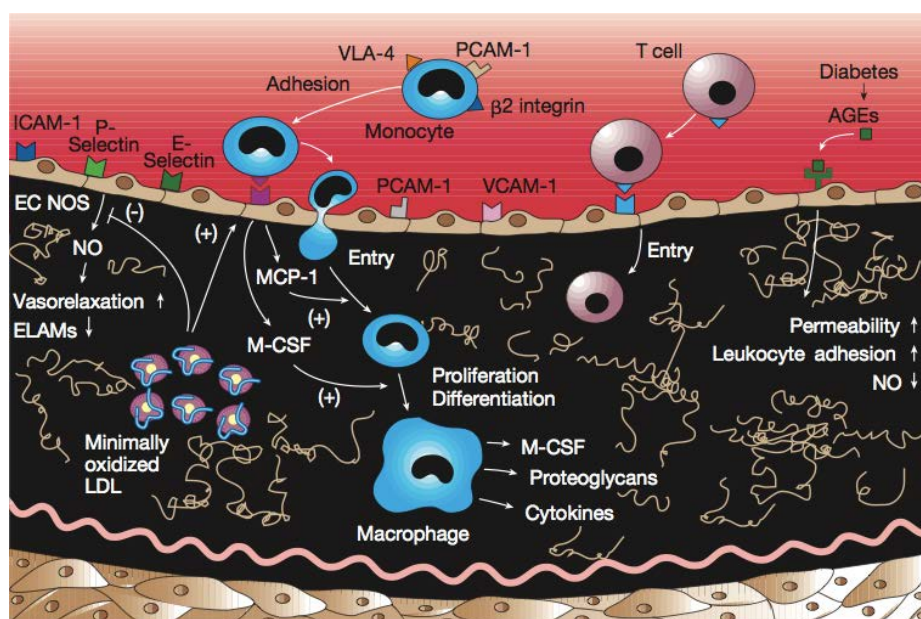


Figure 18. Inflammation in the early stage of atherosclerosis
(Lusis 2000)

4. Foam-cell formation

The m-oxLDL cannot be endocytosed by macrophages through its scavenger receptors; only the highly oxidized LDL (h-oxLDL) can be captured rapidly and massively by macrophages. Therefore, the sub-endothelial LDL, have to undergo a further oxidation to form the highly oxidized LDL (h-oxLDL). This reaction involves not only the ROS produced by macrophages and ECs, but also several enzymes form these cells. Myeloperoxidase is secreted by macrophages; it can use hydrogen peroxide (H_2O_2) and chloride (Cl^-) to generate the highly reactive species hypochlorous acid (HOCl). The LDL oxidized by myeloperoxidase can be recognized by scavenger receptors of macrophage (Delporte, Van Antwerpen et al. 2013). Sphingomyelinase is secreted by ECs; it can stimulate the aggregation of LDL that gives rise to a further retention and uptake of the oxidized LDL by macrophages (Wen, Satchell et al. 2014). In addition, a recent study shows that sphingomyelinase can stimulate the oxLDL-induced macrophages apoptosis through endoplasmic reticulum stress (Zhao, Pan et al. 2016). Secretory phospholipase A_2 (sPLA $_2$), also secreted by ECs, can stimulate the

oxidation of LDL (Shridas and Webb 2014). It has been shown that the inhibition of sPLA₂ exerts a beneficial performance in patients with an acute coronary syndrome (Nicholls, Cavender et al. 2012).

The uptake of h-oxLDL by macrophages is not controlled by the intracellular content of cholesterol and is mediated by several membrane receptors which contain a wide range of ligands. SR-A and CD36, two members of scavenger receptors, play primary role in this process (Zani, Stephen et al. 2015). The expression of these two receptors is regulated by TNF- α (tumor necrosis factor- α) (Hashizume and Mihara 2012) and PPAR- γ (peroxisome proliferator-activated receptor- γ) (Dai, Su et al. 2013).

After the extensive uptake of h-oxLDL, cholesterol (as its ester form) accumulates in macrophages. The cholesterol-engorged macrophages are called foam cells; and the accumulation of foam cells in artery walls, which is easily recognized by light microscopy, is the sign of early atherosclerotic lesions. In the sub-endothelial space, foam cells tend to form layer with the proteoglycan (Thomas, Eijgelaar et al. 2015). Several this layers constitute xanthomas or fatty streaks. WHO defined fatty streak as superficial yellow or yellowish-grey intimal lesions that are stained selectively by fat stains.

In addition, it has been proposed that the sub-endothelial macrophages own several different phenotypes which play multiple roles in the development of atherosclerosis (Leitinger and Schulman 2013). One of the phenotypes is M1-like macrophage. After binding to h-oxLDL, the M1-like macrophages begin to secrete the factors that can maintain chronic inflammation in the lesions and facilitate the recruitment of new monocytes, including pro-inflammatory cytokines (interleukin-1 β , TNF- α), ROS (e.g., myeloperoxidase), plasminogen activators, cathepsins and matrix metalloproteinases (Moore, Sheedy et al. 2013). However, the M2-like phenotype is able to secrete the anti-atherosclerotic factors, such as TGF- β (transforming growth factor- β) and proresolving lipids (Stöger, Gijbels et al. 2012). Moreover, macrophages can secrete apo E. A recent study shows that the apolipoprotein E deficiency in mouse exacerbates the formation of foam cells (Goo, Yechoor et al. 2016).

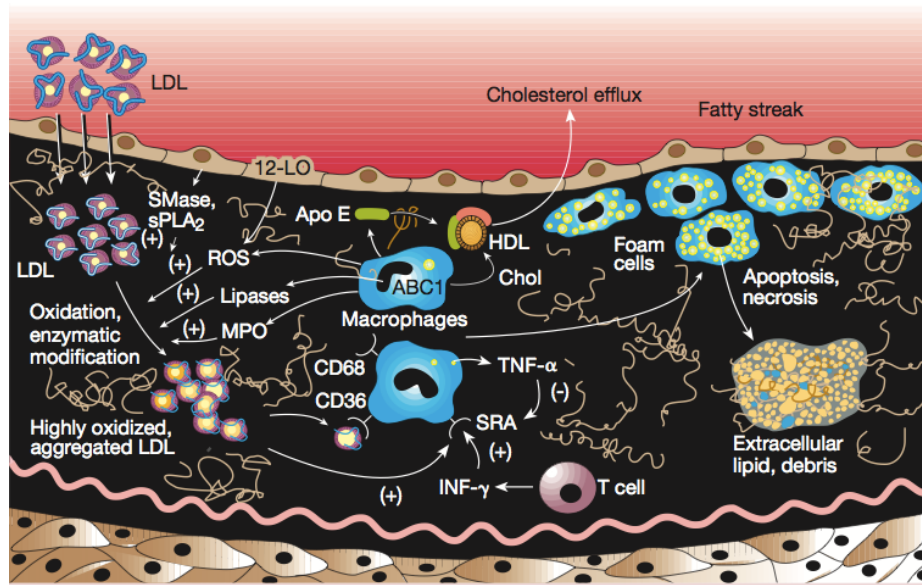


Figure 19. Formation of foam cells
(Lusis 2000)

5. Fibrous plaques and complicated lesions

The initial lesions are asymptomatic. In general, after 40 years, the early lesions begin to evolve into advanced lesions.

Because of the death of foam cells, there are numerous small extracellular lipid pools and cell debris distributed dispersedly in the sub-endothelial space. These lipid pools, which are responsible for pathological intimal thickening, do not lead to evident disruption for the normal structure of arteries when they are isolated (Stary, Chandler et al. 1994, Dalager, Paaske et al. 2007), whereas in some sites, they are able to assemble together to form the necrotic cores and give rise to irreversible damage for the arterial structure. Thereafter, the necrotic core is covered with a fibrous cap, which is composed of SMCs (migrate from medial layer) and the extracellular matrix derived from SMCs; and subsequently a fibrous plaque is formed (Robbins, Hilgendorf et al. 2013). At this stage, macrophages and T cells play important roles in the progression of atherosclerotic lesions. CD40 is expressed on macrophages and T cells. The interaction of CD40 and its ligand CD40L introduces the

secretion of cytokines (e.g. IFN- γ), matrix-degrading proteases and adhesion molecules from macrophages and T cells. IFN- γ , produced by T cells, inhibits the production of matrix by SMCs; the matrix-degrading proteases are produced by macrophages, including collagenases, gelatinases and stromolysin (Libby 2000). They are crucial for the formation of complicated lesions, since they can generate vulnerable plaques with thin fibrous caps. Complicated lesions are defined by WHO as lesions with additional changes or alterations such as hemorrhage, thrombosis, ulceration and calcareous deposits. Among them, thrombosis is the major complication. It is characterized by a rupture of thin fibrous caps and then exposes the thrombogenic lipid material in circulating blood. This results in the formation of thrombus, which occludes the vessel. When the thrombus develops as it obstructs completely the artery, there are acute ischemic heart diseases, such as stable angina, unstable angina, myocardial infarction, and stroke.

d. Alteration of the intracellular transport of cholesterol

Since the formation of foam cells is a characteristic of atherosclerosis, the deregulation of intracellular cholesterol transport in sub-endothelial macrophages is another worth noting point for the progression of atherosclerotic plaque.

Efflux of cholesterol through transporter ABCA1 and ABCG1 is crucial for maintaining cholesterol homeostasis in cells. Macrophages in intima can also release the excessive cholesterol through these two membrane transporters. However, this compensatory mechanism is negligible in the hypercholesterolemia system. Also, apolipoprotein A1 can be oxidized by myeloperoxidase, therefore reducing the capability of HDL to stimulate cholesterol efflux (Shao, Tang et al. 2014, Rosenson, Brewer Jr et al. 2016). In early stage, the excessive cholesterol can be stored in macrophages as cholesteryl ester; whereas in advanced stages, the extensive uptake of oxLDL gives rise to an accumulation of free cholesterol in macrophages (Maxfield and Tabas 2005), which suggests that the free cholesterol is blocked

in late endosomes/lysosomes and cannot be released out of cells (Jerome 2010). Free cholesterol also accumulates in ER and has deleterious effects on cell viability (DeVries-Seimon, Li et al. 2005).

Finally, NPC1, a transport protein localized on LE/Ly membrane, is essential for intracellular transport of cholesterol. It has been shown that in NPC knockout macrophages, there is a decrease of ABC1 transporters expression and an increase of oxidative stress (Zhang, Coleman et al. 2008). In the macrophages incubated with oxidized LDL, the expression of NPC1 is elevated (Yu, Li et al. 2012).

e. Oxysterols in atherogenesis

After 1966 Brook and his colleagues first time discovered oxysterols in atherosclerotic plaques (Brooks, Harland et al. 1966), many studies detected these oxidative products of cholesterol in human coronary and carotid atherosclerotic plaques (Björkhem, Andersson et al. 1994, Brown, Leong et al. 1997, Crisby, Nilsson et al. 1997), suggesting that oxysterols are involved in the formation of atherosclerotic lesions. In normal conditions, the content of oxysterols is very low in macrophages, whereas it can be extremely increased when there are stimuli. In advanced atherosclerotic plaques, 27-HC is the predominant oxysterol and its content is associated with the severity of atherosclerosis (Carpenter, Taylor et al. 1995). 7-KC is the second abundant oxysterol followed by 7 β -HC and 7 α -HC. These oxysterols account for 75-85% of the total oxysterols detected in atherosclerotic plaques from various sites (Kim, Jang et al. 2013). The others are 25-HC, 24-HC and 5,6-epoxycholesterol.

Several oxysterols, such as the non enzymatically formed ones, are thought to play important roles in various aspects of atherogenesis, including dysfunction of ECs, adhesion and transmigration of monocytes, formation of foam cells and fibrous caps, vulnerability of plaques (Poli, Sottero et al. 2009).

Cytotoxicity and apoptosis induced by oxysterols:

Oxysterols perform the cytotoxic effects predominantly on the induction of apoptosis, which contributes to the formation of necrotic core. Two main pathways are involved: the mitochondrial pathway (intrinsic) and the cell death receptor pathway (extrinsic). Caspase-3 is considered to be the most crucial effector caspase; it is able to cleave poly ADP-ribose polymerase, which eventually leads to the morphological and biochemical changes in apoptotic cells (Elmore 2007). In macrophages, caspase-3 is involved in the apoptosis triggered by oxysterols (Lordan, Mackrill et al. 2009). It can be activated by 7-KC (Palozza, Serini et al. 2007), 7 β -HC, 25-HC, and 5,6-epoxycholesterol (Lim, Kang et al. 2003, Rusiñol, Thewke et al. 2004). Bcl family is a kind of apoptotic sensors on mitochondria; the proteins of this family have antiapoptotic effect. In THP-1 macrophages, 7 β -HC, 5,6-epoxycholesterol, and 25-HC suppress the expression of Bcl-2/Bcl-xL protein (Lim, Kang et al. 2003, Lordan, Mackrill et al. 2008). 7-KC promotes different proapoptotic ways associated with the Bax, Bad or Bim proteins (Berthier, Lemaire-Ewing et al. 2004, Palozza, Simone et al. 2011). In addition, 7-KC inhibits the PI3-K/PDK-1/Akt pathway, which protects cells against apoptosis (Vejux and Lizard 2009).

Among the oxysterols found in atheroma plaques, 7-HC and 7-KC are thought to be the most cytotoxic ones (Meynier, Andre et al. 2005). The increment of 7 β -HC can augment the permeability of mitochondrial membrane and stimulate the release of endonuclease G to cell nucleus, which leads to the fragmentation of DNA and death of cells. 7-KC and 7 β -HC can also induce the production of ROS and decrease of cellular antioxidants, therefore inducing a mitochondrial oxidative stress in the sub-endothelial space (Larsson, Baird et al. 2006, Tabas, García-Cardena et al. 2015). The oxidative stress in mitochondria exhibits two main effects: (1) induce the destabilization of lysosomal membrane that proposed to stimulate apoptosis of macrophages (Li, Dalen et al. 2001); (2) activate the NF- κ B pathway that generates chemoattractant factors and therefore leads to the recruitment of monocytes (Wang, Wang et al. 2014). In addition, the increment of ROS in cells stimulates the expression of Egr1 (early growth response protein 1) that regulates the expression of p53 tumor suppressor gene. It has

been shown that 7KC and 7 β -HC effectively induce the overexpression of p53 and thereafter trigger apoptosis (Yuan, Osman et al. 2010). In p53-deleted macrophages, apoptosis is significantly reduced at the early and advanced stage of atherogenesis (Boesten, Zadelaar et al. 2009). In recent studies, the level of 7 β -HC in plasma is considered to be a biomarker for the coronary artery disease associated with a high risk of atherosclerosis (Khatib and Vaya 2014).

Finally, the cytotoxicity of 27-HC depends on its concentration in cells. With low concentration, 27-HC acts as a protector; while at high concentrations, it induces apoptosis (Riendeau and Garenc 2009).

Proinflammatory effect of oxysterols:

Oxysterols can recruit the immune cells, induce the formation of foam cells, and promote the rupture of fibrous caps by stimulating the expression of various inflammatory molecules, including adhesion molecules, growth factors, cytokines and chemokines.

ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) and E-selectin are adhesion molecules involved in the transmigration of monocytes and T cells in intima; many studies have shown that these molecules can be induced by 7-HC and 7-KC (Romeo and Kazlauskas 2008, Dorantowicz, Luczak et al. 2014). IL-8 (interleukin-8) exhibits pro-atherogenic effect by recruiting T cells and monocytes to the atherogenic sites; its production in THP-1 macrophages is regulated by 7 β -HC, 7-KC and 25-HC through a calcium-dependent pathway involving MEK/ERK and AP-1 mediated process (Erridge, Webb et al. 2007, Lemaire-Ewing, Berthier et al. 2009). The cytokine TNF- α (tumor necrosis factor α) is mainly expressed in macrophages; it is able to induce inflammation and apoptosis and plays a key role in atherogenesis, especially in formation of foam cells (Parameswaran and Patial 2010). It has been shown that 7-KC can increase the production of TNF- α in macrophages and amplify inflammatory response in cells (Buttari, Segoni et al. 2013). In addition, 7 β -HC, 7 α -HC or 27-HC can stimulate the secretion of TNF- α in macrophages (Kim, Jang et al. 2013). MMP-9 (matrix metalloproteinase 9) is a member of the zinc-metalloproteinases family; it is involved

in the degradation of extracellular matrix, therefore plays a key role in the rupture of fibrous caps and the formation of advanced atherosclerotic lesions (Fan, Wang et al. 2014). It has been reported that a mixture of oxysterols, which is similar to the components in atherosclerotic lesions, can stimulate the expression of MMP-9 in human monocytic U937 cells but not increase its inhibitors TIMP-1 and TIMP-2 in cells (Gargiulo, Sottero et al. 2011). In addition, MCP-1 (monocyte chemotactic protein-1), also referred to as CCL2, is a small cytokine; it is able to recruit monocytes and T cells to the sites of inflammation (Kolattukudy and Niu 2012). In U937 cell line macrophages, the expression and synthesis of this chemokine can be increased by a mixture of oxysterols (Leonarduzzi, Gamba et al. 2005). This regulation involves the augmentation of phosphorylation of ERK 1/2 (extracellular signal-regulated kinase 1/2) and the nuclear binding of NF- κ B (nuclear factor κ B).

f. Functionality of HDL in atherosclerosis

The most important function of HDL in body is to stimulate the efflux of cholesterol from cells and tissues, then transport cholesterol back to liver. This process is called reverse cholesterol transport. HDL particles and its major protein component apolipoprotein A-I (apo A-I) are able to accept cholesterol from transporters ABCA1 and ABCG1. In atherosclerosis, HDL and apo A-I can attenuate the progression of atherogenesis by stimulating the efflux of excessive cholesterol from macrophages and foam cells. Besides stimulation of cholesterol efflux, due to the complexity of HDL particles, this lipoprotein plays an anti-atherosclerotic role in various aspects, including (1) inhibition of chemotaxis of monocytes, (2) inhibition of monocyte adhesion to endothelial cells, (3) inhibition of LDL oxidation, (4) inhibition of oxLDL-induced endothelial dysfunction and apoptosis, (5) stimulation of endothelial cell proliferation, (6) stimulation of endothelial synthesis of prostacyclin and CNP, (7) stimulation of SMCs proliferation, (8) inhibition of platelet activation, (9) inhibition of factor X activation

and (10) stimulation of activated protein C (Nofer, Kehrel et al. 2002). Among these functions, (1) (2) (3) (4) are the most relevant ones in the formation of foam cells.

HDL protects ECs:

In response to multiple physiological stimuli, ECs produce and secrete various molecules which can regulate vasoconstriction and vasodilatation of arteries, proliferation of SMCs, coagulation and fibrinolysis (A Chistiakov, V Revin et al. 2015). Nitric oxide (NO) is one of the most significant molecules secreted by ECs since it plays a key role in relaxation of arteries and decreases the adhesion and migration of monocytes to endothelium. Oxidized LDL is one of the factors that inhibit the production of NO. It functions as an inhibitor by inducing the displacement of endothelial nitric oxide synthase from caveolae; this action of oxLDL can be prevented by HDL (Brindisi, Duvillard et al. 2013). In addition, endothelial dysfunction is one of the initial factors for atherogenesis; it gives rise to the infiltration of monocytes into sub-endothelial space, the decrease of fibrinolysis and the interference in vasodilatation (Gimbrone and García-Cardeña 2016). It has been shown that endothelial dysfunction can be caused by a substantially decreased concentration of HDL (Sugiura, Dohi et al. 2011). Furthermore, the acetylcholine-mediated vasorelaxation may be affected by oxLDL and this negative action can be reversed by HDL (Matsuda, Hirata et al. 1993). Apart from vasodilation, it has been shown that HDL and apo A-I can prevent the death of ECs caused by oxLDL (Brodeur, Brissette et al. 2008), TNF- α (Riwanto, Rohrer et al. 2013), and the remnants of triglyceride-rich lipoproteins (Speidel, Booyse et al. 1990).

HDL inhibits the adhesion of monocytes to ECs:

The overexpression of adhesion molecules, including VCAM-1, ICAM-1 and E-selectin, on the surface of ECs plays a crucial role in the adhesion of leukocytes to endothelium. It has

been shown that HDL inhibits the expression of these three adhesion molecules stimulated by cytokine (Tran-Dinh, Diallo et al. 2013, Tabet, Vickers et al. 2014, Gomaschi, Ossoli et al. 2015), and prevents the interaction and adhesion of monocytes with endothelium induced by oxLDL (Fan and Karino 2008). However, lipid-free apo A-I and apo A-II cannot perform the protective functions like HDL. In addition, the expression of E-selectin can be activated by the production of sphingosine-1-phosphate (Zhang, Yang et al. 2013). HDL is able to interfere the expression of E-selectin by affecting the turnover of sphingomyelin and its metabolites (Lemaire-Ewing, Lagrost et al. 2012).

HDL acts as an anti-oxidant:

Oxidation of LDL plays a crucial role in the initiation of atherosclerosis, whereas it is extensively decreased by the presence of HDL. The anti-oxidative function of HDL is realized by the inhibition of transition metal ions, the inhibition of 12-lipoxygenase, and the acquisition and transport of some lipids of oxLDL, such as lysophosphatidylcholine and lipid peroxides (Aluganti Narasimhulu, Fernandez-Ruiz et al. 2016). Apo A-I and PON serve as the anti-oxidative molecules in HDL particles. Apo A-I has the capability to reduce the oxidative products of phospholipids and cholesterol esters followed by the formation of oxidized apoA-I that contains methionine sulfoxides at positions 112 and 148 (Rosenbaum, Chaudhuri et al. 2015). However, this modification does not impair the abilities of apo A-I, such as the stimulation of cholesterol efflux and the activation of LCAT.

PON can inhibit the oxidation of LDL by interfering the transition metal ion and by catalyzing the breakdown of oxidized phospholipids of LDL (Watson, Berliner et al. 1995). It also has been shown that the quantity of peroxidized lipids is reduced by the presence of PON in human atherosclerotic lesions (Aviram 2012).

CHAPTER 2:

General presentation of the experimental work

I. Objectives

As detailed in literature review, atherosclerosis is characterized by atheroma plaque formation in arteries. This process is initiated by the penetration of LDL in the sub-endothelium space. LDL undergo oxidative modifications on both protein and lipid moieties and are readily internalized by macrophages through unregulated scavenger receptors. Macrophages then turn into foam cells accumulating high content of cholesterol and oxysterols.

It is mainly believed that the accumulation of oxysterols in atheroma macrophages results from the massive uptake of oxidized LDL that itself contains increased content of oxysterols. Accordingly, oxysterols recovered in atheroma plaques mainly originate from non-enzymatic oxidation, e.g. 7-ketocholesterol, 7 α / β -hydroxycholesterol and cholesterol 5,6-epoxides.

Oxysterol levels in cultured macrophages are very low under normal conditions, but can increase dramatically in response to various perturbations such as excessive cholesterol loading using modified acetylated LDL or upon LPS exposure (Fu, Menke et al. 2001). However, the contribution of cell-mediated generation of oxysterols towards LDL-associated oxysterols is poorly known. Only few study clearly demonstrated the intracellular formation of oxysterols in macrophages upon exposure to modified LDL (Wen and Leake 2007, Yoshida and Kisugi 2010).

In this context, we proposed to consider the following issues:

- **Characterize the species of oxysterols formed in macrophages**
- **Identify the external regulation factors (native and *in vitro* modified lipoproteins)**
- **Identify the cellular regulation factors; in particular evaluate the role of phospholipid BMP**
- **Study the mechanisms of oxysterol efflux**
- **Study the function of diabetic HDL and modified HDL (oxidation, glycation and glycoxidation) on oxysterol efflux**
- **Establish the oxysterols profile of lipoproteins in normal conditions, after modification *in vitro* or *in vivo* (isolated from healthy subjects and diabetic patients)**

II. Materials

a. Lipid standards

7 α -Hydroxycholesterol, 7 β -Hydroxycholesterol, 7-Ketocholesterol, 25-Hydroxycholesterol, 27-Hydroxycholesterol, Stigmasterol: Avanti Polar lipids

b. Radioactive molecules and consumable

[1, 2-³H (N)] Cholesterol (50 Ci/mmol): Perkin Elmer NEN® Radiochemicals

[1, 2-³H] 7-Ketocholesterol (43 Ci/mmol): American Radiolabeled Chemicals

Liquid scintillator Pico-Fluor 15: Packard

c. Consumables for cell culture

RPMI 1640 medium, MEM medium, nonessential amino acids, trypsin, Dulbecco's phosphate-buffered saline (DPBS), penicillin and streptomycin: Life science

Fetal bovine serum (FBS): Corning

Phorbol 12-myristate 13-acetate (PMA); Lipoprotein deficient serum (LPDS): Sigma

d. Other products

Protein assay kit Bradford: Bio-Rad Laboratories

Apolipoprotein A-I: Sigma

Potassium bromide (KBr): Sigma

Solvents: Carlo Erba Reagenti.

e. Biological Materials

1. Culture of cells

The THP-1 cell line:

The human origin THP-1 monocytic cells were grown in 75 cm² flasks in the liquid nutrient medium RPMI supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100U/mL penicillin and 0.1mg/mL streptomycin (complete RPMI). Culturing is carried out in an incubator at 37°C under humid atmosphere with 5% CO₂.

THP-1 cells were seeded in 6-well plates at a density of 1×10⁶ cells/mL in complete medium (2mL/well) and the differentiation was induced by 100nM of PMA. In all cases, the cells were incubated for 48 hours with PMA before any additional processing and PMA was maintained until the end of the experiment.

The RAW 264,7 cell line:

RAW 264,7 murine macrophages were grown in 75 cm² flasks in the liquid nutrient medium MEM supplemented with 10% fetal bovine serum, 2mM L-glutamine, 1% nonessential amino acids, 100U/mL penicillin and 0.1mg/mL streptomycin (complete MEM). Culturing is carried out in an incubator at 37°C under humid atmosphere with 5% CO₂.

RAW 264,7 macrophages were seeded in 6-well plates. After trypsinization and centrifugation, we resuspended each flask cells with 1mL complete MEM medium and then seeded 100μL RAW 264,7 cells in each well. The cells were incubated for 1 day before all the experiments.

2. Lipoproteins

2.1 Preparation of lipoproteins

Blood samples were taken from healthy donors at ESF in Lyon. Plasma is obtained by centrifugation at 900g for 20min. VLDL, LDL and HDL are subsequently isolated from plasma by differential ultracentrifugation (Havel, Eder et al. 1955). The first step is to isolate VLDL by ultracentrifugation at 180 000g for 18h at 15°C. VLDL which float and form a ring on the surface are eliminated. To isolate the LDL, the VLDL-free plasma is adjusted to the density of 1,063 g/mL by addition of potassium bromide (85,7 mg/mL plasma) and centrifuged at 180 000g for 20h at 4°C. LDL are then collected and dialyzed in the buffer with 10mM Tris, 150mM NaCl, pH 7,4 supplemented with 2mM EDTA (antioxidant). The concentration of LDL protein is estimated by the colorimetric assay of Bradford method and LDL are stored at -80°C. HDL is similarly prepared from plasma devoid of VLDL and LDL after adjusting the density to 1,21g/mL (237,5mg KBr per mL of plasma) and centrifuging at 180 000g for 48 h at 4°C. HDL are collected, dialyzed and stored as described for LDL.

2.2 Labeling of LDL with [1,2-³H (N)] cholesterol

An adequate amount of the radioactive tracer in ethanol solution was transferred into a glass tube (in an amount of 40μCi per mg of LDL protein). After evaporation of the solvent under nitrogen stream, the dry residue is taken up in a volume of dimethyl sulfoxide (DMSO, less than 10% of the LDL volume) before addition of LDL, and is incubated overnight at 40°C (dry bath). The labeled LDL are re-isolated by ultracentrifugation as previously described to remove the radioactive molecules that have not been incorporated into LDL particles and then dialyzed in the buffer with 10mM Tris-EDTA, 150mM NaCl, pH 7,4 before use. Aliquots were also taken to determine the LDL protein concentration by the Bradford method. To prepare [³H]cholesterol labeled oxLDL, LDL oxidation is performed after labeling.

2.3 Oxidation of LDL

After transfer LDL in a dialysis bag, the first dialysis in buffer with 10mM Tris, 150mM NaCl, pH 7.4 is carried out overnight at 4°C with stirring, in order to remove EDTA. A second dialysis is 5h at 37°C with stirring in the buffer with 10mM Tris, 150mM NaCl, and 10µM CuSO₄. To eliminate the CuSO₄ and stop oxidation, a third dialysis is realized overnight at 4°C with stirring in the buffer with 10mM Tris, 150mM NaCl, supplemented with 2mM EDTA.

2.4 Glycation of HDL

Native HDL (≈3mg/mL) are incubated in the absence or presence of D-glucose (50mM) for 5 days at 37°C. The incubations are performed in the presence of EDTA (1mM) and BHT (20µM) in order to prevent auto-oxidation. All the preparations contain sodium azide (1mM). After glycation, a dialysis is performed to remove the excessive glucose.

2.5 Oxidation of HDL

After transfer HDL in a dialysis bag, a first dialysis overnight at 4°C with stirring is carried out in buffer with 10mM Tris, 150mM NaCl, pH 7.4, to remove EDTA. A second dialysis for 1 day at 37°C with stirring in the buffer with 10mM Tris, 150mM NaCl and 5µM CuSO₄ was performed. To eliminate the CuSO₄ and stop oxidation, a third dialysis is realized overnight at 4°C with stirring in the buffer with 10mM Tris, 150mM NaCl, supplemented with 2mM EDTA.

III. Methods

a. Radiochemical method

1. Separation and identification of labeled oxysterols derived from [^3H] cholesterol oxidation in LDL

We first developed the chromatographic conditions to properly separate and identify the sterols of interest. To this purpose, we labeled LDL with [^3H] cholesterol and exposed them to oxidative conditions (CuSO_4 10 μM , 5h). Total lipids were extracted according to the Bligh-Dyer method (Bligh and Dyer 1959), and then separated by thin layer chromatography (TLC) on silica gel plates using a mixture: hexane / ethyl ether / methanol / acetic acid (50: 50: 1: 5; v / v / v / v) as solvent (Figure 20), before analysis by a radioactivity reader (Raytest, France).

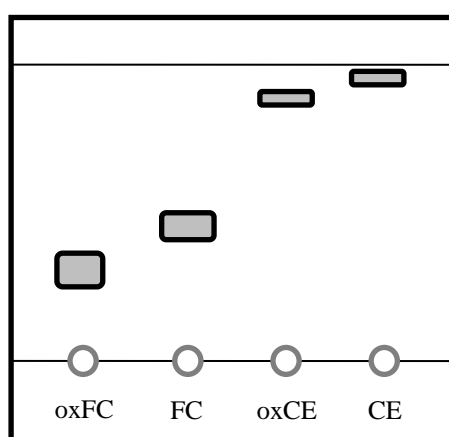


Figure 20. Schematic of a TLC plate after migration
oxFC: oxidized free cholesterol (oxysterols); FC: free cholesterol;
oxCE: oxidized cholesterol esters (oxysterol esters); CE: cholesterol esters

A typical profile of radioactivity is shown in Figure 21. Several radioactive peaks, whose frontal references are below that of cholesterol, co-migrate with cold appropriate standards: 7-hydroxycholesterol, 25-hydroxycholesterol and 7-ketocholesterol. These peaks of radioactivity will be taken as reference to measure the percentage of [^3H]cholesterol oxidation in the following experiments.

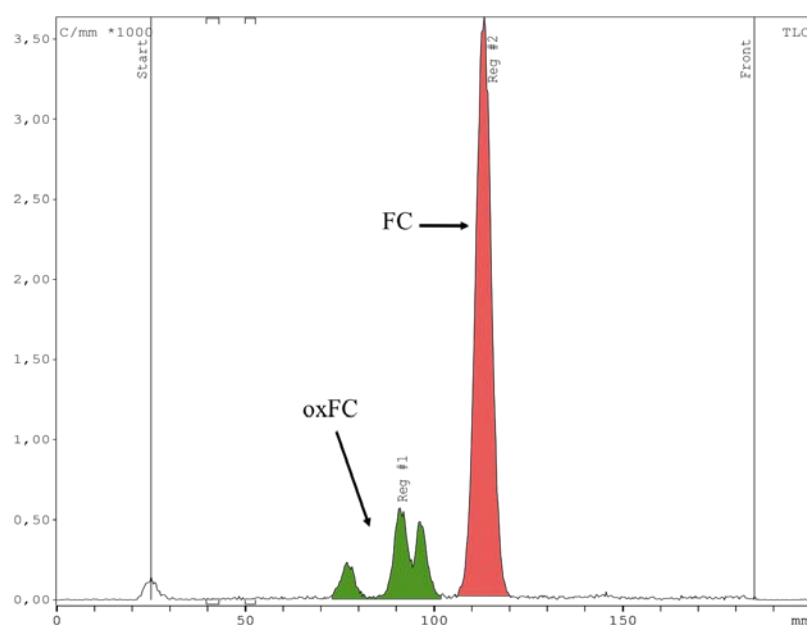


Figure 21. Radioactivity profile obtained for [^3H]cholesterol in oxidized LDL. LDL were labeled with [^3H]cholesterol and then oxidized by copper. After extraction and separation of total lipids by TLC, the radioactivity associated with cholesterol and its oxidation products was measured by the radioactivity reader. FC: free cholesterol, oxFC: oxidized free cholesterol.

2. Separation and identification of labeled oxysterols derived from [^3H]cholesterol oxidation in macrophages

To follow the oxidation of cellular cholesterol, macrophages were pre-incubated with [^3H]cholesterol that can exchange with plasma membrane and label cellular pools (Figure 22).

To monitor the oxidation of cholesterol associated to LDL, cells were incubated with [^3H]cholesterol-labeled LDL (Figure 22).

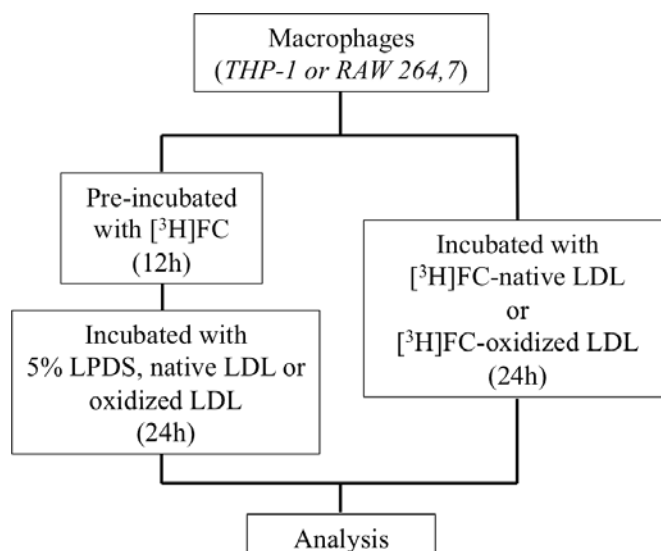
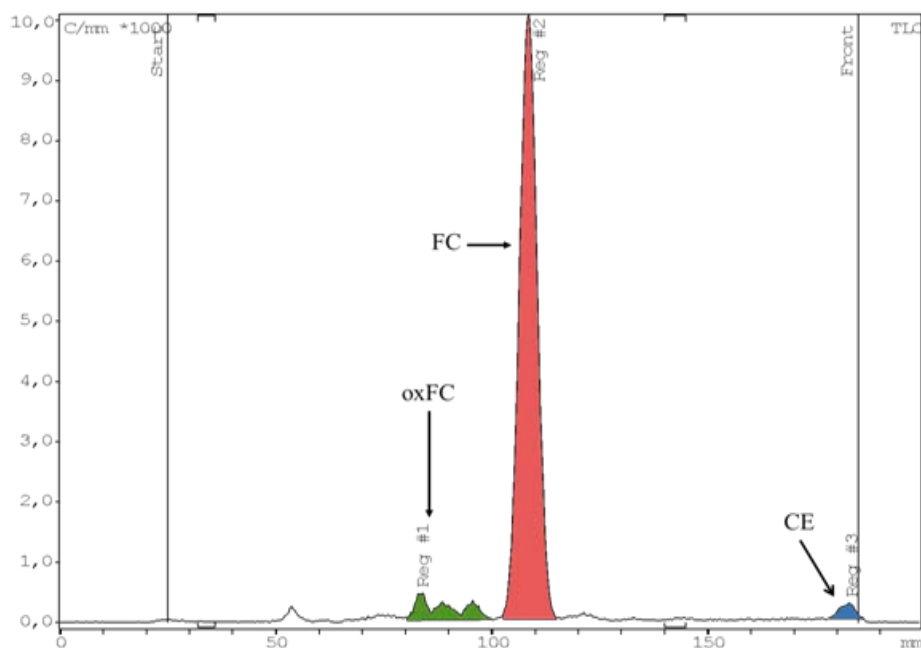


Figure 22. Experimental design (1)

Total lipids from cells and media were extracted by Bligh-Dyer method. Sterols were separated by TLC and the labeled compounds were identified by co-migration with authentic standards (Figures 23 and 24). Identification of oxidized cholesterol esters was confirmed by the disappearance of the peaks after saponification. Cholesterol oxidation was calculated as the percentage of total radioactivity recovered as [³H]oxysterols and [³H]oxidized cholesterol esters (only barely or not detected)

A.



B.

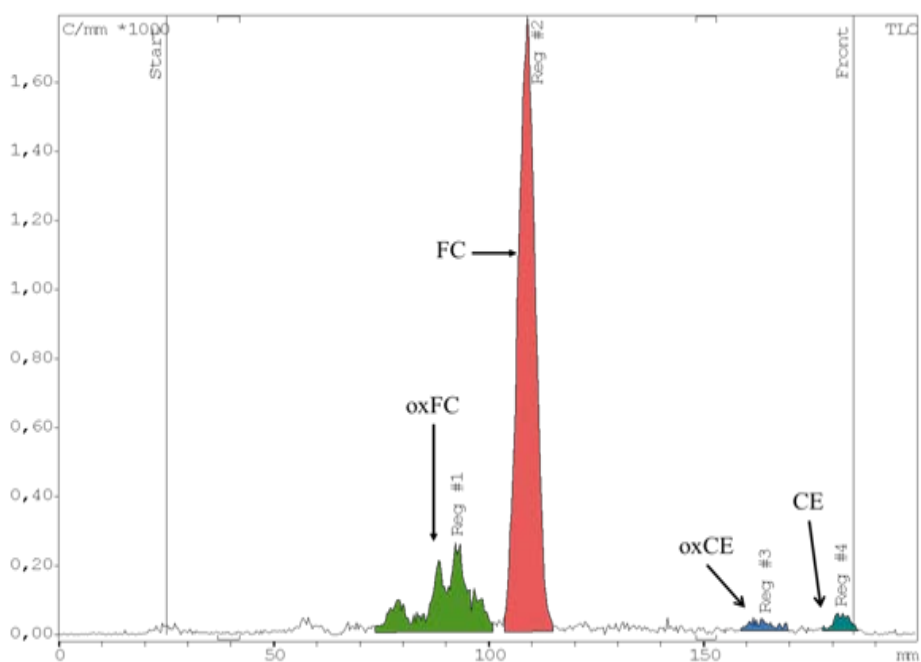
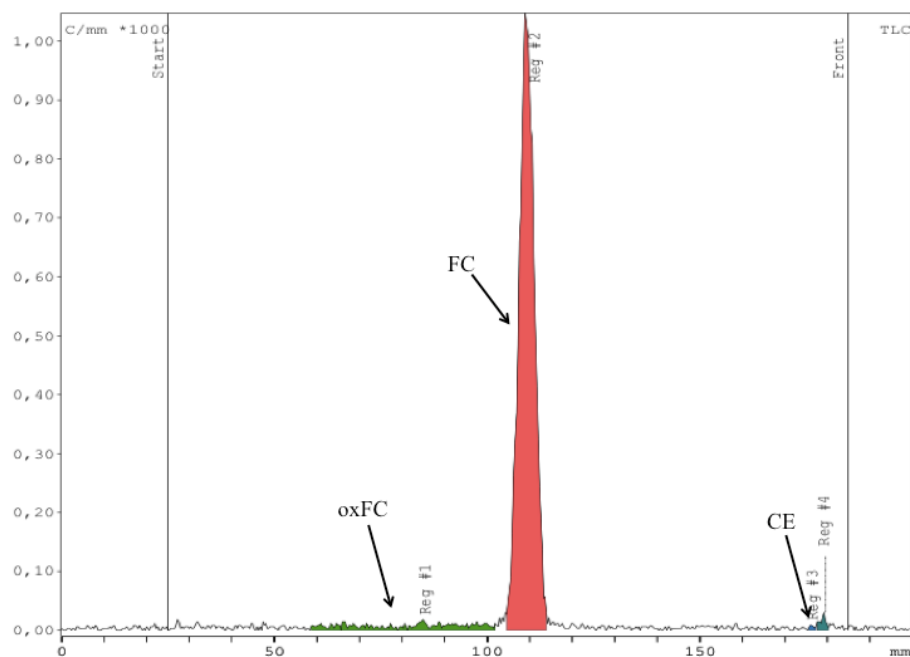


Figure 23. Radioactivity profiles in cells labeled with $[^3\text{H}]$ cholesterol

The cells are pre-incubated with $[^3\text{H}]$ cholesterol for 18 hours, and then incubated in the absence (A) or in the presence of oxidized LDL (B) for 24 hours. After extraction and separation of different classes of lipids by TLC, the radioactivity associated with various sterols is measured by radioactivity reader. oxFC: oxidized free cholesterol, FC: free cholesterol, oxCE: oxidized esterified cholesterol, CE: cholesterol ester

A.



B.

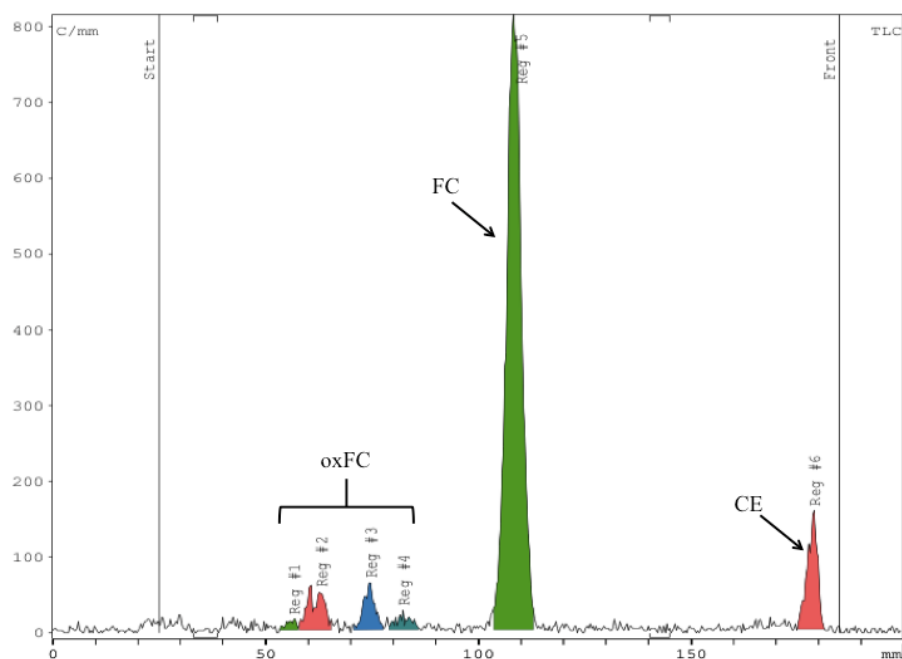


Figure 24. Radioactivity profiles in cells incubated with [^3H] cholesterol labeled LDL

The cells are incubated with [^3H] cholesterol labeled nLDL (A) or with [^3H] cholesterol labeled oxLDL (B) for 24 hours. After extraction and separation of different classes of lipids by TLC, the radioactivity associated with various sterols is measured by radioactivity reader. oxFC: oxidized free cholesterol, FC: free cholesterol, oxCE: oxidized esterified cholesterol, CE: cholesterol ester

3. Measurement of the oxysterol efflux by HDLs and apo A-I

The experiments to measure oxysterol efflux were also performed with radiochemical methods.

To trace the release of sterols derived from LDL (cholesterol and oxysterols), THP-1 macrophages were pre-incubated with [^3H]FC-oxLDL (100 $\mu\text{g/mL}$) for 24 hours. To analyze the efflux of cellular sterols (cholesterol and oxysterols), THP-1 macrophages were pre-incubated with [^3H]7-ketocholesterol or [^3H]cholesterol (0,5 $\mu\text{Ci/mL}$) for 12 hours. 7-ketocholesterol was chosen among other oxysterols as we found that it was predominantly produced in THP-1 macrophages. Cells were then challenged with different HDL (100 $\mu\text{g/mL}$, 6 hours) or apoA1 (10 $\mu\text{g/mL}$, 24h) to stimulate sterol efflux. HDL were extemporaneously prepared, including native HDL, oxidized HDL, control of glycated HDL, glycated HDL, control of glycoxidized HDL, glycoxidized HDL and diabetic HDL (Figure 25).

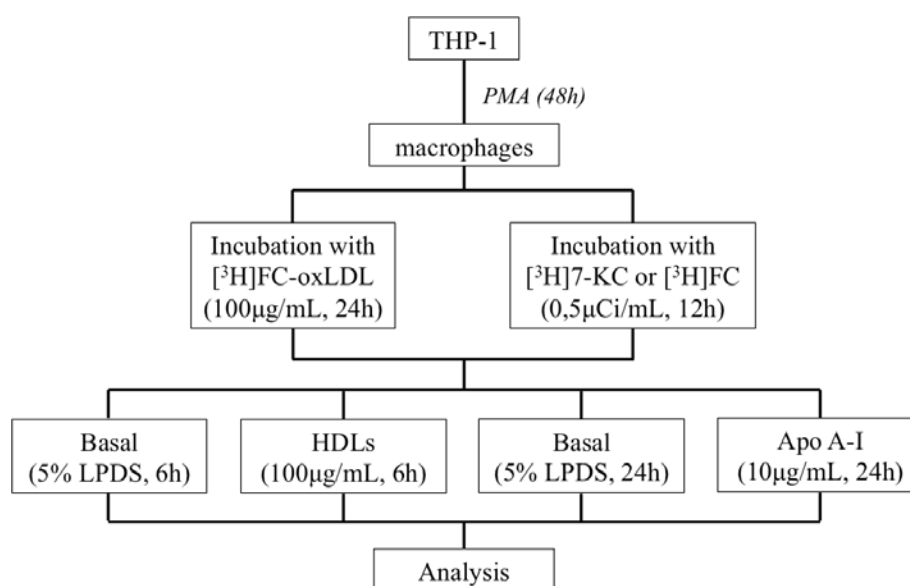


Figure 25. Experimental design (2)

For each sterol, efflux was expressed as the percentage of radioactivity recovered into the medium relative to radioactivity measured in cells plus medium. In experiments using [^3H]FC

or [^3H]7-ketocholesterol to monitor the efflux of cellular sterol, the radioactivity was directly measured in the medium and the cells by scintillation counting. In experiments using [^3H]FC-oxLDL to monitor the efflux of oxLDL-derived sterols, the radioactivity associated to each sterol (cholesterol and oxysterols) was measured by radioactivity analyzer after TLC and converted to DPM according to liquid scintillation counting of total radioactivity in cells or media.

b. Oxysterols quantification by GC-MS/MS

The detection of oxysterols by radioactive labeling allows to estimate the percentage of cholesterol oxidation but not to identify precisely the oxysterol species. An assay method by GC-MS/MS has been developed for both identification and quantification of oxysterols using an internal standard.

1. Oxysterols standards

Several oxysterols of interest, i.e. the species described in oxidized LDL and/or involved in the process of atherogenesis were selected for analysis: 7 α -hydroxycholesterol, 7 β -hydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol. Stigmasterol served as internal standard for quantification, and cholesterol was also analyzed. Typical chromatograms of the oxysterols standards are presented in Figure 26. This method can detect the quantities of ng.

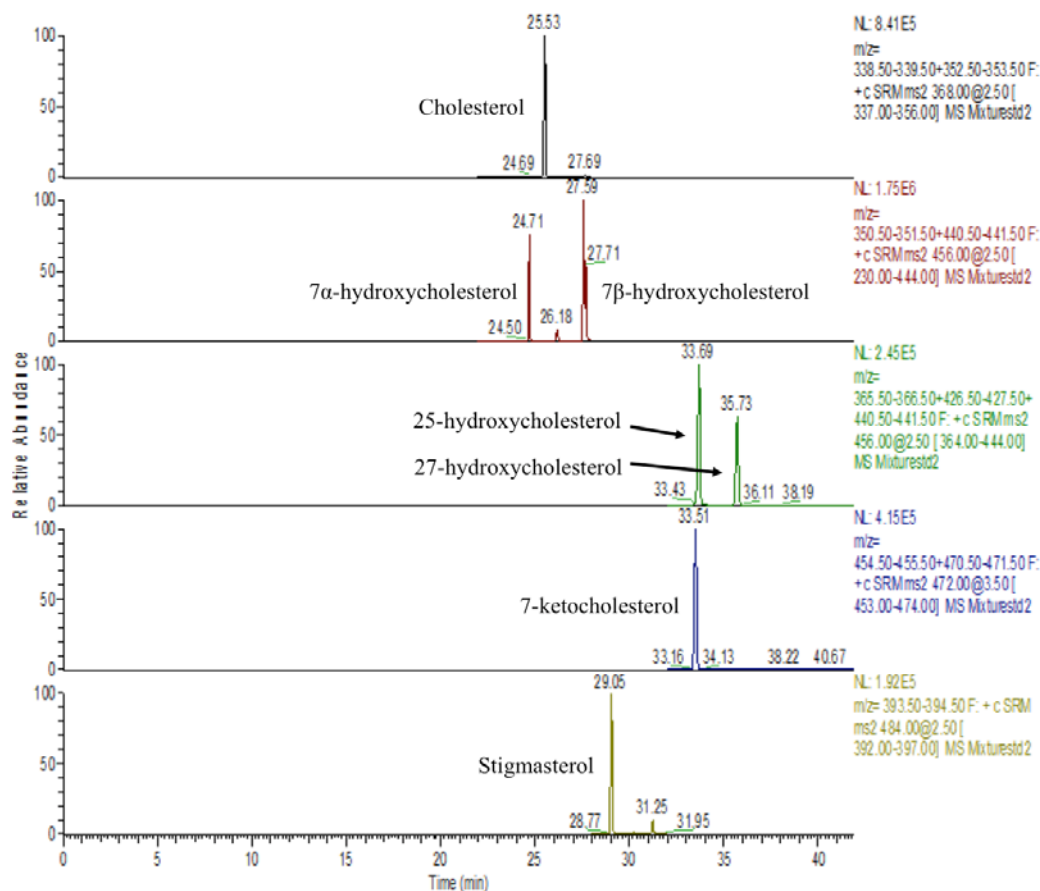


Figure 26. Chromatograms obtained by GC-MS/MS for sterols standards

The retention time (min) is displayed at the top of the peaks, for example 25,53 min for cholesterol. The peaks correspond to 20 ng injected.

2. Oxysterols in macrophages

This method was used to identify and quantify oxysterols in native and oxidized LDL. It was then applied for measuring the production of oxysterols in cultured macrophages.

Typical chromatograms obtained for cells and medium incubated with oxidized LDL are presented Figure 27.

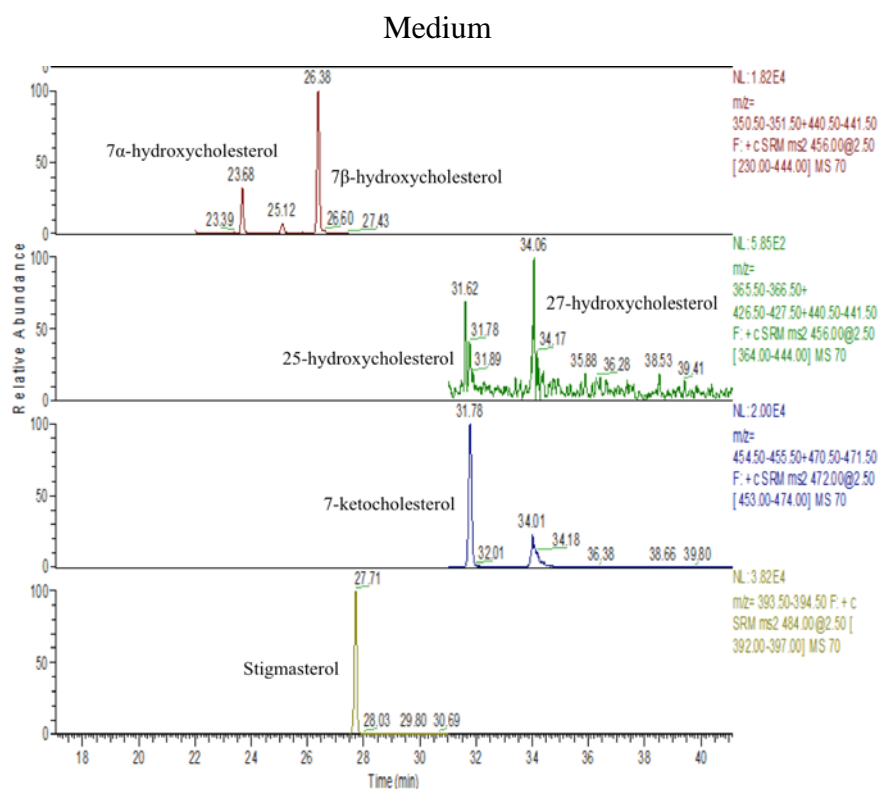
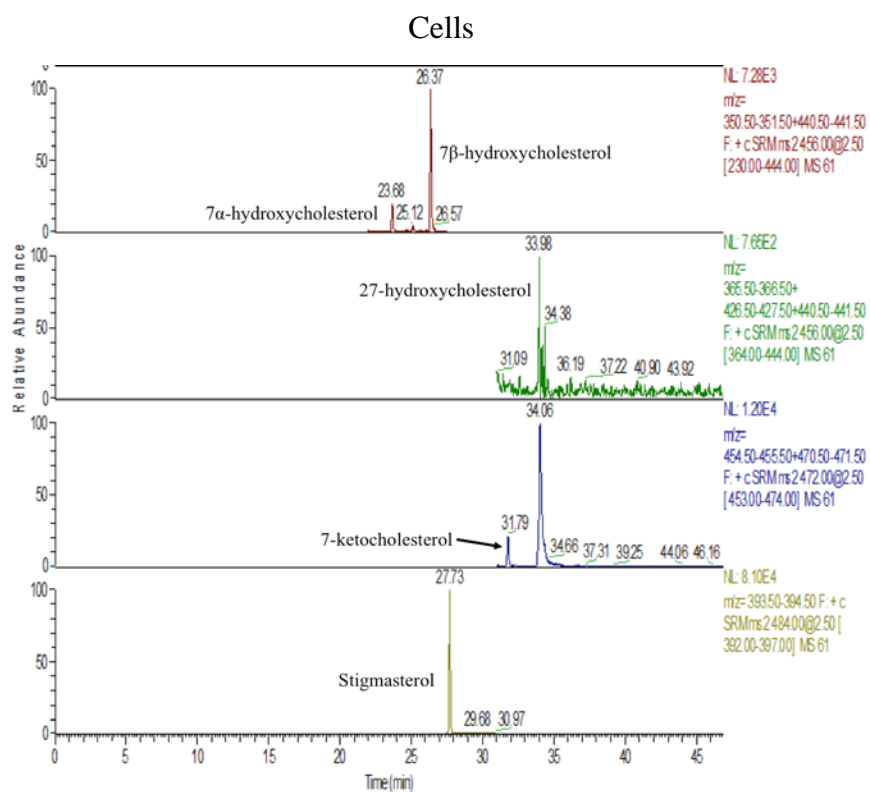


Figure 27. Chromatograms obtained by GC-MS/MS for the experimental condition of oxidized LDL

CHAPTER 3:

Intracellular production of oxysterols in macrophages: regulatory mechanisms and efflux

I. Article 1

Chen, Y., Arnal-Levron, M., Lagarde, M., Moulin, P., Luquain-Costaz, C., Delton, I. (2015). **THP1 macrophages oxidized cholesterol, generating 7-derivative oxysterols specifically released by HDL.** Steroids 99, 212-218.

The aim of this work was to study the intracellular oxidation of cholesterol in THP-1 macrophages and the mechanisms involved in oxysterol efflux.

By using radiochemical and GC-MS/MS analyzes, we showed that both LDL derived-cholesterol and intracellular cholesterol can be oxidized in THP-1 macrophages upon exposure with native or oxidized LDL. Incubation of THP-1 macrophages with native or oxidized LDL induced 2 and 100-fold increase in oxysterol production, respectively. Most oxysterols were generated through non-enzymatic pathway (7-ketocholesterol and 7 α / β -hydroxycholesterol); the enzymatically formed 25- and 27-hydroxycholesterols were recovered in much lower proportions.

Cholesterol is preponderant in plasma membrane but is also present in endoplasmic reticulum and in late endosomes where endocytosed LDL transit. Cellular sites of cholesterol oxidation are not clearly identified but some studies have proposed a role of late endosomes in the oxidation of LDL-derived cholesterol (Wen and Leake 2007). Using pharmacological compound U18666A, which inhibits the transport of cholesterol from late endosomes/lysosomes to endoplasmic reticulum, we could demonstrate that the oxidation of LDL cholesterol mainly occurred in late endosomes in both RAW and THP-1 macrophages.

Finally, we report that both the oxysterols derived from LDL cholesterol and cellular cholesterol were efficiently released by HDL, but not apo A-I.

II. Article 2

Arnal-Levron, M., Chen, Y., Delton-Vandenbroucke, I., Luquain-Costaz, C. (2013). **Bis(monoacylglycero)phosphate reduces oxysterol formation and apoptosis in macrophages exposed to oxidized LDL.** *Biochemical Pharmacology* 86, 115-121.

Previous works of the team have focused on the role of bis(monoacylglycero)phosphate (BMP) in cholesterol homeostasis in macrophages. BMP is a unique phospholipid preferentially found in late endosomal membranes that participates in the intracellular cholesterol metabolism/traffic in macrophages and regulates cholesterol efflux via HDL (Delton-Vandenbroucke, Bouvier et al. 2007, Luquain-Costaz, Lefai et al. 2013). Importantly, using acellular models, the team also reported that BMP could exert protective action against cholesterol oxidation (Bouvier, Berry et al. 2009). Of note, it was shown that late endosomes where BMP is concentrated exhibit high oxidant status and could be a site of cholesterol oxidation (Wen and Leake 2007, Reiners, Kleinman et al. 2011).

The aim of the present study was to assess the capacity of RAW macrophages to generate oxysterols and to evaluate the putative role of BMP in this process, in relation with the oxysterol-induced apoptosis.

Similar to THP-1 macrophages, RAW macrophages produce oxysterols intracellularly after loading with oxidized LDL. Most oxysterols were generated through non-enzymatic pathway (7-ketocholesterol and 7 α / β -hydroxycholesterol) while only low amounts of enzymatically formed oxysterols 25- and 27-hydroxycholesterol were quantified.

The original finding of this paper was to highlight the protective role of BMP against oxysterol-induced apoptosis by reducing their cellular production.

CHAPTER 4:

Functionality of diabetic HDL and modified HDL on oxysterol efflux

I. Introduction

In article 1, we have confirmed that HDL is important for the efflux of oxysterols. It has been reported that oxidatively modified HDL and HDL isolated from diabetic patients are less prone to stimulate cholesterol efflux compared to native HDL and HDL from healthy controls (Nagano, Arai et al. 1991, Bergt, Pennathur et al. 2004, Attia, Nakbi et al. 2007). However, the consequences of such modifications on oxysterol efflux are still unknown.

In the last part of my work, I therefore compared the efficiency of HDL isolated from 9 diabetic subjects vs 9 healthy controls to release oxysterols. As HDL are particles with high complexity and heterogeneity, the components involved in the stimulation of sterol efflux are various and complicated. Besides the alteration of lipid profile, HDL in patients with type 2 diabetes may undergo oxidation, glycation and glycoxidation. Therefore, our second objective was to evaluate the impact of these modifications of HDL on oxysterol efflux.

Oxysterols may play important roles in atherogenesis as they can lead to apoptosis and elevate intracellular oxidative stress. Several studies have reported elevated amounts of oxysterols in plasma from diabetic subjects. However, data regarding classes of lipoproteins are scarce. We have therefore initiated analyses to establish oxysterol profiles of both LDL and HDL isolated from diabetic patients. Data obtained for HDL of normal vs diabetic are exposed in this part, as well as oxysterol profiles of *in vitro* modified HDL. These data will be discussed with respect to HDL efficiency to stimulate sterol efflux.

II. Results:

a. Effects of *in vivo* (diabetic) and *in vitro* (oxidative) modifications of HDL on their ability to stimulate sterol efflux

Efflux of both cellular oxysterols and oxysterols issued from LDL oxidation (as defined in article 1) have been evaluated.

After labeling with [^3H]cholesterol or [^3H]7-ketocholesterol (0,5 $\mu\text{Ci/mL}$, 12h) or [^3H]FC-oxLDL (100 $\mu\text{g/mL}$, 24 hours), cells were challenged with different HDLs (100 $\mu\text{g/mL}$) for 6 hours to stimulate sterol efflux. Efflux was measured as detailed in experimental section (Chapter 2, page 89).

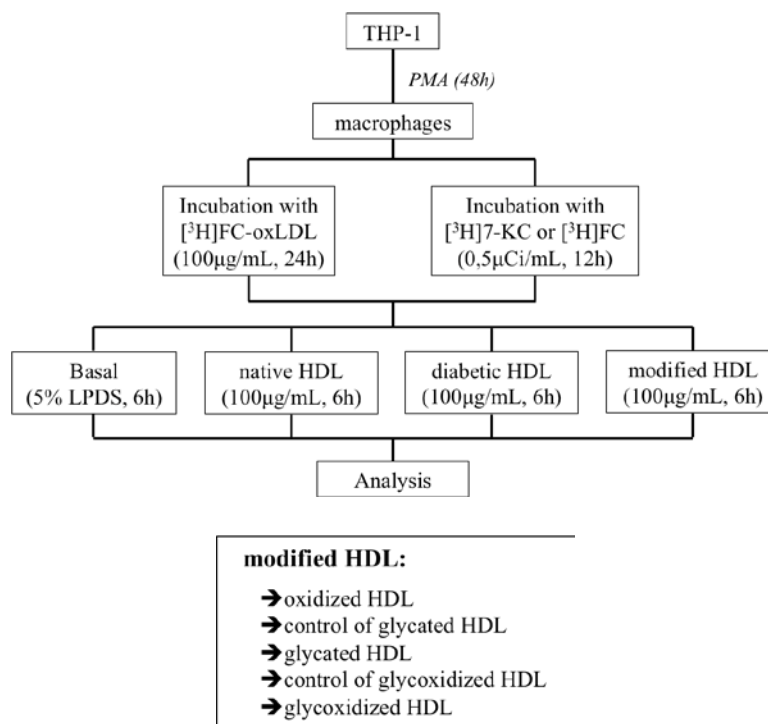


Figure 28. Experimental design (3)

1. *in vivo* modified HDL: diabetic vs normal HDL

Figure 29A shows the efflux of oxLDL-derived cholesterol (FC) and oxysterols (Oxy) stimulated with normal or diabetic HDL after loading with [^3H]FC-oxLDL. Independent of their origin, HDL are much more efficient to efflux oxysterols than cholesterol, which is in agreement with our previous observations (article 1). Of interest, oxysterol efflux was significantly reduced by around 20% with diabetic HDL compared to normal HDL. The trend is also observed for cholesterol efflux without reaching statistical significance. Individual efficiency of normal and diabetic HDL are shown in Figure 29B, highlighting the intra-group variability between HDLs.

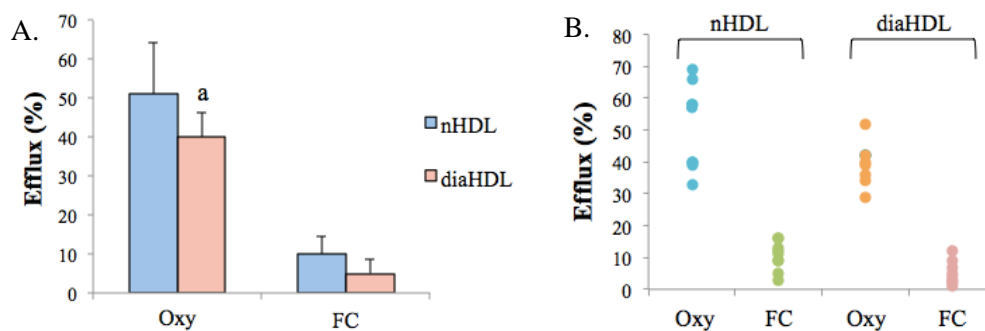


Figure 29. Efflux of oxLDL derived sterols in response to normal HDL and diabetic HDL THP-1 macrophages were loaded with [^3H]FC-oxLDL to generate oxLDL-derived oxysterols. For each individual sterol, efflux is expressed as the percentage of [^3H] radioactivity recovered into the medium from the total [^3H] radioactivity in the medium and the cells, after subtraction of basal efflux (in triplicate wells). A, data are the means \pm SD of 9 subjects. B, individual values (average of triplicate wells). $^a p < 0,05$ nHDL vs diaHDL. Oxy: oxysterols; FC: free cholesterol; nHDL: normal HDL; diaHDL: diabetic HDL.

Similar results were observed when measuring HDL stimulated efflux of 7-ketocholesterol or cholesterol (Figure 30). Independent of HDL, efflux of 7-ketocholesterol was much higher than cholesterol efflux. In addition, it was significantly reduced with diabetic HDL vs normal HDL whereas cholesterol efflux was not changed.

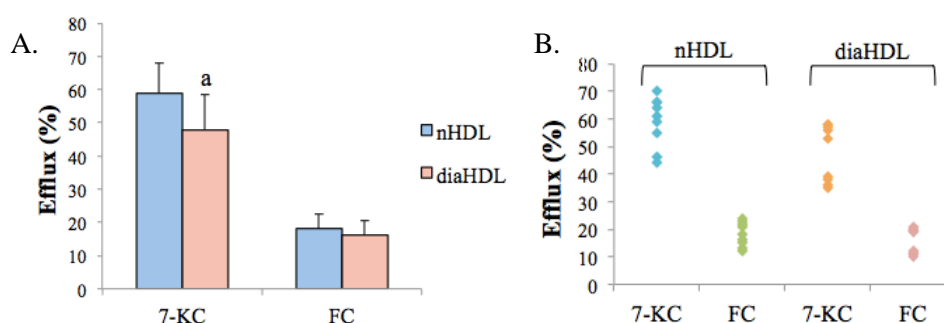


Figure 30. Efflux of cellular sterols in response to normal HDL and diabetic HDL THP-1 macrophages were pre-incubated with [^3H]7-ketocholesterol or [^3H]7-cholesterol to label cellular pools. For each individual sterol, efflux is expressed as the percentage of [^3H] radioactivity recovered into the medium from the total [^3H] radioactivity in the medium and the cells, after subtraction of basal efflux (in triplicate wells). A, data are the means \pm SD of 9 subjects. B, individual values (average of triplicate wells). $^a p < 0,05$ nHDL vs diaHDL. 7-KC: 7-ketocholesterol; FC: free cholesterol; nHDL: normal HDL; diaHDL: diabetic HDL.

Of interest, HDL ability to efflux oxysterols seems to correlate with its ability to efflux cholesterol. This is especially evident for the group diaHDL-7-keto efflux with $R^2=0,93$ (Figure 31C). Correlation is not so clear regarding the group diaHDL-oxLDL derived sterol efflux with $R^2=0,18$ (Figure 31A), even though extremes values of oxysterol efflux indeed correlate with values of cholesterol efflux.

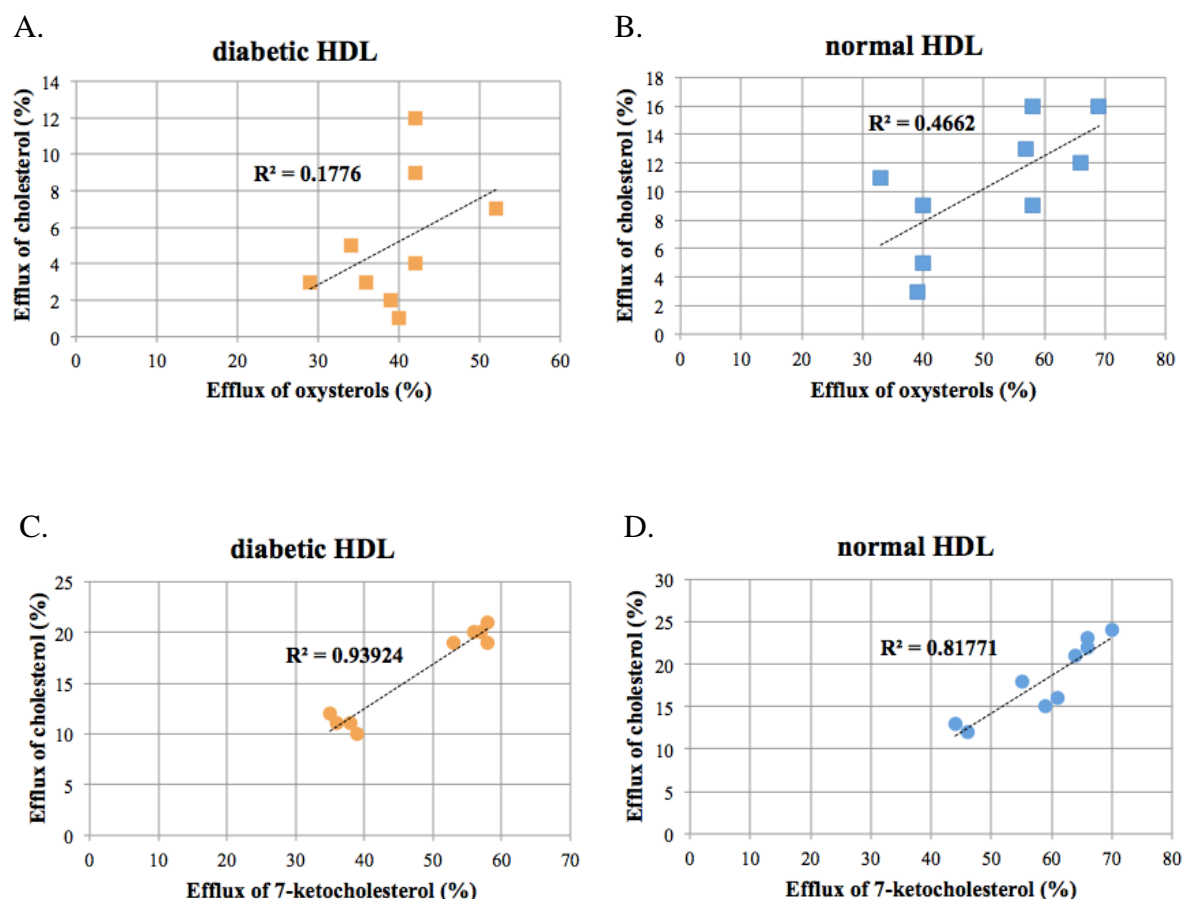


Figure 31. Correlation between oxysterol efflux and cholesterol efflux in response to diabetic HDLs and normal HDLs

Altogether, these results indicate that the capacity of HDL to release oxysterols from macrophages tends to be decreased in patients with type 2 diabetes.

2. *in vitro* modified HDL: normal, oxidized, glycated, and glycoxidized HDL

Oxidized, glycated HDL and glycoxidized HDL were prepared as described in chapter 2. HDL incubated 5 days at 37°C without glucose and HDL incubated 5 days at 37°C without glucose and 1 day without copper serve as control for glycated HDL and glycoxidized HDL, respectively.

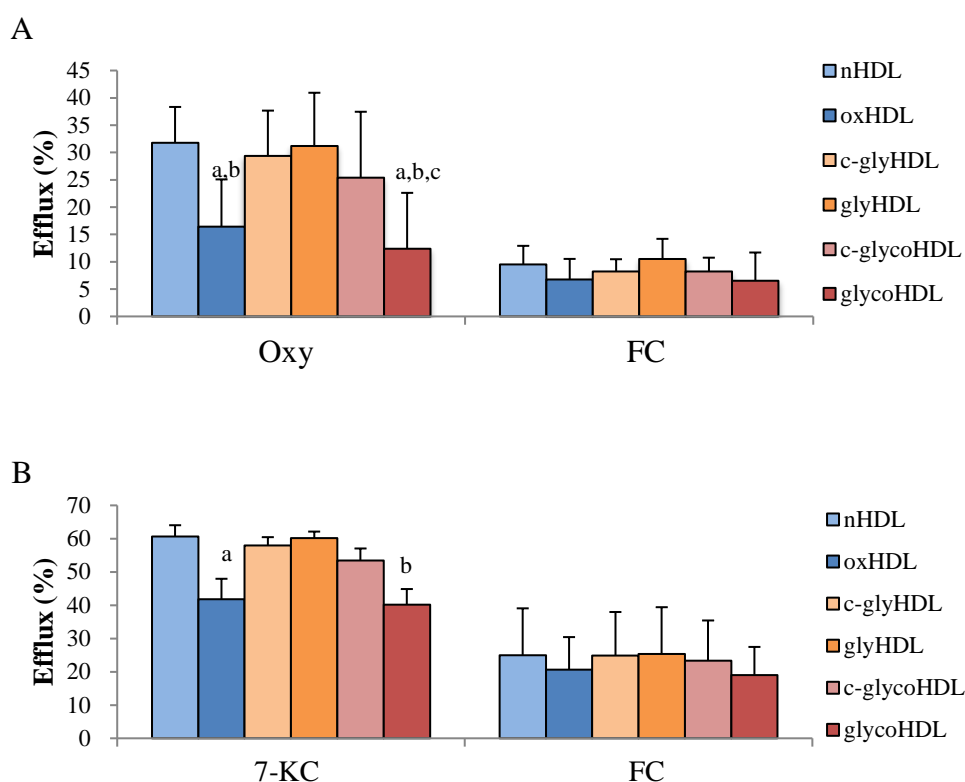


Figure 32. Sterol efflux in response to normal HDL and modified HDLs

THP-1 macrophages were incubated with (A) [³H]FC-oxLDL or (B) [³H]7-ketocholesterol or [³H]cholesterol. For each individual sterol, efflux is expressed as the percentage of [³H]radioactivity recovered into the medium from the total [³H]radioactivity in the medium and the cells, after subtraction of basal efflux. Data are the means \pm SD of 6 independent experiments in triplicated wells. ^a $p < 0,01$ vs nHDL; ^b $p < 0,01$ vs glyHDL; ^c $p < 0,01$ vs c-glycoHDL vs glycoHDL. nHDL: native HDL; oxHDL: oxidized HDL; c-glyHDL: control of glycated HDL; glyHDL: glycated HDL; c-glycoHDL: control of glycoxidized HDL; glycoHDL: glycoxidized HDL.

As shown in Figure 32A, the efflux of oxLDL-derived oxysterols (Oxy) was decreased almost by half with oxidized HDL and glycoxidized HDL compared to their respective control (nLDL, c-glycoHDL). We can also observe a significant reduced efflux by nearly 25% of 7-KC with these modified HDL (Figure 32B). However, for both sources of oxysterols, glycation alone had no impact on HDL efficiency. Unlike oxysterols, efflux of cholesterol was not changed with modified HDL. Altogether, these results indicate that the functionality of HDL on oxysterol efflux is significantly and selectively impaired after oxidative modifications of HDL.

b. Identification and quantification of oxysterols in HDL

HDL from 10 healthy controls, 10 diabetic patients, 4 sets of oxidation and 4 sets of glycoxidation were analyzed for their total (free and esterified) oxysterol content by GC-MS (Lipidomic Analytical Platform, Université de Bourgogne, Dijon).

Table 3. Oxysterol composition of HDLs

healthy HDLs					
	7 α -OH	7 β -OH	7-KC	25-OH	27-OH
C1	0.031	0.050	0.121	0.025	0.060
C2	0.027	0.048	0.131	0.023	0.062
C3	0.037	0.065	0.142	0.025	0.038
C4	0.035	0.060	0.119	0.027	0.042
C5	0.037	0.072	0.153	0.026	0.050
C6	0.036	0.073	0.139	0.026	0.073
C7	0.034	0.064	0.145	0.025	0.045
C8	0.026	0.049	0.102	0.028	0.069
C9	0.034	0.058	0.152	0.024	0.043
C10	0.034	0.074	0.160	0.025	0.047
Mean	0.033	0.061	0.136	0.025	0.053

SD	0.004	0.010	0.018	0.002	0.012
diabetic HDLs					
	7 α -OH	7 β -OH	7-KC	25-OH	27-OH
D1	0.025	0.052	0.092	0.029	0.041
D2	0.041	0.061	0.119	0.027	0.031
D3	0.025	0.040	0.061	0.031	0.023
D4	0.027	0.035	0.076	0.029	0.047
D5	0.023	0.028	0.053	0.028	0.022
D6	0.030	0.033	0.070	0.028	0.037
D7	0.028	0.037	0.075	0.029	0.028
D8	0.056	0.084	0.089	0.025	0.034
D9	0.030	0.051	0.076	0.029	0.027
D10	0.041	0.050	0.085	0.028	0.037
Mean	0.033	0.047	0.079	0.029	0.033
SD	0.010	0.017	0.018	0.002	0.008
oxidized HDLs					
	7 α -OH	7 β -OH	7-KC	25-OH	27-OH
Ox1	4.00	5.67	5.25	0.94	Traces < LOQ
Ox2	27.06	22.51	102.16	1.31	
Ox3	14.37	16.68	32.21	1.62	
Ox4	30.04	25.63	113.46	1.62	
Mean	18.868	17.622	63.271	1.372	-
SD	12.015	8.789	52.797	0.322	-
glycoxidized HDLs					
	7 α -OH	7 β -OH	7-KC	25-OH	27-OH
Go1	6.71	6.03	24.66	0.84	Traces < LOQ
Go2	19.48	16.54	73.48	1.39	
Go3	15.23	17.58	37.19	1.81	
Go4	9.83	10.49	22.85	1.20	
Mean	12.812	12.661	39.543	1.308	-
SD	5.671	5.414	23.508	0.403	-

Oxysterol were analyzed and quantified by GC-MS using deuterium species as internal standards. Data are presented in $\mu\text{g}/\text{mg}$ protein. C, healthy controls; D, diabetic subjects; Ox, oxidized HDL from healthy controls; Go, glycoxidized HDL from healthy controls. 7 α -OH: 7 α -hydroxycholesterol; 7 β -OH: 7 β -hydroxycholesterol; 7-KC: 7-ketocholesterol; 25-OH: 25-hydroxycholesterol; 27-OH: 27-hydroxycholesterol.

As shown in Table 3, HDL from healthy controls contain very low amounts of oxysterols, with 7-ketocholesterol being the most represented. No changes were observed in HDL from

diabetic subjects. By contrast, *in vitro* modifications of HDL generate large amounts of oxysterols, especially 7-derivatives which are produced by non enzymatic routes. 25-OH cholesterol which can also be produced not enzymatically was also significantly increased, although to a much lower rate.

III. Discussion

HDL plays a protective role in atherosclerosis; it has antioxidant and anti-inflammatory properties and the capacity to stimulate the efflux of cholesterol. Our previous works imply that HDL also plays a key role in oxysterol efflux. The patients with type 2 diabetes are more susceptible to atherosclerosis. Many studies have reported the dysfunction of HDL on cholesterol efflux in the type 2 diabetes (Borggreve, De Vries et al. 2003, Tsun, Shiu et al. 2015). However, the consequences in terms of efflux of oxysterols are still unclear. We have also shown that THP-1 macrophages can produce oxysterols either by oxidation of oxLDL-cholesterol or by oxidation of cellular cholesterol. In this work, we measured HDL ability to stimulate efflux of oxysterols from both sources.

Compared with HDL isolated from the plasma of healthy controls, the efflux of oxysterols from both sources was decreased by nearly 20% after the incubation with HDL isolated from the plasma of patients with type 2 diabetes. These results attest to the lower efflux capacity of diabetic HDL towards oxysterols. Other works have reported that cholesterol efflux stimulated by diabetic HDL was lower than healthy control HDL. We also found a trend to decrease cholesterol efflux with diabetic HDL although not statistically significant and weaker than for oxysterols.

Oxysterols can be generated either by enzymatic pathway or by non-enzymatic pathway. As shown in article 1, the quantity of oxysterols is dramatically increased in THP-1 macrophages incubated with oxidized LDL; and most of the oxysterols generated in cells are the non-enzymatically formed oxysterols (mainly 7-KC and 7 β -HC). These oxysterols are also recovered in main proportions in atheroma macrophages and tissue lesions (Kim, Jang et al. 2013). Regarding that these oxysterols may be produced in larger quantities in presence of

increased oxidative stress as generally occurring in diabetic status, the reduced efficiency of diabetic HDL for their removal may contribute to macrophage dysfunctions.

From Figure 29 B and D, we can conclude that not all the normal HDL (efflux of 7-KC: 44-70%; efflux of oxysterols derived from LDL: 33-69%) are more efficient than the diabetic HDL (efflux of 7-KC: 35-58%; efflux of oxysterols derived from LDL: 29-52%) on oxysterol efflux. However, ability of efflux oxysterol and cholesterol seems to be correlated, suggesting that a common mechanism is involved for both sterols. However, since oxysterol efflux was more affected than cholesterol efflux, another distinct mechanism may also exist. Alternatively, the difference between oxysterol and cholesterol may be related to their different cellular distribution and different hydrophobicity.

In type 2 diabetes, HDL undergo modifications, including oxidation, glycation or even glycooxidation. The dysfunction of oxidized HDL on cholesterol efflux has been shown in many studies (Gesquière, Loreau et al. 1997, Bergt, Pennathur et al. 2004, Shao, Tang et al. 2014). However, the effect of glycation of HDL is still controversial. Several reports indicate that the non-enzymatic glycation of HDL leads to the impaired cholesterol efflux (Duell, Oram et al. 1991, Low, Hoang et al. 2012); whereas other studies shows that glycation has no consequences (Rashduni, Rifici et al. 1999, Passarelli, Shimabukuro et al. 2000). Thus, the second aim of our work is to evaluate the impact of the modifications of HDL on oxysterol efflux. We found that after oxidation and glycooxidation, HDL are less efficient on stimulating the efflux of both cellular and LDL-derived oxysterols, compared to respective control HDL. By contrast, glycation alone cannot alter the functionality of HDL on oxysterol efflux (Figure 32), indicating that oxidation status is crucial. As a matter of fact, oxidation and glycooxidation of HDL as applied in our experimental conditions led to a severe decrement of vitamin E and a much severer increment of MDA (malonaldehyde) which is a marker for lipid peroxidation (Lê, El Alaoui et al. 2015). Of interest, there is no significant differences between the functionality of oxidized HDL and glycooxidized HDL on efflux of 7-KC (oxHDL: 42%; glycoHDL: 40%) or oxysterols derived from LDL (oxHDL: 16%; glycoHDL: 12%). It was reported that the glycation of HDL can impair the activity of paraoxonase (Ferretti, Bacchetti et al. 2001) and reduce LCAT activity (Fournier, Myara et al. 1995), causing higher

susceptibility of HDL for oxidation. Our observations suggest that the oxidation rate in oxHDL is enough to reach the threshold to impact oxysterol efflux so that higher oxidation rate in glycoxidized HDL would not have more impact. More surprisingly, we found that both oxidation and glycoxidation had not impact on cholesterol efflux, which may suggest that oxidative status of HDL is not a determinant for cholesterol or that this condition is much more critical for oxysterol efflux.

Works from our laboratory showed that oxidation and glycoxidation significantly diminished the proportions of several polyunsaturated fatty acids (PUFA) especially linoleic and arachidonic acids in phospholipids of HDL and generated oxidized PL containing hydroxylated fatty acids derived from these PUFA (Lê, El Alaoui et al. 2015). They also indicated that functionality of HDL in terms of platelet aggregation was modified after enrichment with these hydroxylated fatty acids. Since the acceptor phospholipids play an essential role in oxysterol efflux (Gelissen, Rye et al. 1999), the altered phospholipids composition triggered by oxidation may be one of the reason why oxidative and glycoxidative modifications lead to the dysfunction of HDL on oxysterol efflux (Morel 1994).

Abnormal composition of HDL generally refers to the CE/TG ratio, CE/PL ratio and the quantity of apo A-I or other proteins in HDL₂ or HDL₃ particles (Nobecourt, Jacqueminet et al. 2005, Gordon, Davidson et al. 2013, Kopecky, Genser et al. 2015). In our work, we determined the oxysterols composition in different HDLs. Oxysterols could be detected at only very low levels in control healthy subjects and no increase was observed in HDL from diabetic patients. This suggests that despite the potential elevated oxidative status in diabetic patients, HDL are highly protected against cholesterol oxidation; in addition, this means that oxysterol content in HDL does not affect efflux ability. Analyses of oxysterol content in LDL will be informative in this respect. In contrast, *in vitro* oxidative and glycoxidative modifications of HDL led to huge oxysterol production. Most of the oxysterols are generated through non-enzymatic pathway, especially 7 α / β -hydroxycholesterol and 7-ketocholesterol. Such a huge production of oxysterols may indicate high oxidation rate for these modified HDL.

Further investigations will be necessary to unravel the mechanisms responsible for weaker oxysterol efflux efficiency in modified and diabetic HDLs.

CHAPTER 5:

Discussion, conclusion and perspectives

The entire work of this thesis depicts the characterization of the oxysterols generated in macrophages and the role of HDL in their efflux.

In the progress of atherogenesis, the LDL in the sub-endothelial space of artery wall undergo oxidative modification, thereby leading to the formation of oxidized LDL (oxLDL). The oxidation triggers the modification of apo B-100 and lipids, including PL and cholesterol. Certain oxidative products of cholesterol, such as 25- and 27-HC, have beneficial performance on intracellular homeostasis of cholesterol. They can decrease biosynthesis and uptake of cholesterol by preventing the activation of SREBP and accelerating the degradation of HMG-CoA reductase; they can also serve as the ligands of LXRs to stimulate the efflux of cholesterol, or increase the intracellular cholesterol storage as esters (Brown and Jessup 2009). However, several oxysterols, including 7-KC and 7 α / β -HC, exert cytotoxicity and increase intracellular oxidative stress (Khatib and Vaya 2014). After massive uptake of oxLDL, the sub-endothelial macrophages are over-loaded with cholesterol esters and form the foam cells. Many studies have reported the presence of oxysterols in foam cells (Hayden, Brachova et al. 2002) and in atherosclerotic lesions (Garcia-Cruset, Carpenter et al. 1999).

In articles 1 and 2, we demonstrate that both cellular cholesterol and cholesterol derived from LDL undergo intracellular oxidation in human THP-1 and murine RAW 264,7 macrophages. In particular, cholesterol from oxLDL was more prone to oxidation compared to cholesterol associated to native LDL. This means that in addition to carry oxysterols, oxLDL promotes intracellular oxidation of cholesterol, in a kind of deleterious loop. The vast majority of the oxysterols produced by macrophages upon oxLDL exposure are the non-enzymatically formed oxysterols; the most abundant one is 7-KC, followed by 7 β -HC and 7 α -HC. 27-HC and 25-HC, two enzymatically formed oxysterols, can also be found although in much lower proportion. The predominant oxysterol in advanced atherosclerotic plaques is 27-HC (Carpenter, Taylor et al. 1995), the second significant is 7-KC and followed by 7 β -HC and 7 α -HC (Kim, Jang et al. 2013). Comparing these two profiles, the change on oxysterols quantities may imply variable metabolism of oxysterols at different stages of atherosclerotic

lesions formation or the specificities of cell lines.

We could also demonstrate that oxidation of LDL cholesterol mainly occurred in late endosomes. This compartment confers high oxidative status due to its high content in catalytically active iron. Moreover, the enzymes that catalyze the formation of oxysterols, including 27-hydroxylase, 24-hydroxylase and 25-hydroxylase, are located in other compartments, such as mitochondria, endoplasmic reticulum and Golgi. These two parameters are consistent with the predominant formation of 7-derivatives oxysterols in macrophages.

7-KC and 7 β -HC are the most toxic oxysterols in the progression of atherosclerosis. Macrophages can attenuate their cytotoxicity by esterification or exporting them to extracellular acceptors. Our results clearly indicate that oxysterol efflux can be stimulated by HDL, but cannot be promoted by apo A-I. This is the case for both cellular oxysterols and oxLDL-derived oxysterols. These results indicate a selectivity of acceptors in sterol efflux and reveal the important role of HDL in oxysterol export. We can also make the hypothesis that the efflux of oxysterols required ABCG1 but not by ABCA1. ABCA1 and ABCG1 are the critical cholesterol transporters in macrophage membrane; ABCA1 transfers cholesterol uniquely to apoA-I/lipid-poor apoA-I, whereas ABCG1 transports cholesterol to HDL particles. It has been proposed that ABCA1 and ABCG1 work sequentially in cholesterol efflux (Vaughan and Oram 2006). Cholesterol can be released concurrently with phospholipid through ABCA1 to apoA-I (Smith, Le Goff et al. 2004). However, for oxysterols, phospholipid in the extracellular acceptors is a requirement for its efflux (Gelissen, Rye et al. 1999). Therefore, the inability of apo A-I to efflux may suggest that 1) the quantity or types of phospholipids exported from macrophages to apo A-I are not sufficient or suitable for oxysterol efflux; 2) the sequential work model is not tenable for oxysterols. Gelissen and his colleagues have shown that the phospholipid/apoA-I ratio after the incubation of lipid-free apo A-I with acLDL-loaded cells was only 3, while this ratio in the phosphatidylcholine/apoA-I disc, which can successfully accept 7-KC, was more than 60, which indicates that the quantity of phospholipids in extracellular acceptors is determinant for oxysterol efflux (Gelissen, Rye et al. 1999). The types of phospholipids are different among cells and at different stages of atherogenesis; so they may

also be a factor that influences oxysterol export from macrophages.

HDL plays a key role in anti-atherosclerosis. Because of its components complexity, HDL has antioxidation and anti-inflammatory capacities and can promote the export of excessive cholesterol from foam cells. However, in type 2 diabetes, there are functional deficiencies of HDL; and the patients with type 2 diabetes are more susceptible to atherosclerosis. The dysfunction of diabetic HDL in cholesterol efflux has been shown in many publications (Borggreve, De Vries et al. 2003, Tsun, Shiu et al. 2015); but the functionality of diabetic HDL in oxysterol efflux from macrophages is still an interesting question for us. Our study with only 9 diabetic subjects reveals a lower efficiency of diaHDL to release oxysterols. Other assays will be performed to strengthen this trend. After oxidative and glycoxidative modification, HDL were less efficient to promote the efflux of oxysterols compared to the non-modified HDL. By contrast, glycation alone had no effect and there were almost no differences between oxidized and glycoxidized HDL. We may therefore correlate the dysfunction of diabetic HDL to their degree of oxidation, while glycation does not appear to be determinant.

Impaired cholesterol efflux was also observed with diabetic HDL, although to a lower extent than for oxysterols. In addition, cholesterol efflux was not changed with oxidized and glycoxidized HDL. The quantity of oxysterols in macrophages or foam cells is much lower than cholesterol, but it is very significant, in respect to their deleterious effects. We can conclude that the retention of 7-oxysterols triggered by the dysfunctional HDL may play a crucial pro-atherosclerotic role in the patients with type 2 diabetes.

At last, we focused on the abnormal compositions of diabetic HDL and the HDL with oxidative and glycoxidative modifications. Previously, much attention was paid to the altered CE/TG ratio, the abnormal PL/CE ratio, the loss of apoA-I or other proteins in HDL₂ or HDL₃ particles (Nobecourt, Jacqueminet et al. 2005, Gordon, Davidson et al. 2013, Kopecky, Genser et al. 2015). There is no doubt that these parameters are helpful to some degree in determining the efficiency of HDL, but we are still curious about the presence of oxysterols in these HDL particles. Our results indicate that HDL contains very low amounts of oxysterols

even in diabetic subjects, suggesting high anti-oxidant protection. However, oxysterols can be recovered under more stressful oxidative conditions. Analyses on both diabetic LDL and LDL with oxidative and glycoxidative modifications will be informative in this respect. Referring to the lower efficiency of diaHDL to efflux oxysterols, oxysterol content in HDL does not seem to be involved in their efflux ability. This point however needs to be confirmed.

In conclusion, our works attest to the dramatically production of 7-ketocholesterol and 7 β -hydroxycholesterol, two most toxic oxysterols, in oxLDL-loaded macrophages, the preponderant role of HDL in oxysterol efflux and the dysfunction of type 2 diabetes HDL in oxysterol efflux. These results may imply another pro-atherosclerotic role of oxysterols (especially 7-KC and 7 β -HC) in type 2 diabetes.

Finally, our results also raise the question of which specific mechanism is involved in oxysterol efflux. Several tracks are to be followed, including the role of intracellular transporters (in particular ABCG1), membrane receptors (SR-BI) or specific components of HDL (oxidized proteins or lipids).

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FOLIO ADMINISTRATIF

THESE DE L'UNIVERSITE DE LYON OPEREE AU SEIN DE L'INSA LYON

NOM : CHEN

DATE de SOUTENANCE : 21 Octobre 2016

Prénoms : YINAN

TITRE : Characterization of oxysterols produced in macrophages and mechanisms of regulation

NATURE : Doctorat

Numéro d'ordre : 2016LYSEI102

Ecole doctorale : Interdisciplinaire Sciences-Santé

Spécialité : Biochimie

RESUME :

Les macrophages jouent un rôle clé dans l'athérosclérose. Après la captation massive des LDL oxydées (oxLDL), les macrophages sous-endothéliaux sont chargés en cholestérol et se transforment ainsi en cellules spumeuses qui contribuent à la formation de la plaque d'athérome. Les oxystérols, produits d'oxydation du cholestérol, sont retrouvés en quantité importante dans les oxLDL. Au niveau cellulaire, ils sont impliqués dans la régulation de l'homéostasie du cholestérol, l'induction du stress oxydatif cellulaire et de la cytotoxicité. Notre travail montre que le cholestérol, associé aux LDL et d'origine cellulaire, est fortement oxydé par les macrophages lors d'une exposition aux oxLDL. Les principaux produits d'oxydation sont le 7-cétocholestérol et 7 α / β -hydroxycholestérol. De plus, nous démontrons que ces oxystérols sont exportés hors des macrophages via les HDL, mais pas l'apoA1. Nous avons aussi caractérisé les oxystérols formés dans les HDL suite à des modifications oxydatives et les HDL issues de patients diabétiques. Nous avons en outre montré que ces modifications sont associées à une diminution de la capacité des HDL à exporter les oxystérols.

MOTS-CLÉS : low density lipoprotein, cholesterol, oxysterols, atherosclerosis, high density lipoproteins

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