Cell Growth and Cholesterol Esters

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Dedication

To Giovanni, Mario and Laura
CONTENTS

1. Overview—Intracellular Cholesterol Homeostasis: Old and New Players ................................................. 1

Sandra Dessì and Barbara Batetta

Acquisition of Cholesterol by Peripheral Cells ................................................................. 1
Endogenous Synthesis ........................................................................................................... 1
Exogenous Uptake: The LDL Receptor Pathway .............................................................. 2
Mechanisms of Cholesterol Homeostasis ........................................................................... 4
Involvement of MDR1-P-gp in Cholesterol Transport ..................................................... 4
Sterol Regulatory Systems in the ER .................................................................................. 6
Metabolic Fate of Cytoplasmic Lipid Droplets ................................................................ 8
Efflux of Cholesterol .......................................................................................................... 8
HDL Metabolism .................................................................................................................. 8
HDL Receptors .................................................................................................................... 9
Conclusions .......................................................................................................................... 10

2. Role of Mevalonate Derivatives in Cell Cycle Progression ............................................. 13

Sandra Dessì

Cholesterol and Cell Growth ............................................................................................. 13
Role of Mevalonate in DNA Synthesis ............................................................................. 16
Loss of Feedback Control of Cholesterol Synthesis in Malignant States .............................................. 16
Mevalonate and Protein Prenylation .................................................................................. 17
Protein Prenylation ........................................................................................................... 17
Family of GTP-Binding Proteins (G Proteins) .................................................................... 18
Ras Superfamily ................................................................................................................ 18
The Role of Farnesylation in Ras Function ......................................................................... 18
Ras Encoded G Protein Signaling Cascade ......................................................................... 18
Role of Cholesterol in the Formation of Rafts and Signal Transduction ........................... 20
Conclusions ........................................................................................................................ 21


Sandra Dessì and Barbara Batetta

Cholesterol Esters Regulation in Vivo ................................................................................ 25
Cholesterol Esters and Cell Growth in Experimental Animals ........................................ 27
Cholesterol Ester Metabolism in Experimental Models
  Involving Normal Liver Growth Activation ....................................................................... 28
Cholesterol Ester Metabolism in Experimental Models
  Involving Extrahepatic Growth Activation ....................................................................... 29
The Inhibition of Cholesterol Esterification Suppress the Proliferative Capacity of an Organ .............................................................................................. 30
Cholesterol Esterification during Tumor Growth ................................................................ 30
Alterations of Cholesterol Esterification in Proliferating Tissues Are Associated with Peculiar Changes of Lipid Metabolism in the Plasma Compartment ........................................... 31
Conclusions ........................................................................................................................ 33
4. Cholesterol Metabolism in Human Tumors

Sandra Dessì and Barbara Batetta

Lipoprotein Metabolism and Human Cancer
General Pathway of Lipoprotein Metabolism: An Overview
Hypocholesterolemia and Human Tumors
Lipoprotein Metabolism in Hematologic Neoplasms
Lipoprotein Metabolism in Solid Tumors
Serum Lipid Profiles in Non-Tumoral Human Proliferative Disease
Cholesterol Metabolism in Tumoral Tissues

Conclusions

5. The Mobilization of Cholesterol Released at Sites of Tissue Injury

Robert Kisilevsky and Shui-Pang Tam

Serum Amyloid A (SAA)
What Is the Physiologic Function of Acute Phase SAA?
The Influence of SAA on Macrophage Cholesterol Metabolism during Inflammation
SAA, Atherogenesis, Unstable Angina, and Prognosis of Myocardial Infarction

6. Cholesterol Esters and Cell Growth in Human Lymphocytes:
Possible Implication of P-gp Modulators

Francesca Sanna, Marirosa Putzolu and Barbara Batetta

Introduction
Proposed Roles of P-gp in Cell Physiology
Time-Dependent Changes in Cholesterol Ester Synthesis and MDR1 and ACAT Gene Expressions in Lymphocytes Stimulated to Growth by PHA
P-gp Inhibition Suppresses Proliferation of PHA Stimulated Lymphocytes
In PHA Stimulated Lymphocytes, MDR1 Gene Expression Is Not Correlated to a MDR Phenotype
Progesterone Treatment Increased Raft-Cholesterol Content in PHA-Stimulated Lymphocytes

Conclusions

7. Cholesterol Esterification and MDR1-P-gp in Lymphoblastic Leukemia Cells: Functional Relationships

Rosa Rita Bonatesta and Barbara Batetta

Introduction
Altered Cholesterol Balance during Tumoral Growth
Positive Correlation between Growth Rate and Cholesterol Esterification in CEM and MOLT4 Cell Lines
A Role for P-gp in the Lipid Transport of Leukemia Cells

Conclusions
8. MDR-1, Cell Growth and Cholesterol Esterification ........................... 81
   Alessandra Pani and Sandra Dessì
   Multidrug Resistance ................................................................. 81
   P-gp and MDR ............................................................................ 81
   P-gp Substrates and Antagonists .................................................. 83
   P-gp Tissue Localization and Physiologic Function(s) ..................... 84
   P-gp in Cell Death and Cell Growth ............................................. 86
   P-gp Involvement in Intracellular Cholesterol Trafficking .......... 86
   P-gp, Cell Growth and Cholesterol Esterification ....................... 89
   Conclusions ............................................................................... 92

9. Involvement of Cholesterol Ester Cycle in the Progression
   of Atherosclerosis ...................................................................... 98
   Maria Franca Mulas and Sandra Dessì
   Structure of the Normal Arteries ................................................. 98
   Lesions of Atherosclerosis .......................................................... 99
   Pathogenesis of Atherosclerosis .................................................. 100
   Plaque Rupture ......................................................................... 101
   Roles of Cell Proliferation and Cholesterol Ester Accumulation
     in the Pathogenesis of Atherosclerosis .................................... 101
   Proliferating Cells in the Artery Wall .......................................... 102
   Cholesterol Ester Accumulation .................................................. 104
   Cholesterol Ester Cycle ............................................................... 104
   Cholesterol Efflux .................................................................... 105
   Possibility of a Link between Cholesterol Esterification
     and Cell Proliferation during Atherogenesis ............................ 106
   Conclusions ............................................................................... 107

10. Role of Cholesterol Esterification in the Modulation
    of Vascular Smooth Muscle Cell (VSMC) Cycle ......................... 111
    Maria Franca Mulas and Sandra Dessì
    Human Vascular Proliferative Diseases ..................................... 111
    VSMC Proliferation .................................................................. 112
    Cell Cycle Regulation of Vascular Muscle Cell Proliferation .... 113
    Molecular Regulation of Proliferation and Potential
        Therapeutic Applications ....................................................... 114
    VSMC Cycle and Cholesterol Esterification Pathway .................. 114
    Possible Mechanisms by Which Cholesterol Esterification
        May Regulate VSMC Proliferation ....................................... 116
    Hypothetical Model by Which Cholesterol Esterification
        Induces VSMC .................................................................... 117
    Conclusions ............................................................................... 119
11. MDR, Cell Growth and Cholesterol Esterification: Implications for Cancer Therapy

Alessandra Pani and Sandra Dessì

Anti-Cancer Chemotherapy .............................................................. 123
Variables of the Cancer Response to Chemotherapy .................. 124
MDR and Cancer .............................................................................. 125
MDR Modulators in Clinical Trials .................................................. 126
Clinical Significance of P-gp Expression in Cancer ..................... 127
Other Mechanisms by Which P-gp Inhibitors May Block Cancer Progression ................................................................. 128
Conclusions ....................................................................................... 129

Index .................................................................................................. 135
In recent years, understanding the molecular mechanisms involved in intracellular cholesterol homeostasis has radically changed to include an increasing number of structurally diverse receptors and carriers. The latest additions have led to the implication of cholesterol in fundamental cell functions such as cellular signaling and growth regulation. It appears that, at least in some instances, adaptive regulation of cholesterol metabolism does not protect cells indefinitely. Changes in this fine homeostatic regulation may occur leading to pathologic consequences. The challenge of this book has been to provide a useful point of reference on the mechanisms that link cholesterol esters to cell growth and division. Particular attention has been dedicated to the alterations in cholesterol esterification in two important proliferative processes such as cancer and atherosclerosis.

The data presented in this book provide in vivo and in vitro evidence of a strong relationship between cholesterol esterification and rate of cell proliferation, and suggest that changes in the cholesterol esterification pathway might represent fundamental events in developmental growth processes. Although much progress has been made in tumor and atherosclerosis research, and remarkable therapeutic successes have been achieved in both these pathologies, several questions on the mechanisms underlying changes in cholesterol metabolism and cell proliferation remain to be answered. Therefore, the data acquire particular significance in view of the possibility that the overall process of cholesterol esterification could play a key role in regulating cell proliferation.

We hope that this book provides valuable information not only for physicians, but also for teachers and students. As for the Editors, we would like to look at this book as a milestone of a path started some seven years ago when two friends, even though not so young any more, still deeply fascinated by science and the potentiality of biology, put together their scientific competence to face important matters such as the pharmacological control of pathological proliferative processes from a novel perspective.

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Intracellular Cholesterol Homeostasis: Old and New Players

Sandra Dessì and Barbara Batetta

Cholesterol is an extremely important biological molecule being a major component of cell membrane as well as a precursor for the synthesis of a number of essential vitamins, steroid hormones and bile acids. It is for this reason that an adequate supply of cholesterol must be constantly insured to all cells of an organism. This is achieved mainly by two ways, either cholesterol is synthesized de novo within the cell, or it is supplied by an extra-cellular source. Nevertheless, since cholesterol overaccumulation may be toxic, the cells have developed a series of complex responses that work together to control the intracellular levels of cholesterol. These multiple responses are referred to as mechanisms of cholesterol homeostasis and make cholesterol one of the most controlled molecule inside the cells. Although regulation of cholesterol homeostasis was recognized nearly 30 years ago, over the intervening years several new mechanisms have been discovered. This chapter focuses on common and novel themes of cholesterol homeostasis paying particular attention to the complex network of proteins involved in the regulation of intracellular cholesterol trafficking.

Acquisition of Cholesterol by Peripheral Cells

Cholesterol is a biological molecule of vital importance for mammalian cell structure and function. Lacking cholesterol invariably leads to cell death. Unless specialized cells, such as hepatocytes which require cholesterol for the synthesis of lipoproteins and bile acids and steroidogenic cells for steroid hormone production, other cells demand cholesterol for the proper functioning of cell membranes where it has a major role in regulating fluidity of the lipid bilayer.

The cellular requirement of cholesterol is satisfied mainly from two sources:
- endogenously, by synthesis from acetyl-coenzyme-A (CoA) through mevalonate;
- exogenously, from receptor-mediated uptake of low-density lipoproteins (LDLs)

Endogenous Synthesis

Cholesterol synthesis occurs in the cytoplasm and microsomes from the two-carbon acetyl group of acetyl-CoA (for review see ref. 1).

The process has five major steps:
1. Acetyl-CoAs are converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase.
2. HMG-CoA is converted to mevalonate by HMG-CoA reductase (HMG-CoA-R). The reaction catalyzed by this enzyme is the rate limiting step of cholesterol biosynthesis, this enzyme being subject to complex regulatory controls.
3. Mevalonate is converted to the isoprene based molecule, isopentenyl pyrophosphate (IPP), with the concomitant loss of CO₂.
4. IPP is converted to squalene.
5. Squalene is converted to cholesterol in the endoplasmic reticulum (ER).

From its site of synthesis, cholesterol is transported to other cellular destinations, prevalently plasma membranes, where 70-90% of cellular cholesterol resides. It is reported that cholesterol reaches the cell surface within 10-20 min after synthesis in the ER. Recent evidences indicate caveolae as the initial site on the cell surface where new cholesterol appears. Caveolae are distinctive, flask-shaped invaginations of the plasma membrane found in many cells. In contrast to coated pits, which are constitutively endocytosed, caveolae remain attached to the plasma membrane with their release being affected by unknown signal. They have a characteristic lipid composition, rich in cholesterol and glycosphingolipids, and are associated with the presence of a 22 kD protein called caveolin-1. It is also reported that caveolin-1 is required for the translocation of newly synthesized cholesterol from the ER directly to caveolae. The arrival of new cholesterol in caveolae is followed by the immediate movement of the sterol to noncaveolae membrane and possibly out of the cell.

The fate and the transport of newly synthesized cholesterol in mammalian cells is shown in Figure 1.1.

Exogenous Uptake: The LDL Receptor Pathway

Exogenous cholesterol is mainly obtained by the internalization of cholesteryl-rich lipoproteins via LDL receptor pathway. This pathway is known since 1973 and its discovery led to the award of the Nobel Prize to Brown and Goldstein in 1985.

It is well known that cholesterol is a hydrophobic molecule quite insoluble in water. Thus, it cannot pass from the liver and/or the intestine to the cells simply dissolved in blood and in extracellular fluid. Instead it is carried in tiny droplets of lipoprotein. The most abundant cholesterol carriers in human are the LDLs.

LDL particles are spheres with a core containing mainly cholesterol esters covered with a single layer of phospholipid molecules with their hydrophilic heads exposed to the watery fluid (e.g., blood) and their hydrophobic tails directed into the interior. Over a thousand molecules of cholesterol are bound to the hydrophobic interior of LDL particles. A protein, called apolipoprotein B-100 (ApoB-100) is exposed at the surface of each LDL particle. The first step in acquiring LDL particles is their binding to LDL receptors that are localized at the cell surface in pits coated with clathrin, a protein involved in the formation of transport vesicles from membranes. LDL receptor is a cell surface glycoprotein with a molecular weight of 164 kDa having a site that recognizes and binds to the ApoB-100 on the surface of the LDL. Once LDL binds the receptor, ligand and receptor are collected in the coated pits, and internalized to form coated vesicles. The clathrin coat is then removed and the uncoated vesicle fuses with endosomes to form an early sorting endosome. Here, ATP-dependent proton pumps lower the pH which causes the LDL to separate from its receptor.

The vesicle then pinches apart into two smaller vesicles: one containing LDLs (late endosome); the other containing receptors (endocytic recycling compartment, (ERC)). The ERC returns to and fuses with the plasma membrane, turning inside out as it does so. In this way the LDL receptors are returned to cell surface for reuse. The late endosome fuses with lysosomes to form a late endosome/lysosome where the LDL undergoes digestion. This results in degradation of apoproteins and hydrolysis of cholesterol esters by a specific acid cholesterol esters hydrolase, (aCEH) in fatty acids and free cholesterol. This process, apparently complex, is extremely necessary for that cholesterol may be used by cell for membrane structure purpose. Cholesterol esters, the form by which cholesterol is found in LDL particles, are, in fact, highly non polar molecules and for this solubility characteristic they can not be able to become a
Overview—Intracellular Cholesterol Homeostasis: Old and New Players

Free cholesterol liberated into lysosome is promptly transported at cell surface caveolae from where it may be used by the cells for the synthesis of new membrane and/or for the normal membrane turnover. It has been found that a maximum of 40-50 min is required for transport of LDL-cholesterol from lysosomes to plasma membrane. Thus, intracellular transport of LDL derived free cholesterol begins at clathrin-coated pits and terminates at cell surface caveolae.

Recently, it has been speculated that a protein called NPC1 is involved in free cholesterol efflux from the late endosome/lysosome and that the NPC1 protein is responsible for transport of cholesterol to the trans Golgi network and then to plasma membranes. This idea comes from the discovery that cells obtained from patients with a rare, fatal, and presently untreatable disease.
autosomal recessive disorder (Niemann Pick type C1 disease) accumulate massive amounts of free cholesterol in late endosomes which also expand, filling with whorls of membrane.\textsuperscript{13,14} More recently, however, Lange and Coll.\textsuperscript{15} provided evidence that functional NPC1 protein is not required for the exit of cholesterol from NPC1 lysosomes, being the rate of movement of cholesterol from lysosomes to plasma membrane in NPC1 cells at least equal to that observed in normal cells. They conclude that, the build up of cholesterol in NPC1 lysosomes was not a physiological response to cholesterol overload. Rather, it results from an imbalance in the brisk flow of cholesterol among membrane compartments. Based on these results the mechanisms mediating free cholesterol transport from lysosomes to the plasma membrane remain to be established. The LDL receptor pathway is summarized in Figure 1.2.

**Mechanisms of Cholesterol Homeostasis**

Within the cell, the distribution of cholesterol is highly compartmentalized, with most of the cellular cholesterol concentrated in the plasma membranes.

However, to preserve the integrity of cells, membrane FC mass must be maintained within narrow limits. The excess cholesterol is then stored as cholesterol esters in form of cytoplasmic lipid droplets. The esterification process is catalyzed by a membrane bound enzyme located in the ER called acyl-coenzyme A: cholesterol acyltransferase (ACAT). To maintain optimal content of cholesterol within the cells, the LDL uptake process, the endogenous sterol synthesis process, and the sterol esterification process are coordinately regulated. This is achieved by a series of complex responses referred to as mechanisms of cholesterol homeostasis.

When plasma membrane free cholesterol exceeds a threshold level, cholesterol beyond the cell’s need is rapidly transported to the ER where homeostatic proteins reside.\textsuperscript{16} Although cholesterol transport from the plasma membrane to ER seems to represent a crucial point of homeostatic activities, it is still unclear how sterols are transported to the ER and how this transport is regulated.

Possible mechanisms that have been involved, include aqueous diffusion, vesicle-mediated transport, and soluble carriers, which may work together or separately to mobilize cholesterol within the cell. More recent evidences raise the possibility of a mechanistic role for MDR1-P-glycoprotein (P-gp) in cholesterol transport.\textsuperscript{17-19}

**Involvement of MDR1-P-gp in Cholesterol Transport**

P-gps (140-180 kDa) are members of the large ATP-binding cassette (ABC) superfamily of transport proteins also called traffic ATPase. They are composed of two homologous halves joined by a flexible linker region, each one containing six transmembrane domains and an ATP binding-utilization domain. ATP-binding and hydrolysis appear to be essential for the proper functioning of P-gps. One member of P-gps family, encoded by MDR1 gene was originally identified for its ability to confer resistance against unrelated cytotoxic drugs in tumor cells. This phenomenon termed “multidrug resistance” represents today one of the main causes of cancer therapy failure\textsuperscript{20} and will be discussed in more detail in the next chapters.

Although, MDR1-P-gp is over-expressed in tumors following chemotherapy, it is also normally expressed in many different tissues raising the question of the physiologic function(s) of this protein. In addition to the prospected roles for P-gp in outward translocation of substrates,\textsuperscript{21-24} several recent studies suggest a function for MDR1-P-gp in the trafficking of sterol within cells. A series of amphiphilic agents, known to modulate MDR activity, blocked transport of cholesterol substrate from the plasma membrane to ER in a cultured rat hepatoma cell line.\textsuperscript{16} Likewise, in CaCo2 cells and in a human hepatoma cell line, compounds known to inhibit MDR1 P-gp also inhibited the movement of cholesterol from the plasmamembrane to the ER.\textsuperscript{25} Using a series of steroid hormones, Debry et al\textsuperscript{18} found that inhibition of cholesterol
Fig. 1.2. The LDL receptor pathway.

- The first step in acquiring LDL particles is their binding to LDL receptors (LDL-R) that are localized at the cell surface in pits coated with clathrin.
- Once LDL binds the receptor, ligand and receptor are collected in the coated pits, and internalized to form coated vesicles. The clathrin coat is then removed and the uncoated vesicle fuses with endosomes to form an early sorting endosome. Here, ATP-dependent proton pumps lower the pH which causes the LDL to separate from its receptor.
- The vesicle then pinches apart into two smaller vesicles: one containing LDLs (late endosome); the other containing receptors (endocytic recycling compartment (ERC)). The ERC returns to and fuses with the plasma membrane, turning inside out as it does so. In this way the LDL receptors are returned to cell surface for reuse.
- The late endosome fuses with lysosomes to form a late endosome/lysosome where the LDL undergoes digestion. This results in degradation of apoproteins and hydrolysis of cholesterol esters (CE) by a specific acid cholesterol esters hydrolase, (aCEH) in fatty acids and free cholesterol (FC).
- Most of the LDL-bound cholesterol released from the lysosome rapidly emerges at cell surface caveolae, from where it may be used by the cells for the synthesis of new membrane and/or for the normal membrane turnover or it is transported to ER for the esterification by ACAT.
- Cholesterol flux in and out of the ER may serve to regulate ACAT and other cholesterol sensors in the ER. Under high cholesterol trafficking, a dynamic cholesterol-cholesteryl ester cycle exists.
Cell Growth and Cholesterol Esters

trafficking from the plasma membrane to the ER correlated directly with the hydrophobicity of each steroid and its potency in reversing the effect of P-gp on drug accumulation. Although the mechanism by which P-gp may affect cholesterol trafficking within cells is currently unknown, overall, these data support an additional physiologic function for P-gp in cholesterol transport from the plasma membrane to the ER.

Sterol Regulatory Systems in the ER

As mentioned above, cellular cholesterol levels are controlled by a diverse set of homeostatic activities that are all located in the ER. These include enzymes for sterol biosynthesis (e.g., HMGCoA reductase) and esterification (acyl-CoA: cholesterol acyltransferase, ACAT) as well as precursors for transcription factors that control the expression of other regulatory elements. It seems that all cholesterol homeostatic proteins are orchestrated by a common regulatory signal: the level of ER cholesterol in their vicinity. The expression of genes for the LDL receptor, HMGCoA-synthase, HMGCoA reductase and other regulatory proteins that control the expression of other regulatory elements is under the control of the ER, such as farnesyl diphosphate synthase and squalene synthase, is under the positive control of a family of ER membrane-bound proteins called sterol regulatory element-binding proteins (SREBPs), of which three different isoforms are currently recognized. They activate genes involved in the synthesis of cholesterol and its uptake from plasma lipoproteins by acting as transcription factors after proteolytic cleavage. This cleavage is regulated by a polypeptide-sequence binding protein called SREBP cleavage-activating protein (SCAP), which forms complexes with SREBPs in membranes of the ER and also serves as a sterol sensor, losing its activity when sterols overaccumulate in the ER.

When cholesterol levels in the ER are low, the NH2-terminal domains of the SREBPs are released from ER membranes by two sequential proteolytic cleavages, catalyzed by two different proteases, site-1 protease (S1P) and site-2 protease (S2P). S1P is a membrane-bound serine protease that cleaves the SREBPs at a leucine-serine bond within a hydrophilic loop that projects into ER lumen, dividing the SREBPs in two halves. SCAP facilitates cleavage of SREBPs by S1P. After the two halves of the SREBP have separated, a second protease, designated S2P, cleaves the NH2-terminal intermediate fragment at a site that is just within its membrane-spanning domain. Active fragments of SREBP leave the ER membrane and translocate to the nucleus where they bind to a 10 bp sterol regulatory element-1 (SRE-1) sequence contained in the promoters of sterol-regulated genes and activate gene transcription. On the contrary, when cholesterol levels in the ER rise, the proteolytic release of SREBPs from ER membranes is blocked and SERBP remains membrane-bound. As a result of these events, transcription of sterol-regulating genes declines, and sterol synthesis and uptake are suppressed.26-27

Current evidence indicates that cholesterol blocks the proteolytic release process by selectively inhibiting cleavage by S1P. S2P does not appear regulated directly by sterols since this enzyme cannot act until the two halves of SREBP have been separated through the action of S1P.

The build-up of cholesterol in the ER also results in an increased rate of cholesterol esterification by the ER-bound enzyme, ACAT, which converts excess free cholesterol to cholesterol esters that accumulate in the cytoplasm as cholesteryl esters droplets. To minimize interference by ACAT in the sterol translocation process between the ER and the plasma membrane, significant esterification by ACAT does not occur unless the cholesterol concentration in the ER exceeds a certain critical threshold that is higher than that required to induce SREBPs. Based on these observations it has been proposed that the main role of ACAT is to guard against excessive buildup of cholesterol in the ER.28

Figure 1.3 depicts the current knowledge about the two-step proteolytic cleavage of membrane-bound SREBPs.
Overview—Intracellular Cholesterol Homeostasis: Old and New Players

Fig. 1.3. Sequential proteolytic cleavage of SREBPs.

- The molecular basis of coordinate cholesterol regulation has been unraveled by the finding, made in the laboratory of Brown and Goldstein\textsuperscript{26,27}, that sterol regulatory element binding proteins (SREBPs) serve as sterol-specific transcription factors.
- The precursor forms of SREBPs exist as integral membrane proteins in the endoplasmic reticulum (ER); a protein called SCAP interacts with the SREBP in the ER. The N-terminal segment of the SREBP precursor contains the domain necessary for recognizing the SRE in order to activate transcription; this segment is held from entering the nucleus by an anchor segment formed by two transmembrane sequences and a short loop segment in the ER lumen.
- When cells are deprived of cholesterol, the SREBP precursor undergoes two sequential proteolytic cleavages. The first cleavage occurs within the lumen of ER, producing an intermediate that contains the SRE-recognition segment but remains attached to the ER through the first transmembrane domain. This cleavage, by the protease designated as Site-1 protease (S1P), is sensitive to cholesterol. SCAP is believed to be the sterol sensitive component involved in the S1P cleavage step. A second cleavage occurs within the first transmembrane domain of the intermediate, by Site-2 protease (S2P), that is insensitive to sterol, causing the release of the mature SREBP from the membrane.
- After the second cleavage, the mature SREBP (active fragment) leaves the ER membrane and enters the nucleus, where it activates target genes controlling cholesterol synthesis and uptake.
Metabolic Fate of Cytoplasmic Lipid Droplets

Thus, ACAT is the enzyme responsible for the intracellular formation of cholesterol esters from cholesterol and long-chain fatty acids derived from acyl CoA and requires ATP for its synthesis. Beside its role in cholesterol detoxification, cholesterol esters are believed to be of physiological relevance also because they represent the form by which cholesterol is stored inside the cells, and thus a pool of cholesterol promptly available for cellular needs.28 Cholesterol esters droplets, despite their quiescent appearance in electron micrographs, are metabolically active, undergoing continuous hydrolysis and re-esterification in an apparently futile cycle that wastes ATP; a process that has been termed the “cholesteryl ester cycle”.29 The enzyme responsible for the hydrolytic phase of the cholesterol ester cycle is a neutral cholesterol ester hydrolase (nCEH) located in the cytoplasm nearby the outer face of ER membrane. nCEH is distinct from lysosomal aCEH involved in LDL degradation, in that it localizes in the cytosol, it has neutral pH optima, it can be activated by cAMP and it hydrolyzes CE endogenously formed by ACAT.30

Until now not much information is available on the fate of free cholesterol released from hydrolysis of cholesteryl ester lipid droplets. It has been reported that if the free cholesterol generated by hydrolysis is not removed efficiently from the CE cycle it undergoes re-esterification by ACAT, without a change in cellular CE mass. Alternatively, it can move between the intracellular pool(s) and the plasma membrane where it may be re-utilized by the cells or become available for efflux via extracellular acceptor particles.

In summary it seems that, according to cellular needs, free cholesterol generated from hydrolysis of CE in droplets moves to the ER, from where it again becomes a substrate for ACAT or it is transported to the plasma membrane. In the latter compartment, cholesterol may be utilized for membrane biogenesis and turnover or it is eliminated from the cells via high density lipoprotein (HDL) efflux.

Efflux of Cholesterol

Efflux of cholesterol is the first step in “reverse cholesterol transport”, a process firstly proposed by Glomset in 1968,31 by which excess cholesterol is removed from peripheral cells and delivered to the liver for excretion from the body. For most types of cells this is the only mechanism available for removal of cellular cholesterol, being cells unable to catabolize it. It is thought that small, nascent, lipid poor HDL particles and other forms of phospholipid-rich-HDL are the referred acceptors of cellular cholesterol.

HDL Metabolism

HDL metabolism has been awarded much attention because the HDL plasma concentration negatively correlates with atherosclerosis. Growing evidence indicates that the protective effect against atherosclerosis relates to the HDL-facilitated reverse cholesterol transport of cholesterol from extrahepatic tissues to the liver.

HDLs represent a heterogeneous group of lipoprotein particles, which can be divided in subfractions, mainly HDL2 and HDL3, with different protein and lipid composition, continuously modulated by lipases, lipid transfer proteins and receptors.32 They are secreted from the liver or the small intestine as discoidal nascent particles (HDLn), mostly comprising phospholipid and apolipoprotein A-1 (APO-AI). Similar HDL particles can be also directly generated in the plasma compartment following the lipolysis of triglyceride-rich lipoproteins. HDLn promote the removal of excess free or unesterified cholesterol from cells of peripheral tissues. Cholesterol that is transferred to nascent HDL is esterified by the plasma-enzyme lecithin cholesterol acyl transferase (LCAT) to cholesteryl esters, which, by virtue of their hydrophobicity, move into the core of the HDL particle giving rise to the