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Cholesterol

Good, Bad and the Heart

Edited by Madan L. Nagpal

CHOLESTEROL - GOOD, BAD AND THE HEART

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http://dx.doi.org/10.5772/intechopen.72468
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Notice

First published in London, United Kingdom, 2018 by IntechOpen
eBook (PDF) Published by IntechOpen, 2019
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

Cholesterol - Good, Bad and the Heart
Edited by Madan L. Nagpal

p. cm.

Print ISBN 978-1-78923-586-9

Online ISBN 978-1-78923-587-6

eBook (PDF) ISBN 978-1-83881-688-9

Meet the editor

Dr. Madan L. Nagpal has been a research associate professor at the Department of Microbiology and Immunology, University of South Carolina, Columbia, SC, and a research chemist at the Dorn Veterans Medical Center, Columbia, SC, USA, for more than 10 years. He has published several scientific articles. He was awarded Research and Productive Scholarship 1990 by the University of South Carolina. He was funded as coinvestigator by the National Institutes of Health (NIH) and VA Merit Review grants. Dr. Nagpal contributions include development of technologies in understanding the gene regulation in steroid hormone biosynthesis and the characterization of *Bacillus* species. He is a member of the American Society of Cell Biology, American Association of Advancement of Science, Endocrine Society, American Association for Laboratory Animal Science, International Who's Who Professionals, and Marquis Who's Who in America.

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Preface

In the food, the diet, and the body, one often hears the term "cholesterol" and asks "what is this cholesterol?" In this book entitled *Cholesterol - Good, Bad, and the Heart*," now you got the answers given by the experts in the field. Moreover, you can explore more by reading the references/citations given in the articles of each chapter. It is still an emerging field and lot more is being discovered. You will be amazed how much knowledge is already there in this book on cholesterol.

The book contains eight chapters. The first section covers the structural and functional features of cholesterol in Chapters 1 to 5.

In Chapter 1, Zhiwei Y. et al. describe cholesterol in various forms, as high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and cholesteryl ester transfer protein (*CETP*), the molecules that play central roles in the transport of cholesterol. HDL-C has been well known as antiatherogenic (good cholesterol), whereas LDL-C (bad cholesterol) is considered to be a major cause of atherosclerotic cardiovascular diseases. In Chapter 2, Zhang Weizhan describes cholesterol balance regulation by endocrine hormones and regulation of lipid metabolism in humans and rats and its linkage with genetic differences. In Chapter 3, Hissa B. and Pontes B. explore the role of cholesterol in cellular processes, such as modulating actin architecture and cellular contractility, lipid rafts, and membrane heterogeneity. In Chapter 4, Gungor Banu focusses on biosensors as analytical tools to measure cholesterol with very high precision, sensitivity, and speed and thus overcomes the disadvantages of the earlier procedures. In Chapter 5, Bikram H.A. shows that cholesterol is an important and useful parameter for distinguishing between pleural fluid exudates and transudates.

The second section includes Chapters 6 to 8 on cholesterol and the heart. In Chapter 6, Eyup A. et al. give a comprehensive review of cholesterol hypothesis and epidemiology of atherosclerosis. In Chapter 7, Yuan Y. et al. discuss the role of dietary cholesterol as a risk factor in the pathogenesis of nonalcoholic fatty liver disease. In Chapter 8, , Cardoso ML. et al. describe biochemical, phenotypic, and neurophysiological characteristics of the mouse model of Smith-Lemli-Opitz syndrome (SLOS), an inborn error of metabolism in cholesterol biosynthesis.

You will realize that cholesterol is an essential and extremely important building block of cell membranes and thus serves vital functions in the body. It is also a precursor for the synthesis of steroid hormones, bile acids, and vitamin D. What makes it good or bad is the type of lipoprotein that binds to it. One is high-density lipoprotein, or HDL, and the cholesterol bound to it is the HDL-cholesterol, and it is the good cholesterol (antiatherogenic), whereas the low-density lipoprotein, or LDL-bound cholesterol, LDL-cholesterol, is the bad cholesterol (atherogenic). LDL-cholesterol contributes to fatty buildups and narrows blood vessels and raises the risk for heart attack and stroke. HDL-cholesterol protects against heart attack and stroke.

I convey my appreciation to all the contributing authors and to the IntechOpen team, particularly, Romina Rovan, the Publishing Process Manager, for her generous help, guidance, and support in the preparation of this book.

I believe this book will be incredibly powerful and useful in teaching to give new perspectives on cholesterol.

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University of South Carolina, USA

Section 1

Cholesterol Features: Structural and Functional

Chapter 1

Structural Basis and Functional Mechanism of Lipoprotein in Cholesterol Transport

Zhiwei Yang, Dongxiao Hao, Yizhuo Che,
Lei Zhang and Shengli Zhang

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76015

Abstract

Lipoprotein transports lipids in circulation and is primary driver/modulator of atherosclerosis. Highly dynamics of lipoprotein conformations are crucial to lipid transport along the cholesterol transport pathway, where high-density lipoprotein (HDL), low-density lipoprotein (LDL) and cholesteryl ester transfer protein (CETP) are major players in lipid digestion & transport and the plasma cholesterol metabolism. This chapter covered how do HDL, LDL and CETP induce the metabolisms during cholesterol transport, and summarized recent process in the spatial information of the three lipoproteins, especially the elevations of plasma HDL and LDL, and shine a light on the assembly processes of lipoprotein particles and the substrates dynamics exchanges, for an in-depth understanding on the correlation between various lipoprotein classes and cardiovascular risk.

Keywords: lipoproteins, structure–function relationship, cholesterol transport, reverse cholesterol transport (RCT), lipoprotein particle metabolism

1. Introduction

Cardiovascular disease (CVD), a leading cause of mortality in many developed and developing countries [1], roots in the evolvement of atherosclerosis which is associated with profound disturbances of cholesterol metabolism. To some degree, these metabolism disturbances attribute to the net movement of cholesterol among blood and peripheral tissues. For instance, cellular cholesterol uptake is increased in atherosclerosis, while cholesterol efflux is downregulated [2]. Lipoproteins (consists of apolipoproteins, phospholipid and cholesterol) play an

important role in the transport of cholesterol [3]. Based on density and size, lipoproteins can be classified as ultra-low- (chylomicrons), very low- (VLDL), intermediate- (IDL), low- (LDL), and high- density lipoproteins (HDL) [4]. The last two might be the significant sections of cholesterol transport and metabolism: (1) LDL could transfer lipids into the blood vessel walls, and contribute to the atherosclerosis, which causally be associated with CVD and all-cause mortality; (2) HDL could remove the lipids and carry them back to the liver, being regarded as "good" one [5, 6]. Hence, the lipoprotein-mediated cholesterol metabolism (cholesterol transport) has aroused great attention and showed the benefit for the in-depth understanding of CVDs, as well as the prevention and treatment of CVDs.

As shown in **Figure 1**, the lipoprotein-mediated cholesterol metabolism can be divided into exogenous and endogenous pathways [7]. Exogenous pathway is one of crucial ways to transport cholesterol to the body tissues (chylomicrons → VLDL → IDL → LDL) [8, 9], under the co-action of lipoprotein lipase (LPL) and hepatic lipase (HL) [10, 11]. While the higher plasma LDL level might drive the process of atherosclerosis [12]. Endogenous pathway delivers cholesteryl esters back to the liver, working cooperatively in a concurrent manner with ATP-binding cassette transporter A1 (ABCA1) [13], enzyme lecithin-cholesteryl acyltransferase (LCAT) [14], as well as HDL receptors scavenger receptor B1 (SR-BI) [15] or other unidentified HDL receptor (HDLR) [16]. It is widely accepted that HDL protein particles alleviate atherosclerosis with better cardiovascular health (reverse cholesterol transport, RCT) [6, 17, 18]. Besides, cholesteryl ester transfer protein (CETP) does a heteroexchange of triglycerides and cholesteryl esters between VLDL/ LDL and HDL, with the lessen of cholesterol eliminations [19, 20]. Therefore, the functions of HDL, LDL and CETP play the important roles during the cholesterol transport (lipoprotein particle metabolism), and pharmacological inhibition of CETP is being regarded as a way to prevent CVDs [19, 20].

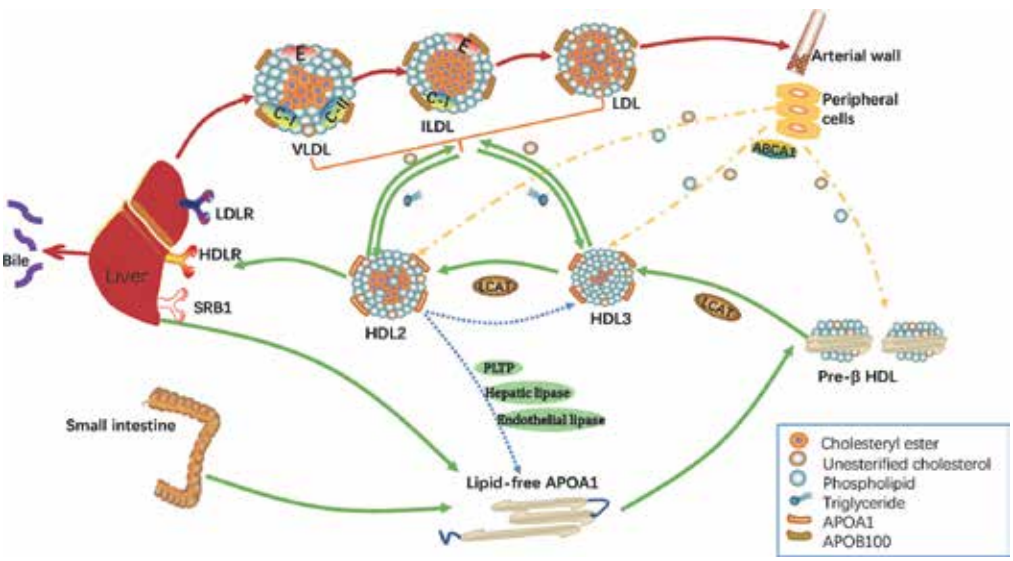

Figure 1. Lipoprotein-mediated cholesterol metabolism in human body.

To best of our knowledge, there are scant reviews elaborating the structure–function relationship of lipoproteins albeit the schematic illustrating is oncoming clear. A comprehensive understanding in this regard was endeavored, and then bioavailability that is closely related with cholesterol transport was discussed. In this chapter, we will summarize the recent achievements towards the structural basis and functional mechanism of lipoproteins in cholesterol transport, mainly focusing on functions of HDL, LDL and CETP, conformation dynamics of lipoprotein particles, and substrates dynamics exchanges.

2. Structure and function of HDL

HDL, a plasma lipoprotein, plays an important role in cholesterol metabolism [21–23], with several potentially anti-atherogenic properties (remove cholesterol from macrophages) [24–26]. Knowing the assembly mechanism and spatial information is of great importance to mediate cholesterol transport. HDLs exit three main steadier state during the cholesterol transport process: lipid-free apoA-I (apoA-I, the major protein component of HDL particles), discoidal and spherical HDL, with highly heterogeneous and differences of density, size, shape, as well as composition of lipid and protein.

2.1. Lipid-free apoA-I

Structure of full-length lipid-free apoA-I (28-kD, 243 residues) at native states still remains unclear due to its high flexibility. The initial X-ray crystal structure revealed that N-terminal truncated (Δ(1–43)) lipid-free apoA-I features "horseshoe-shape" antiparallel helical dimers [27], being regarded as a vital initial model ("double-belt" model) for comprehending the structure of apoA-I on HDL subclasses (**Figure 2b**) [28]. Subsequent crystal organization of lipid-free Δ(1–43)apoA-I accommodated a four-helix bundle [29–31]. However, the structural information is out of step with some physical biochemical measurements, hinting the conformation dynamics of lipid-free apoA-I. The crystal structures of the N- and C-terminally truncated

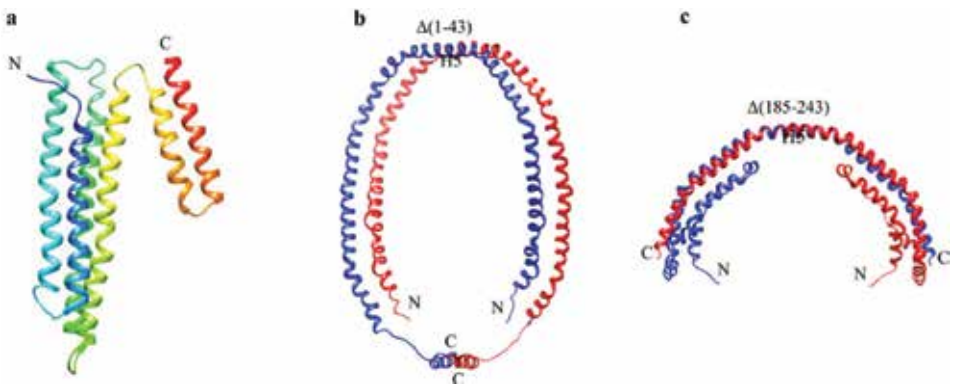

Figure 2. Three structures of lipid-free apoA-I: (a) full-length lipid-free apoA-I, [36] (b) N-terminal truncated Δ(1–43) apoA-I dimer, [27] and (c) C-terminal truncated Δ[185–243] apoA-I dimer [32].

proteins presented antiparallel helical dimers, with inherent properties (e.g., 5/5 repeat register, **Figure 2b** and **c**) in the lipid-bound and intermediate states [27, 32]. Amphipathic α-helix enables apoA-I to stabilize all HDL subclasses via the conformation change, and N-terminal two thirds constitute a dynamic, four-helix bundle, and the helical segments unfold and refold in seconds. While the C-terminal third, an intrinsically disordered domain, mediates initial binding to phospholipid surfaces. These structural motifs are important for the remodeling of apoA-I during the formation of various HDL particles. Nowadays, there remains some confusions for the structure of full length free apoA-I, especially the dynamic conformations in solutions. The dynamic helical structure is unfolding and refolding in seconds, and the helices bundle at the N-terminal of apoA-I is far more stable than could be achieved in isolation, with mutually stabilizing interactions [33, 34]. The highly dynamic apoA-I molecules are capable of adopting an array of conformations through remodeling HDL that is crucial to lipid transport during the RCT process. Further studies show that mutations in apoA-I induce varied types of dyslipidemias [35].

2.2. Discoidal HDL

Human plasma HDL is high heterogeneous, and exists as a short-lived heterogeneous substrate for LCAT in human plasma. Hence, reconstituted HDL particle (rHDL) is a powerful in vivo model system to study its structure and function, with most of the properties of native lipoprotein complexes (e.g., LCAT activation, lipid transfer, and receptor binding) [37–39]. Based on the crystal structure of Δ(1–43)apoA-I, [27] the original double-belt model features two antiparallel monomers, where each helix 5 segments directly oppose each other [40, 41], and the closely contact involved hydrophobic face of amphipathic α-helix with the fatty acid acyl chains [42]. In refined "looped belt" model, N- and C-terminal 40–50 residues doubled back as the "belt and buckle" [43], and residues 134–145 were coincide with a looping region, resulting in partial opening of the parallel belts. It is consistent with the accession between LCAT and the cholesterol and phospholipid acyl chains [44], With the aid of mass spectrometry (MS) and rHDL, lipid-free and lipid-bound apoA-I structures were solved at 104 Å resolution, and resulted in a "solar flares" model, where C-terminal of both apoA-I molecules interacted with each other, and 159–178 loop might be the LCAT binding site, with reduced deuterium exchange [45, 46], Different from normal discoidal shape, double superhelix (DSH) apoA-I model [47] has an open helical shape, with the similar interface interaction between two apoA-I molecules (5/5 double-belt). While, the DSH model is not stable, and could rapidly collapse to a disc-shaped structure during the molecular dynamics (MD) simulations [48].

In according to the rapid growth of transmission electron microscopy (EM) technique, the directly imaging particle's structure can be performed on individual particles, in order to preferably investigate lipoprotein structures. Negative stain EM combined with cryo-EM tomography have been applied to uncover the discoidal shape of apoA-I/HDL particles (both plasma HDL and 7.8, 8.4, 9.6 nm of rHDLs) [49, 50]. In these rHDL particles, the double belt was formed in an antiparallel fashion, with a gross "right-to-right" rotation of the helices after lipidation. The nonhelical regions in lipid-free apoA-I (residues 45–53, 66–69, 116–146, and 179–236) change conformation from random coil to α-helix, to adjust a hydrophobic interior

[34, 46]. Above descriptions were further confirmed by the structures of reconstituted discoidal HDL particles via nuclear magnetic resonance (NMR), electron paramagnetic resonance (EPR) and transmission electron microscopy (TEM) methods [51]. Based on the structures of lipid-free and lipid-bound apoA-I, we can speculate that the monomeric apoA-I forms a helix bundle in which the C-terminal domain binds the lipid to form a helical structure (**Figure 3**). Discoidal HDL are stabilized by two apoA-I molecules wrapped around the edge of the disc in an antiparallel, double-belt arrangement so that the hydrophobic PL acyl chains are protected from exposure to water [52]. These apoA-I molecules are in a highly dynamic state and adapt to discs of different sizes by certain segments forming loops that detach reversibly from the particle surface.

2.3. Spherical HDL

Due to the complexity of spherical HDL particles in human plasma, the spherical HDL structures are rarely known compared with lipid-free apoA-I and discoidal HDL. Recent developments in native and reconstituted spherical HDL supported a trefoil model, using by the elegant chemical cross-linking and mass spectrometry [53]. In this model, half of each apoA-I molecule in the double-belt arrangement is bent 60° out of the plane of the particle, suggesting the hinging of the Δ(1–43)apoA-I molecule is occur near residues 133 and 233 [53] which is different from the hinging of the full-length protein conformation, meanwhile, trefoil model is assumed to occur near residues 65 and 185 [54]. Determined by small angle neutron scattering method, the helical dimer with a hairpin (HdHp) model was proposed, associated with the intramolecular interactions within the hairpined apoA-I [55].

The first LpA-I HDL model at molecular level was proposed, with only apoA-I fractions isolated from human plasma [56]. These isolated human plasma HDL particles range in diameter from 8.8 to 11.2 nm and contain 3–5 apoA-I molecules. It was found that apoA-I adopts intermolecular interactions in plasma HDL which is very similar to those of the double-belt and trefoil models derived from reconstituted systems. Thus, apoA-I might adopt a common structural organization, characterized by distinct intermolecular contacts, regardless of size and shape or natural versus synthetic method of production [57]. Furthermore, circulating

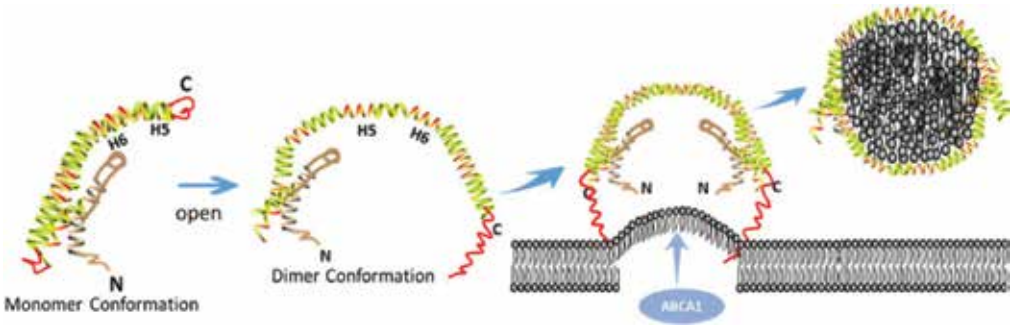

Figure 3. The monomer open conformation transfer to dimer conformation of apoA-I (intermediate state) and final HDL state in solution regulated by the H5 region.

sHDL contains similar amount of core lipid in reconstituted sHDL and has obviously less surface lipid monolayers, indicating that the apoA-I package on native spheres is much closer than the typical recombinant particles [46]. When a HDL disc alters to a sphere (LCAT converts free cholesterol to cholesteryl ester), global apoA-I conformation does not change significantly between particles of different shapes or origins, with similar protein–protein contacts.

3. Structure and function of LDL

In normal human body, there are about 70% plasma cholesterol contained in LDLs, and the endocytosis of cholesterol-rich LDLs is mediated by LDL Receptor (LDL-R) on the surface of body cell. Hence, LDLs work as the vehicle for cholesterol transportation between liver and cells to maintain a constant cholesterol supply in human body [58, 59]. In some abnormal conditions, LDL might induce over-accumulation of cholesterol to form foam cells, resulting in the development of atherosclerosis [60]. The apo-B48 (apoprotein B48) and apo-B100 (apoprotein B100) located in surface of LDL particles tend to interact with extracellular material, which make LDL particles easy to bind with blood vessel intima [61]. The oxidation-LDL can promote lipoproteins aggregation [62, 63] and provoke inflammation by recruiting the circulating monocytes to the site followed invade the vessel wall and differentiate into macrophages, to finally produce atherosclerotic plaque [62, 64–66]. Cryo-EM combined with single particle technology and small angle scattering model reconstruction technology have been effectively applied to analyze the LDL structures, and molecular components [67]. LDLs include difference in density (~1.019–1.063), shape, size (diameter ~18–25 nm), surface charge and chemical composition [68]. A general consensus is that LDLs particles all have two compartments, an amphipathic surface phospholipid monolayer which surrounded by one single copy of apoB-100, and a hydrophobic lipid-cholesteryl esters core [69]. The structure and physical function of LDLs predominantly depend on the core-lipid composition and the conformation of the apoB-100 [70, 71].

3.1. Lipid core of LDL

Lipid core of LDL mainly consists of cholesteryl esters, some triglycerides, and some free-cholesterol. Structural changes of LDL are strikingly related to physiological temperature [72]. Lipids located in core show order arranged to a liquid-crystalline phase below the critical temperature, indicated by the results of X-ray and neutron small angle scattering technology, with the transition temperature of 15~35°C [73, 74]. Besides, the overall structure of LDL is a classical spherical particle when core structure is composed of radial cholesteryl esters arranged into a concentric spherical shell [75, 76]. However, the core-located lipids present in the liquid-crystalline state within an ellipsoidal shape particle revealed by the cryo-EM data [76, 77]. It seems reasonable to speculate that the change of temperature might indirectly change the shape of LDL particles from roughly spherical to ellipsoid [67]. Many efforts have been made to explore the structure of LDL at different temperatures, such as 4, 6 [77–79] and 37°C [80].

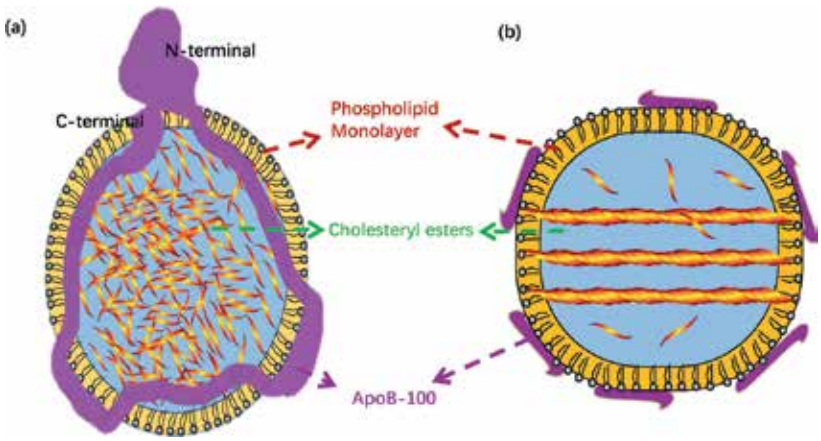

Figure 4. Overall structure and core structure of LDL above (a) or below (b) the critical temperature.

3.2. apoB-100 in LDL

ApoB-100 (4536 residues, ~20% of overall LDL) is the only protein component of LDL, and wrapped around the phospholipid monolayer on the surface of LDL particle, with an irregular ring shape. N- and C-terminus of apoB-100 touch each other, with the formation of a protruding globular structure at N-terminal [81]. A more generally accepted structural model of apoB-100 is "pentapartite" structure, which generated by molecular simulations. In this model, apoB-100 has five consecutive functional domains, NH2-βα1-β1-α2-β2-α3-COOH [79]. As shown in **Figure 4**, a new LDL reconstruction in which lipid core is revealed an organized three-layer structure by using the single particle approach, including a pair of "paddles" configurations with several long "fingers" extensions which have similar length and interval [82].

4. Structure and function of CETP

CETP acts as a medium between lipoproteins for elevating plasma LDL-C (or VLDL-C) level and lowering HDL-C level [19]. A series of CETP inhibitors have been investigated in clinical, such as torcetrapib, dalcetrapib, evacetrapib, and anacetrapib [83–85]. However, current inhibitors represent the turbulent beginning of CETP inhibition and an increased mortality rate related to off-target effects and lack of efficacy [86–88]. Accompanying adverse effects call for a deeper exploration of the mechanism for CETP-mediated lipid transfer.

CETP is a hydrophobic transfer protein composed of 476 amino acids and reveals a so-called banana-shape (the size is 135 × 30 × 35 Å, see **Figure 5**) [20]. Its crystal structure includes two different β-barrel structures in N- and C- terminal respectively, and a central β-sheet with an ~60 Å-long hydrophobic central cavity, which can hold two phospholipids and two cholesterol molecules. Moreover, the two phospholipid molecules that located in two pores near the central domain expose the hydrophilic terminal to the aqueous environment and hydrophobic terminal to the hydrophobic cavity. Because of its special function to transfer cholesterol

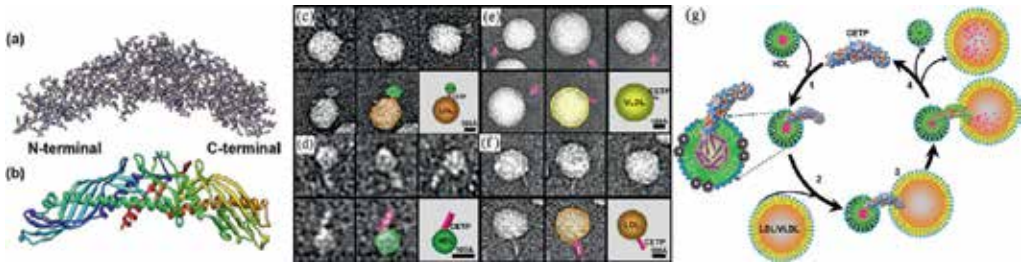

Figure 5. The crystal structure of CETP (PDB: 2OBD) and three-dimensional density maps of CETP binging lipoproteins. (a) Atom figure of CETP. (b) Secondary structure of CETP. (c) Ternary complexes of HDL-CETP-LDL in cryo-EM micrographs. (d)~(f) the CETP insert into HDL, VLDL, LDL respectively in cryo-EM micrographs. (g) (color online) the tunnel model of CETP-mediated lipid transfer [89].

esters between HDL and LDL (or VLDL), the way of CETP interacts with lipoproteins is extremely essential. CETP shows a high binding affinity for nascent HDL and other lipoproteins to cover the lipoproteins surfaces owing to its proper curvature radius. They proposed a lipid transport mechanism, shuttle model. In this mechanism, the CETP in turn covers the surface of LDL (or VLDL) and HDL to swap LDL-cholesterol esters (or VLDL-cholesterol esters) with HDL-triglycerides. These steps are constantly recycled until the completion of the transport process, in which cholesterol esters move from LDL (or VLDL) to HDL [20]. This model based on the hydrophobic cavity of CETP and its feasibility of binding to lipoproteins, explains the mechanism of CETP-mediate lipid transfer reasonably, but there are not complex of CETP binding to lipoproteins in the cryo-EM micrographs intuitively to verify the authenticity of the model.

Zhang et al. [89] studied human recombinant CETP with cryo-EM by using an optimized negative-staining (OpNS) EM protocol [49, 90]. Applied the single-particle techniques, they obtained the 3D structure of CETP and the complexes of CETP binging to lipoproteins. In the 3D-map of complexes, they discovered the HDL-CETP binding structure which appears to be formed by N-terminal of CETP insert into HDL and the HDL-LDL (or HDL-VLDL) is formed by C-terminal of CETP insert into HDL (or LDL) (**Figure 5**c~f). This conclusion was later confirmed by Geraldine et al. by using large-scale atomistic molecular dynamics [91]. The measurement of the protrusion from the lipoproteins surface shows that ~48 Å of the tapered N-terminal end of CETP penetrates the HDL surface and ~25 Å of the C-terminal end of CETP penetrates the LDL surface (~20 Å of the C-terminal end of CETP penetrates the VLDL surface) reaching the lipid–rich, lipoproteins core. Furthermore, Zhang et al. proposed the tunnel model of lipid transfer mediated by CETP [89, 92, 93]. In this model, both CETP terminals finish penetrating surface sites on lipoproteins, N-terminal to HDL and C-terminal to LDL (or VLDL). Then neutral lipids, including cholesterol esters and triglycerides, transfer through the hydrophobic tunnel at the core of the CETP (**Figure 5**).

However, there are some discrepancies with the tunnel model mentioned above. Matthias et al. used the experiments which involve three monoclonal antibodies to demonstrate that the antibodies binding on both ends of CETP do not inhibit CETP's function of transshipment cholesterol esters, but the antibodies on the middle does [94]. In their research they supposed that the formation of the ternary tunnel complexes is not a mechanistic prerequisite by CETP

to perform its functions. Hence, the real mechanism of CETP-mediated lipid transfer still remains to be studied and verified.

5. Conclusion

In this chapter, we briefly summarized the functional mechanism and structural basis of lipoproteins (e.g., HDL, LDL and CETP) in cholesterol transport, as well as their structural dynamics during the transport process. Furthermore, the latest developments in the plasma lipoprotein (HDL and LDL) elevations were summarized, especially the conformational changes of lipoprotein particles. Due to the incapability of the current assays and highly heterogeneous of lipoprotein particles, the function of lipoprotein in cholesterol transport remains elusive with regard to many important questions, such as how the lipoprotein particle assembles and how the assembly modulates the neutral lipids dynamic exchanges at the molecular level. Cryo-EM coupled with MD simulations have revealed several important mechanisms of CETP-mediated lipid exchange and metabolism with all-atom detail [89, 95]. Further researches could pay more attention to simultaneously monitor the dynamic structural change of lipoproteins and the dynamic mechanism of lipid transfer, especially the internal motivation of physical mechanism during the process of lipid transport.

Acknowledgements

Project supported by the National Natural Science Foundation of China under Grant No. 11374237, 11504287, 11774279 and 11774280, Fundamental Research Funds for the Central Universities (xjj2017029), China Postdoctoral Science Foundation (2017 M613147) and Shaanxi Province Postdoctoral Science Foundation (2017).

Conflict of interest

The authors have declared that no competing interests exist.

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Chapter 2

Hormonal Regulation of Cholesterol Homeostasis

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76375

Abstract

Cholesterol homeostasis is tightly regulated by a group of endocrine hormones under physiological conditions. Hormonal dysregulation is often associated with disturbed cholesterol homeostasis, resulting in many clinical disorders including atherosclerosis, fatty liver and metabolic syndrome. Circulating hormones regulate cholesterol metabolism by altering levels of relative genes either through their interactions with nuclear receptors or by interfering with bile acid signaling pathways. A better understanding of hormonal regulation of cholesterol metabolism would improve our likelihood of identifying effective and selective targets for the intervention of disturbed cholesterol. In this review, we discuss selected hormones critical for the cholesterol balance, including thyroid hormone, sex hormones, growth hormone, glucagon and irisin. We focus our discussion on the most recent advance in clinical epidemiology, animal mechanistic studies and the clinical application.

Keywords: cholesterol, thyroid hormone, sex hormones, growth hormone, glucagon and irisin

1. Introduction

Cholesterol is mainly composed of low-density lipoprotein (LDL), very-low-density lipoprotein (VLDL) and high-density lipoprotein (HDL). It plays a critical role in membrane biogenesis and steroid hormone biosynthesis. The disturbed plasma cholesterol is associated with many diseases, such as cardiovascular disease, diabetes and hepatic steatosis. Cholesterol is either uptaked exogenously from the diet or synthesized endogenously within cells. The liver is the major organ for cholesterol de novo synthesis which involves 19-step complex biochemical process. The rate-limiting enzyme is 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase. Sterol regulatory element-binding protein 1c (SREBP-1c) is the master regulator

of cholesterol by stimulating the transcription of LDL and HMG-CoA. LDL receptor (LDLr) is responsible for importing LDL from extracellular to intracellular environment for metabolism. Cholesterol is the primary source for biogenesis of steroid hormones. In turn, many hormones exert critical effects on cholesterol synthesis or metabolism. This occurs through the direct effect of these hormones on regulation of the expression or activity of HMG-CoA reductase, SREBP-1c or LDLr. In this chapter, we will discuss the regulatory role of several interesting hormones in cholesterol metabolism.

2. Thyroid hormone

2.1. Thyroid hormone and thyroid hormone receptors

Thyroid hormones (THs) include thyroxine (T4) and triiodothyronine (T3). They are synthesized and secreted by the thyroid gland. T4 is the major secreted hormone, while T3 has a higher affinity for TH receptors (TRs). T3 is considered as the active and more potent TH. T4 could be converted to T3 through a deiodination process catalyzed by deiodinases. TH regulates a number of biological functions including growth, development and metabolism in almost all tissues [1]. TH exerts these effects through binding to TRs which are expressed on different cells and tissues. TRs have two isoforms, TRα and TRβ, which are encoded by the THRA and THRB genes, respectively, in humans. Each TR isoform has several splice products, TRα1 (α2) and TRβ1 (β2). TRα1 and TRβ1 are ubiquitously expressed, while TRβ1 is the major TR existed in the liver. TRβ2 is expressed in the hypothalamus, the pituitary gland and the developing brain [2]. TRs are ligand-activated transcription factors, belonging to the family of nuclear receptors (NRs). It can bind to DNA sequences called TH-responsive elements (TREs) together with the retinoid X receptor alpha (RXR-α). In the absence of TH, TRs bind with corepressors, e.g., nuclear receptor corepressor and silencing mediator for retinoid and thyroid hormone receptor (NCOR2), suppressing the transcriptional activity. In the presence of TH, the binding induces a conformational change of TRs, releasing the corepressors and recruiting several co-activators to enhance the transcriptional activity. Since TRs associate with corepressors without ligand binding, it could decrease the transcriptional activity of the target genes. Therefore, it should be cautious to compare the data from animal models in which TRs are genetically deleted with the models with low levels of circulating THs, such as hypothyroidism or thyroidectomy [3].

2.2. Role of TH in cholesterol metabolism

There is substantial evidence linking TH status with cholesterol or lipid metabolism. Thyroid dysfunction exerts an important effect on the cholesterol level. Hypothyroidism patients typically have elevated plasma cholesterol and increased lipid accumulation in the liver. TH supplement can normalize this lipid dysregulation. THs promote cholesterol synthesis through inducing HMG-CoA reductase and farnesyl pyrophosphate gene expression [1]. THs markedly decrease the expression of apoB-100, the major protein of LDL, while increasing the expression of apo A-I, the major protein of HDL. In addition, THs increase LDLr gene expression. LDLr mediates the uptake of LDL from blood to the liver. Rat LDLr promoter contains two functional TREs. THs could directly bind to the TRE and upregulate

the LDLr gene expression [4]. THs may also regulate the clearance of circulating remnant lipoproteins. Hepatic low-density lipoprotein receptor-related protein 1 (LRP1) is a receptor for remnant lipoproteins. Hepatic LRP1 protein expression and function are reduced in the hypothyroidism mouse model. T3 supplement partially normalizes its protein expression level [5]. THs also promote the cholesterol elimination by increasing conversion and secretion of cholesterol into bile acids. In this process, cholesterol 7α-hydroxylase (Cyp7A1), the enzyme in the cytochrome P450 family, is responsible for catalyzing the rate-limiting reaction in the degradation of cholesterol. Cyp7A1 is a direct TR target gene with TREs in its promoter region [6]. ATP-binding cassette (ABC) transporters G5 (ABCG5) and G8 (ABCG8) form a heterodimer that limits intestinal absorption and facilitates biliary secretion of cholesterol. Mice homozygous for disruption of Abcg5 demonstrate a significant reduction in basal biliary cholesterol secretion. T3 treatment does not increase the cholesterol secretion in Abcg5−/− mice as in the wild-type control mice. This observation suggests that THs induce secretion of cholesterol, largely dependent on the ABCG5/G8 transporter complex [7]. THs also modulate gene expression via micro-RNAs. In a human hepatic cell line, THs decrease sterol O-acyltransferase 2 (SOAT2 or ACAT2), the enzyme crucial for the hepatic secretion of cholesterol esters, via miR-181d [8].

T3 also upregulates LDLr gene expression by activating the expression of the sterol regulatory element-binding protein-2 (SREBP-2) and scavenger receptor class B1 (SR-B1) [9]. Cholesteryl ester transfer protein (CETP) mediates the exchange of cholesteryl esters from HDL to the VLDL and from total triglyceride (TG) to the opposite direction. THs could increase the activity of CETP to influence HDL metabolism [10]. In addition, THs stimulate the lipoprotein lipase (LPL) and hepatic lipase (HL) levels, catabolizing the TG-rich lipoproteins.

2.3. Interaction with other transcription factors

In addition to the direct action on the cholesterol-related genes, TRs also cross talk with many nuclear receptors to regulate their transcriptions. It shares the same DNA-binding site (direct repeat 4) with liver X receptor (LXR). Activation of TRβ1 by T3 upregulates mouse LXRα, but not LXRβ, mRNA expression in the liver at the transcriptional level [11]. TRβ1 is the major TR mediating the TH effects on plasma cholesterol. ATP-binding cassette transporter A1 (ABCA1) is important for HDL assembly and transporting cholesterol back to the liver for excretion. TR forms a heterodimer with retinoid X receptor (RXR) and binds to the DR-4 element of ABCA1 promoter, suppressing its transcription [12]. The apolipoprotein AV gene (APOA5) is a key determinant of the plasma triglyceride level. It affects the plasma TG level through promoting lipolysis of TG-rich lipoproteins and removal of their remnants [13]. TR-β mediates the effects of THs on the activation of APOA5 gene. Administration of TR-β-selective agonist increases apoAV and diminishes triglyceride levels [14]. In addition, TR-β may compete with LXR/RXR heterodimers for binding to the DR-4 element in the CYP7A1 promoter [15]. TR-β but not TR-α KO mice completely lost the induction effects of T3 on Cyp7a1 gene, confirming the critical role of TR-β in mediating the TH effect on cholesterol metabolism [16].

Taken together, TH regulates the serum cholesterol level in multiple crucial steps including stimulating its hepatic synthesis, serum uptake and the intrahepatic conversion to bile acids. The physiological level of TH is essential for maintaining the cholesterol homeostasis.

3. Sex hormones

It is well recognized that premenopausal females have better lipid profiles than males and are more protected from hypercholesterolemia-related diseases, such as cardiovascular diseases. Lipid screening has found that premenopausal women are associated with a lower level of LDL cholesterol and a higher level of HDL cholesterol. After menopause, the gender difference of lipid profiles disappears, and women even have higher-level LDL compared to age-matched men [17]. Estrogen replacement therapy would improve lipoprotein profiles in postmenopausal women [18]. Sex hormones, especially estrogen, account for the gender difference of cholesterol profiles.

3.1. Estrogen and estrogen receptors

The predominant and most important biologically relevant form of estrogen is 17β-estradiol (E2). Both women and men produce E2 through aromatization of androgen. In premenopausal women, estrogen is mainly synthesized in the ovaries. While in postmenopausal women and men, it is primarily converted from testosterone by aromatase (encoded by CYP19 gene) in extragonadal tissues such as adipose tissue, adrenal glands, bones, etc. [19]. There are at least three types of estrogen receptors, ER-α, ER-β and membrane-bound receptor G protein-coupled ER (GPER, also known as GPR 30). ER-α and ER-β are the classic estrogen receptors and are mainly expressed in the cytosol. Upon estrogen binding, ER-α and ER-β form homo- or heterodimers and bind to estrogen response element (ERE) in the downstream target genes, to initiate or suppress the transcriptional activity. The GPER and membrane-associated ER-α and ER-β variants are expressed in the plasma membrane. They mainly exert actions via non-genomic signaling. This membrane-initiated signaling involves protein kinase A (PKA), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) signaling pathways [20–22].

3.2. Role of estrogens in cholesterol homeostasis

The influence and mechanism of estrogens on cholesterol metabolism have been investigated for a long time. Studies by Cypriani et al. in 1988 demonstrated that estrogens induced HMG-CoA reductase and subsequent cholesterol synthesis in breast cancer cell line [4]. Later, it was found that HMG-CoA reductase gene promoter contains an estrogen-responsive element-like sequence at position-93 (termed Red-ERE). And, estrogen induction of HMG-CoA reductase gene is dependent on the Red-ERE. The induction activity of estrogens occurs in the breast cancer cells but not in hepatic cells, indicating differential regulation of HMG-CoA reductase by estrogens in a tissue-specific manner [23]. Aromatase is an enzyme responsible for the key step in the biosynthesis of estrogens. Aromatase knockout (ArKO) mice display increased intra-abdominal adipose tissue and lipid droplet accumulation in the liver. Total cholesterol and LDL are also elevated in these transgenes [24]. Supplement of estrogens in both ArKO mice and rats with ovariectomy (OVX) normalizes LDL and total cholesterol levels, confirming the important role of estrogens in the lipid homeostasis in both males and females [25]. Hormone replacement therapy (HRT) increases the expression of leucocyte ABCA1 gene,

which mediates the efflux of cholesterol to the HDL particles, leading to the subsequent increase in the HDL cholesterol level [26]. Estrogens thus play an important role in the modulation of the total cholesterol level by reducing LDL and concurrently increasing HDL.

The beneficial role of estrogens on cholesterol metabolism is mediated through nuclear and extranuclear ER-α and ER-β, as well as GPER. Genetic deletion of ER-α in mice results in upregulation of the genes involved in hepatic lipid biosynthesis and downregulation of the genes involved in lipid transport, indicating that estrogens act via ER-α to regulate lipid metabolism [27]. ER-α KO and ER-α/β double KO mice showed increased serum cholesterol and smaller LDL particles, but not in ER-β single KO mice [28]. Therefore, ER-α plays a more prominent role than ER-β. The roles of GPER in the regulation of metabolism are only beginning to emerge, which gains more attentions. GPER knockout mice exhibit impaired cholesterol homeostasis manifesting significantly a higher LDL level but a normal HDL level, suggesting that GPER mainly regulates LDL metabolism [29]. And, human individuals with a hypofunctional GPER P16L allele are associated with elevated plasma LDL. In vitro study shows that activation of GPER by the agonist upregulates hepatic LDLr expression [30]. The role of GPER signaling in cholesterol or metabolic control remains unclear and needs more further investigations [31]. In summary, estrogens protect against increases in the plasma cholesterol level mainly by activating ER-α and GPER.

3.3. Androgens

The human androgens include dehydroepiandrosterone, androstenedione, testosterone and dihydrotestosterone (DHT). Testosterone can be converted to DHT via 5α-reductase. Testosterones and DHT are active androgens, because they are the only androgens capable of binding to androgen receptors (ARs) to exert biological functions. AR is mainly expressed in the prostate, skeletal muscle, liver and central nervous system (CNS). Like ERs, AR is a member of the steroid and nuclear receptor superfamily. Ligand binding induces a conformation change of AR, leading to recruitment of cofactor proteins and transcriptional machinery and subsequent regulation of the target genes' transcription.

The effect of androgen on cholesterol is still not conclusive. Clinical studies show that androgen deficiency, such as in old men, is associated with increased risks of dyslipidemia, higher serum cholesterol and LDL levels [32]. Another study has found that AR antagonists might be useful in the treatment of obesity in men [33]. In the animal studies, dihydrotestosterone (DHT) treatment in castrated obese mice decreases LDL secretion and increases the expression of hepatic scavenger receptor class B member 1 (SR-1B) which is important in regulating cholesterol uptake from HDL. It also decreases the enzyme cholesterol 7α-hydroxylase which participates in bile formation and cholesterol removal. In another study using an orchidectomized Sprague–Dawley (SD) rat model, DHT treatment causes decreased lipid accumulation and cholesterol synthesis by increasing expression of carnitine palmitoyl transferase 1 and phosphorylation of HMG-CoA reductase via an AR-mediated pathway [34]. However, this finding in animals contradicts a clinical study showing that a single dose of testosterone injection increases the total cholesterol level by 15% through stimulating the hepatic expression of HMG-CoA reductase [35]. These contradictory results indicate a complex role of androgen on the cholesterol homeostasis in the liver.

4. Growth hormone

4.1. Growth hormone and growth hormone receptors

Growth hormone (GH) is secreted by the somatotroph cells of the anterior pituitary gland under neural, hormonal and metabolic control. GH regulates postnatal growth, as well as lipid, glucose and energy metabolism. The molecular mechanism of GH action is relatively complicated. It affects metabolism through direct or indirect action via insulin-like growth factor-1 (IGF-1) or antagonism of insulin action. GH receptor (GHR) is a member of the cytokine receptor superfamily. Upon binding to GH, GHR activates the cytoplasmic tyrosine kinase Janus kinase 2 (Jak2) and then recruits members of the signal transducer and activator of transcription (STAT) family of transcription factors. Phosphorylated STATs translocate into the nucleus and modulate the transcription of multiple target genes, including IGF-1, ALS and suppressor of cytokine signaling (SOCS) [36]. In addition to the Jak2/STAT signaling pathway, GHR can activate the Src tyrosine kinase signaling pathway and cross talk with insulin and IGF-1 signaling pathways.

4.2. Role of GH in cholesterol and lipid metabolism

There exists a negative relationship between obesity and GH. Enormous evidence supports that GH alters lipid metabolism. Clinical studies have shown a significant association between lower serum GH levels and non-alcoholic fatty liver disease (NAFLD). Hypopituitary patients with GH deficiency are more prone to NAFLD than control subjects [37–39]. GH supplementation has been shown to improve the NAFLD and the metabolic dysfunction [40, 41]. In rodent studies, high-fat diet feeding and obesity suppress pulsatile GH secretion [42]. In turn, chronic GH treatment ameliorates hepatic lipid peroxidation and improves lipid metabolism in high-fat diet-fed rats [43].

Hypophysectomy is a surgery process in which the pituitary gland (hypophysis) is removed, leading to an impairment of GH secretion. This model is used for investigating the GH function in animals under pathophysiology conditions. Increase of hepatic LDLr and hypocholesterolemia induced by estrogens is completely attenuated in hypophysectomized rats. Only GH supplementation is able to restore this effect of hypophysectomy. Further, GH treatment on the gallstone patients stimulates the expression of hepatic LDLr by twofold, leading to subsequent decrease in serum cholesterol by 25%. This study indicates that GH secretion is critical for the control of plasma LDL levels in humans [44]. GH is also important for the synthesis of bile acids by maintaining the normal activity of cholesterol 7α-hydroxylase. Hypophysectomized rats show significantly reduced activities of HMG-CoA reductase and cholesterol 7α-hydroxylase and hence an inhibition of cholesterol and bile acid biosynthesis. GH substitution restores the enzymatic activity of 7α-hydroxylase and increases the fecal excretion of bile acids [45]. Treatment of LDLr-deficient mice with GH reduces their elevated plasma cholesterol and triglyceride levels by stimulating the activities of HMG-CoA reductase and cholesterol 7α-hydroxylase [46]. GH thus regulates plasma lipoprotein levels and bile acid metabolism by altering hepatic LDLr expression and the enzymatic activity of cholesterol 7α-hydroxylase, respectively.

GHR is present in the liver and critical for the hepatic lipid metabolism. Laron dwarfism is a disorder characterized by an insensitivity to GH due to a genetic mutation of GHR. These male patients manifest NAFLD in adults [47]. Liver-specific deletion of GHR in mice leads to increased circulating free fatty acids and fatty liver as a result of increased synthesis and decreased efflux of triglyceride [48]. Binding of GH to GHR activates JAK2-STAT5 signaling pathway and modulates a number of target genes. Among these, altered expression of CD36, PPARγ and PGC1α/β, along with fatty acid synthase, lipoprotein lipase and very-low-density lipoprotein receptor (VLDLr) contributes to the hepatic lipid metabolism process [49, 50]. All these findings suggest that hepatic GH signaling is essential for the regulation of intrahepatic lipid and cholesterol metabolism.

5. Glucagon

Glucagon is a 29-aa peptide hormone secreted from the pancreatic islet alpha cells in response to low glucose. It is a well-known counter-regulatory hormone to insulin, mainly stimulating hepatic glucose production by increasing glycogenolysis and gluconeogenesis and concurrently inhibiting glycogen synthesis. Glucagon also affects hepatic cholesterol metabolism. The relationship between glucagon and cholesterol has been investigated since the 1950s [51]. The portacaval shunt surgery in a 6-year-old girl with the homozygous form of familial hypercholesterolemia disorder has been reported to significantly reduce LDL and cholesterol synthesis 5 months after surgery. This alteration is associated with a marked elevation of bile acids and the glucagon level, indicating that glucagon may improve hepatic lipid metabolism [52]. In the animal study, infusion of glucagon into the hyperlipidemic rat reduces circulating VLDL apoprotein and serum TG levels. It is due to the inhibition of incorporating amino acid into the apoprotein by glucagon [53]. Chronic glucagon administration in rats significantly reduces serum cholesterol and triglyceride levels but not in the liver. The internal secretion of cholesterol and cholesterol transformation into bile acids measured by an isotope balance method are strikingly increased, suggesting that glucagon stimulates cholesterol turnover rate [54]. Studies by Rudling et al. have found that injection of glucagon increases LDL binding to the LDLr in a dose-dependent manner and concomitantly decreases cholesterol and apoB/E in LDL and large HDL particles in rats. Moreover, the induction of LDLr by glucagon is not due to increased mRNA levels, indicating a novel posttranscriptional regulatory mechanism present in the liver [55]. In humans, glucagon administration represses cholesterol 7α-hydroxylase (CYP7A1) mRNA expression by increasing the PKA phosphorylation of HNF4a and reducing its ability to bind with the CYP7A1 gene, thus inhibiting bile acid synthesis [56].

Glucagon receptor, encoded by the GCGR gene, is a seven-transmembrane protein and belongs to the class II guanine nucleotide-binding protein (G protein)-coupled receptor superfamily. They are abundantly expressed in the liver and kidney. In the liver, glucagon receptors are mainly located in hepatocytes, with a small number expressed on the surface of Kupffer cells [57]. Mice with a null mutation of the glucagon receptor (*Gcgr*−/−) display low blood glucose and markedly elevated the plasma LDL level. Serum total cholesterol and HDL are not significantly changed in *Gcgr*−/− mice [58]. *Gcgr*−/− mice are more prone to develop

hepatosteatosis following high-fat diet feeding [59]. Several glucagon receptor antagonists (GRA) have been developed to reduce hepatic glucose overproduction and improve the overall glycemic status. However, some GRAs including MK-0893 have been shown to dose-dependently increase LDL in T2DM patients. In the rodent preclinical trial, blockade of glucagon receptor using various GRAs elevates plasma LDL-c and total cholesterol. This is caused by increased cholesterol absorption instead of the change in cholesterol synthesis or secretion [60]. Taken together, these results suggest that glucagon plays a hypolipidemic effect through its glucagon receptors, making it an interesting and attractive pharmaceutical agent for the treatment of dyslipidemia and obesity.

6. Irisin

Irisin is a newly identified hormone encoded by the gene fibronectin type III domain-containing protein 5 (FNDC5). It is secreted into the circulation as a cleaved protein product and induced by exercise [61]. Irisin is proposed to mediate the metabolic benefits of exercising by promoting the browning of subcutaneous adipose tissue, reducing visceral obesity and improving glucose and cholesterol metabolism. Circulating the irisin level is negatively associated with fat mass, fasting glucose and dyslipidemia, as well as intrahepatic TG contents in humans [62, 63]. A higher baseline irisin level is associated with the metabolic benefits of diet-restricted treatment on human weight loss [64]. Lentivirus-mediated FNDC5 overexpression or subcutaneous perfusion of irisin promotes lipolysis and reduces hyperlipidemia in obese mice [65]. Irisin is negatively associated with HDL cholesterol and large HDL particles in adults with higher cardiovascular risk [66]. In addition, the serum irisin level is significantly higher in the NAFLD patients than in normal subjects [67]. Elevation of saliva irisin is positively related to total cholesterol [68]. Subcutaneous infusion of irisin decreases body weight, plasma total, VLDL, LDL, HDL cholesterol in diet-induced obese mice. The hepatic levels of total and esterified cholesterol are also reduced. These alterations are associated with significant reduction in the expression of the genes important for cholesterol synthesis, including *Srebp2*, HMG-CoA reductase (*Hmgcr*), the liver X receptor α (*Lxr*α, *Nr1h3*) and HMG CoA synthase (*Hmgcs*) in the liver and primary hepatocytes. Further experiments demonstrate that irisin inhibits cholesterol synthesis in hepatocytes through the activation of AMPK and SREBP2 [69]. As a novel hormone, evidence supporting the critical role of irisin in the regulation of cholesterol or lipid metabolism is still limited. More studies are needed to clarify the role of FNDC5/irisin in the lipid homeostasis under physiological and pathological conditions.

7. Conclusion

Cholesterol balance is regulated at multiple steps, including the biosynthesis, uptake, intracellular transport and conversion to bile acids for excretion. Hormones affect cholesterol biosynthesis and uptake by altering the transcription of genes critical for these biological processes (**Table 1**). Novel identified hormones are constantly added into the list implicated

	Origin	Biosynthesis	Uptake	Secretion	Conversion to bile acid
Thyroid hormone	Thyroid	↑	↑		↑
Sex hormone					
Estrogen	Ovary	↑		↑	
Androgen	Testis		↑	↑	↑
Growth hormone	Pituitary	↑	↑		↑
Glucagon	Islet α cells			↑	↓
Irisin	Skeletal muscle	↓			

Table 1. Effect of hormones on cholesterol metabolism.

in cholesterol balance process. Identification of hormonal receptor agonist/antagonist and understanding the hormonal regulatory mechanisms would help to identify potential effective and selective targets for the control of cholesterol dysfunction.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (81500619), Natural Science Foundation of Guangdong Province (2016A030310040), Shenzhen Science and Technology Project (JCYJ20160422091658982, JCYJ20150324140036854), Shenzhen Peacock Plan (KQTD20140630100746562,827–000107) and Natural Science Foundation of SZU (201567).

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Chapter 3

Role of Membrane Cholesterol in Modulating Actin Architecture and Cellular Contractility

Barbara Hissa and Bruno Pontes

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76532

Abstract

Atherosclerosis is a chronic inflammatory process that initiates with accumulation of apolipoprotein B containing lipoproteins (LPs) in the subendothelium (intima), especially in areas where the laminar flow is disturbed. LP retention triggers an inflammatory response leading to activation of endothelial and vascular smooth muscle cells that culminates with recruitment of leukocytes. Atherosclerosis is the leading cause of vascular disease worldwide being its major clinical manifestations ischemic heart disease, ischemic stroke, and peripheral arterial disease. Even though a lot has been done to unravel the role of turbulent flow and mechanotransduction for atherosclerosis development, little is known about the role of plasma membrane (PM) cholesterol in this process. This chapter is going to be focused on exploring what has been done so far to decipher the role of PM cholesterol in regulating actin architecture, cellular mechanical properties, and cellular contractility in muscle and nonmuscle cells.

Keywords: cholesterol, actin, myosin, cytoskeleton, contractility

1. Introduction

The role that cholesterol plays in cardiovascular diseases is widely known and studied [1]. However, less appreciated is the importance that cholesterol has in orchestrating other important cellular functions, such as cellular contractility and cytoskeleton organization. Cellular contractility is the ability of a cell to exert mechanical work on a substrate or on a neighboring cell due to actomyosin cytoskeleton enzymatic activity [2]. In muscle and nonmuscle cells, most of the cholesterol content is localized at the plasma membrane [3] where it can partition into microdomains called lipid rafts. Lipid rafts are highly dynamic regions of the plasma membrane

that contain sphingolipids and cholesterol and are responsible for compartmentalizing and regulating several intracellular signaling events [4–6]. One way of studying the importance of cholesterol for a specific cellular function is to decrease its concentration by either interfering directly with its synthesis, through the mevalonate pathway, or by chelating the molecule directly through the use of cyclodextrins [7]. However, depending on how one does the cholesterol depletion, the effects on cellular contractility can be opposite. Differences in muscle versus nonmuscle cell contractile behavior are observed upon cholesterol depletion using cyclodextrins. Muscle cells get impairment in their contractile machinery [8] whereas nonmuscle cells get more contractile [9]. This book chapter gives an overview about how cholesterol is organized at the plasma membrane and how its depletion changes cellular contractile properties.

2. Lipid rafts and membrane heterogeneity

Even though the Cell Theory started to be developed in the nineteenth century [10], it was not until the first quarter of the twentieth century that the idea of a membrane encompassing the cell was experimentally observed. In 1924, the Dutch physiologists Gorter and Grendel elegantly demonstrated, for the first time, the existence of a lipid bilayer surrounding red blood cells of various animals [11]. They isolated erythrocytes from humans and different mammals (rabbit, dog, guinea pig, sheep, and goat), extracted their lipids using acetone, and let those lipids spread on an air-water interface of a Langmuir-Adam apparatus. By knowing the number and area of the erythrocytes used in their experiment, they concluded that those cells were surrounded by a layer of lipids whose thickness was equivalent to two molecules [11], hence a lipid bilayer. The Gorter and Grendel model for cellular plasma membrane considered only the lipid nature of this cellular component and, because of that and due to other experimental and theoretical inaccuracies, it failed in explaining satisfactorily experimental results for membrane thickness [12], membrane tension [13], membrane electrical capacitance [14], and membrane permeability [15].

In order to explain those membrane properties, another model, called the paucimolecular model, was proposed by Danielli and coworkers in 1935 [12, 16, 17]. By examining the surface tension of a single drop of mackerel egg oil, Danielle and Harvey found that the value they measured was lower than the equivalent obtained for nonliving pure water-oil systems. They hypothesized that the difference observed for surface tension in living versus nonliving water-oil systems was due to the fact that the plasma membrane not only contained lipids but also proteins adsorbed in the lipid bilayer [16]. In the same year, Danielli and Davson [17] extended the paucimolecular model in order to explain permeability experiments. In that model, the layer of proteins adsorbed on top of a lipid film was able to discern size of molecules and charge of ions that were penetrating the membrane. This lipid film containing adsorbed proteins was considered to be relatively stable with mosaics consisting of practically impenetrable regions and hydrated areas where anions could move through [17].

For the next 30 years, the paucimolecular model was the most accepted one among the scientific community. However, with the advancement of microscopy techniques and structural studies, a new and more robust model, named the fluid mosaic model, was proposed in 1972 by Singer and Nicolson [18]. According to that model, integral transmembrane proteins are

http://dx.doi.org/10.5772/intechopen.76532

arranged in the plasma membrane of living cells such that the polar regions are facing the aqueous phase and the hydrophobic regions are embedded on a viscous phospholipid bilayer and those proteins are able to move freely on that two-dimensional, approximately homogeneous fluid "sea" of phospholipids [18]. One year later, in 1973, Bretscher published a Science paper in which he discusses overall membrane organization based on evidences collected from experiments performed in red blood cells [19]. According to that paper, the plasma membrane of mammalian cells was not as simple as depicted by the fluid mosaic model. Some of the integral proteins span the membrane and their glycosylation is responsible for locking them at the membrane impeding their migration to the cytoplasm. Another important contribution from this paper is that proteins not only interact with the outer layer of the plasma membrane but also with the inner layer, and membrane proteins are a subtype of cytoplasmic proteins that are not secreted [19]. In the same year, Yu and collaborators, also performing experiments in red blood cells, showed that when those cells are incubated with the nonionic detergent Triton X-100, there are some fractions of the cellular proteins that are resistant to the detergent extraction and seem to form oligomeric complexes with some of the lipid components, which were preferentially composed by nonglycosylated proteins and sphingolipids [20].

2.1. Lipid rafts

The idea of possible membrane microdomains started to be speculated in the early 1970s [20, 21] and experimentally demonstrated in 1982 by Karnovsky [22], who showed that there were multiple phases in the lipid environment of a membrane. One type of microdomain can be formed by cholesterol and sphingolipids [23]. These microdomains were already shown to be present in cell membranes [24]. In 1988, after several experimental demonstrations, Simons and van Meer called these microdomains as lipid rafts [25]. Thus, lipid rafts are defined as small, heterogeneous, and highly dynamic microdomains enriched in cholesterol, glycosphingolipids, and proteins that are much more organized than the surrounding lipid bilayer [26]. These membrane microdomains serve as organizing clusters capable of influencing several cellular processes such as membrane trafficking and neurotransmission [26].

The most striking difference between lipid rafts and the plasma membrane from which they are derived from is the lipid composition. Experiments have shown that rafts contain much more cholesterol than the surrounding bilayer [27, 28]. Cholesterol, therefore, works as a sort of "dynamic glue" that maintains the raft together [29], serving as a molecular spacer and filling the empty spaces between sphingolipids [30]. One of the main challenges when studying lipid rafts in living cells is their size. They are small microdomains ranging from 10 to 200 nm, below the classical diffraction limit of the optical microscope [28]. The first studies in the field considered methods to extract and separate rafts from the surrounding membrane. The procedure would take advantage of lipid raft resistance to nonionic detergents. When detergents are added to cells, the fluid membrane will dissolve while the lipid rafts may remain intact and could be extracted [31]; however, the validity of this methodology has been called into question due to ambiguities in the lipids and proteins obtained after extraction [32]. Other methods, based on synthetic membranes, were also used, however with many drawbacks. Firstly, synthetic membranes either lack or have lower protein concentration when compared to cell membranes [26]. Secondly, it is very difficult to simulate, in synthetic membranes, the membrane-cytoskeletal interactions that occur in cell membranes, although

some recent studies have been able to overcome these limitations [33–35]. Finally, another problem includes the lack of natural asymmetry between the bilayer leaflets [36].

Although lipid rafts present sizes below the classical diffraction limit of the optical microscope, fluorescence microscopy has been extensively used in the field. For example, fluorophores conjugated to cholera-toxin B-subunit, which binds to the raft constituent ganglioside GM1, is used. Also, lipophilic membrane dyes (such as Laudran) that either partition between rafts and the surrounding membrane or change their fluorescent properties in response to membrane phase are used. Finally, lipid rafts can also be fluorescently labeled in cells after genetic expression of fluorescent fusion proteins [35].

Another methodology, which has been widely used in the study of lipid rafts, is the manipulation of cholesterol contents in membranes. Sequestration (using filipin, nystatin, or amphotericin), depletion and removal (using methyl-β-cyclodextrin, MβCD), or inhibition of cholesterol synthesis (using 3-hydroxy-3-methyl-glutaryl-coenzyme A, HMG-CoA, reductase inhibitors) are great examples of how cholesterol can be manipulated in lipid raft studies [26]. Several questions, however, have been raised against the effectiveness of the experimental design when disrupting lipid rafts. Acute methods of cholesterol depletion, which disrupt the rafts, can also disrupt another lipid, called PI(4,5)P2, which plays an important role in cytoskeletal regulation [37]. Thus, the loss of a particular cellular function after cholesterol depletion cannot necessarily be attributed only to raft disruption, since other processes are also being affected.

Despite these limitations, more sophisticated methods have been applied in order to fight against the problems of small size and dynamic nature of lipid rafts. These methods include single particle and molecule tracking using very sensitive CCD cameras together with total internal reflection microscopy. These combined techniques provide information of the diffusion coefficient of particles in the membrane and also reveal membrane corrals, barriers, and sites of confinement [38]. Finally, other optical techniques have been used to elucidate other features of lipid rafts: fluorescence correlation spectroscopy, to gain information of fluorophore mobility in the membrane [39]; fluorescence resonance energy transfer, to detect when fluorophores are in close proximity [40], and optical tweezers, to give information about the membrane mechanical parameters [8, 41]. In the future, it is expected that other super-resolution microscopy techniques, such as stimulated emission depletion microscopy [42] or various forms of structured illumination microscopy may overcome the problems imposed by the diffraction limit.

Apart from the different imaging methods, research over the last decades have demonstrated the existence of two types of rafts: (1) planar lipid rafts (also known as noncaveolar or glycolipid rafts) and caveolae. Planar rafts are known to be continuous with the plane of the plasma membrane (not invaginated) and contain flotillin proteins. Caveolae are flask shaped invaginations of the plasma membrane that contain caveolin proteins. Both types are enriched in cholesterol and sphingolipids. Flotillin and caveolins can either recruit or separate other molecules from lipid rafts and caveolae, respectively, thus playing an essential role in signal transduction [43].

2.2. Caveolae

Caveolae are plasma membrane invaginations with a diameter ranging from 60 to 80 nm and were first identified in the early 1950s by electron microscopy [44]. These invaginations are

expressed in various cell types such as smooth muscle, fibroblasts, endothelial cells and adipocytes, among several others. Their functions are diverse and include endocytosis, calcium signaling as well as regulation of various cell signaling pathways [45].

The major constituent of caveolae is caveolin1 [46], followed by two other isoforms: caveolin2 [47] and the muscle-specific caveolin3 [48]. All three caveolin proteins share a common topology with both their N and C terminal domains in the cytoplasm and a long hairpin transmembrane domain. All three types of caveolin are formed inside the cells, more precisely in the Golgi apparatus, as monomers [49]. However, as soon as they enter in the secretory pathway, they start to be structured as oligomers [50]. For caveolin1, for example, its exit from the Golgi apparatus is accelerated upon addition of cholesterol [49]. The oligomerization ability of caveolin1 is crucial for caveolae formation [51]. Caveolin2 has also been implicated in caveolae formation [52], and although caveolin1 null mouse shows a significant decrease in caveolae assembly, they are still present in the caveolin2 mouse [53]. In muscle, caveolin3 is crucial for caveolae formation. Mutations or loss of caveolin3 result in dystrophic phenotypes [54, 55].

Caveolin expression at the plasma membrane is not the only inducer of caveolae formation. Cholesterol extraction has been extensively shown to disrupt caveolae at the plasma membrane [46] since it is required for caveolin incorporation into raft domains at the plasma membrane, a critical event for caveolae formation [56].

Although caveolins and cholesterol were initially thought to be necessary and sufficient for caveolae formation, several studies have shown additional molecular players called cavins. This protein family has four different members already described: cavin-1 (also called PTRF) [57], cavin-2 (also called SDPR) [58], cavin-3 (also called SRBC) [59], and the muscle-specific cavin-4 (also called MURC) [60]. These four proteins are essential to caveolae formation and functions. Thus, caveolae formation is a highly complex and regulated cellular process. It has been estimated that ~150–200 caveolin monomers are necessary to associate with ~50–60 cavins in order to form a single caveola [61, 62]. Moreover, caveolae architecture was recently proposed to be a dodecahedron formed by cavins aligned with their vertices and also in the caveolin oligomers located at each of the pentagonal faces [61, 62].

As already mentioned, caveolae represent a subdomain of lipid rafts [43]. Confocal microscopy has shown that the distribution of GM1, a well characterized raft marker, do not merge with caveolin1 [63]. Another raft marker, flotillin, defines noncaveolar rafts and merges with GM1 [63]. Thus, rafts exhibit a heterogeneous distribution over the plasma membrane changing between caveolar (invaginated) and noncaveolar (planar) regions.

3. Actomyosin cytoskeleton: the contractile machinery of muscle and nonmuscle cells

The cytoskeleton constitutes a dynamic network of filaments that exists in the inner space of a cell. This network not only provides scaffolding but is also responsible for transporting organelles, generating and transducting mechanical forces. The cytoskeleton maintains cellular organization by linking together several cellular components in such a way that it

mediates communication across the entire cell and, therefore, has a tremendous impact on cellular functions [64]. Three main filaments constitute the cytoskeleton, each one with its distinct protein composition and function: the microtubules, intermediate filaments, and microfilaments.

Microfilaments, also known as actin filaments, are ~7 nm in width. They are primarily composed of actin, the most abundant protein in cells. Actin filaments can create a huge number of arrays, such as bundles, two-dimensional networks, and three-dimensional gels. These different structural organizations are controlled by several actin-binding proteins and are found, for example, at the leading edge of a moving cell, particularly in filopodia and lamellipodia (**Figure 1B**), which causes the actin filaments to be the primary cytoskeletal component to drive cell motility [64]. Actin filaments also allow the cell to probe or sense its microenvironment. More stable networks of actin filaments, known as stress fibers, allow cells to brace against the underlying surface [65]. Thus, microfilaments can either be alone, as simple filaments, or together with the myosin filaments, which are part of the actomyosin contractile apparatus, in muscle and nonmuscle cells. Myosin filaments, associated with actin filaments, use ATP hydrolysis to exert forces against stress fibers during cytoskeletal contractility [65].

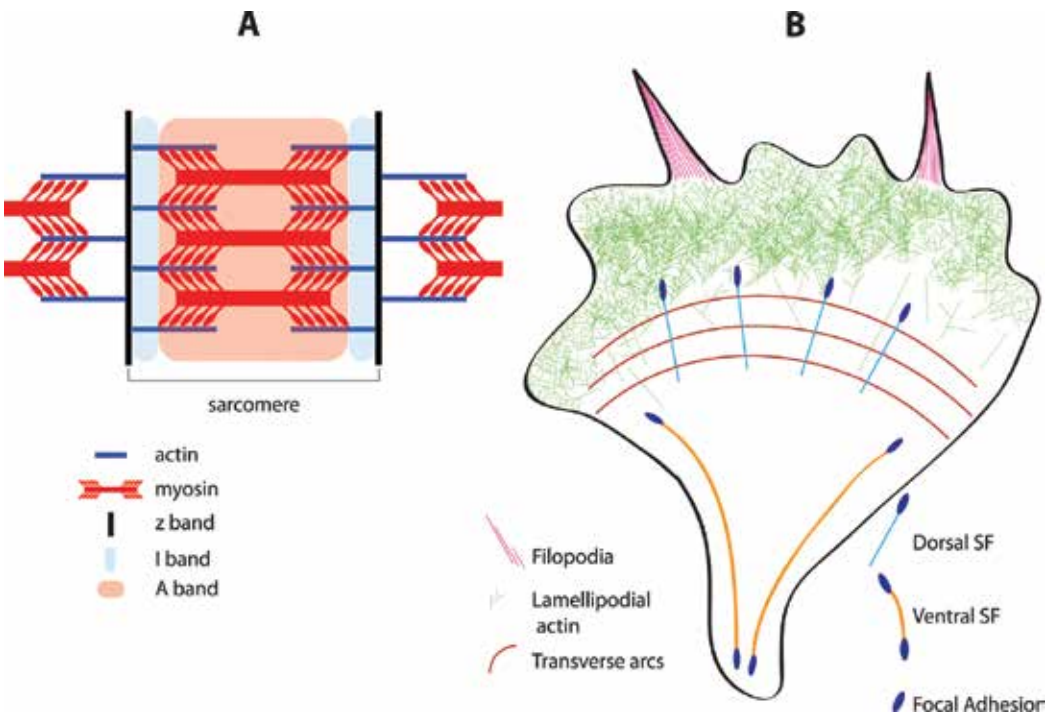

Figure 1. Actomyosin cytoskeleton schematic of striated muscle cell (A) and smooth muscle/nonmuscle cells. Striated muscle cells have the sarcomeric organization, which is shown in A and consists of actin and myosin filaments overlapping. Smooth muscle and nonmuscle cells (B) have different populations of actin stress-fibers that can be found in distinct parts of the cell. There are stress fibers that form filopodia (pink), lamellipodia (green), contractile transverse arcs (red), dorsal stress fibers (light-blue) and ventral stress fibers (orange) that terminate into one or two focal adhesions (navy-blue).

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The actomyosin contractile machinery is relatively well conserved, despite some differences in organization and dynamics among different cell types. Actin filaments are polarized as barbed ends (fast-growing end) and pointed ends (slow-growing ends) and serve as scaffold for myosin filaments, which vary in size ranging from a few heads, in nonmuscle cells, to hundreds of heads in thick filaments of striated muscle cells. The myosin filaments drive the translocation of actin filaments toward their barbed ends. This event can trigger either the contraction or extension of two actin filaments [64].

The most well understood example of a contractile actomyosin apparatus is found in striated muscles and is called sarcomere. The sarcomeres are what give the striated muscles their appearance. It is known that a sarcomere is defined as a segment between two neighboring Z-lines. The Z-lines (from the German "Zwischenscheibe") are "dark" lines that appear in both extremities of a sarcomere (**Figure 1A**). They act as anchoring points for actin filaments. Surrounding two Z-lines, there are two regions called I-bands, regions of actin filaments that are not superimposed by myosin filaments (**Figure 1A**). Between the two I-bands is an A-band, which contains the entire length of myosin filaments and part of actin filaments that extend from I-bands (**Figure 1A**). The barbed ends of actin filaments are localized at the Z-line. The myosin filaments are segregated toward the pointed ends of actin filaments. Several other proteins are present and allow the stability of a sarcomere. The interaction between actin and myosin filaments in the A-band of a sarcomere is responsible for muscle contraction (**Figure 1A**) [64].

The contraction starts when a motor neuron releases acetylcholine, a neurotransmitter that binds to a postsynaptic nicotinic acetylcholine receptor on the muscle fiber, causing a change in receptor conformation and allowing an influx of sodium ions followed by postsynaptic action potential initiation. The action potential travels through T-tubules until it reaches the sarcoplasmic reticulum, where it activates voltage-gated L-type calcium channels. The initial inward flow of calcium from the L-type calcium channels activates ryanodine receptors, which releases a huge amount of calcium ions from the sarcoplasmic reticulum toward the cytoplasm of muscle cells. This mechanism is called calcium-induced calcium-release [64]. Inside muscle cells, the protein tropomyosin covers the myosin binding sites of actin filaments in the sarcomere. In order to allow contraction, tropomyosin must be moved from its original place. Initially, tropomyosin is attached to the actin filaments, covering myosin binding sites. When calcium ions enter in the muscle cell cytoplasm, they immediately bind to troponin-C and trigger a change in the structure of tropomyosin. This change in conformation forces tropomyosin to reveal the myosin-binding sites on actin filaments and allows myosin filaments to pull antiparallel actin filaments together. Muscle contraction ends when calcium ions are pumped back from the muscle cell cytoplasm into the sarcoplasmic reticulum, allowing the contractile machinery to relax [64].

The actomyosin cytoskeleton in nonmuscle and smooth muscle cells is organized in similar ways, both different from sarcomeres of striated muscles. Nonmuscle and smooth muscle cells use myosin to generate contractility during migration, cytokinesis, as well as cell-cell and cell-matrix junctions, for example [66]. Nonaligned actomyosin networks, with actin filaments and clusters of bipolar myosin filaments interacting with each other at their ends, represent the simplest contractile machinery in nonmuscle and smooth muscle cells, especially

in cytoskeletal regions that do not have stress fibers [67, 68]. Smooth muscle and nonmuscle cells also contain more organized actomyosin bundles, such as transverse arcs, radial stress fibers, peripheral bundles, and ventral stress fibers (**Figure 1B**) [65]. Transverse stress fibers or arcs (**Figure 1B**) are formed after reorganization of lamellipodial actin filaments [69] during lamellipodium retraction [70]. This process is driven by myosin filaments, which become co-aligned with actin filaments and form stacks separated by alfa-actinin [68]. Radial stress fibers (**Figure 1B**), on the other hand, are anchored to focal adhesions in one end. Myosin molecules are recruited to the tips of focal adhesions, where nascent radial stress fibers start to form [71]. Contractility of the radial-transverse-stress fiber network leads to the formation of ventral stress fibers (**Figure 1B**) attached to focal adhesions on both ends [72]. Also, actomyosin filaments from nonmuscle and smooth muscle cells are highly dynamic when compared to striated muscle cells. Both actin and myosin can frequently undergo turnover or cycles of assembly/disassembly [73, 74].

Based on all above-described features, the most striking differences between stress fibers and sarcomeres are: (1) the molecular composition is cell-specific [75], (2) stress fiber contraction is regulated by phosphorylation of myosin light chain (pMLC) (see pMLC regulation in Section 4), while sarcomere contraction is regulated by troponin switching [76], (3) stress fibers are approximately one order of magnitude thinner, less organized (with different directions and lengths) and with less coordinated contraction when compared to sarcomeres [75], (4) the magnitude of the force they produce is different, while the stress fiber contractile forces come from individual cells and are applied, through focal adhesions, to the extracellular environment in which these cells are located [77], the sarcomeres from striated muscles can transmit contractile forces over macroscopic lengths. Finally, (5) striated muscles can rapidly contract and relax based on action potentials and Ca^{2+} release [64, 78] while stress fibers from smooth muscle and nonmuscle cells respond much slower and do not depend solely on electrical pulses [78].

While striated muscle cells present different actomyosin organization and features when compared to nonmuscle and smooth muscle cells, these three cell types share a common actomyosin structure: the actin cortex, also known as cell cortex or actomyosin cortex. This is a thin and highly disordered contractile actomyosin network underling the plasma membrane of cells [79]. It was first discovered in large cells, like amoeba and animal eggs and subsequently, extrapolated to all animal cells [79]. Non-adherent cells [80], cells during mitosis [81], or cells performing amoeboid-like migration [82] present a well distributed and uniform actin cortex. Cells spread over flat surfaces, although more difficult to be observed, also present a cortical layer of actomyosin, as shown by electron microscopy [83]. The actomyosin appears as an isotropic network parallel (and some perpendicular) to the plasma membrane with a width of 20–250 nm [83, 84]. Numerous actin-binding proteins have already been described to be part of the actin cortex [84], most of them are classical actin-binding proteins; however, little is known about how actin cortex is assembled.

The actin cortex plays a major role in cell mechanics as the main determinant of cell surface tension [79]. Biophysical methods like micropipette aspiration and membrane tether pulling assays (using either optical tweezers or atomic force microscopy) have been used to measure cell surface mechanics [85]. Micropipette aspiration is a suitable technique to measure

the overall cellular tension, which is a combination of the tension in the plasma membrane together with the tension in the underlying actin cytoskeleton [86]. Moreover, membrane tether pulling assays also bring information about the membrane itself and its attachment with the cortical cytoskeleton [86, 87]. During bleb formation, for example, a momentary separation between the plasma membrane and the actin cortex occurs [88].

Biophysical methods show that the membrane-cortex attachment is the major determinant in cell surface tension [89, 90] and that different cells have different surface tension values, indicating that there may have different mechanisms to maintain surface tension homeostasis among cells [90]. Modifications of specific cross-linking proteins, whose function is to link the plasma membrane to the actin cortex, can induce changes in cell surface tension [91–94]. Also, actin filament disruption or myosin inhibition can reduce cell surface tension [90, 95–97]. Changes in membrane composition, particularly in cholesterol content, have also been shown to influence cell surface tension. MβCD causes an increase in tension in embryonic kidney cells [98], fibroblasts [9] and cardiomyocytes [99]. This increase is not only due to changes in membrane composition, but it also affects the actomyosin cytoskeleton. In fibroblasts, MβCD treatment shows an increase in stress fiber formation [9] whereas cardiomyocytes show sarcomeric disorganization together with contraction abnormalities [99].

4. Cellular contractility in nonmuscle cells: the role of Rho and pleiotropic effects of statins

In order to divide, migrate, and undergo tissue morphogenesis, cells change shape and exert forces either on the substrate that they are attached to or on the neighboring cells. Nonmuscle cells generate contractile stresses via molecular motors, such as myosin, that are able to convert chemical energy into mechanical work [2]. Myosin activity is controlled through phosphorylation of its light chain via myosin light chain kinase (MLCK) [100, 101] which, in turn, is activated by Rho kinase ROCK and the small GTPase Rho A upstream. In 1985, the Rho gene was first isolated, from abdominal ganglia of the Aplysia, and identified as a member of the Ras family [102]. In 1990, after injecting a constitutively active form of Rho (Vall4rho), Paterson and collaborators verified that active Rho is able to cause changes in cellular morphology inducing formation of stress fibers [103]. In 1992, Ridley and Hall showed that active RhoA induces formation of stress fibers and focal adhesions upon growth factor stimulation [104]. Being a GTPase, Rho can switch back and forth between its active state, when bound to GTP, and its inactive state, when bound to GDP. The switching process is finely regulated by guanine nucleotide exchange factors (GEFs), which promote activation, and GTPase-activating proteins (GAPs), which promote inactivation. There are approximately 60 GEFs and 70 GAPs that were already identified in the human genome [105]. In order to be activated, Rho goes through some posttranslational modifications that are essential to induce Rho migration toward the plasma membrane, where it gets activated. Prenylation is a post-translational modification that is known to be pivotal for Rho translocation to the membrane [106]. Protein prenylation is essentially an insertion of a prenyl group, which is a hydrophobic group, to the c-terminal of a protein. That way, the protein has a lipid anchor that allows

it to stay membrane bound. There are two types of prenylation: farnesylation and geranyl-geranylation, which are regulated by farnesyltransferase and geranylgeranyltransferase I, respectively [107]. In the case of RhoA, the protein gets geranyl-geranylated before it goes to the membrane to get activated and trigger downstream effectors [108, 109] (**Figure 2**).

Both isoprenoids, farnesyl pyrophosphate and geranylgeranyl pyrophosphate, are synthetized by the mevalonate pathway. Interestingly, cholesterol is the end product of this pathway [110] (**Figure 3**). Therefore, by manipulating the mevalonate pathway, one can perturb both cholesterol synthesis and prenylation of important target proteins such as RhoA, and, as a consequence, cellular contractility (**Figure 3**).

The rate-limiting step of the mevalonate pathway is regulated by the enzyme HMG-CoA reductase. The activity of HMG-CoA reductase is precisely governed by the amount of cholesterol available. There are basically two different sources of cholesterol in the body: the exogenous one (obtained through intestinal absorption of cholesterol from the diet) and the endogenous one (through the *de novo* synthesis via the mevalonate pathway), being the endogenous source down regulated when enough cholesterol is obtained from nutrition [111]. During the early 1970s, a lot of effort was put into identifying pharmacological candidates that were able to reduce the HMG-CoA reductase activity especially in patients with high LDL cholesterol. In 1984, on a National Institutes of Health (NIH) Consensus Conference for Coronary Primary Prevention Trial, it was demonstrated the importance of a balance diet and drug treatment in order to lower LDL-cholesterol to prevent coronary heart disease [112]. After 1987, statins, that are essentially very specific drug inhibitors of HMG-CoA reductase activity, started to be prescribed for patients with high cholesterol

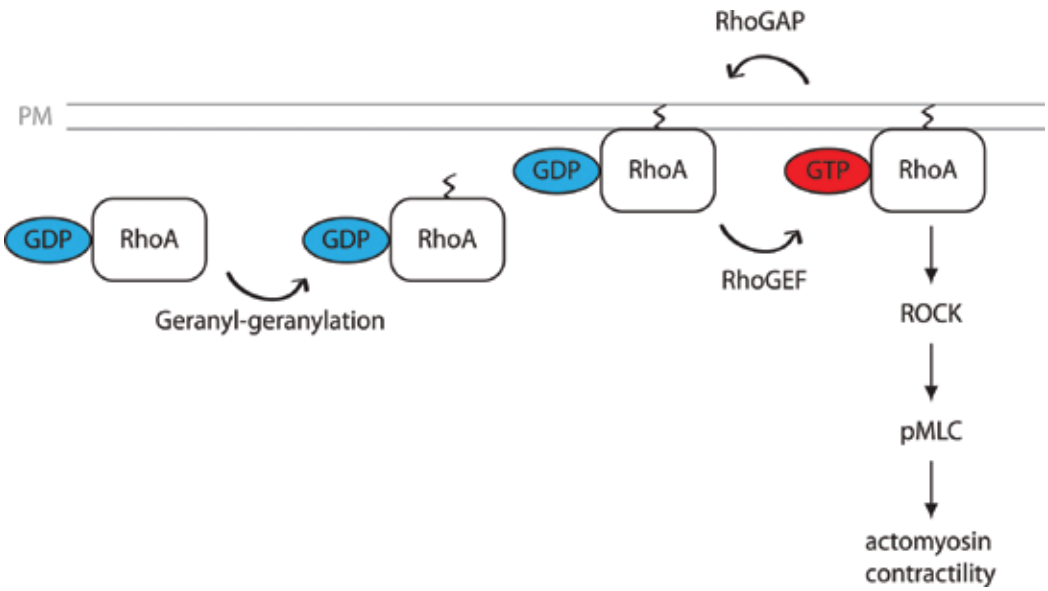

Figure 2. RhoA requires geranyl-geranylation in order to go to the membrane and be activated by RhoGEFs. Active RhoA triggers actomyosin contractility by inducing ROCK phosphorylation of myosin light chain (pMLC).

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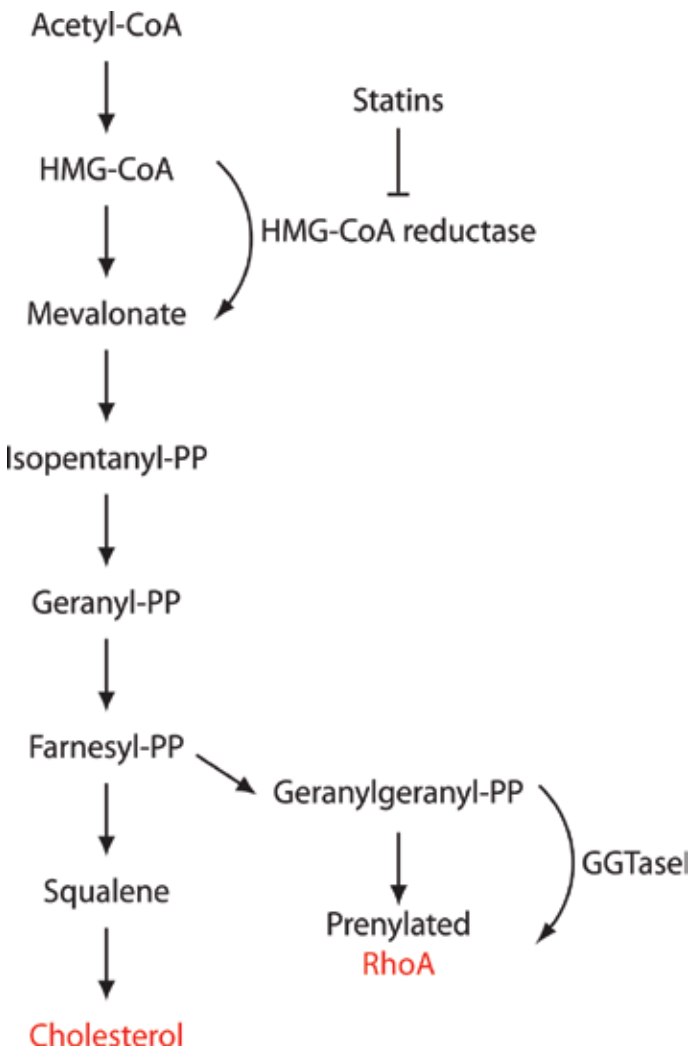

Figure 3. Simple schematic of the mevalonate pathway showing that posttranslational modifications pivotal for RhoA (red) activation and cholesterol (red) synthesis are part of the same intracellular pathway.

values [113]. By reducing the activity of HMG-CoA reductase, statins not only lower the amount of circulating cholesterol but also the amount of isoprenoid, both synthesized the mevalonate pathway, as one can see in **Figure 3**. Active RhoA as well as RhoA downstream effectors, such as ROCK, are inhibited upon statin treatment [114]. In endothelial cells, for example, a combination of flow and simvastatin exposure led to cell rounding and disorganization of the actin cytoskeleton [115]. In order to mimic atherosclerosis and aging effects on vessel walls, endothelial cells were plated in a series of substrates with low (physiological) and high stiffness values. High stiffness substrates increased both RhoA and ROCK activities. However, upon simvastatin incubation, contractility was abrogated in those cells [116].

Interestingly, when cholesterol is directly depleted by MβCD, an opposite trend is observed regarding nonmuscle cellular contractility. Human skin fibroblasts, after MβCD treatment, showed a reduction in the mobility of plasma membrane proteins being that reduction in motion a direct result of cytoskeleton reorganization [117]. It was also shown, for bovine aortic endothelial cells, that MβCD-dependent cholesterol depletion increased cortical stiffness [118]

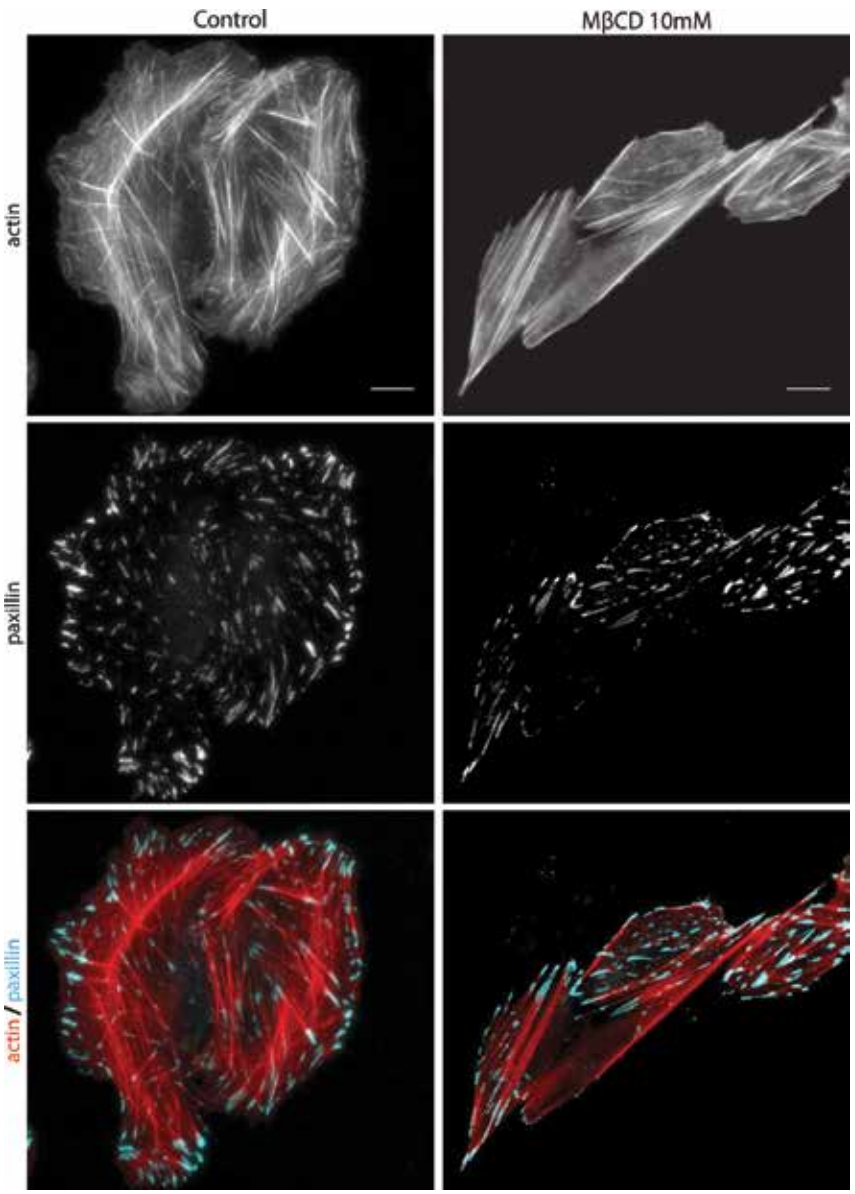

Figure 4. Fixed human osteosarcoma cell line U2OS, labeled for actin (red) paxillin (cyan), a focal adhesion protein. Notice the change in stress fibers between control and cholesterol depleted cells and how aligned the stress fibers get in the latter. Scale bar 10 μm.

as well as adhesion energy between membrane and cytoskeleton, which decreased the lipid diffusion coefficient [119]. Serum starvation followed by cyclodextrin-mediated cholesterol depletion increased stress fiber formation and RhoA activation in an osteoblast cell line [120]. Later on, those results were also corroborated by our group in a murine fibroblast cell line [41]. Similar features can also be observed in an osteosarcoma cell line U2OS (**Figure 4**) in which cholesterol depletion led to stress fiber formation and reorganization of actin cytoskeleton. More studies need to be performed in order to understand why different manipulations in cholesterol content trigger opposite results regarding cellular contractility.

5. Cellular contractility in muscle cells: interplay among Ca^{2+}, sarcomeres and cholesterol

Even though statins had been shown to be relatively safe and to promote health benefits to patients with high risks of cardiac diseases, there are some side effects and risks associated with statin therapy. Myotoxicity is one of the most adverse side effects, being the most common clinical outcomes: myosite, myalgia, and rhabdomyolysis [121]. In vitro studies performed on single muscle fibers isolated from rat skeletal muscle showed that fluvastatin and pravastatin led to contractility impairment and vacuolization of the muscle after 72 h of treatment and cell death after 120 h. Those changes in cellular morphology and contraction were proven to be dependent on geranyl-geranylation of GTPases since concomitant incubation of fluvastatin and geranylgeranyl pyrophosphate attenuated the deleterious effects of statins [122]. In vivo and in vitro treatment with simvastatin also led to contractile dysfunction, actin cytoskeleton disruption and apoptosis of smooth muscle cells [123].

Regarding the effects of direct cholesterol depletion mediated by MβCD on muscle cells, our group demonstrated, using primary cell culture of neonatal rat cardiomyocytes, that a lower cholesterol content increased the contraction rate of those cells and also led to defects in cell relaxation [8]. Moreover, cholesterol depletion increased the Ca^{2+} cytoplasmic concentration and Ca^{2+} sparks during contraction. This phenotype can be attributed to changes in caveolin3 and L-type Ca^{2+} channels distribution across the plasmalemma and hyperactivation of cAMP-dependent PKA activity. Cholesterol-depleted cardiomyocytes also present aberrant myofibrils due to calpain (a Ca^{2+} sensitive protease) activation. By using high-quality confocal microscopy and quantitative data analysis, this work has set in stone the role of cholesterol in regulating cardiomyocyte contractile behavior [8]. Other groups have also shown, for adult rat cardiomyocytes, that cholesterol depletion due to MβCD incubation changed localization of caveolin-3 from a raft to a nonraft membrane fraction changing MAPK signaling and increasing contractility and intracellular Ca^{2+} concentration [124]. Adult murine cardiomyocytes treated with MβCD also presented impairment in the T-tubule system and intercalated discs, which reinforces the role of cholesterol in regulating cardiac contractility [125]. More studies need to be performed in order to understand why MβCD-driven cholesterol depletion in nonmuscle cells increase contractile behavior whereas in muscle cells the same treatment tend to abrogate cellular contractility in several levels.

6. Conclusions

Cholesterol is a very important lipid that controls several cellular processes. This chapter describes how cholesterol is organized in cellular membranes and how it regulates and orchestrates the contractile machinery in muscle and nonmuscle cells. Cholesterol and RhoA protein prenylation share the same synthetic route: the mevalonate pathway. By lowering cholesterol concentration, using either chelating agents, such as MβCD, or inhibitors of HMG-CoA reductase, such as statins, one can observe opposite effects on actin cytoskeleton organization and contractile behavior. Cellular treatments with statins lead to a less-contractile profile, since this drug depletes the amount of prenylated RhoA, which, in turn, is the main upstream regulator of contractility in nonmuscle cells. On the other hand, MβCD-mediated cholesterol depletion induces RhoA activation, stress fiber formation, and increase in cortical stiffness pointing toward a more contractile behavior. In muscle cells, the results are even more intriguing: treatments with either statins or MβCD lead to myofibril disorganization, increase of contraction rate and defects in cell relaxation and in the ability of cells to handle intracellular Ca^{2+}. The reason why muscle and nonmuscle cells behave differently regarding cholesterol depletion is not completely understood and further investigation needs to be performed in order to elucidate this paradigm.

Acknowledgements

This work was supported by the Brazilian agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Rio de Janeiro (FAPERJ).

Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 4

Vascular Inflammation and Genetic Predisposition as Risk Factors for Cardiovascular Diseases

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76016

Abstract

Atherosclerosis previously defined as an obstructive disease leads to fatty deposits in the arterial wall. Nowadays, according to the best of our knowledge, specific cells, molecular mechanisms, and genes play crucial roles in the pathogenesis of the disease. Inflammatory reaction contributes to atherosclerotic lesion formation, since fatty streak leads to a plaque erosion or rupture. Experimental and clinical studies have shown that besides well-known risk factors, such as smoking, hypertension, diabetes, and dyslipidemia, genetic variations in certain locuses affect the disease burden. A common genetic variability at the apoE locus has been shown to be associated with a risk for cardiovascular disease. In many studies, a higher cardiovascular risk has been associated with the presence of the apo ε4 allele, whereas the apo ε2 allele has been protective. Recent studies stated that pro-inflammatory cytokines increase the binding of low-density lipoprotein (LDL) to endothelium and smooth muscle cells, so inflammatory response solely increases lipoprotein accumulation within the vessel wall. As a conclusion, cholesterol accumulation leads to atherosclerotic plaque via several mechanisms. Genetic predisposition and inflammatory process may affect disease severity.

Keywords: vascular inflammation, apoE, Lp-PLA2, LDL subtypes, endothelial dysfunction

1. Introduction

Cholesterol is one of the important molecules of the organism due to its strong relationship with cardiovascular disease. In clinical practice, the term of lipids is mainly used instead of lipoprotein metabolism because of its association with atherosclerosis. Certain lipoprotein fractions lead to the deposition and retention of cholesterol in the vessel wall causing atherosclerosis. Different guidelines recommend that lowering plasma cholesterol level by diet or drugs results in the reduction in cardiovascular disease.

Cholesterol is a hydrophobic macromolecule, transported in the circulation by lipoproteins such as chylomicrons, very low-density lipoprotein (VLDL), and high-density lipoprotein (HDL). Low-density lipoprotein (LDL) and intermediate-density lipoprotein (IDL) are synthesized from VLDL. Cholesterol, which is taken from the diet, is absorbed from the intestine and transported via chylomicrons to the liver. In the liver, endogen lipids with cholesterol are packaged into VLDL and secreted to bloodstream. LDL is responsible for peripheral cholesterol transport. Lipoproteins contain different proportions of lipids and proteins and have different physical and chemical properties. Apolipoproteins (apo) are the protein components of lipoproteins. Each lipoprotein class differs by its apoprotein content and proportions. ApoB100 is the main apoprotein of LDL and VLDL. ApoA-I and ApoA-II are the main apoproteins of HDL. ApoC-I, apoC-II, apoC-III, and apoE are present in all lipoproteins in different proportions. These apoproteins have various functions and help in the metabolic pathways of lipoprotein metabolism such as the activation of specific enzymes and the stabilization of the lipoprotein structure and recognized by cell surface of specific cells/ tissues. Among different types of apolipoproteins, ApoE has a crucial importance due to its link with cardiovascular diseases.

1.1. ApoE and its relationship with cholesterol metabolism

Apolipoprotein (apo) E is synthesized mainly by the liver, and less is produced by other cell types such as macrophages. It has a great biological and biomedical importance in lipid metabolism such as cholesterol transportation, triglyceride metabolism, and lipoprotein metabolism. It is a structural component of plasma chylomicrons, very low-density lipoproteins (VLDL) and high-density lipoproteins (HDL), and a ligand for apo B/E (LDL) receptor and LDL receptor-related protein (LRP) [1]. It also facilitates the interlocation of lipoproteins with proteoglycans. ApoE consists of 299 amino acid residue and has three common isoforms apoE2, apoE3, and apoE4 which differ structurally by two amino acid substitutions at residues 112 and 158: ApoE2 (Cys112, Cys158), ApoE3 (Cys112, Arg158), and ApoE4 (Arg112, Arg158) [2]. Each isoform is encoded by three different apoE alleles (ε2, ε3, and ε4), resulting in six different genotypes (E2/2, E3/2, E4/2, E3/3, E4/3, and E4/4) [3] (**Figure 1**).

ApoE consists of two functional domains joined by a flexible hinge region: an amino-terminal domain that contains a highly positively charged receptor-binding region composed mainly of arginine and lysine residues, and a carboxyl-terminal domain which includes a lipid-binding region. Substitutions of two amino acid residues in the three ApoE isoforms significantly alter their receptor-binding and lipid-binding affinities and lead to differences in lipid metabolism [4]. ApoE2 has a lower-binding affinity for low-density lipoprotein (LDL) receptors compared to ApoE3 and ApoE4. ApoE3 and ApoE4 bind similarly to the LDLR; however, compared to apoE3, apoE4 reduces plasma cholesterol less in humans which makes ApoE4 pro-atherogenic lipoprotein than others. Further, ApoE2 and ApoE3 preferentially bind to small, phospholipid-enriched high-density lipoproteins (HDL), whereas ApoE4 preferentially binds to larger, triglyceride-enriched lipoproteins [5, 6]. ApoE isoforms have different binding affinities to different lipoproteins such as ApoE4 that exhibits a better lipid-binding ability with the VLDL particle than apoE3, whereas apoE3 binds preferentially to high-density lipoprotein (HDL) [7].

http://dx.doi.org/10.5772/intechopen.76016

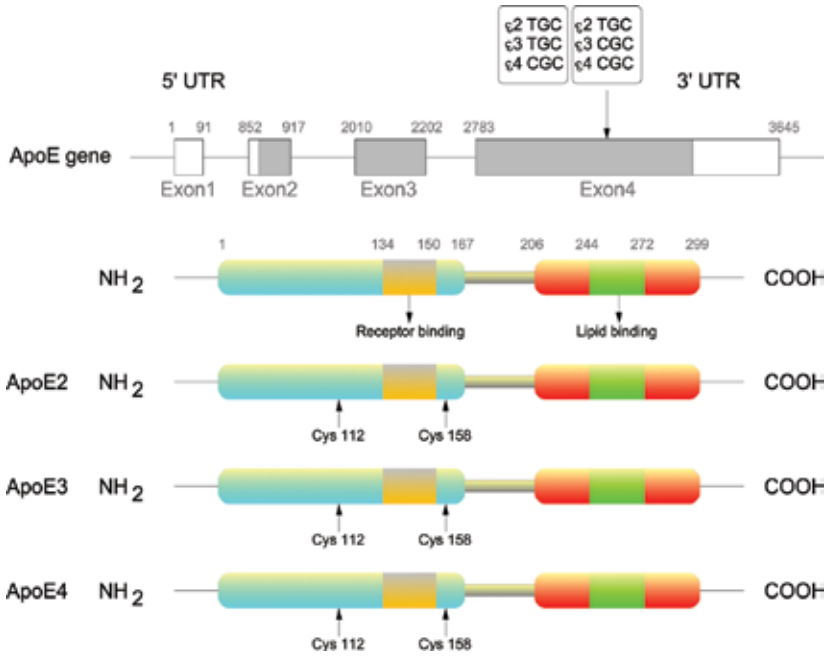

Figure 1. The schematic representation of the structural and functional domains of human apolipoprotein E (ApoE) isoforms.

The three-dimensional structure of apoE differs by Cys-Arg substitution, which causes changes in lipoprotein binding. The human apoE molecule contains the LDLR recognition site in the N-terminal helix bundle domain (residues 1–191) and initiates lipid binding by a C-terminal domain (residues 192–299). The substitution Cys and Arg residues, which differentiates apoE3 and apoE4 in the helix bundle domain, leads to different organizations of the segment spanning residues 261–272 which plays a critical role in the interaction with lipid surfaces; this structural change is the basis for the preferential binding of apoE4 to VLDL than of apoE3 [8–10]. The relative lipid- and lipoprotein-binding abilities of apoE3 and apoE4 have important consequences for the distribution of cholesterol between the VLDL and HDL fractions of plasma [11].

To date, several epidemiological studies have investigated the relationship between apoE polymorphism and coronary artery disease (CAD) risk, with a significant association [12–17]. It has been shown that apoE polymorphism may contribute to the plasma LDL cholesterol level and apolipoprotein B concentration in the various populations. The effects of apoE genotypes on coronary risk might also be explained as not only the receptor affinity or lipid levels but also apolipoprotein concentrations affecting the CAD phenotype. To the best of our knowledge, it has been shown that the ε2 allele is associated with lower levels of total plasma cholesterol (TC), LDL cholesterol (LDL-C), and apolipoprotein B (apo B) and with elevated levels of triglycerides (TG) as compared to the ε3 allele. An elevated level of TG is related with the impaired clearance of remnant particles containing apo ε2, which might be due to a defective receptor recognition of those particles. Reduced levels of apo B and LDL cholesterol in E2/2 and E2/3 individuals are results of impaired conversion of the intestinal VLDL particles to LDL by interfering with normal lipolytic processing [18, 19]. On the other hand, the ε4 allele

is associated with higher levels of total and LDL-C and apo B because particles with carriers of that allele have a faster catabolic rate than the ε3 counterparts [20]. Meta-analysis has been emphasized that ε2 carriers had a 20% reduced coronary risk as compared to ε3/ε3 genotype [21]. The precise mechanism may be explained by the binding affinity of apoE2 isoform to heparin and small, phospholipid-enriched HDL. These effective binding mechanisms enhance remnant lipoprotein metabolism and also reverse cholesterol transport [5–22]. Further, apo E2 isoforms bind to LDL receptors much more weakly than apo E3 or apo E4 counterparts. Apo E4 isoforms are also related with an increased cholesterol absorption and statin hyporesponsiveness. However, meta-analysis has been suggested that ApoE genetic testing contributes to little information for statin treatment [23].

Numerous effects of apoE genotypes on coronary risk might also be explained by influences on additional lipid-related phenotypes such as lipoprotein subtypes, markers of inflammation, immunity, or oxidative status.

Recently, the atherogenicity of LDL and HDL subclasses and their relationship to coronary heart disease has taken more attention. Various studies revealed that small, dense LDL is more atherogenic and associated with an increased risk of CHD [24, 25] with a high triglyceride level [26]. Researchers reported confusing results about apoE genotypes and LDL, HDL subtypes. Some studies stated a relationship between ε2 allele and smaller LDL particles compared to ε4 allele carriers [27, 28]; others reported contradictory results [29, 30]. As we know that the relationships between apo E polymorphism, serum lipids, and CHD were differed due to different ethnicities, lifestyles, diet habits, and even to age. Epidemiological studies showed that men have more atherogenic profile than women with a low level of HDL cholesterol and an increased triglyceride level. The incidence of first cardiovascular events is also higher among young men and is increasing very fast along with age, than in women [31]. The effect of apo E allelic variants on lipids and lipoprotein particle sizes has been studied in many populations [28, 30, 32, 33]. Several studies suggested that the apo E polymorphism influences lipoprotein particle size and might indirectly increase the CHD risk differently for each gender. Topic and his friends stated that apo ε carriers and its relationship with lipoprotein subtypes differed among sex; men with ε2 allele had the smaller LDL particles and a higher TG/HDL-C ratio [1]. Further, Dobiasova and Frohlich proposed that the Log (TG/HDL-C) be called "atherogenic index of plasma" and used as a marker of plasma atherogenicity because this ratio showed a strong inverse correlation with LDL size [34]. Some researchers have reported an increased LDL-C concentration and a decreased LDL size in subjects with ε4 allele [30]. On the women side, studies showed an association of the ε4 allele with smaller HDL particle and a higher frequency of small HDL phenotype and Framingham Risk Score ("intermediate"). Further, the presence of the ε4 alleles is found to be the independent factor for HDL size variation in women. Others reported different results with ε4 allele; carrier women had the small HDL particles which relates with the severity of CHD [27, 35, 36].

1.2. Endothelial dysfunction and cardiovascular diseases

Despite the strong evidences about lipoproteins and apolipoproteins, it has been shown that chronic oxidative stress and inflammatory changes in the vascular tissue play a crucial role in

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coronary atherosclerosis pathogenesis. Endothelium controls the normal vasomotor balance, the inhibition and stimulation of smooth muscle cell proliferation, migration, thrombogenesis, and fibrinolysis. When these functions of endothelium are impaired, endothelial dysfunction occurs and leads to damage of the wall. Damage to the endothelium promotes substantial events and provokes atherosclerosis by increasing endothelial permeability, platelet aggregation, and leukocyte adhesion.

The early lesion of atherosclerosis results in the focal accumulation of lipoproteins in the intimal layer of the artery. The intimal layer contains smooth muscle cell which is embedded in the extracellular matrix, so lipoprotein accumulation often associates with proteoglycans of the arterial extracellular matrix such as heparin sulfate, keratan sulfate, or chondroitin sulfate. These proteoglycan molecules increase the retention of lipoprotein particles in the arterial bed and permit their chemical modification. The extracellular bed of arterial wall is particularly susceptible to oxidative modification. The modification of lipids leads to hydroperoxides, lysophospholipids, oxysterol, and oxidized phospholipids formation which are other stimulators of arterial lesion progression. On the other hand, the apoprotein part of the lipoproteins may undergo similar chemical modification which performs irregular protein moieties that have a pro-inflammatory role during lesion development. The irregular changes of the arterial wall might also activate mononuclear phagocytes. These phagocytes as well as vascular endothelial and smooth muscle cells can produce reactive oxygen species when activated. Then, reactive oxygen species can induce smooth muscle cell growth and trigger inflammatory response.

The second step of the lesion formation is leukocyte recruitment which is the main cell of atheroma of the mononuclear lineage: monocytes and lymphocytes. The vascular endothelium synthesizes certain biomolecules for a regular vascular function. Various adhesion molecules or receptors are synthesized on the endothelial cell surface for leukocytes such as vascular adhesion molecule I (VCAM-I), intercellular adhesion molecule I (ICAM-I), and P-selectin. In normal arteries, the laminar shear stress suppresses the expression of adhesion molecules and stimulates adequate nitric oxide (NO) to maintain vasodilatation. Further, NO production can control adhesion molecules expression such as VCAM-I and show an anti-inflammatory effect. Reactive oxygen species may also react with NO, reduce NO bioavailability, and improve vascular damage indirectly by disrupting adhesion molecule express. In addition to relationship between adhesion molecules and NO, in vitro studies have shown that this relationship is attenuated by apoE [37, 38]. Recently, Ma et al. reported a reduced VCAM-1 and ICAM-1 gene expression in the whole aorta of hyperlipidemic mice at the sub-physiological levels of plasma apoE [39]. As a result of an increased adhesion of certain key molecules to endothelial cells, this leads to the penetration of monocytes and lymphocytes to the subendothelial layer. Besides modified lipoproteins, mediators of inflammation, cytokines can also regulate adhesion molecule expression and promote leukocyte recruitment. For example, VCAM-I and ICAM-I expressions on endothelial cells are stimulated by cytokine interleukin-I (IL-1) and tumor necrosis factor α (TNF-α). As mentioned earlier, the different pathways perform a cumulative effect on endothelial dysfunction and lesion formation. In the intima, the recruitment of mononuclear phagocytes differentiates to macrophages which promote the lipid-loaded foam cell formation. Foam cells have two different features: they either can go to apoptotic pathway or produce cytokines and growth factors which lead to further complicated cellular events

in complicated lesion. Growth factors (platelet growth factor, fibroblast growth factor, etc.) and cytokines again stimulate smooth muscle cell proliferation and induce extracellular matrix formation. Among numerous growth factors, some of them solely trigger interstitial collagen production by smooth muscle cells. These mediators induce lesion progression by inducing transformation of fatty streak into a more complicated lesion with a high fibrous tissue and extracellular matrix by using either paracrine or autocrine pathway. As well as the effect of traditional risk factors and local mediators of various cell types, coagulation and thrombosis also contribute to lesion progression.

Endothelium serves as a barrier between circulating blood and its surrounding tissue. A transmembrane receptor tissue factor (TF) is expressed by blood vessels. An injury of the vascular endothelium leads to exposure of TF and activates the clotting cascade. The imbalance of hemodynamics might also effect the normal activity of endothelial cells. Endothelial cells control platelet function via the synthesis and secretion of Von Willebrand factor (vWF). The vWF is an important glycoprotein in the regulation of hemostasis, stored also in platelets, and can be released as a response to prothrombotic and inflammatory factors. It is a bridge between platelet adhesion and coagulation. VWF contains functional domains for collagen, platelet (GPIb binding), and factor VIII binding. At the site of injury, VWF recognizes collagen in the subendothelial matrix, steer platelet adhesion via collagen, and GPIb-binding sites. Its level increases in plasma as a result of endothelial damage. vWF increases platelet adhesion to subendothelial layer and contributes to thrombus formation [40, 41].

The other anticoagulant functions of the endothelium are inhibition of coagulation by TFPI, keeping balance between thrombin- thrombomodulin secretion and regulation of coagulation factors by antitrypsin. On the other hand, the endothelium controls the activation and regulation of fibrinolysis by the secretion of tPA and PAIs. tPA is released in response to thrombin which is controlled by PAI-1. Various complex functions of endothelium are controlled strictly with high fidelity to maintain vascular health.

Based on the robust relationship between lipoprotein metabolism and basic endothelium functions, it has been recommended that different types of inflammatory reactions might be involved in the initiation and progression stages of atherosclerosis and contribute to vascular events.

Numerous epidemiologic studies reported that 20% of the coronary events occurred in the absence of the classical risk factors: hyperlipidemia, diabetes, hypertension, and smoking. In this situation, a question raises whether traditional risk factors are adequate to predict CVD risk. To solve this issue, new biomarkers are proposed for daily practice for better identification of the risk, including hemostatic system markers (the best identified ones, tissue plasminogen activator inhibitor—PAI-I, tissue plasminogen activator—tPA, fibrinogen, von Willebrand Factor—vWF, etc.) and inflammatory markers (high sensitive C reactive protein—HsCRP, lipoprotein-associated phospholipase II—Lp-PLA2, myeloperoxidase, pentraxin III—PTX3, serum amyloid A, etc.). Together with these, interleukins, inflammatory cytokines, adhesion molecules, homocysteine, and heat shock proteins can be used as appropriate. All these factors participate in the atherosclerotic process and show an abnormal behavior in individuals at high risk, or suffered from a cardiovascular event.

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1.3. Vascular inflammation as a risk factor for cardiovascular diseases

Among the wide range of markers, C-reactive protein (CRP) was recommended as a comprising marker for the evaluation of CVD risk in 2009 by the common declaration of the Laboratory Medicine Practice Guideline of National Academy of Clinical Biochemistry (NACB) and American Heart Association and the CDC (AHA/CDC) [42]. CRP is an acute-phase protein, which is primarily produced in the liver during acute inflammation or infection. CRP is stimulated by interleukin 6 (IL-6) and also detected at local sites of inflammation or injury. It is not specific for vascular inflammation. Then, more sensitive types of CRP have been developed to detect the small changes of the protein, called hs-CRP. Since hs-CRP has been increased, the predictive accuracy of risk evaluation with other risk-scoring systems is still inadequate. Hs-CRP levels are known to be systemic inflammatory marker and increased by infection and tissue damage, malignancies, obesity, aging, hypertension, diabetes mellitus, smoking, and other cardiovascular risks [43].

As described earlier, the increased expression of endothelial adhesion molecules, which trigger subendothelial penetration of LDL, is more susceptible to oxidation and stimulates inflammatory cytokines. Great attention has been recently given to myeloperoxidase (MPO), released systemically and locally by activated leukocytes. It has been shown that this enzyme is present in atherosclerotic lesions with higher concentrations and also contributes to LDL oxidation by different mechanisms via radical and non-radical mechanisms either lipid or apoprotein moieties [44]. Moreover, MPO limits the bioavailability of nitric oxide (•NO) and contributes to endothelial dysfunction [45, 46]. Unlike CRP, MPO is more involved in different stages of atherosclerosis such as foam cells, endothelial dysfunction and apoptosis, the activation of matrix metalloproteinases, and the expression of tissue factor which could address the patients with vulnerable plaques, and the potential burden of such plaques in clinical practice [47]. The members of the inflammatory cytokine family IL-6 and tumor necrosis factor (TNF)-α, released from the main cells of the plaque, vascular smooth muscle cells, endothelial cells, monocytes, and macrophages, are highly involved in atherosclerosis [48]. Ridker and colleagues reported from 14,916 healthy male; blood IL-6 concentrations were significantly elevated in individuals who had myocardial infarction as compared with those who did not [49]. Another prospective study reported a relationship between IL-6 levels and the incidences of ischemic cardiac disease, stroke, and heart failure events from middle-aged participants [50]. The other important player of the inflamed plaque is chemokines, leading to the recruitment of leukocytes to the damaged area of the arterial wall, as well as other systemic inflammatory markers, particularly monocyte chemoattractant protein 1 (MCP-1), which is again non-specific for interpreting CV risk [51].

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2) or platelet-activating factor acetylhydrolase is a unique pro-inflammatory biomarker, specific for vascular inflammation and atherosclerosis [52, 53]. Lp-PLA_2 was discovered in 1980, and it was classified as a Ca^{2+}-independent PLA_2 [54] produced by a wide range of inflammatory and non-inflammatory cells [55] (**Figure 2**).

Lp-PLA_2 shows a positive correlation with CV events by various scientific and clinical studies [56–58]. Lavi et al. found that patients with early coronary atherosclerosis had higher

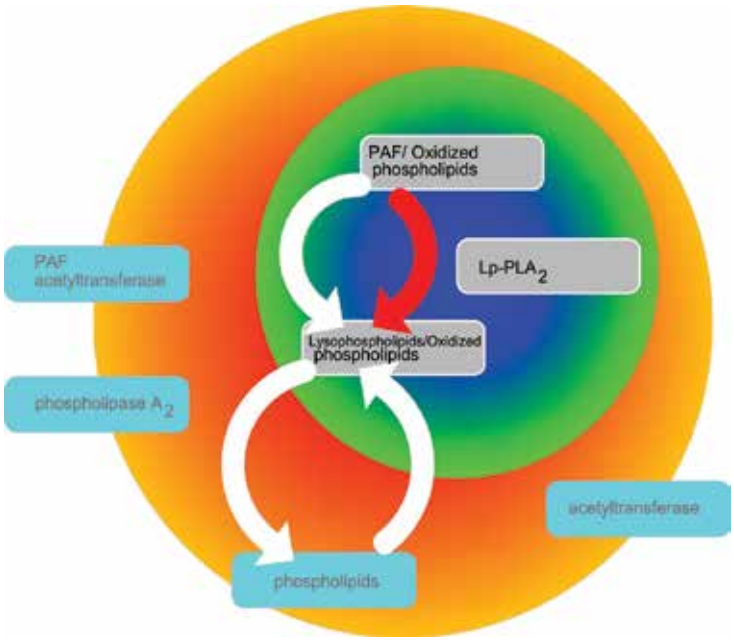

Figure 2. Relationship of Lp-PLA2 action with phospholipids. Platelet-activating factor (PAF) is an active phospholipid related to many pathologic and physiologic reactions. The PAF is formed through two reactions: firstly, the cytosolic phospholipase A2 (cPLA2) acts on membrane phospholipids producing lysophospholipids; then, the lysophospholipids are modified by PAF acetyltransferase, Thus, PAF concentration is modulated by Lp-PLA2 activity.

lysophosphatidylcholine when compared with control subjects [59]. Herrmann et al. showed that carotid artery plaques of patients with cardiac events presented higher Lp-PLA2, lysophospholipids, macrophage, and collagen content when compared to patients without events [60]. Kuniyasu et al. demonstrated that oxLDL and, particularly, the lysophosphatidylcholine present in this particle enhance the plasminogen activator inhibitor-1 expression [61].

Moreover, the difference in the distribution and association of Lp-PLA_2 activity and index with apoB containing lipoproteins across lipoprotein subfractions has also been reported [62]. Further, Lp-PLA_2 has been recommended as an adjunct to traditional risk factors for individuals at a moderate or a high CV risk by the Adult Treatment Panel III (ATP III) guideline [63].

Lp-PLA_2 is synthesized mainly by macrophages of atherosclerotic plaque, then enters the circulation, and binds to LDL, HDL, and Lp(a). In the atherosclerotic plaque, Lp-PLA_2 hydrolyzes oxLDL into Lyso-PC and oxidized nonesterified fatty acids (oxNEFAs), both of which have a pro-inflammatory role (**Figure 3**). The degradation products, Lyso-PC and oxNEFAs, hydrolyzed by Lp-PLA_2 play crucial roles on the development of atherosclerosis. Both Lyso-PC and oxNEFAs induce the recruiting of leukocytes, upregulating inflammatory cytokine such as TNF-a and IL-6, amplifying oxidation, and increasing matrix metalloproteinase expression. During the process, the presence of OxLDL, as well as lysophospholipids and oxNEFAS, stimulates the growth of the plaque [64, 65]. As mentioned earlier, Lp-PLA2 resides on different types of lipoproteins, so dyslipidemia effects the enzyme mass and activity and alters its

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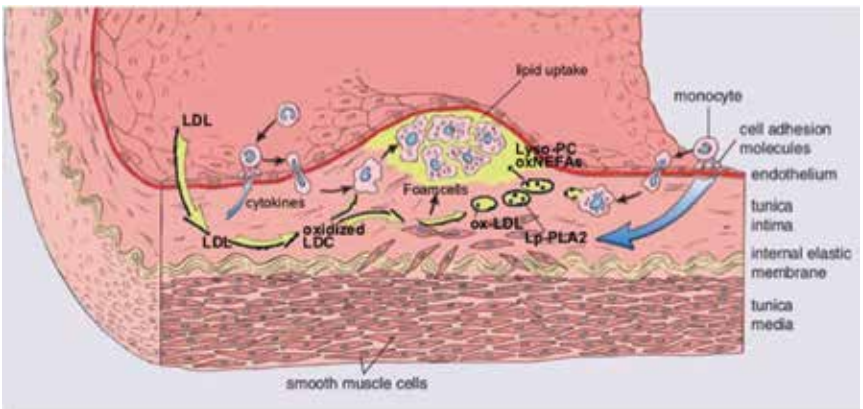

Figure 3. The role of Lp-PLA$_2$ on atherosclerotic plaque.

distribution between apo B- and apo AI-containing lipoproteins, as reported by Tsimihodimos et al. The same study also demonstrated inducing the increase of HDL-Lp-PLA2 activity and the reduction of LDL-Lp-PLA$_2$ activity by atorvastatin treatment [66].

Lp-PLA$_2$ quantitatively reflects the degree of inflammatory reaction of the plaque in the plasma. Lp-PLA$_2$ measurement can be classified into enzyme activity and enzyme mass; however, Berglund and colleagues recommended an integrated measure of Lp-PLA$_2$ activity and mass as Lp-PLA$_2$ index which showed an independent predictor of CAD in different ethnicity [58]. Since the first report, many epidemiological studies and meta-analysis have also proved the significant associations between Lp-PLA$_2$ atherogenesis and CV-risk stratification [52, 67, 68]. A meta-analysis of 32 clinical studies evaluated the association of Lp-PLA$_2$ mass or activity with the future risk of CHD, emphasizing strongly that Lp-PLA$_2$ is a reliable indicator for the future CV-risk assessment. The study also revealed that the association of the enzyme activity with lipid markers is stronger than the association with mass [69]. Further, Gungor et al. demonstrated an association between apoE genotype and Lp-PLA$_2$, for the first time. The Lp-PLA$_2$ index, an integrated measure of Lp-PLA$_2$ mass and activity, was higher in apo E4 carriers irrespective of ethnicity and underlines the importance of assessing the relationship between genetic predisposition and inflammation, in the assessment of cardiovascular disease risk [70]. The genetic variation of Lp-PLA$_2$ activity and mass and relationship with 13 common single nucleotide polymorphisms (SNPs) of the PLA2G7 gene was investigated in the community-based Framingham Heart Study. The study reported that Lp-PLA2 activity is influenced by variation in the genomic region of PLA2G7. Further, it has been underlined that different pathophysiological roles of Lp-PLA$_2$ activity and mass conveyed different clinical outcome. The strong association is seen for Lp-PLA$_2$ activity with cardiovascular risk factors compared to Lp-PLA$_2$ mass [71].

Pentraxin 3 (PTX3) is a protein from acute-phase reactant family. It belongs to long pentraxins and possesses numerous properties in the field of inflammation. Pentraxin 3 transcription is upregulated by tumor necrosis factor and interleukins (IL-1) in different cell types such as endothelial cells, phagocytes, smooth muscle cells, and fibroblasts which are involved in the different stages of atherosclerosis. PTX3 represents a specific and sensitive marker connecting inflammation with CVD.

During inflammation, the blood vessel produces large amounts of PTX3; its high level in circulation related with pathological conditions affects cardiovascular system. Recently, epidemiological and clinical data showed that PTX3 is a valid biomarker for atherosclerosis [72] and its high plasma levels were found to be related with the severity of coronary atherosclerosis [34]. It has been demonstrated that PTX3 increases the tissue factor (TF) expression in mononuclear and endothelial cells. The increased level of TF activates the coagulation cascade and causes the thrombus formation [73]. PTX3 might also bind to growth factor 2 (FGF2) and interfere with plaque stability via effect of the proliferation and migration of smooth muscle cells [74]. Based on such findings, PTX3 is more specific for coronary plaque instability than for atherosclerosis. In addition, the elevated plasma level of PTX3 has been found in patients with high systolic and diastolic blood pressure levels [75]. On the other hand, in patients with acute myocardial infarction, PTX3 was shown to be produced by the neutrophils penetrating into unstable coronary plaques. This underscored the fact that PTX3 might be more accurate predictor of cardiovascular events after myocardial infarction than other markers [76].

As conclusion, it is well known that atherosclerosis is a chronic inflammatory disease of the vessel wall. This chapter reviewed the relationship between atherosclerosis and the various effects of different inflammatory biomarkers and possible roles of genetic predisposition on the development and progression of coronary artery diseases (**Figure 4**). Atherosclerotic disease mostly starts

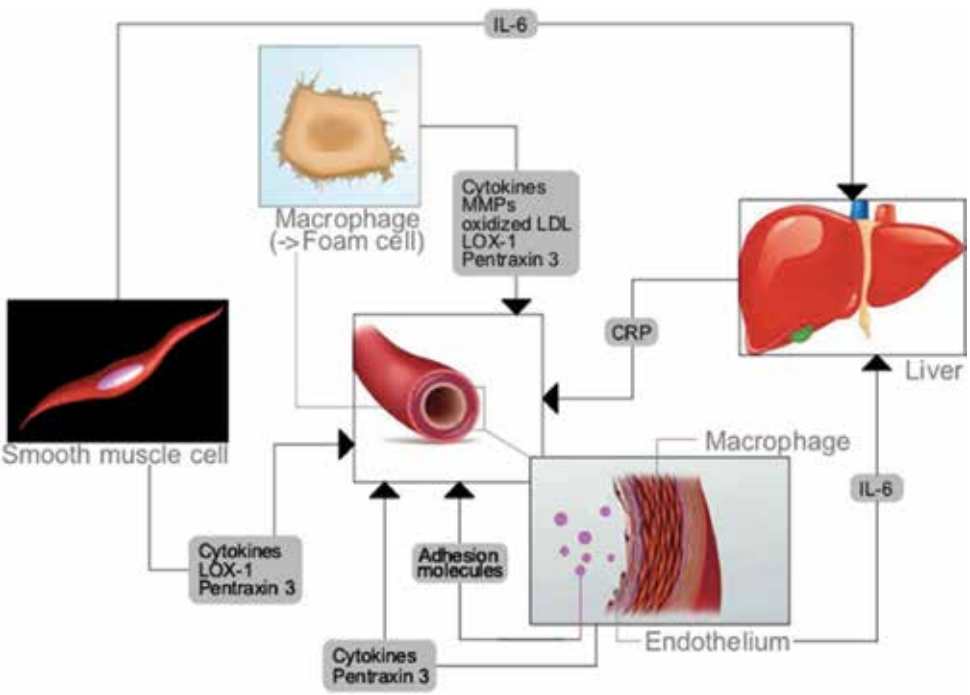

Figure 4. Inflammatory markers secreted from various cells in atherosclerotic lesion. Pro-inflammatory markers such as cytokines, pentraxin-3, MMPs, and LOX-1 are produced by macrophages, endothelial cells, and vascular smooth muscle cells in atherosclerotic lesion. CRP is mainly produced in the liver stimulated by IL-6. CRP indicates C-reactive protein; IL-6, interleukin-6; LDL, low-density lipoprotein; LOX-1, lectin-like oxidized LDL receptor-1; and MMP, matrix metalloproteinase.

as asymptomatic, however, when it gives symptoms; the life quality is effected significantly and sometimes it will be life threatening. In these circumstances, the early detection of the disease or prediction of the individuals with a high CV risk becomes very important. The evaluation of a CV risk or a disease progression by an accurate biomarker either vascular inflammation or genetic markers would be promising and underscores their diagnostic importance in clinical practice.

Acknowledgements

The author would like to thank Zeynep Erdem and Berna Kavas for reviewing the English of this chapter and Gizem Ozturk for drawing the figures.

Conflict of interest

The author declares that there is no conflict of interest.

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Chapter 5

Role of Pleural Fluid Cholesterol in Pleural Effusion

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76370

Abstract

Pleural effusion occurs when formation and accumulation of pleural fluid exceeds its absorption. It indicates an imbalance between pleural fluid formation and its removal. Pleural fluid accumulates in settings of increased hydrostatic pressure, increased vascular permeability, decreased oncotic pressure, increased intrapleural negative pressure and decreased lymphatic drainage. On the basis of pathophysiology, pleural effusion can be transudates or exudates. It is important to establish an accurate etiological diagnosis so that the patient may be treated in a rational manner. Using Light's criteria may need other extra investigations to differentiate transudates and exudates but using pleural fluid cholesterol (pCHOL) will help to diagnose them with only the pleural fluid analysis. Moreover the albumin or protein gradient will need serum as well as the pleural fluid investigations and will have more financial burden than just investigating pleural fluid cholesterol. Pleural cholesterol is thought to be derived from degenerating cells and vascular leakage from increased permeability. Thus pleural fluid cholesterol is one of the important investigations that can distinguish exudates from transudates. Routine use of pleural fluid cholesterol for classifying pleural effusion should be encouraged to improve the accuracy, sensitivity and specificity.

Keywords: pleural effusion (PE), pleural fluid cholesterol (pCHOL), transudates, exudates, pleural fluid lactate dehydrogenase (pLDH), serum lactate dehydrogenase (sLDH), congestive heart failure (CHF)

1. Introduction

Pleurae are the continuous membranes of the serous pleural sac that invest and enclose the lungs. They are called parietal and visceral pleura. The visceral pleura is also called the pulmonary pleura that closely covers the lung and is adherent to its surfaces. The parietal pleura adhere to the diaphragm, mediastinum and the wall of the thorax. It consists of costal

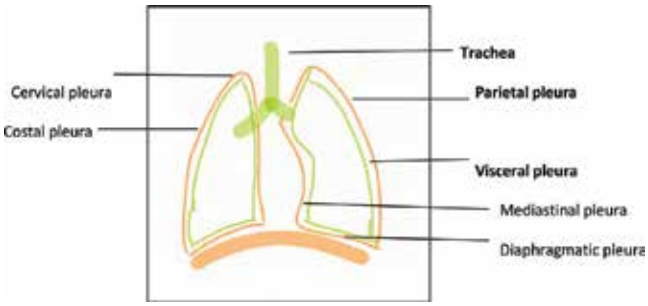

Figure 1. Schematic diagram of pleura and pleural cavity.

pleura, mediastinal pleura, diaphragmatic pleura and cervical pleura. The pleural space lies between the lung and the chest wall and is bounded by the parietal and visceral membranes. It contains a thin layer of fluid that serves as a coupling system called pleural fluid. A pleural effusion (PE) is present when there is an excess fluid in the pleural space. It indicates an imbalance between pleural fluid formation and its removal. It is important to establish an accurate etiological diagnosis so that the patient may be treated in a rational manner.

The pleural space is a real, not potential, space that is approximately 10–20 μm wide and extends completely around the lung to the hilar root [1, 2]. When air or fluid collects between the two layers, the pleural cavity expands. The schematic diagram for pleural cavity and pleurae is in **Figure 1**.

Pleural fluid is formed from the systemic vessels of the pleural membranes at an approximate rate of 0.6 ml/h and is absorbed at a similar rate by the parietal pleural lymphatic system. Normally, the pleural spaces contain approximately 0.25 ml/kg of low protein liquid. Disturbances in either formation or absorption result in the accumulation of excess pleural fluid [3].

The volume of pleural fluid is small, approximately 0.1–0.2 ml/kg in different studies. From parietal pleural capillaries, there is constant movement of fluid into the pleural space at a rate of 0.01 ml/kg bodyweight/h. There is a balance of the formation (entry) and absorption (exit) of the pleural fluid. The resultant homeostasis leaves 5–15 ml of fluid in the normal pleural space [4]. For pleural effusion to be there must be an increase in entry rate or a reduction in exit rate.

The parietal pleura has a hydrostatic pressure similar to that of the systemic circulation (30 cm H_2O), whereas that of the visceral pleura depends on the pulmonary circulation (10 cm H_2O). Oncotic pressure is similar in both (25 cm H_2O), but the pressure within the pleural cavity is affected by the gravity gradient. Thus, the pleural space is heterogeneous with a nondependent portion in which Starling forces favor outpouring of fluid into the cavity and the parenchymal capillaries [5].

2. Pathophysiology

The mechanism of pleural liquid formation is that the liquid originates from the systemic vessels of the pleural membranes, not from the pulmonary vessels [6]. It means that pleural

liquid is interstitial fluid of the systemic pleural microvessels. There are three major considerations that support this hypothesis [7]:

i. The systemic vessels (of both parietal and visceral pleural membranes) are adjacent to the pleural space and are much closer to the pleural space than are the pulmonary vessels.

ii. The low pleural liquid protein concentration (1 g/dl) and ratio to the plasma protein concentration (0.15 g/dl) are consistent with a filtrate from high-pressure systemic vessels. Large particles will be sieved and relatively restrained compared to the liquid if liquid and protein are filtered at high-pressure and high flow across a semi permeable membrane. Thus, plasma proteins, being large, will be retarded much more than the liquid in their movement across a membrane, and the protein concentration of the resultant filtrate will be low. On the other hand, if liquid and protein are filtered at low pressure and low flow, proteins are retarded less, and the protein concentration of the resultant filtrate is higher. Filtrates from low-pressure pulmonary vessels, e.g., lung lymph, have a high protein concentration (4.5 g/dl) and ratio (0.7) compared to filtrates from systemic vessels and to pleural liquid. Of note in this argument, pleural liquid formation is described as high flow, whereas its measured rate is relatively slow (0.01 ml/kg/h). However, it is the filtration at the systemic microvessels that is described as high as or at least higher than filtration across pulmonary microvessels. Some of that filtrate is reabsorbed into the low-pressure post capillary venules, and some is removed by bulk flow via the local lymphatic vessels. It is only the remainder that then moves into the low-pressure pleural space.

iii. In situations where systemic pressure varies, the pleural liquid protein concentration varies in concert. For example, systemic hypertensive rats have a lower pleural liquid protein-to-plasma protein concentration ratio than do normotensive rats (0.42 versus 0.55), even though their pulmonary pressures are the same [8]. During development from the fetus to the adult, systemic blood pressure generally rises and pulmonary pressure falls. In a study in sheep, the pleural protein ratio decreased with development, as would be expected if the pleural liquid originated from the high-pressure systemic vessels [9].

2.1. Increased fluid entry

Excess liquid filters out of microvessels based on a balance of hydrostatic and osmotic forces across a semi permeable membrane [1, 6]. These forces are well described in the Starling equation, in which the hydrostatic forces that filter water out of the vessel are balanced by osmotic forces that reabsorb water back into the vessel [10, 11].

$$\text{Flow} = k \times \left[(Pmv - Ppmv) - s\,(\pi mv - \pi pmv)\right]. \quad (1)$$

In this equation, k is liquid conductance of the microvascular barrier, Pmv and Ppmv represent hydrostatic pressure in the microvascular and perimicrovascular compartments, respectively, is the reflection coefficient for total protein and ranges from 0 (completely permeable) to 1 (completely impermeable), and πmv and πpmv represent protein osmotic pressure in microvascular and perimicrovascular liquids, respectively and s is Staverman's reflection coefficient. In normal micro vessels, there is ongoing filtration of a small amount of low protein liquid. The flow can increase with changes in various parameters of the Starling equation.

Increase in permeability: An increase in flow can be due to increases in either liquid conductance (an increase in k) or protein permeability (a decrease in reflection coefficient). If the endothelial barrier becomes more permeable to liquid and protein, for example, there will be an increase in flow of a higher protein liquid. Because absorption does not alter the protein concentration of pleural liquid, pleural liquid with a high protein concentration indicates its origin from a circulation across an area of increased permeability.

Increase in microvascular pressure: An elevation in venous outflow pressure induces the elevation of microvascular pressure (Pmv). Increases in arterial pressure are less likely to be transmitted to the microvessels because of the high precapillary resistance and autoregulation of arteriolar tone.

Elevations in either systemic venous pressure (affecting the parietal pleura) or pulmonary venous pressure (affecting the visceral pleura) can lead to an increase in pleural liquid formation and the development of a pleural effusion. As vascular permeability is unchanged in this setting, the increased flow is associated with a greater sieving of proteins, leading to a filtrate with a lower protein concentration than normal (with a pleural liquid-to-plasma protein ratio of less than 0.15). Of course, most effusions formed due to increased microvascular pressures, i.e., transudative effusions, have a pleural liquid-to-plasma protein ratio much higher than this, between 0.4 and 0.5. This fact demonstrates that most liquid must arise from a source other than the systemic circulation of the pleural membranes. The likely source is the large non-systemic circulation adjacent to the pleural space, namely the pulmonary circulation of the nearby lung. In the normal state, lung interstitial liquid, e.g., lymph, filtered from the low-pressure pulmonary circulation has a protein concentration ratio [12] (lung to plasma protein concentration ratio) of 0.7, but with increased flow due to increased pulmonary microvascular pressures, this ratio falls to 0.4–0.5. This lung interstitial oedema liquid then is the likely source of the majority of the hydrostatic pleural effusion [13].

The way lung liquid reach the pleural space is that when the rate of filtrate formation exceeds the absorptive capacity of the lung lymphatics, the filtrate accumulates in the peribronchovascular spaces (“cuffs”) [14]. Once in these interstitial spaces, the liquid is not accessible to lung lymphatics [15].

Thus, although the lymphatics are undeniably important in removing liquid as it is filtered from the pulmonary circulation, they cannot account for the clearance of already established oedema from the lung [16]. This interstitial oedema probably leaves the lung by flowing down pressure gradients along the interstitial spaces (interlobular septae, peribronchovascular bundles and visceral pleura) of the lung toward either the mediastinum or the pleural space. The entry of large amounts of lung interstitial liquid into the pleural space will elevate the overall protein concentration of the pleural liquid, giving a ratio of 0.40–0.50, the expected range for a transudative effusion [16].

Decrease in pleural pressure: A decrease in pleural pressure, as seen with significant atelectasis, may alter the balance of forces described in the Starling equation by reducing the pressures surrounding the nearby micro vessels. This decrease in perimicrovascular pressures

(Ppmv) can enhance filtration across the microvascular barrier of a low protein liquid (with a pleural liquid-to-plasma protein ratio of less than 0.15).

Decrease in plasma osmotic pressure: Hypoproteinemia (due to hypoalbuminemia) will decrease the plasma oncotic pressure (πmv), thereby increasing the forces favoring filtration until the balance is restored. By itself, hypoproteinemia can probably induce small effusions with a low protein concentration. In addition, hypoproteinemia can lower the threshold for effusion formation when other Starling forces are changed. In a study of hospitalized patients with AIDS, for example, hypoproteinemia alone was the apparent cause of 19% of all pleural effusions [17]. Together with other factors, a lower plasma protein concentration may have contributed to effusion formation in many more patients, because, in general, all patients with effusions had a lower plasma albumin concentration than those without effusion (2.5 versus 3.4 g/dl).

2.2. Decreased fluid exit

A decrease in exit rate reflects a reduction in lymphatic function. Because lymphatic function is poorly understood, much of this discussion is speculative. Unlike blood vessels, lymphatic vessels have one-way valves and propel lymph using both their own rhythmic contractions and the respiratory motions of the chest wall. In addition, flow is affected by lymphatic patency, availability of liquid, and the pressures influencing filling (pleural pressure) and emptying (systemic venous pressure) of lymphatics [18–20].

Intrinsic factors: A number of factors can interfere with or inhibit the ability of lymphatic's to contract, including:

- Cytokines and products of inflammation (e.g., endotoxins)
- Endocrine abnormalities (e.g., hypothyroidism)
- Injury due to radiation or drugs (e.g., chemotherapeutic agents)
- Infiltration of lymphatics by cancer
- Anatomic abnormalities (e.g., yellow nail syndrome)

Extrinsic factors: Multiple extrinsic factors can inhibit lymphatic function although the lymphatics themselves are normal. These include:

- Limitation of respiratory motion (e.g., diaphragm paralysis, lung collapse and pneumothorax)
- Extrinsic compression of lymphatics (e.g., pleural fibrosis and pleural granulomas)
- Blockage of lymphatic stomata (e.g., fibrin deposition on pleural surface and pleural malignancy)
- Decreased intrapleural pressure (e.g., trapped lung caused by a fibrous rind on the visceral pleura)

Normal pleural fluid resembles water in appearance and clarity, and is odorless [20]. Its chemical composition is summarized in **Table 1**.

Pleural effusion is present when there is excess accumulation of pleural fluid due to its exceeding formation on pleural fluid absorption. At normal circumstances, pleural fluid entering the pleural space from the capillaries in the parietal pleura is removed by the lymphatics which can absorb 20 times more fluid than is formed.

Fluid can enter the pleural space from the interstitial spaces in the visceral pleura or through the diaphragmatic pores from the peritoneal cavity. So pleural effusion will develop in two circumstances:

1. When there is excess formation of pleural fluid from parietal pleura, interstitial spaces from the lung and peritoneal cavity.
2. When there is inability of removal of pleural fluid by the lymphatics.

Local factors: There is change in the pleural surface permeability due to which the exudative pleural effusion occurs.

Systemic factors: There is increase in pulmonary capillary wedge pressure (PCWP) or decrease in oncotic pressure that result in alteration of formation and absorption of pleural fluid as in transudative pleural effusion.

Translocation of fluid: Small pores in diaphragm act as pathways for peritoneal fluid to enter into the pleural cavity as in hepatic hydrothorax. It may be massive even without marked ascites.

Parameters	Value
Volume	0.1-0.2ml/kg
Cells	1000-5000/mm3
Mesothelial cells	3- 70%
Monocytes	30-75%
lymphocytes	2-30%
Granulocytes	10%
Protein	1-2gm/dl
Albumin /protein	50-70%
Glucose	as in plasma
Lactate Dehydrogenase	< 50% of plasma

Table 1. Normal composition of pleural fluid.

The basis in which accumulation of pleural fluid occurs are: increased hydrostatic pressure, increased vascular permeability, decreased oncotic pressure, increased intrapleural negative pressure and decreased lymphatic drainage.

Pleural effusion may be of two types depending upon the underlying pathology, i.e., transudative and exudative. The causes of transudative and exudative pleural effusion are summarized in **Tables 2** and **3**, respectively.

Transudate will be clear fluid with low protein while exudates will have cloudy fluid with high protein. Exudates have a ratio of protein in pleural fluid and serum >0.5; ratio of LDH in pleural fluid and serum >0.6 and pleural fluid LDH > 2/3rd of upper limit of serum LDH. Protein in transudate is less than 2.5 g/dl while exudates have higher values [21].

Transudative pleural effusion is usually due to the increased hydrostatic pressure that is caused by congestion in the capillaries, e.g., in heart failure and there is formation of pleural fluid from the increased venous pressure of the pleural membranes. However in case of exudates, there is vascular leakage of fluid due to increased permeability as a result of inflammation.

1.	Increased hydrostatic pressure	Congestive Heart Failure
		Superior vena cava syndrome
		Pericardial effusion
		Constrictive cardiomyopathy
		Massive pulmonary embolism
2.	Decreased capillary Oncotic pressure	Cirrhosis of Liver
		Nephrotic syndrome
		Malnutrition
		Protein losing enteropathy
		Small Bowel disease
3.	Transmission from Peritoneum	Any cause of ascites
		Peritoneal Dialysis
4.	Increased capillary permeability	Small pulmonary emboli
		Myxoedema
5.	Miscellaneous	Urinothorax
		Acute atelectasis
		Wet Beriberi
		Idiopathic

Table 2. Transudative pleural effusion.

1. Respiratory causes	Parapneumonic effusion
	Tuberculosis
	sarcoidosis
	Parasitic infections
	Pulmonary embolism
	Trapped lung
2. Gastrointestinal causes	Pancreatitis
	Postoperative
	Intrabdominal abscesses
	Posttransplant of liver
	Esophageal perforation
	Endoscopic variceal sclerotherapy
3. Cardiac causes	Post Myocardial Infarction
	Constrictive pericarditis
	PostPericardiotomy
4. Occupational	Asbestosis
5. Traumatic	Hemothorax
6. Post surgical	Coronary artery bypass surgery
7. Autoimmune causes	Systemic lupus erythematosus
	Rhematoid pleurisy
	Drug induced lupus
	Sjogren syndrome
	Wegener's granulomatosis
	Chrug strauss Syndrome
8. Endocrine causes	Hypothyroidism
	Ovarian hyperstimulation syndrome
9. Renal related	Uremia
	Peritoneal dialysis
10. Malignancies and complications	Mesothelioma
	Metastases
	Superior vena caval obstruction
11. Drug induced	Bromocriptine,Dantrolene, Nitrofurantoin, Amiodarone,etc
12. Lymphatic cause	Chylothorax

13. MISCELLANEOUS	Amyloidosis
	Iatrogenic injury
	Radiation therapy
	Yellow nail syndrome

Table 3. Exudative pleural effusion.

3. Clinical features

The clinical features of pleural effusion depend on the amount, the rate of accumulation of fluid and the underlying cause. In acute cases, the symptoms appear suddenly. Patients may present with shortness of breath, pleuritic pain, cough and constitutional symptoms. Dyspnea may result from compression of lung tissue and from mechanical alterations in the respiratory muscles as the fluid changes their length-tension relationship. There will be associated symptoms related to the etiology of the pleural effusion. So careful elicitation of history in cases of pleural effusion may streamline the physician toward the etiological aspect of pleural effusion.

Physical examination reveals decreased respiratory movements on the affected side and displacement of mediastinum to the opposite side. If there is an associated collapse of lung or fibrosis, the trachea may be central or may even be pulled to the same side depending on the degree of collapse or fibrosis. Tactile fremitus may be decreased to absent but may also be increased toward the top of large effusion. Percussion reveals dull to flat note over the fluid.

Auscultation reveals decreased to absent breath sounds but bronchial breath sounds may be heard near top of large effusion. Pleural rub can also be heard and sometimes crackles above the level of effusion. Frequently, there are E to A changes (egobronchophony) at the upper fluid border where underlying lung parenchyma is compressed.

4. Diagnostic clues for exudates from transudates

Light et al. in 1972 found a criteria to have sensitivity and specificity of 99% and 98%, respectively, for differentiating transudative and exudative PE (ratio of protein in pleural fluid and serum >0.5; ratio of LDH in pleural fluid and serum >0.6 and pleural fluid LDH > 2/3rd of upper limit of serum LDH) [21]. But the other investigators could only reproduce specificities of 70–86% using light's criteria. Also it is found that 25% of patients with transudates pleural effusion are mistakenly identified as having exudative effusion by Light's criteria.

Most transudates have absolute total protein concentrations below 3.0 g/dl (30 g/l), although acute diuresis in heart failure can elevate protein levels into the exudative range [22–24].

If one or more of the exudative criteria are met and the patient is clinically thought to have a condition producing a transudative effusion, the difference between the protein levels in

the serum and the pleural fluid should be measured. If this gradient is >31 g/l (3.1 g/dl), the exudative categorization by these criteria can be ignored because almost all such patients have a transudative pleural effusion [25]. About only 75% of cases, the etiology of pleural effusion can be established with the clinical presentation, biochemical parameters and fluid cytology. Despite extensive diagnostic work up in about 20% of pleural effusion, the etiology remains unknown [26].

From meta-analysis, Heffner et al. has identified pleural effusion of exudative type with at least one of the following condition [27]:

- Pleural fluid protein >2.9g/dl
- Pleural fluid cholesterol >45 mg/dl (1.16 mmol/l)
- Pleural fluid LDH > 2/3rd of upper limit of serum

Roth et al. [28] found that despite the high sensitivity of Light's criteria (100%), these criteria had a low specificity (72%). Using an albumin gradient of 1.2 g/dl or less to indicate exudates and greater than 1.2 g/dl to indicate transudates, 57 of the 59 patients (41 exudates; 18 transudates) were correctly classified. Two patients with malignant effusions were misclassified as having transudates.

In 2003 National medical journal of India, one article published by Guleria R of AIIMS, New Delhi [29] found that for exudative pleural effusion, pleural fluid cholesterol ≥60 mg/dl has 92% accuracy, 88% sensitivity and 100% specificity; however, Light's criteria was 98% sensitive and 80% specific.

Evaluation through pleural fluid cholesterol only can avoid the financial burden and double pricks (serum and pleural fluid) in anxious patients to go through the series of tests to confirm the exudative pleural effusion.

In a study done in Nepal by Hamal et al. [30], pleural fluid cholesterol (pCHOL) is highly correlated than protein ratio (pleural fluid protein/serum protein) with clinical diagnosis for exudates. It is found that in transudates, parapneumonic, tubercular and neoplastic pleural effusion, pCHOL levels were 0.53 ± 0.28, 1.81 ± 0.59, 2.08 ± 0.58 and 1.58 ± 0.65 mmol/l, respectively. With a classifying threshold of 1.16 mmol/L, pCHOL has a sensitivity of 97.7% and specificity of 100% for diagnosis of exudates with accuracy of 98.3%.

Pleural cholesterol is thought to be derived from degenerating cells and vascular leakage from increased permeability. Though the cause of the rise in cholesterol levels in pleural exudates is unknown, two possible explanations have been put forward.

According to the first, the cholesterol is synthesized by pleural cells themselves for their own needs [31] (extrahepatic synthesis of cholesterol is now known to be much greater than was once thought, depends on the metabolic needs of cells, and is in dynamic equilibrium with cholesterol supply by LDL and cholesterol removal by HDL) [32] and the concentration of cholesterol in pleural cavity is increased by the degeneration of leukocytes and erythrocytes, which contain large quantities.

The second possible explanation is that pleural cholesterol derives from plasma; some 70% of plasma cholesterol is bound to low density, high molecular weight lipoproteins (LDL) and the rest to HDL or very low density lipoproteins (VLDL) and the increased permeability of pleural capillaries in pleural exudate patients would allow plasma cholesterol to enter the pleural cavity.

The cause of the increased cholesterol concentration is unknown, but two hypotheses are available [33, 34]: (A) cholesterol production by different cells has been recognized and it is possible that destruction of white and red blood cells in pleural effusion can cause an increase in the fluid cholesterol level. (B) Increased pleural permeability causes cholesterol concentrations to increase.

Measurement of pleural cholesterol >45 mg/dl has been used to improve the accuracy of differentiating transudative and exudative effusion [35].

Another study done in Catholic University hospital, Santiago, Chile [36] Marina Costa found sensitivity and specificity of following parameters for exudative pleural effusion as 98 and 82% (criteria by Light et al.), 90 and 100% (pCHOL >45 mg/dl) and 99 and 98% (by pCHOL+ pLDH >200 IU/l), respectively.

A study done by Hamm et al., mean cholesterol level in malignant effusions was 94 mg/dl, 76 mg/dl in inflammatory effusions and 30 mg/dl in the transudates. Using a dividing line of 60 mg/dl to separate the exudates from transudates, only 5% were incorrectly classified. Elevated cholesterol levels in exudates seem to be independent of serum levels [34].

Using pleural fluid, cholesterol levels at a cut-off point of greater than 60 mg/dl and/or total protein at a cut-off point of greater than 3 g/dl for distinguishing transudates and exudates, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV), were 100% in a study done by Patel and Choudhury [37].

Brett reviewing Eid et al. CHEST 2002 Nov, most but not all, exudative effusions in CHF patients have causes other than heart failure. The authors believe that, in some cases with no apparent cause other than CHF, transudates might be `converted' into exudates by traumatic taps (which lead to increased pleural fluid lactate dehydrogenase—itself a criterion for an exudate) or by aggressive dieresis (which might transiently increase protein and LDL cholesterol concentrations in pleural fluid). In patients with previous bypass surgery, persistent impairment of lymphatic clearance might predispose to exudative effusions [38].

Pleural fluid cholesterol is better than Light's criteria for the differentiation of transudates and exudates and is less cumbersome as it does not require a simultaneous blood sampling. Cut-off value of pleural fluid cholesterol for differentiating transudates and exudates should be 45 mg/dl [39]. In this study, the sensitivity, specificity, positive predictive value and negative predictive value of the pleural fluid cholesterol (cut-off >45 mg/dl) were 97.06, 94.74, 97.06 and 94.74%, respectively, for identifying exudates.

NT-proBNP has been shown to correctly diagnose congestive heart failure as a cause of most effusions that have been misclassified as exudates by Light's criteria. Use of this test may therefore avoid repeated invasive investigations in patients where there is a strong clinical

suspicion of cardiac failure. The cut-off value however, varied widely from 600 to 4000 pg/ml (with 1500 pg/ml being most commonly used), and most studies excluded patients with more than one possible etiology for their effusion [40].

The findings in a study done by Mehdi Kashmiri showed taking a value of pleural cholesterol >55 mg/dl and pleural/serum cholesterol >0.3 to define exudative effusion resulted in less erroneous classification with a sensitivity of 93%, a specificity of 100%, a positive predictive value (PPV) of 100% and an accuracy of 95.2%. Using Light's criteria gave a sensitivity of 95%, a specificity of 95%, a PPV of 97.6% and an accuracy of 95.2%. Using cholesterol in differentiating exudate from transudate was especially useful in patients with congestive heart failure who received diuretics [41].

There are other biochemical parameters other than pleural fluid cholesterol to identify the exudative pleural effusions. The difficulties in classifying pleural fluid effusion are wiped away with few parameters other than cholesterol.

It has been observed that increase in uric acid level was present in pleural fluid of transudative pleural effusion than exudative pleural effusion. The optimum cut-off level for pleural fluid uric acid was 5.35 mg/dl with sensitivity of 89.32% and specificity of 92.60% [42]. Increase in uric acid in pleural fluid can be regarded to be a manifestation of tissue hypoxia [43]. Most of the patients with reasons to produce transudative effusion had oxidative stress or hypoxemia to explain the increased uric acid synthesis. The respiratory tract, indeed, remains a major target of oxidative damage caused by both endogenous and exogenous processes [44, 45]. The major causes of tissue damage associated with chronic inflammatory lung disease are the reactive species produced by phagocytes.

Metintas et al. [46] stated that the binding of uric acid is minimal to plasma protein and it is diffuse freely to different compartments. They suggested that the increase permeability, due to change in pleural-capillary pressure in formation of transudate, is the cause of the increase of uric acid levels in pleural fluid. So all these factors explains why uric acid level increases in transudative condition than exudative one.

In cases where no cause for an exudative effusion can be identified or CHF suspected, the sequential application of the fluid LDH, followed by the serum to pleural fluid protein (SF-P) and then the serum to pleural fluid albumin (SF-A) gradients, may assist in reclassifying pleural effusions as transudates [47].

Leers Mathie P.G. from Netherlands [48] found that combination of the parameters: pleural cholesterol and pleural LDH had accuracy of 98%, sensitivity of 98% and 95% specificity for diagnosing exudative pleural effusion compared that calculated by light's criteria being accuracy of 93%, sensitivity 100% and specificity 73%.

5. Conclusion

A pleural effusion (PE) is present when there is an excess fluid in the pleural space. It indicates an imbalance between pleural fluid formation and its removal. Pleural fluid is formed from the systemic vessels of the pleural membranes at an approximate rate of 0.6 ml/h and is

absorbed at a similar rate by the parietal pleural lymphatic system [6]. Pleural fluid accumulates due to local factors, systemic factors or translocation of fluid. At normal circumstances, pleural fluid entering the pleural space from the capillaries in the parietal pleura is removed by the lymphatics which can absorb 20 times more fluid than is formed.

Pleural fluid accumulates in settings of increased hydrostatic pressure, increased vascular permeability, decreased oncotic pressure, increased intrapleural negative pressure and decreased lymphatic drainage. On the basis of pathophysiology, pleural effusion can be transudates or exudates. It is important to classify the pleural fluid for diagnosis and appropriate management. Transudates occur when the mechanical factors influencing the formation or reabsorption of pleural fluid are altered, like a decrease in plasma or elevated systemic or pulmonary hydrostatic pressure. Exudates results from inflammation or irritation or other disease processes involving pleura resulting in increased permeability.

Light et al. found criteria to have sensitivity and specificity of 99 and 98%, respectively, for differentiating transudative and exudative PEs (ratio of protein in pleural fluid and serum >0.5; ratio of LDH in pleural fluid and serum >0.6 and pleural fluid LDH >2/3rd of upper limit of serum LDH) [20]. It is found that 25% of patients with transudates pleural effusion are mistakenly identified as having exudative effusion by Light's criteria. In cases of heart failure on diuretic therapy, the transudative pleural effusions have high protein. Pleural cholesterol is thought to be derived from degenerating cells and vascular leakage from increased permeability. The cause of the increased cholesterol concentration is unknown, but two hypotheses are available: one states that cholesterol production by different cells has been recognized and it is possible that destruction of white and red blood cells in pleural effusion can cause an increase in the fluid cholesterol level and second relates with increased pleural permeability that causes cholesterol concentrations to increase.

Pleural fluid cholesterol as proposed by Heffner's meta-analysis can diagnose exudative pleural effusion without need of serum values. This can avoid the financial burden and double pricks (serum and pleural fluid) in anxious patients to go through the series of tests to confirm the exudative pleural effusion. With a classifying threshold of 1.16 mmol/l, pCHOL has a sensitivity of 97.7% and specificity of 100% for diagnosis of exudates with accuracy of 98.3% compared to Light's criteria (98% sensitivity and 82% specificity). pCHOL is highly correlated than protein ratio with clinical diagnosis for exudates [29]. Moreover in pleural effusion with etiologies as transudates, parapneumonic, tubercular and neoplastic pleural effusion, pCHOL levels were 0.53 ± 0.28, 1.81 ± 0.59, 2.08 ± 0.58 and 1.58 ± 0.65 mmol/L, respectively.

Study done by Leers Mathie PG, it was found that pleural cholesterol and pleural LDH had accuracy of 98%, sensitivity of 98% and 95% specificity for diagnosing exudative pleural effusion compared that calculated by light's criteria being accuracy of 93%, sensitivity 100% and specificity 73% [47].

It is concluded that pCHOL has a better sensitivity, specificity and accuracy in differentiating transudates and exudates than the parameters of Light's criteria. This also avoids the plasma protein and gradients, sLDH, pleural fluid protein and LDH. Therefore it is more efficient, easier and more cost effective method to differentiate exudates from transudates. This study also suggests that determination of pCHOL should be in routine practice in cases of pleural effusion.

Conflict of interest

There is no conflict of interest in this chapter.

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Cholesterol and the Heart

Chapter 6

Role of Cholesterol as a Risk Factor in Cardiovascular Diseases

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76357

Abstract

Cardiovascular disease is the most common cause of death in adult population in the world. The disease includes numerous problems, many of which are related to a process called atherosclerosis. Atherosclerosis is a condition that develops when a substance called plaque builds up in the walls of the arteries. This plaque narrows the arteries, making it harder for blood to flow through. If a blood clot forms, it can stop the blood flow. This can cause a heart attack or stroke. There are many risk factors associated with cardio vascular disease (CVD). While some risk factors cannot be changed, such as family history, some of them can be modified with treatment such as abnormal blood lipid and sugar levels, obesity, smoking, and high blood pressure. Research makes it clear that abnormal blood lipid (fat) levels have a strong correlation with the risk of coronary artery disease, heart attack and coronary death. Cholesterol plays detrimental roles in the pathogenesis of atherosclerosis and CVD. In this chapter, we aim to summarize the relationship between blood cholesterol levels and CVD.

Keywords: cholesterol, cardiovascular disease, atherosclerosis

1. Introduction

Atherosclerotic cardiovascular disease is a group of disease which contains coronary artery disease, carotid artery disease, upper and lower extremity disease, and renal arterial diseases. The main cause of atherosclerotic cardiovascular diseases is the atherothrombotic process that occurs with atherosclerotic plaque rupture. Atherosclerosis is a chronic lipid-associated inflammatory disease concomitant with intimal thickening, especially involving bifurcation regions where endothelial damage is particularly high. Hypertension, hyperlipidemia,

diabetes, and smoking are the main risk factors for atherosclerosis. Fibrinogen, hsCRP, and interleukin-6 (IL-6) which are markers for inflammation have been found elevated in atherosclerosis. Atherosclerotic diseases are also common with systemic inflammatory diseases such as lupus and rheumatoid arthritis.

2. Pathogenesis of atherosclerosis

Atherogenesis starts early in life. Subintimal lipoprotein accumulation and leukocyte adhesion occurs as a result of increased endothelial permeability due to endothelial damage. Intimal neovascularization is seen with the migration of vasa vasorum into the intima. There is a structure in the luminal side of the atherosclerotic plaque that is called fibrous cap which contains molecules such as smooth muscle, collagen, and elastin. External elastic lamina is placed adjacent to the atherosclerotic plaque and tunica media and the lipid core is found between these two structures and it is made from the cholesterol crystals, smooth muscle cells, vascular structures, and foam cells.

Atherosclerotic plaques create luminal stenosis in the advanced stage. The first study in this subject was conducted by Glagov et al. in autopsy material of a patient with 136 left main coronary artery (LMCA) lesions. In this study, there is a positive correlation between internal elastic membrane area and plaque area, and luminal stenosis is prevented by expansion of the compensator at the atheromatous load of less than 40% [1]. In the REVERSAL trial, statin therapy was performed in patients with asymptomatic coronary artery disease. Atheroma volume, percentage of atheroma volume, and atheromatous change in the diseased segment were evaluated and progression of coronary atherosclerosis was observed in the pravastatin receiving group compared to baseline but no progression of atherosclerosis was observed in the atorvastatin group [2].

3. Lipoprotein structure

The lipid core that carries triglycerides and cholesterol esters has a hydrophobic structure and is coated with polar capsules which contain apolipoproteins, phospholipids, and nonesterified cholesterol crystals. When the lipoproteins were classified according to their migration rates in lipoprotein electrophoresis, the band closest to the origin formed the chylomicron band; low-density lipoprotein (LDL) in the beta band, very-low-density lipoproteins (VLDL) in the pre-beta band, and high-density lipoprotein (HDL) in the alpha band, respectively.

Chylomicron is synthesized in liver from dietary fat molecules. Since chylomicrons and VLDL molecules larger than 70 nanometers cannot reach the subintimal region through the transcytotic transport system, chylomicrons do not have atherogenic potential. But chylomicron remnants are atherogenic and cannot be removed from circulation when they are present in high quantities [3]. Hydrolysis of the chylomicrons with lipoprotein lipase results in the formation of VLDL. The majority of VLDL is converted to LDL. Chylomicrons are attached to ApoB48. Chylomicron remnants, LDL, and VLDL are connected to apoB100 and are called non-HDL cholesterol.

http://dx.doi.org/10.5772/intechopen.76357

4. Atherosclerosis and cholesterol hypothesis

The hypothesis of cholesterol suggests that lipids play a major role in the development of atherosclerosis. The 4S trial (Scandinavian Simvastanin Survival Study) showed that while there was significant reduction in total cholesterol level, LDL cholesterol level, and decrease in major coronary events, HDL cholesterol level was elevated in simvastatin receiving group [4]. After 4S study, REVERSAL, ASTEROID, and SATURN studies revealed that parallel plaque regression was observed with aggressive lipid-lowering therapy and reduction in major cardiovascular events was achieved. These similar studies have proven the relationship between hyperlipidemia and atherosclerosis [2, 5, 6].

Statins reduce macrophages and extracellular lipid accumulation in atherosclerotic plaque region and increase the content of collagen in the extracellular matrix which result in intimal calcification. Statins also stabilize inflammation and coagulation cascade after plaque rupture.

5. LDL and total cholesterol

LDL is the particle that is responsible for transporting cholesterol to tissues. Cholesterol transportation is achieved by binding of the LDL receptor and apoB. There are three separate fractions of LDL: LDL (large/floating), IDL, and small dense LDL. The most atherogenic LDL is small dense LDL.

In the WOSCOP trial and the AFCAPS/TeXCAPS trial which used pravastatin and lovastatin, respectively, the effect of hyperlipidemic therapy on the primary prevention of coronary artery disease was shown [7, 8]. The ASCOT-LLA study was terminated early in hypertensive individuals because atorvastatin significantly reduced nonfatal MI and CAD-induced mortality [9]. Similarly, the CARDS study was terminated early in diabetic individuals because atorvastatin decreased 37% in major cardiovascular events and 48% in stroke [10].

LIPID study compared low-dose and high-dose atorvastatin in patients with stable coronary artery disease and mortality was similar in both groups, but there was a significant decrease in major cardiovascular events in the high-dose atorvastatin group. The HPS study has shown that statin therapy protects high-risk patients with LDL cholesterol levels below 116 mg/dL [11]. CARE study with pravastatin in acute MI and MIRACLE study with atorvastatin in USAP or MI have shown early initiation of statins have a positive affect [12, 13].

In the ASTEROID trial, high-dose statin therapy (rosuvastatin 40 mg/day) was shown to reduce 53% LDL cholesterol, 15% increase in HDL cholesterol, and regression in 78% atheroma [5].

Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors inhibit the PCSK9 protein, which is effective in LDL receptor synthesis and provide 50–70% reduction in LDL-C levels.

In the ACCELERATE study, it was shown that the CETP inhibitor (Evacetrapib) did not reduce major cardiovascular events despite a 39% reduction in cholesterol level [14].

6. HDL

HDL is a molecule that is antioxidant, antiinflammatory, antiapoptotic and increases macrophage cholesterol excretion and endothelial healing. The removal of cholesterol from the body by the liver via HDL is called reverse cholesterol transport. ABCA-1, ABCG-1, and SR-B1 are effective in reverse cholesterol transport.

ApoA1 and ApoA2 are mainly found in the structure of HDL, and also HDL includes apoCs, ApoE, apoD, apoJ, lecithin-cholesterol acyltransferase (LCAT), serum paraoxonase (PON1) and platelet-activating factor acetylhydrolase (PAF-AH) molecules. Enzymes carried by HDL prevent oxidative modification of LDL.

Pentraxin 3 (PTX-3) in HDL controls leukocyte level. Defective PTX-3 was associated with large atherosclerotic plaques and higher level of inflammation [15, 16].

The association between low HDL and atherosclerotic cardiovascular disease was first shown by the Framingham study. Hypertension, diabetes mellitus, elevated total cholesterol, low HDL cholesterol, smoking, and age is considered as risk factor for coronary artery disease. The association between a low HDL cholesterol and atherosclerosis has been proven, but the increase in HDL has not been associated with a reduction in the incidence of atherosclerotic cardiovascular disease. Due to HDL being a molecule that prevents inflammation, some changes in HDL structure occur in chronic inflammatory processes.

7. Lipoprotein (a)

Lipoprotein (a) (Lp (a)) consists of an LDL molecule bound to apolipoprotein (a). Lipoprotein (a) is structurally similar to plasminogen and is thought to play a role in atherothrombosis with antifibrinolytic properties. In a study with patients with normal LDL and elevated Lp(a) levels, it was determined that increased Lp(a) levels was associated with high cardiovascular risk [17].

Cholesterol ester transfer protein (CETP) is responsible for transferring cholesterol esters. CETP inhibitors are associated with increased HDL and decreased LDL levels. In the study conducted with anacetrapib, there was no significant difference in mortality despite a significant increase in HDL and a significant decrease in non-HDL cholesterol compared to placebo [18].

8. Atherogenic dyslipidemia

In atherogenic dyslipidemia which is the result of an increase in triglyceride levels, triglyceride content is increased. The primary source of triglycerides is the VLDL. While LDL molecules are more easily oxidized, HDL molecules are more easily eliminated from the kidneys. Metabolic syndrome, type 2 diabetes, insulin resistance, abdominal obesity, and polycystic ovary syndrome are associated with atherogenic dyslipidemia.

In the case of atherogenic dyslipidemia, since chylomicrons have no effect on atherosclerosis, non-HDL cholesterol level is used rather than triglyceride level. Although levels of

LDL, VLDL, and chylomicron residues can be determined by detecting Apo B levels, there is limited access and standardization for the detection of Apo B level. In the ESC dyslipidemia guide, non-HDL cholesterol calculation is recommended instead of measuring ApoB levels in the presence of hypertriglyceridemia. (Class 2a) In a study conducted by Puri et al., the level of non-HDL cholesterol rather than LDL cholesterol significantly correlated with atheromatous progression when the triglyceride level rises above 200 mg/dl. In the NICE guideline, all individuals are focused on evaluating non-HDL cholesterol exclusively from LDL cholesterol.

9. Familial hypercholesterolemia

Familial hypercholesterolemia is a metabolic disorder that occurs as a result of the absence or lack of LDL receptors in the liver. Since LDL molecules are removed from the circulation, very high LDL levels and premature atherosclerosis are observed. Familial hypercholesterolemia is thought to be approximately 1/500 of the homozygous form and approximately 1/1 million of the heterozygous form. Tendon xanthomas are pathognomonic signs for familial hypercholesterolemia. There is also an increase in the frequency of corneal arcus, xanthelasma.

10. Sitosterolemia

Sitosterol is a plant-derived molecule and its structure resembles cholesterol. Cytosterolemia is a progressive disease with an increase in the absorption and a decrease in biliary secretion of cholesterol and sitosterol molecules. Sitosterolemia is also called pseudohomozygous familial hyperlipidemia.

Recommendations for ESC 2016:

- Total cholesterol should be used to predict cardiovascular risk via the SCORE system. (1-C)
- LDL-C should be used primarily in screening, diagnosis, risk estimation, and treatment. (1-C)
- HDL-C should be used in the Heart Score algorithm. (1-C)
- TG provides additional information in the risk estimation. (1-C)
- Non-HDL cholesterol should be considered as a risk indicator, especially in individuals with high triglyceride levels. (1-C)
- ApoB should be considered as an alternative risk marker in patients with high triglyceride values. (2a-C)
- Lp (a) may be considered in individuals with high-risk, early family history of CVD and in the reclassification of individuals with borderline risk. (2a-C)
- ApoB1/ApoA1 ratio can be considered as an alternative analysis in risk prediction. (2b-C)

- The ratio of non-HDL cholesterol/HDL cholesterol can be considered as an alternative, but the HDL cholesterol used in the HEART SCORE provides a better risk estimate. (2b-C)
- LDL cholesterol is the main treatment target. (1-A)
- When available, apoB should be an alternative to non-HDL-C. (2a-C)
- Lp(a) should be recommended in selected case at high-risk, for reclassification at borderline risk, and in subjects with a family history of premature CVD. (2a-C)
- TC may be considered but is usually not enough for the characterization of dyslipidemia before initiation of treatment. (2a-C)
- HDL cholesterol and non-HDL cholesterol/HDL cholesterol levels are not recommended as treatment targets (Class 3).

11. Epidemiology

More than 30% of worldwide deaths are thought to be cardiovascular based and the frequency tends to increase due to changes in lifestyle and prolonged life. According to AHA 2016 statistics, in the United States, one in every 42 seconds loses his/her life due to cardiovascular reasons [19]. In Europe, the cardiovascular mortality rate is 4.1 million a year. A total of 1.8 million deaths, in other words 20% of all deaths, are due to ischemic heart disease. This is followed by cerebrovascular events with an annual death of 1.1 million. According to ESC data, 1.5 million deaths before the age of 75 and 710,000 deaths before the age of 65 are cardiovascular sources; half is due to coronary artery disease [20]. Deaths in all age groups, 51% of women and 42% of men are cardiovascular.

12. Coronary artery disease and cholesterol

Acute coronary syndrome is a clinical event that occurs when the coronary blood flow is reduced by thrombus on the rupture plaque and the myocardial oxygen requirement cannot be met. Acute coronary syndrome is broad spectrum which contains STEMI, nonSTEMI, unstable angina pectoris, and sudden cardiac death. In many cases, the thrombosis process begins with plaque rupture. Up to 25% of cases of acute coronary syndromes can begin with plaque erosion. Lymphocyte and macrophage activation and the inflammatory response is accompanied by atherothrombosis. There are clinical differences according to coronary collateral reserve and obstruction severity. This process occurs after a plaque rupture and is called Type 1 MI.

Atherosclerotic plaques that play an essential role in acute coronary syndrome are divided according to their structural characteristics: Plaque structure is with thin fibrous cap, dense necrotic core, high inflammatory cell density, and low smooth muscle content; it is called vulnerable plaque. Vulnerable plaque increases with hypertension, diabetes mellitus, elevated LDL, decreased HDL, and elevated ACE. Conversely, stabilized plaques with thick fibrous caps, poor necrotic cells, and dense extracellular matrix with low inflammatory content are observed in individuals with low risk factors (**Figure 1**).

http://dx.doi.org/10.5772/intechopen.76357

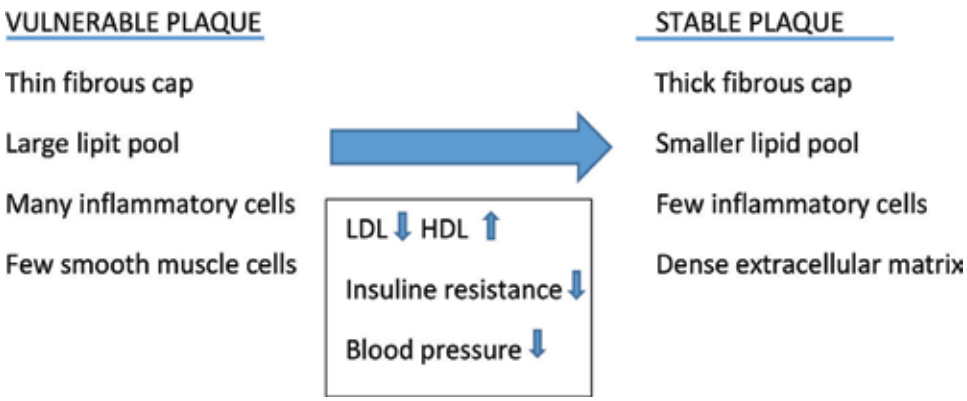

Figure 1. Structural differences between vulnerable and stable plaques.

In a meta-analysis involving 90,056 patients, a reduction of 38.6 mg/dl in LDL was shown to be associated with a 20% reduction in major cardiovascular events [21]. In MIRACLE study: increased plaque stability with statin therapy reduces death, incidence of acute coronary syndrome and frequency of recurrent coronary ischemia [13]. In the PROVE IT TIMI-22 trial, atorvastatin 80 mg and pravastatin 40 mg were compared and it was determined that high-dose statin therapy was more effective than low-dose statin therapy in reducing cardiovascular events. The 2017 ESC STEMI guidelines recommended that high-dose statin therapy was independent of cholesterol level. In the FOURIER study, it has been shown that the addition of the evolocumab in patient with LDL level ≥ 70 mg/dl, despite the use of high dose statin, is associated with a decrease in cardiovascular deaths [22].

13. Coronary calcium score

The coronary calcium score began to be used in the 1990s and the method was prepared by Agatson et al. Zero coronary calcium score has a high negative predictive value. It is the most commonly used method. In the 2016 ESC Guidelines for Cardiovascular Disease Prevention, the use of coronary calcium scoring has been proposed for predicting cardiovascular risk in individuals with a SCORE risk threshold of 5–10%.

CARDIA study showed a correlation between elevated LDL or non-HDL cholesterol and coronary calcium score [23].

14. Peripheral arterial diseases and cholesterol

Peripheral arterial disease is a concept that involves diseases of arteries other than coronary arteries. It most commonly occurs as a result of atherosclerotic process. In addition to atherosclerosis, vasculitis, and injuries, trapping syndromes are also effective in the formation of peripheral arterial disease. Approximately one-third of the individuals with peripheral artery

disease are accompanied by coronary artery disease. Peripheral artery disease should be considered as equivalent to coronary artery disease risk. Deaths are mostly of cardiac origin.

According to the REACH study, 3-year vascular-induced deaths were more common in patients with peripheral arterial disease than in those with coronary and carotid artery disease [24]. The use of statin has reduced both symptoms and cardiovascular mortality in a variety of studies on peripheral arterial disease [25, 26].

15. Carotid diseases, stroke, and cholesterol

Ischemic stroke should be investigated in two groups as embolic and thrombotic stroke.

Smoking and age are the most important risk factors for carotid atherosclerosis. The atherosclerotic plaque is located in the bifurcation area and often extends on the outer wall of the carotid bulb.

When stenotic plague increases, the risk of emboli increases. Carotid stenosis is defined as a stenosis of 50% or more in the extracranial portion of the internal carotid artery. In addition to the luminal narrowing, the lesion's edge irregularity, the presence of intraplate plaque hemorrhage, whether the lesion is unilateral or not, also determines the severity of the disease. Symptomatic carotid stenosis is the occurrence of symptoms related to carotid stenosis in the last 6 months.

In the heart protection study with simvastatin, a reduction of 39 mg/dL at the LDL level resulted in a 20% reduction in major cardiovascular events, 25% reduction in stroke, and 38% reduction in ischemic stroke [11].

In the SPARCLE trial (stroke prevention by aggressive reduction in cholesterol levels), patients who had stroke and TIA within the last 1–6 months were evaluated for 5 years. In patients receiving high-dose atorvastatin, a reduction of 43% in LDL levels resulted in a 20% reduction in major cardiovascular events and a 16% reduction in stroke. Despite the increase in hemorrhagic stroke rates in the high-dose statin group, there was no difference in lethal hemorrhagic stroke [27, 28].

It has been suggested that statin therapy initiated after stroke also improves neurological function with a decrease in infarct area. According to the information obtained from the meta-analyses, the use of statin before and after stroke is associated with improvement in neurological function. However, there was a relationship between statin therapy and hemorrhagic transformation in cases treated with thrombolytic therapy [29].

Carotid intima media thickness is a subclinical atherosclerosis indicator and it is recommended to use it in addition to classical cardiovascular risk indicators, especially in individuals with hypertensive middle cardiovascular risk (SCORE risk 1–5%). Values above 0.9 mm or values above normal 75th percentile should be considered pathological. According to the American Society of Echocardiography, these individuals should be considered as having increased CV risk. Individuals between 75 and 25% have expected cardiovascular risk. Individuals below the 25th percentile have low cardiovascular risk [30].

http://dx.doi.org/10.5772/intechopen.76357

16. Renal artery stenosis hypertension and cholesterol

Renovascular hypertension is about 5% of all hypertension cases. In the presence of peripheral artery disease, the frequency of renal artery stenosis reaches up to 14%. There is an increase in the frequency of renal artery stenosis and peripheral artery disease association in the presence of diffuse peripheral artery disease [31]. Atherosclerotic renal artery disease is the most common cause of renovascular hypertension. Atherosclerotic renal artery disease is often defined as having ≥60% stenosis in the osteal or proximal one-third of the renal artery. The second most common cause is fibromuscular dysplasia in younger individuals with no atherosclerotic risk factors. There is a "string of beats" view at the distal one-third of the renal artery. Renal artery stenosis can be tolerated by autoregulation mechanisms until the renal perfusion pressure reaches 70 mmHg. Renal revascularization has not been shown to reduce hypertension, renal, or cardiovascular events. Antihypertensive therapy, antiplatelet therapy, and statins are the main treatments.

17. Lower extremity peripheral artery diseases and cholesterol

A common cause of lower extremity peripheral artery disease is atherosclerosis. It is common in men who have cigarette use at a young age. Diabetes and smoking are the most common causes of amputation in peripheral artery diseases. In the atherosclerotic process, progressive narrowing of the vessel wall occurs. Clinical signs are observed in the later stages of the disease. Clinical disease severity is determined by Fontaine and Rutherford classifications. There are studies that argue that the ankle brachial index (ABI) used in lower extremity diseases should be used as a risk factor for coronary artery disease. When ABI is above 0.9, it is considered normal but below 0.40 is considered as serious disease.

The 2017 ESC guidelines for peripheral arterial disease recommended LDL cholesterol lowering to 70 mg/dL or 50% reduction in LDL levels in patients with an initial LDL level of 70–135 mg/dL. Studies in lower extremity arterial disease patients have shown that statin therapy decreases all-cause mortality and cardiovascular mortality.

18. Aortic aneurysm and cholesterol

Aneurysm is defined as enlarging the diameter of artery, local or diffuse, by 50% or more relative to normal. According to localization, it is divided into thoracic and abdominal. Aortic aneurysms are 80% in abdominal location [32]. It is a chronic disease associated with inflammation of the aortic wall. It is suggested that the vessel is formed as a result of elasticity and power loss of the aortic wall after occlusion of vasa vasorum.

In the population with abdominal aortic aneurysm, association with other atherosclerotic cardiovascular diseases was frequently observed. The presence of abdominal aortic aneurysm was frequently associated with other atherosclerotic cardiovascular diseases. Smoking, age,

and male sex increases the risk of aortic aneurysm. While intimal atheroma and thrombosis process are present in both diseases, elastin fragmentation and adventitial chronic inflammation are limited to aortic aneurysms [33, 34].

In the tromsø study, there was a relation between the intima media thickness and the incidence of coronary artery disease and abdominal aortic aneurysm, but no correlation with aortic diameter [35]. The relationship between lipid level and aortic aneurysm has not been clearly elucidated [36]. The data for the studies are based on the similarity of risk factors for atherosclerosis and aortic aneurysm risk factors.

19. Retinal vascular diseases and cholesterol

Hyperlipidemia is associated with retinal vascular diseases. In old age, structures called "druzen" in tissue are similar to atherosclerotic lesions. Ischemic optic neuropathy can be seen as a result of stenosis in the retinal arteries and venules. In the ACCORD Eye trial, although strict treatment for diabetes and hyperlipidemia was beneficial, there was no significant benefit from strict blood pressure regulation [37, 38].

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Chapter 7

The Role of Cholesterol in the Pathogenesis of Hypertension-Associated Nonalcoholic Steatohepatitis

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.76199

Abstract

Dietary cholesterol is a crucial risk factor for nonalcoholic steatohepatitis (NASH). Our recent studies indicated that high cholesterol intake was associated with the pathogenesis of hypertension-associated NASH. We developed a novel hypertensive rat model of NASH by feeding stroke-prone spontaneously hypertensive rats (SHRSP5/Dmcr) a high fat and cholesterol (HFC) diet. Histological features resembling human NASH were observed in this model. Furthermore, we investigated the kinetics of cholesterol in the rats fed an HFC diet and determined that suppression of bile acid (BA) detoxification led by HFC feeding results in cytotoxic BA accumulation in hepatocytes, which induces inflammatory response and liver damage. Sex differences in fibrogenesis were also observed in this model, and we found this was associated with a different ability in BA detoxification. Since SHRSP5/Dmcr rats are hypertensive, we investigated the role of hypertension in NASH progression by comparing NASH development among SHRSP5/Dmcr rats, spontaneously hypertensive rats and their original strain, Wistar Kyoto, with normal blood pressure. HFC diet induced more severe hepatic fibrosis in the hypertensive strains compared with the normotensive one. In conclusion, dietary cholesterol plays an essential role in the pathogenesis of NASH, and the combined action of cholesterol and hypertension further aggravates its progression.

Keywords: cholesterol, hypertension, nonalcoholic steatohepatitis, spontaneously hypertensive rat, Wistar Kyoto rat, CYP7A1, kinetics of bile acids, gender differences in fibrogenesis

1. Introduction

High dietary cholesterol intake may lead to increased risk of diseases such as cardiovascular disease and diabetes [1, 2]. Although the recommendation to restrict daily dietary cholesterol

intake (300 mg) was removed from the 2015–2020 Dietary Guidelines for Americans [3], it is still recommended that individuals minimize cholesterol consumption. Animal foods such as egg yolk, meats, dairy products, fish, and poultry are major sources of dietary cholesterol. Meanwhile, dietary cholesterol is not found in plant foods. Instead, many plants contain phytosterols, which are chemically similar to cholesterol, and can therefore compete with it and decrease its absorption in the intestinal tract [4]. The effect of dietary cholesterol on plasma cholesterol levels remains undetermined, since the body may suppress endogenous cholesterol synthesis in response to additional cholesterol ingestion [1]. Some studies have suggested that dietary cholesterol increases serum total cholesterol (TC), low-density lipoprotein (LDL) cholesterol, as well as the ratio of LDL to high-density lipoprotein cholesterol [5–8], which are considered to be associated with risk of vascular diseases.

Dietary cholesterol is also linked to the pathogenesis of nonalcoholic fatty liver disease (NAFLD)/nonalcoholic steatohepatitis (NASH) [9, 10]. NAFLD is one of the most common chronic liver diseases worldwide and comprises a spectrum of liver damage, from simple steatosis (a benign non-progressive condition) to NASH, the advanced form that may progress to hepatic cirrhosis or hepatocellular carcinoma [11]. The pathological characteristics of NASH include steatosis, hepatocellular ballooning, lobular inflammation, and hepatic fibrosis. Cholesterol may contribute to NASH development by being catabolized in the liver into bile acids (BAs), which are hepatotoxic and cause liver damage [12]. Li et al. demonstrated that dietary cholesterol exacerbates liver damage and hepatic inflammation in mice fed a high-fat diet [13]. Subramanian et al. reported that an LDL receptor-deficient mouse fed a high-fat, high-carbohydrate diet was a good animal model of NAFLD/NASH, and showed that dietary cholesterol worsened hepatic steatosis and inflammation in this model [9].

In addition, NAFLD/NASH was described as a hepatic manifestation of metabolic syndrome, and its development was associated with hypertension, obesity, diabetes, and hyperlipidemia [14, 15]. Some studies have shown an increased prevalence of NAFLD/NASH among hypertensive patients [16–18]. Using spontaneously hypertensive (SHR) rats fed a choline-deficient diet as a hypertensive animal model of NASH, and its normotensive control, the Wistar Kyoto (WKY) rat [19], Ikuta et al. revealed that hypertension enhances the progression of NASH. We previously developed a novel animal model of hypertension-associated NASH by feeding stroke-prone spontaneously hypertensive5/Dmcr (SHRSP5/Dmcr) rats a high fat and cholesterol (HFC) diet [20]. Further studies from our group suggested that dietary cholesterol may have a potential effect on the development of hypertension-associated NASH (unpublished).

In this chapter, we will discuss the crucial role of dietary cholesterol in the progression of hypertension-associated NASH.

2. The development of a novel animal model of NASH and the mechanism underlying the progression of NASH in this model

The mechanisms of the pathogenesis of NASH are not completely understood, partly due to a lack of ideal animal models with histological patterns that resemble human NASH. Matsuzawa et al. showed that an HFC diet (an atherogenic, high-fat diet containing 1.25%

cholesterol and 60% fat) induced steatohepatitis, cellular ballooning, and fibrosis in the livers of male C57Bl/6J mice [21]. We previously established an HFC diet-induced NASH model using hypertensive SHRSP5/Dmcr rats [20].

SHRSP5/Dmcr rats are the fifth substrain of the stroke-prone spontaneously hypertensive (SHRSP) rat [20, 22], which is derived from the SHR strain [23]. To establish this strain, SHRSP rats were fed an HFC diet for 1 week, then those with high serum cholesterol levels (600–900 mg/dL in females and 300–600 mg/dL in males) were selected for brother–sister inbreeding. Selective inbreeding was repeated and offspring with increased hypercholesterolemic responses were obtained. Although the SHRSP5/Dmcr rats, formally known as arteriolipidosis-prone rats, were developed as an animal model of arteriosclerosis, marked enlargement and an abnormal whitish color of the liver were noted in the 47th generation. These findings prompted our studies on HFC diet-induced liver damage in this strain.

In order to determine whether the HFC diet-fed SHRSP5/Dmcr strain was a suitable model of NASH, we investigated hepatic histopathological changes following HFC feeding [20]. Male SHRSP5/Dmcr rats at 10 weeks of age were fed either an HFC (35.3% crude lipid and 5% cholesterol) or control diet (4.8% crude lipid and no additional cholesterol) for 2, 8, and 14 weeks. We found that the HFC diet induced microvesicular steatosis and lymphocyte infiltration at 2 weeks. Macrovesicular steatosis, ballooned hepatocytes with eosinophilic Mallory-Denk bodies, and multilobular necrosis were observed in the livers of rats fed an HFC diet at 8 weeks. The severity of steatosis and hepatocyte ballooning was further increased at 14 weeks. Meanwhile, a progressive deterioration of hepatic fibrosis occurred during HFC feeding. Slight pericellular and perivenular fibrotic changes, bridging fibrosis, and end-stage honeycomb fibrosis were observed at 2, 8, and 14 weeks, respectively. In addition, the HFC diet induced a progressive increase in indicators of liver damage, including serum levels of alanine transaminase (AST), aspartate transaminase (ALT), and γ-glutamyltranspeptidase (γ-GTP). Matteoni et al. classified human NAFLD into four types according to histological analysis of liver biopsy specimens: type 1, fatty liver alone; type 2, fat accumulation and lobular inflammation; type 3, fat accumulation and ballooning degeneration; and type 4, fat accumulation, ballooning degeneration, and hepatic fibrosis [24]. The histological characteristics observed in the liver of the SHRSP5/Dmcr strain at 2, 8, and 14 weeks of HFC feedings were very similar to those in type 2, type 3 or 4, and type 4 human NAFLD, respectively. Therefore, all pathological stages of NAFLD can be observed in the SHRSP5/Dmcr strain during HFC feeding. In addition, obesity, insulin resistance, and diabetes were not observed in this model. Therefore, it represents an excellent model of NAFLD/NASH without obesity and diabetes, and is useful for studying the pathogenesis and therapeutics of this disease.

We further investigated the molecular mechanisms underlying the progression of HFC-induced NASH in the SHRSP5/Dmcr strain [25]. Rats were fed either an HFC or control diet for 2, 8, and 16 weeks, and expression of genes involved in inflammation and hepatic fibrosis was evaluated. Tumor necrosis factor α (TNF-α), a proinflammatory cytokine, was reported to be upregulated in the livers of NASH patients [26]. We showed that the HFC diet increased the hepatic expression of TNF-α in SHRSP5/Dmcr rats at all time points. Nuclear factor κB (NF-κB; p50/p65) and inhibitor of κBα, the proteins involved in NF-κB signaling, which is regulated by TNF-α and plays an important role in inflammatory response, were also upregulated by the HFC diet. Hepatocyte injury and inflammation led to hepatic fibrosis via hepatic stellate

cell (HSC) activation, which results in the production and deposition of extracellular matrix (ECM) [27]. The HFC diet induced the upregulation of transforming growth factor-β1 (TGF-β1), a profibrotic cytokine that promotes HSC activation, prior to the appearance of obvious hepatic fibrosis (at 2 weeks). Its upregulation was also observed at subsequent stages (at 8 and 16 weeks). Expression of alpha smooth muscle actin (α-SMA) and platelet-derived growth factor-B, involved in hepatic fibrosis, were elevated at 8 weeks of HFC feeding, indicating extensive activation of HSC at this time point. Alpha-1 type I collagen, the major component of ECM, was produced by activated HSC (myofibroblast) and was markedly elevated at 8 and 16 weeks, corresponding to the appearance of extensive liver fibrosis observed at the same time points.

In order to investigate the role of dietary cholesterol in the pathogenesis of HFC diet-induced NASH in SHRSP5/Dmcr rats, we compared hepatic histological changes induced by a high fat (HF) diet and those by an HFC diet (unpublished). As described above, the HFC diet induced severe steatosis, lymphocyte infiltration, ballooned hepatocytes, and fibrosis in the livers of the rats. In contrast, HF feeding only led to mild hepatic steatosis and lymphocyte infiltration, while liver fibrosis was not observed. It was suggested that dietary cholesterol may play a key role in the transition from simple steatosis to fibrotic steatohepatitis, the progressive stage, during the progression of NAFLD/NASH.

3. The role of hypertension in the progression of NASH

SHRSP5/Dmcr rats are hypertensive, making this strain an ideal model in which to study the correlation between hypertension and NASH. In our previous study, we investigated the mechanism underlying the development of hypertension-associated NASH using three strains of a rat: normotensive WKY, hypertensive SHR and SHRSP5/Dmcr [28]. As mentioned previously, SHRSP5/Dmcr was established from the SHRSP strain, which was derived from SHR strain that was developed from normotensive WKY rats by selective inbreeding of the rats with spontaneously high systolic blood pressure in normal conditions [29]. Male rats with a blood pressure of 150–175 mmHg persisting for more than 1 month, and females with a blood pressure of 130–140 mmHg were mated, and the offspring with high blood pressure (over 150 mmHg persisting for more than 1 month) were selected for further inbreeding. The severity of hypertension was elevated from generation to generation, and all the rats from the third to sixth generation developed spontaneous hypertension by 15 weeks of age. Since the SHR and WKY originated from the same parental outbred Wistar rats, the WKY strain was used as the normotensive control for the SHR and SHRSP5/Dmcr strains. The blood pressure in the adult male rats of the three strains, WKY, SHR, and SHRSP5/Dmcr, were 130, 235, and 180 mmHg, respectively [28].

In our study, the normotensive WKY strain, and two hypertensive SHR and SHRSP5/Dmcr strains were fed either the HFC or control diet for 8 weeks. Changes to liver pathology and expression of proteins associated with inflammation and oxidative stress were determined [28]. We evaluated serum levels of AST, ALT, and γ-GTP, and confirmed that mild liver damage occurred in the hypertensive strains in the absence of HFC feeding, suggesting that hypertension may be a risk factor for chronic liver disease. The HFC diet induced more severe lobular inflammation and hepatic fibrosis in the hypertensive strains compared with the normotensive

http://dx.doi.org/10.5772/intechopen.76199

strain. The severity of the hepatic fibrosis observed in the SHRSP5/Dmcr strain was even higher compared with that of the SHR strain. The HFC diet induced elevation of serum inflammatory cytokines, TNF-α and TGF-β1, in the hypertensive strains, whereas an increase in TGF-β1 was not observed in the normotensive rats. The combination of TNF-α and TGF-β1 may trigger a more severe inflammatory response in the hypertensive rats by regulating the activation of downstream inflammatory signaling such as NF-κB and mitogen-activated protein kinase (MAPK) pathways. Increased activation of NF-κB and MAPK (p38 and JNK) signaling occurred in the hypertensive strains, which may have contributed to the more severe lobular inflammation observed in these rats. In addition, oxidative stress, defined as an imbalance between the production of reactive oxygen species (ROS) and their elimination by antioxidant defenses, may lead to cellular injury and chronic inflammation [30]. An increase in oxidative stress in NASH patients was previously reported [31, 32]. We measured serum thiobarbituric acid reactive substances levels and found that oxidative stress was significantly elevated in hypertensive strains fed an HFC diet but not in normotensive rats (unpublished data). Meanwhile, in hypertensive rats, the HFC diet suppressed the nuclear factor erythroid 2-related factor 2 (Nrf2)/Kelch-like ECH-associated protein 1 (Keap1) pathway, involved in antioxidative defenses [33]. We also found that hepatic levels of superoxide dismutase-1 (SOD-1) [25] and SOD-2 [28], that contribute to antioxidant defense by catalyzing the dismutation of superoxide anions [34], were decreased in hypertensive SHRSP5/Dmcr rats fed the HFC diet. The decrease in SOD-2 expression induced by HFC feeding was not observed in normotensive WKY and hypertensive SHR strains [28]. This could suggest that an increase in oxidative stress and a lower antioxidative capacity may trigger a more severe inflammatory response and liver damage in hypertensive rat strains following HFC feeding, compared with normotensive strains.

4. The role of cholesterol in the development of hypertension-associated NASH

As previously stated, dietary cholesterol intake is considered a risk factor for NAFLD/NASH. The liver is a crucial organ implicated in the regulation of cholesterol metabolism, including the synthesis and secretion of cholesterol, as well as the synthesis of BAs from cholesterol (a major pathway for hepatic cholesterol catabolism) and BA detoxification [35]. Disturbed cholesterol homeostasis in the liver is thought to be associated with the pathogenesis of NAFLD/NASH [35]. Our study showed that the HFC diet increased serum and hepatic levels of TC in the hypertensive SHR and SHRSP5/Dmcr strains, as well as the normotensive WKY strain [28]. It is worth noting that the increase in hepatic TC levels in the hypertensive rats was significantly lower than those in the normotensive WKY strain. Therefore, we postulated that more cholesterol was consumed for the synthesis of BAs in the livers of the hypertensive rats. In addition, serum TC levels in the hypertensive strains fed the control diet were markedly lower compared with those of the normotensive WKY strain, suggesting that the dysregulation of cholesterol metabolism may play an important role in the progression of hypertension-associated NASH.

In order to investigate the kinetics of cholesterol during the development of HFC-induced NASH in our hypertensive SHRSP5/Dmcr rat model, we evaluated the expression of proteins

involved in de novo cholesterol synthesis, cholesterol uptake from bloodstream in the form of LDL, cholesterol secretion into blood in the form of very-low-density lipoprotein, and BA synthesis and detoxification [36].

4.1. De novo cholesterol synthesis and its uptake from blood

Excessive intake of cholesterol may suppress de novo cholesterol synthesis via a feedback mechanism dependent on the transcriptional factor sterol regulator element-binding protein 2 (SREBP-2) [35]. SREBP-2 resides in the endoplasmic reticulum and remains there when cholesterol is abundant in hepatocytes; however, SREBP-2 is activated in response to low levels of cholesterol and translocated to the nucleus, where it triggers the expression of various genes, including low-density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR). HMGCR is the rate-limiting enzyme for cholesterol biosynthesis. Our study showed that HMGCR was downregulated in the livers of SHRSP5/Dmcr rats during consumption of the HFC diet (2, 8, and 14 weeks), although SREBP-2 expression remained unchanged [36]. It was proposed that additional signaling, except SREBP-2, may be required for cholesterol synthesis in our rat model. The HFC diet decreased the expression of LDLR and LDLR-related protein 1, which are required for clearing cholesterol-contained lipoproteins from the blood by the liver [37]. Therefore, excessive intake of dietary cholesterol led to accumulation in the liver and consequently resulted in suppression of cholesterol synthesis and uptake.

4.2. BA synthesis and excretion

There are two major pathways of BA synthesis. The classic pathway is initiated by cholesterol 7 alpha-hydroxylase (CYP7A1), the rate-limiting enzyme, followed by the catalytic action of sterol 12 alpha-hydroxylase (CYP8B1) [38]. On the other hand, the initial step in the alternative (acidic) pathway is catalyzed by sterol 27-hydroxylase (CYP27A1), followed by oxysterol 7alpha-hydroxylase (CYP7B1). The major primary BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA), are produced from cholesterol in the liver, while the secondary BAs, lithocholic acid (LCA) and deoxycholic acid (DCA), are generated from CDCA and CA in the intestines, respectively. After synthesis, conjugation of Bas is required for effective transport and detoxification [39]. BAs are conjugated with amino acids (taurine or glycine) or sulfate, mediated by BA coenzyme A synthase and BA amino acid transferase, and sulfotransferase (SULT2A1), respectively. Some BAs are glucuronidated by UDP-glucuronosyl *N*-transferases (UGT1A1, 2B4, and 2B7). Amino acid-conjugated BAs are excreted from the liver into the bile canaliculi via the bile salt export pump (BSEP), an ATP-binding cassette (ABC) transporter protein located in the canalicular membrane of hepatocytes [40]. Multidrug-resistant protein 2 (MRP2) is another ABC transporter implicated in the transport of sulfated or glucuronidated BAs to bile, while MRP3, located in the basolateral membrane of hepatocyte, is responsible for the transport of BAs from the liver to the blood. In addition, bile acid-activated nuclear receptors (a group of transcriptional factors), such as farnesoid X receptor (FXR), pregnane X receptor (PXR), and constitutive androstane receptor (CAR), are implicated in the regulation of BA metabolism, including synthesis, transport, and detoxification [39]. Several studies have reported that activation of FXR, PXR, and CAR inhibits transcription of the CYP7A1 gene in hepatocytes, and therefore suppress BA synthesis [41–43]. Activation of FXR and PXR also induces expression of the BA transporter proteins, BSEP and MRP2 [44–46].

Increased levels of hepatic BA were observed in NASH patients and were correlated with inflammation and fibrosis in the liver [47]. In our SHRSP5/Dmcr model, the HFC diet increased hepatic levels of CYP7A1 but decreased levels of CYP8B1, while CYP27A1 was downregulated and CYP7B1 was upregulated [36]. We used ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) to further determine the hepatic levels of 21 types of BA in rats fed the HFC or control diet [48]. The HFC diet significantly increased total BA levels in the liver at 2 weeks, but decreased it at 8 weeks. We also investigated the composition of the total BA in the rats' livers. In the total BA pool, the relative proportions of CDCA species, which are hydrophobic and show high cytotoxicity [49], were markedly elevated at 8 and 14 weeks, whereas hydrophilic CA species, with lower toxicity, were significantly decreased at 14 weeks. The ratio of total CA to CDCA was prominently reduced by HFC feeding at 8 and 14 weeks. Most BAs (about 90% of the total) in the livers of rats fed the control diet were taurine-conjugated. In contrast, glycine-conjugated BAs were predominant in HFC-fed rats. In addition, canalicular transporters, BSEP and MRP2, were reduced in the livers of the rats during HFC feeding (2, 8, and 14 weeks), whereas MRP3, the basolateral transporter, was significantly increased at 8 and 14 weeks [36]. Therefore, the accumulation of total BAs in the rats' liver at 2 weeks of HFC feeding may have resulted from suppressed BA excretion to the bile duct, mediated by BSEP and MRP2 transporter proteins. Meanwhile, the decrease in total BA levels in the liver at 8 weeks may have been triggered by an increase in MRP3-mediated BA excretion to the blood. Furthermore, we demonstrated that the ratio of CA to CDCA was negatively correlated with liver injury (macrovesicular steatosis, serum ALT levels, and fibrotic area), whereas total glycol-BA/total tauro-BA was positively correlated. Therefore, the accumulation of BAs at 2 weeks of HFC-feeding, led by dysregulated BA synthesis and excretion, may trigger liver damage during the initial stages of NAFLD/NASH. Furthermore, a decrease in nuclear FXR, PXR, and CAR was observed in the livers of rats following HFC feeding. The downregulation of these nuclear receptors may be responsible for the increase in CYP7A1, as well as the decrease in BSEP and MRP2.

4.3. BA detoxification

Toxic BA accumulation in the liver induces hepatocyte injury, and BA hydrophobicity is correlated with cytotoxicity [12]. The order of BA hydrophobicity was reported to be CA < CDCA < DCA < LCA [12]. Hydrophobic BAs are potent inflammatory agents, whereas the hydrophilic BAs are anti-inflammatory [38]. Hydrophobic BAs stimulate ROS generation in hepatic mitochondria and lead to oxidative stress, hepatocyte apoptosis, and subsequent liver damage [50, 51]. BAs with detergent properties may also induce damage in hepatocyte membranes by binding to membrane components and disrupting the integrity of the plasma membrane [12, 52].

BA metabolism is tightly regulated to prevent the retention of excessive BAs in the liver [12]. Sulfation and glucuronidation of BAs, catalyzed by SULT2A1 and UGT, respectively, are major detoxification pathways of Bas [53, 54]. These reactions increase the solubility of BAs, enhance their fecal and urinary excretion, and reduce their toxicity. In addition, the nuclear receptors, PXR and CAR, protect hepatocytes from BA toxicity by regulating the transcription of genes involved in BA detoxification, including SULT and UGT [55, 56]. Our study showed that the HFC diet impaired BA detoxification by inducing the downregulation of PXR and CAR and further suppressing SULT2A1-catalyzed sulfation and UGT-catalyzed glucuronidation in the hypertensive SHRSP5/Dmcr rats [36].

5. CYP7A1

Our previous study showed that dysregulated expression of enzymes involved in BA synthesis led to the accumulation of BA in the livers of SHRSP5/Dmcr rats fed an HFC diet [36]. We further investigate the role of CYP7A1 in the pathogenesis of hypertension-associated NASH, and evaluated its hepatic levels in hypertensive SHR and SHRSP5/Dmcr rats, and the normotensive WKY strain [28]. Constitutive CYP7A1 levels were markedly higher (over 300-fold) in the hypertensive strains compared with those in the normotensive WKY strain. Upregulation of CYP7A1 may result in an excessive accumulation of toxic BAs, such as hydrophobic BAs, which may lead to oxidative stress and liver damage. In addition, Kamisako et al. showed that the Nrf2 pathway may regulate the expression of genes associated with BA synthesis and fatty acid metabolism, including CYP7A1 [57]. Our study showed increased activation of Nrf2 signaling in the livers of hypertensive rats fed a control diet compared with the normotensive WKY, which might be the responsible for the overexpression of CYP7A1 in the hypertensive strains [28].

6. Gender differences in NASH development

The prevalence and severity of human NAFLD/NASH varies with gender and age [58]. Yatsuji et al. studied 193 Japanese patients with NASH (86 women and 107 men) and showed a predominance of the disease in women over 50 years old, yet a greater prevalence in men aged 30–40 years [59]. Williams et al. reported that NAFLD patients were more likely to be male, older, and hypertensive [60]. The incidence of NAFLD/NASH is higher in men than premenopausal women (less than 50 years of age), while this immediately increases in women after menopause. Therefore, sex hormones such as estradiol may influence gender differences in NASH. In our study, we regarded female SHRSP5/Dmcr rats aged 12–24 weeks to correspond to the menopausal age in women. We also found female rats were less susceptible to HFC diet-induced liver damage compared with males [61]. Hence, our rat model may be useful for studies into gender differences in HFC-induced NASH. In order to investigate the related mechanisms, mature female and male SHRSP5/Dmcr rats (10 weeks old) were fed either an HFC or control diet for 2, 8, and 14 weeks. The severity of hepatic fibrosis was markedly lower in the female rats compared with the males. Although HFC feeding significantly reduced serum estradiol levels in female rats at 2 weeks, these levels were still much higher in females compared with males during HFC feeding, suggesting that this female hormone may contribute to the gender difference in NASH. In addition, only minor gender differences were noted in the expression of CYP7A1, CYP8B1, CYP27A1, and CYP7B1, the enzymes involved in BA synthesis, as well as MRP3 and BSEP, the proteins associated with BA transport. On the other hand, the enzymes implicated in BA detoxification, UGT and SULT2A1, as well as the nuclear receptors, CAR and PXR, were significantly suppressed in the male rats fed the HFC diet, whereas expression of these proteins was only slightly changed in females following HFC feeding. Since estradiol, which markedly decreases in women after menopause, may stabilize CAR and PXR proteins [61, 62], these results suggested a stronger capacity of BA detoxification associated with higher estradiol levels may be responsible for the resistance to HFC-induced liver damage and hepatic fibrosis in female rats compared with males.

http://dx.doi.org/10.5772/intechopen.76199

7. Treatment of NAFLD/NASH

NAFLD/NASH is related to poor lifestyle, including unhealthy diet habit and lack of exercise, which may, in turn, lead to excessive weight gain. Therefore, dietary intervention and exercise, targeted at weight loss, are the primary therapies for obesity-related NAFLD/NASH [63]. Vilar-Gomezet al. evaluated the effect of weight loss through lifestyle modifications on the improvement of NASH-related histologic features [64]. The study included 293 patients with NASH who followed a recommended lifestyle over 52 weeks to reduce body weight, including a low-fat, hypocaloric diet (750 kcal per day) and walking (200 min per week). Among these patients, 30% lost ≥5% of their weight at 52 weeks, 25% showed resolution of steatohepatitis, and 47% showed reduced nonalcoholic fatty liver disease activity score (NAS). This study also reported that the extent of weight loss was associated with histologic improvement. A higher proportion of patients with ≥5% weight loss had NASH resolution compared with those with ≤5% weight loss. Furthermore, 45% of patients with ≥10% weight loss showed regression of hepatic fibrosis.

Although NAFLD/NASH is closely linked with obesity and diabetes, it may also occur in the absence of these diseases [65]. As described before, the hypertensive SHRSP5/Dmcr rat represents a good model of NAFLD/NASH without obesity and diabetes [20]. We used this model to investigate the efficacy of dietary intervention for improving HFC-induced NASH [66]. Rats were fed an HFC diet for 2 weeks (before the appearance of hepatic fibrosis) or 8 weeks (after the appearance of fibrosis), then subsequently fed a control diet for 6 or 12 weeks. We found that dietary intervention prior to the appearance of fibrosis markedly improved steatosis and suppressed the HFC-induced increase in serum AST, ALT, and TC. On the other hand, dietary intervention after the appearance of fibrosis was unable to suppress the increase in serum ALT and hepatic TC. Although the dietary intervention (in both cases) reset the increased expression of fibrosis-relative proteins, TGF-β1 and α-SMA, it only slightly reduced the fibrotic area compared with continuous HFC feeding. Taken together, dietary intervention was able to completely or partially improve steatosis, inflammation, and cholesterol accumulation in the livers of rats fed an HFC diet, although this was not enough to improve hepatic fibrosis.

In addition, several pharmacological agents use in the treatment of NASH, including vitamin E and pioglitazone, have been tested [67, 68]. Oxidative stress and insulin resistance are considered as key factors implicated in the progression of NASH, and are, therefore, attractive targets for the treatment of NASH [69]. Sanyal et al. tested the efficacy of vitamin E, a lipid-soluble antioxidant, and pioglitazone, an insulin sensitizer, in NASH patients without diabetes [69]. The 247 patients included in this study received 800 IU vitamin E (84 subjects), 30 mg pioglitazone (80 subjects), or placebo (83 subjects) daily for 96 weeks. Both vitamin E and pioglitazone were associated with improvements in hepatic steatosis and lobular inflammation, as well as a reduction of serum AST and ALT, compared with the placebo. However, neither drug had a significant effect on hepatic fibrosis. In conclusion, lifestyle intervention (controlled dietary intake as well as exercise) may be the first choice for NAFLD/NASH treatment and should be optimized, while pharmacological management can be used as an auxiliary method, and should be further tested in large studies with long-term outcomes.

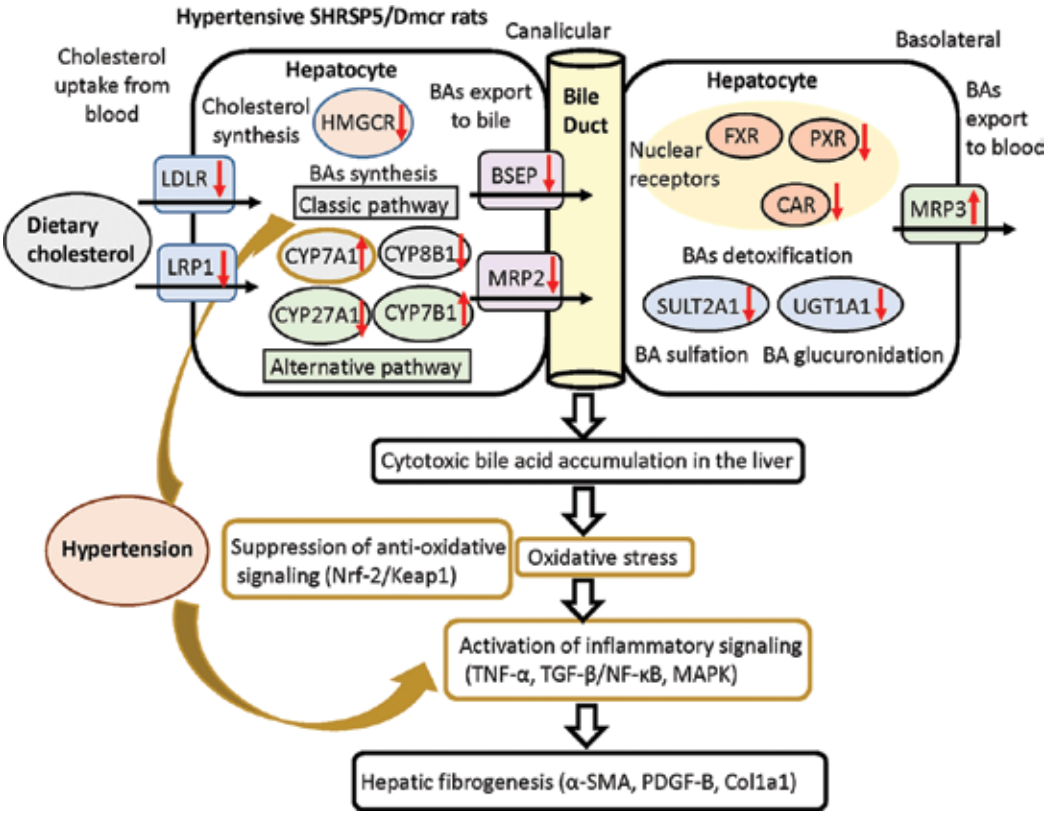

Figure 1. Possible mechanism underlying pathogenesis of HFC diet-induced fibrotic steatohepatitis in hypertensive SHRSP5/Dmcr rats [25, 35, 47]. In response to cholesterol accumulation in the liver triggered by HFC feeding, de novo cholesterol synthesis and its uptake were suppressed, indicated by a reduction in HMGCR, as well as LDLR and LPR1. The HFC diet induced dysregulated BA synthesis (upregulated CYP7A1 and CYP7B1, as well as downregulated CYP8B1 and CYP27A1) and export (downregulated BSEP and MRP2, as well as upregulated MRP3), and led to BA accumulation in hepatocytes. In addition, the HFC diet suppressed BA detoxification by decreasing the expression of nuclear receptors (PXR and CAR), and further downregulating SULT2A1 and UGT1A1, BA detoxification enzymes. Furthermore, cytotoxic BA accumulation in hepatocytes-induced oxidative stress, which activated inflammatory signaling (TNF-α, TGF-β/NF-κB, MAPK) and resulted in hepatitis. Hepatic inflammation-induced upregulation of fibrosis-related genes (α-SMA, PDGF-β, Col1a1) and led to hepatic fibrosis. Additionally, hypertension enhanced the deterioration of HFC-induced fibrotic steatohepatitis by upregulating CYP7A1, further leading to BA accumulation in hepatocytes and increased oxidative stress. On the other hand, hypertension induced the suppression of anti-oxidative signaling (Nrf-2/Keap1) following HFC feeding. Therefore, elevated oxidative stress and suppressed anti-oxidative capacity triggered a more severe inflammatory response in the hypertensive rats fed an HFC diet, as indicated by increased activation of inflammatory signaling (TNF-α, TGF-β/NF-κB, MAPK). BA, bile acid; HMGCR, 3-hydroxy 3-methyl-glutaryl-coenzyme A reductase; LDLR, low density lipoprotein receptor; LRP1, LDLR-related protein 1; CYP7A1, cholesterol 7α-hydroxylase; CYP8B1, sterol 12α-hydroxylase; CYP27A1, sterol 27-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; BSEP, bile salt export pump; MRP2, multidrug resistance-associated protein 2; MRP3, multidrug resistance-associated protein 3; FXR, farnesoid X receptor; PXR, pregnane X receptor; CAR, constitutive adrostane receptor; SULT2A1, sulfotransferase 2A1; UGT1A1, UDP-glucoronosyltransferase 1A1; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor; NF-κB, nuclear factor kappa B; MAPK, mitogen-activated protein kinase; α-SMA, α-smooth muscle actin; PDGF-B, platelet-derived growth factor subunit B; Col1a1, alpha 1 type 1 collagen; Nrf-2, nuclear factor erythroid 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1.

8. Conclusions

In our previous study, we established a novel model of fibrotic steatohepatitis by feeding hypertensive SHRSP5/Dmcr rats an HFC diet. Histological features resembling human NASH were observed in the rats, suggesting that this model is useful for studying hypertension-associated NASH. We compared NASH development among hypertensive strains (SHRSP5/Dmcr and SHR) and the normotensive WKY strain, and showed that hypertension accelerates progression of HFC-induced NASH by elevating BA synthesis (CYP7A1), inducing increased activation of inflammatory signaling (MAPK and NF-κB), and suppressing signaling associated with antioxidant defense (Nrf2/Keap1). To elucidate the role of cholesterol in NASH development, we investigated the kinetics of cholesterol in this model, and found that the HFC diet induced dysregulation of BA synthesis and suppression of BA detoxification, therefore resulting in cytotoxic BA accumulation in hepatocytes, which further induced oxidative stress, followed by activation of signaling involved in hepatic inflammation and fibrosis (**Figure 1**). Sex differences in fibrogenesis were also observed in this model and were associated with a different sensitivity to BA toxicity. More sustained expression of nuclear receptors, CAR and PXR, and the enzymes involved in BA detoxification, UGT and SULT, contributed to the stronger resistance to HFC-induced liver damage in female rats compared with males. In conclusion, our studies demonstrate that dietary cholesterol may play a crucial role in the progression of NASH-associated hypertension and provide a basis for NAFLD/NASH treatment involving restriction of cholesterol intake.

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Chapter 8

Quantitative Proteomic Analysis of Skeletal Muscle Detergent-Resistant Membranes in a Smith-Lemli-Opitz Syndrome Mouse

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.78037

Abstract

Smith-Lemli-Opitz syndrome (SLOS) is an inborn error of metabolism affecting the last step of cholesterol biosynthesis. It is characterized by a deficiency of the enzyme 7-dehydrocholesterol reductase and accumulation of 7-dehydrocholesterol (7DHC) in cells and body fluids. Given the similarities between 7DHC and cholesterol, 7DHC can be incorporated into cell membranes in lieu of cholesterol. Nevertheless, due to their structural differences and distinct affinity to other membrane components, this substitution alters membrane properties and one can expect to find abnormalities in membrane protein composition. In order to identify differences in membrane proteins that could facilitate our understanding of SLOS physiopathology, we isolated detergent-resistant membranes (DRMs) from the skeletal muscle of *Dhcr7*$^{T93M/T93M}$ mice and C57/BL6 controls and performed comparative proteomic analysis using iTRAQ for peptide quantification. A total of 133 proteins were identified in the DRM fraction: 17 (13%) proteins demonstrated increased expression in SLOS mice, whereas, 21 (16%) showed decreased expression. Characterization of functional point of view and bioenergetics pathway and transmembrane transport responded to the major differences between the two groups of animals.

Keywords: skeletal muscle, detergent-resistant membranes (DRMs), Smith-Lemli-Opitz syndrome, SLO, comparative proteomics, mouse model, *Dhcr7*$^{T93M/T93M}$

1. Introduction

Smith-Lemli-Opitz syndrome (SLOS), OMIM #270400, is one of the nine known disorders associated with altered post-squalene cholesterol biosynthesis [1, 2]. This autosomal recessive genetic disease was first described in 1964, as a syndrome of cognitive impairment and multiple malformations [3]. Thirty years were needed to further characterize the disorder as a metabolic disease and identify the underlying enzymatic defect. SLOS is caused by deficiency of 7-dehydrocholesterol redutase (7-DHCR, 3-hydroxysteroid redutase, EC.1.3.1.21) which catalyzes the conversion of 7-dehydrocholesterol (7DHC) to cholesterol, the terminal step of Kandutsch-Russell pathway [4, 5]. Consequently, SLOS patients typically show increased 7DHC and decreased serum and tissue cholesterol levels [5]. In 1998, mutations of the 3β-hydroxysterol Δ7-reductase gene (*DHCR7*) were shown to cause SLOS [6–8] and more than 154 *DHCR7* mutations have been so far identified in SLOS patients [9]. The development of animal models has improved the understanding of SLOS physiopathology and provided material for *in vivo* and *in vitro* investigation of biological consequences of cholesterol deficiency. In 2001, two mouse models with null mutations in *Dhcr7* gene have been created by homologous recombination [10, 11]. The malformations in the null mice are very mild compared to what would be seen in a null human infant (i.e., SLO type II). The mutant mice died within the first 24 h of extra-uterine life. Later, a mouse model with a milder phenotype was developed [12]. This hypomorphic mouse has a missense mutation equivalent to the human p.T93M, previously identified in SLOS patients often with Mediterranean heritage [13–15]. Like the majority of SLOS patients, the SLOS mouse models manifest a deficiency in cholesterol biosynthesis resulting in low levels of cholesterol in serum and tissues [11].

Effective cholesterol biosynthesis is especially critical at certain stages during development and continues to be important throughout life [16], since cholesterol is an essential lipid with multiple functions. Cholesterol is a major lipid component of membrane microdomains, which are crucial cell-surface dynamic structures responsible for many cellular signaling and communication events [17]. Membrane domains can form through a number of mechanisms involving lipid-lipid and protein-lipid interactions. One type of membrane domain is the cholesterol-dependent membrane raft [18]. Properties of these membrane domains have been primarily inferred from the study of detergent-resistant membranes (DRMs), composed by the non-ionic-detergent insoluble, low-buoyant density membranous fractions of cells [19]. Although it was initially thought that such microdomains enriched in cholesterol exist exclusively in the plasma membrane, increasing evidence suggests that similar lipid microdomains (sometimes referred as raft-like microdomains) are also present in internal organelles [20–22] with some of them being involved in the crosstalk between organelles [23].

Proteomics constitutes a powerful tool to study complex biological mechanisms and to identify alterations in protein expression induced by changes in the environment, drugs, or disease states. As such, proteomics is now widely employed to help understand pathological processes induced by the disease [24–27]. The effect of an inborn error of cholesterol synthesis on skeletal muscle has not previously been reported. In this chapter, we report a comparative analysis of protein expression in skeletal muscle DRMs isolated from *Dhcr7* T93M homozygous mutant

http://dx.doi.org/10.5772/intechopen.78037

mice (*Dhcr7*$^{T93M/T93M}$) and wild type controls (*Dhcr7*$^{+/+}$) controls. We analyzed sterols by GC–MS and we used amine-reactive isobaric tagging reagents (iTRAQ) for quantitative subcellular proteomics. We found the altered expression of key muscle proteins involved with bioenergetics, membrane transport and Ca^{2+} homeostasis.

2. Material and methods

2.1. Materials

Butylhydroxytoluene (BHT), N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA), triethylammonium bicarbonate (TEAB), trifluoroacetic acid (TFA), α-cyano-4-hydroxycinnamic acid (α-CHCA), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), protease inhibitor cocktail, formic acid and urea were purchased from Sigma (Karlsruhe, Germany). RC DC Protein Assay kit for protein quantification was from BioRad lab (Hercules, USA). The iTRAQ kit was purchased from Applied Biosystems (Foster City, CA). Sequencing grade modified trypsin (bovine) was from ABSciex (ABSciex, USA). HPLC-grade acetonitrile (ACN, Riedel, Seelze, Germany) and Milli-Q grade water were also used. Rabbit raised polyclonal anti-caveolin 1 (ab2910) and anti-annexin A2 antibodies were purchased from Abcom, Cambridge, UK. Analytical reagent grade chemicals were used unless stated otherwise.

2.2. Animals

The T93M mutation [12] was backcrossed into C57/BL6 for three generations. Homozygous (T93M/T93M) mice are viable and fertile [16]. Control C57/BL6 mice were obtained from IBMC Animal Centre from Oporto University.

All the animals were housed in plastic cages with free access to water and food (cholesterol free —chow -Mucedola, Ref: 4RF21). The animals were handled and maintained in controlled conditions according to international standards. Animals were euthanized using deep isoflurane anesthesia when they were 10 days old. Gastrocnemius and soleus muscles were dissected, submerged in ice-cold buffer (TRIS, HCl, pH 7.4, 10 mM mercaptoethanol, 0.28 M sucrose) and immediately frozen at −80°C.

2.3. DRMs extraction

Detergent-resistant membranes (DRMs) were isolated using cold Triton X-100 treatment followed by sucrose gradient centrifugation. In order to minimize individual variation, samples were analyzed as pools of several animals. The procedure was adapted from Kim et al. [28]. Briefly, the samples were allowed to thaw slowly on ice, and tissue from three mice (approximately 300 mg) was minced with scissors, mixed with 700 μL of cold (4°C) lysis buffer (25 nM HEPES-HCl, pH 6,5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail) and homogenized 30 times with a Poter homogenizer. The sample was maintained in

an ice bath during homogenization and then incubated for 30 min at 4°C. Aliquots of this homogenate were collected for protein quantification and sterol analysis.

The resulting extract was mixed with an equal volume of cold sucrose 80% (w/v) to give a final sucrose concentration of 40%, transferred to the bottom of a ultra-centrifuge tube (Ultra-clear 14 × 89 mm, Beckman Ref 344059) and overlaid carefully with 6.0 mL 30% and 3.0 mL 5% cold sucrose solutions, containing 25 nM HEPES-HCl, pH 6.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100. Discontinuous sucrose gradients were centrifuged for 20 h, at 187,000 g on a Sorvall Ultra Pro 80 UC, swinging bucket SW41 at 4°C. The DRMs fraction, which collects at the interface of the 5 and 30% sucrose layers, was isolated, washed with 9 mL of modified HEPES buffer and centrifuged 30 min at 49,500 g at 4°C. After centrifugation, the supernatant was discarded and the pellet containing purified DRMs was suspended in phosphate buffered saline (PBS) and stored frozen at −80°C until further proteomic or sterol analysis.

2.4. Sterols analysis

For extraction of neutral sterols, 50 μL of sample was mixed with 50 μL of BHT 0.05% in methanol (antioxidant), 50 μL of epicoprostanol 0.10 mmol/L (internal standard) and saponified at 60°C for 1 h by 16 adding 1 mL of 4% (w/v) KOH in 90% ethanol. The samples were then diluted with 1 mL of deionized water and the lipids were extracted twice with 2 mL of hexane. The pooled hexane extracts were dried under a gentle nitrogen stream at room temperature and derivatized with 50 μL BSTFA in 50 μL pyridine at 60°C for 1 h [29]. The trimethylsilylether derivatives of sterols were separated by GC–MS with a Supelco 28471_U SLB-5MS column (30 m × 0.32 mm i.d., 0.25 μm film thickness). The injector temperature was set at 270°C, and the splitless injection mode was used. The initial column temperature was 180°C for 1 min and programmed to increase at a rate of 20°C/min to 250°C and then increased again at 5°C/min rate till 300°C. The carrier gas was helium at a linear constant flow rate of 40 cm/s. The interface was programmed to 280°C, the quadrupole to 150°C and the ionization source to 230°C. After a solvent delay of 2.5 min, the eluted sterols were identified by their retention times (comparing with commercial standards) and respective mass spectra and quantified by selected ion monitoring (**Table 1**). The method was linear and reproducible for the range of amounts assayed.

Sterol	Retention time		m/z	
Epicoprostanol	13.02	**370**	257	355
Cholesterol	13.72	**329**	353	368
7DHC	14.02	**325**	351	366
Desmosterol	14.10	**343**	327	
Sitosterol	14.80	**357**	396	486
Lathosterol	15.80	**255**	443	458

The m/z ions selected for sterol quantification are printed in bold.

Table 1. Sterol parameters.

2.5. Proteomic profile

2.5.1. Pellet solubilization and protein quantification

Each assay involved four sample pools: a wt-BL6 control and a *Dhcr7*$^{T93M/T93M}$ and their duplicates. For protein extraction, the DRMs pellets were treated with 10 volumes (w/v) of solubilization buffer (7 M urea, 2 M thiourea, 1% (w/v) CHAPS, 1% (w/v) Triton X-100, 1% (v/v) ampholytes (3–10) and 1 mM TCEP), sonicated briefly, then incubated for 10 min at room temperature under agitation. Clear cell lysates were obtained by centrifugation at 12,000×*g* for 30 min at 4°C and saved for analysis. Insoluble material was discarded.

The protein concentration was determined by RC DC Protein Assay kit, using BSA to generate the calibration curve.

2.5.2. Sample preparation for mass spectrometry analysis

Reduction, alkylation and digestion steps were performed according to the protocol provided by the manufacturer. Briefly, 100 mg of DRM protein was mixed with TEAB buffer (1 M, pH 8.5) and an enhancer of enzymatic digestion RapiGest (Waters) to give a final concentration of 0.5 M and 0.1%, respectively. Samples were then treated with: (1) a reducing agent—TCEP 5 mM—used to break disulfide bonds within and between proteins, for 1 h at 37°C and (2) a cysteine (sulfhydryl group) blocking reagent—S-methyl methanethiosulfonate (MMTS) 10 mM—for 10 min, at room temperature. Then, 2 mg of trypsin was added to each sample and the digestion was performed for 18 h at 37°C. The digested tryptic specimens were dried using a Speed-Vac. iTRAQ labeling was carried out according to the instructions provided by the manufacturer. *Dhcr7*$^{T93M/T93M}$ mice DRMs samples were marked with iTRAQ Tags 116 and wt-BL6 with114 and the duplicates with 115 and 113, respectively. The labeled samples were combined in pairs and dried in a Speed-Vac. The peptides were separated by reverse-phase liquid chromatography as previously published [30].

2.5.3. LC-MS/MS analysis

Peptide mass spectra were obtained in the mass range 700–4500 Da on a MALDI-TOF/TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA) in the positive ion reflector mode. The obtained spectra were processed and analyzed by the ProteinPilot® software (v4.0 AB Sciex, USA), which uses an algorithm for protein/peptide identification based on the comparison of MS/MS data against the SwissProt protein database [31].

Protein clustering was performed according to biological and molecular functions derived from the PANTHER classification system.

2.6. Immunocytochemistry assay

Immunocytochemistry assays were performed in order to confirm the presence of (1) caveolin-1, a protein marker of a subtype of membrane microdomains which are rich in cholesterol, designated caveolae (anti-caveolin-1 antibody dilution 1:500) and (2) annexin 2 another protein associated with enriched cholesterol microdomains, that was found increased in the SLO

mouse model (antibody dilution 1:2000). A sequential incubation with a secondary biotinylated anti-rabbit antibody was performed and diaminobenzidine (which stains brown) was employed as chromogen. Skeletal muscle samples were then counterstained with hematoxylin.

3. Results and discussion

3.1. Sterols

Sterols were extracted from two pools of biological samples: muscle homogenates and muscle DRMs, and then analyzed by GC-MS. Results are presented as the average ratios between sterols amounts. We observed a markedly elevated cholesterol/7DHC ratio in muscle homogenates from wild-type animals, reflecting the large amount of cholesterol and minimal levels of its precursor (7DHC), in controls. This ratio was also much greater than one in *Dhcr7*$^{T93M/T93M}$ mice, indicating that affected mice have significant residual 7-DHCR enzymatic activity and are thus capable of producing significant amounts of cholesterol (**Figure 1A**).

Desmosterol is a cholesterol precursor by an alternative biosynthetic route, and there are no significant differences in desmosterol levels between controls and affected animals. This is also reflected in the 7DHC/desmosterol ratio. This parameter showed an enrichment of *Dhcr7*$^{T93M/T93M}$ animals' DRMs in 7DHC (**Figure 1B**). The ratio 7DHC/cholesterol also indicates that 7DHC is preferentially incorporated in membrane microdomains (**Figure 1C**). These findings corroborate the previous ones published by Rakheja and Boriack, based on liver analysis of SLOS patients, which showed that 7DHC accumulates in hepatic DRMs [32]. Furthermore, while wt-BL6 controls have essentially only cholesterol in DRMs, affected mice present a mixture of cholesterol and 7DHC (**Figure 2**).

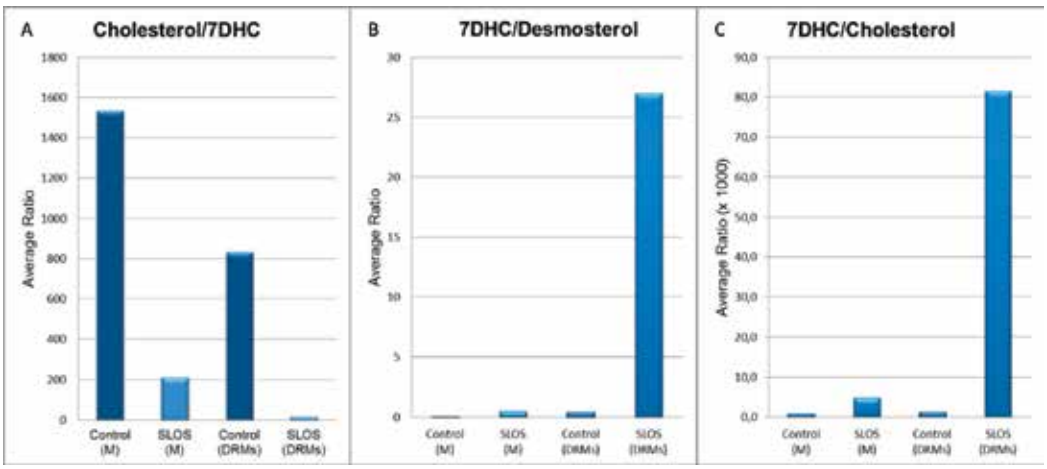

Figure 1. Average ratios between the amounts of: cholesterol and 7DHC (A), 7DHC and desmosterol (B) and 7DHC and cholesterol (C), extracted from skeletal muscle homogenates (M) and DRMs of wt-BL6 controls and hypomorphic *Dhcr7*$^{T93M/T93M}$ mice pooled samples.

http://dx.doi.org/10.5772/intechopen.78037

3.2. Proteomics

In order to explore the protein changes on sarcolemma, due to decreased 7-DHCR activity, we analyzed DRMs utilizing iTRAQ labeling and LC-MS/MS.

A total of 133 unique proteins were identified. Those identified based on a single peptide and those with a protein score less than 2.5 fold were excluded. Then a cut-off of 30% was applied to iTRAQ average ratios allowing us to select proteins with an important variation in SLOS mice relatively to controls. Differential protein expression was specific and not just a general finding. Caveolin-1, a protein known to be expressed in cholesterol-rich membrane microdomains did not show differential expression (**Figure 3**).

Of the 133 identified proteins, we observed an altered expression of 38 (29%) proteins. Increased and decreased expression was observed for 17 and 21 proteins, respectively (**Table 2**). The replicate samples demonstrated a strong positive correlation (r = 0.90) and indicated good reproducibility (**Figure 4**).

Most proteins showing an altered expression in DRMs preparations were found to participate in at least one of three main cellular processes: membrane trafficking, energy production and Ca^{2+} homeostasis.

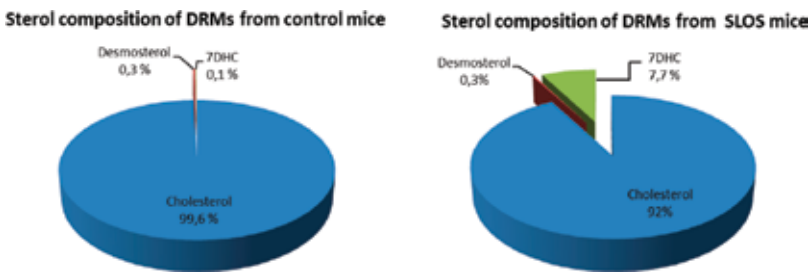

Figure 2. Comparative analysis of sterol composition of DRMs extracted from skeletal muscle of wt-BL6 controls and hypomorphic *DHCR7*$^{T93M/T93M}$ (SLOS) mice.

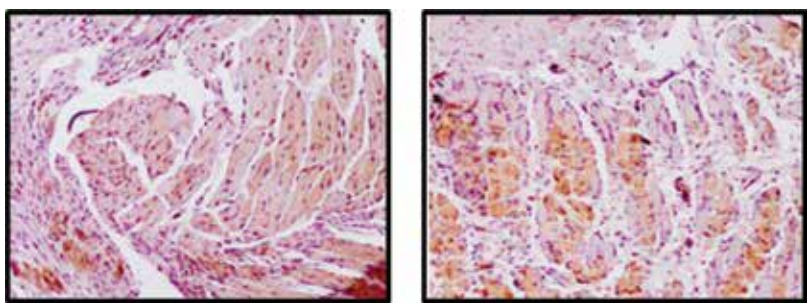

Figure 3. Immunohistochemical stain for caveolin-1 (×10) shows no significant differences between *Dhcr7* $^{93M/93M}$ (on the left) and wt-BL6 (on the right) skeletal muscle samples.

Total score	Sequence coverage %	Accession #		Name	Peptides (95%)	SLOS/wt-BL6
12.5	37	P07356	ANXA2_MOUSE	Annexin A2	15	6.2
6.6	29	P16858	G3P_MOUSE	Glyceraldehyde-3-phosphate dehydrogenase	4	5.2
51.2	50	Q8R429	AT2A1_MOUSE	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1	85	3.9
11.6	53	P07310	KCRM_MOUSE	Creatine kinase M-type	12	2.7
6.7	41	P27573	MYP0_MOUSE	Myelin protein P0	10	2.0
18.5	37	Q8K2B3	DHSA_MOUSE	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	18	1.9
17.4	59	P51881	ADT2_MOUSE	ADP/ATP translocase 2	24	1.9
2.5	9	P09055	ITB1_MOUSE	Integrin beta-1	3	1.9
29.5	56	P56480	ATPB_MOUSE	ATP synthase subunit beta, mitochondrial	35	1.8
4.1	46	Q9D3D9	ATPD_MOUSE	ATP synthase subunit delta, mitochondrial	4	1.6
14,5	14	O09165	CASQ1_MOUSE	Calsequestrin-1	22	1.6
4,6	31	Q8C7E7	STBD1_MOUSE	Starch-binding domain-containing protein 1	7	1.5
10.0	17	P70302	STIM1_MOUSE	Stromal interaction molecule 1	7	1.5
25.4	70	P48962	ADT1_MOUSE	ADP/ATP translocase 1	39	1.5
6.4	33	Q8VEM8	MPCP_MOUSE	Phosphate carrier protein, mitochondrial	9	1.4
19.1	59	Q03265	ATPA_MOUSE	ATP synthase subunit alpha, mitochondrial	27	1.3
3.0	47	Q9CQQ7	AT5F1_MOUSE	ATP synthase subunit b1, mitochondrial	14	1.3
4.0	19	Q5U458	DJC11_MOUSE	DnaJ homolog subfamily C member 11	3	0.8
23.3	45	Q8BMK4	CKAP4_MOUSE	Cytoskeleton-associated protein 4	26	0.8
5.5	50	Q9CR68	UCRI_MOUSE	Cytochrome b-c1 complex subunit Rieske, mitochondrial	9	0.7
4.2	82	P99028	QCR6_MOUSE	Cytochrome b-c1 complex subunit 6, mitochondrial	13	0.7
5.0	73	P56391	CX6B1_MOUSE	Cytochrome c oxidase subunit 6B1	9	0.6
5.1	2	Q8VDD5	MYH9_MOUSE	Myosin-9	5	0.6
4.4	53	Q9DCS9	NDUBA_MOUSE	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	14	0.6
4.2	7	P99024	TBB5_MOUSE	Tubulin beta-5 chain	7	0.6
20.1	46	P08121	CO3A1_MOUSE	Collagen alpha-1(III) chain	25	0.6
26.0	56	Q9CZ13	QCR1_MOUSE	Cytochrome b-c1 complex subunit 1, mitochondrial	38	0.6
6.5	60	P97450	ATP5J_MOUSE	ATP synthase-coupling factor 6, mitochondrial	19	0.6
36.1	62	Q01149	CO1A2_MOUSE	Collagen alpha-2(I) chain	47	0.6
5.3	15	Q9Z239	PLM_MOUSE	Phospholemman	5	0.5

Total score	Sequence coverage %	Accession #		Name	Peptides (95%)	SLOS/wt-BL6
4.5	59	Q9CPQ1	COX6C_MOUSE	Cytochrome c oxidase subunit 6C	6	0.5
66.9	35	P13542	MYH8_MOUSE	Myosin-8	71	0.5
10.1	57	Q9D6J5	NDUB8_MOUSE	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial	13	0.5
3.0	29	Q99LY9	NDUS5_MOUSE	NADH dehydrogenase [ubiquinone] iron-sulfur protein 5	2	0.5
5.9	13	P68134	ACTS_MOUSE	Actin, alpha skeletal muscle	5	0.4
8.8	46	P19783	COX41_MOUSE	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	12	0.4
2.6	3	P68369	TBA1A_MOUSE	Tubulin alpha-1A chain	2	0.2
3.0	4	Q08857	CD36_MOUSE	Platelet glycoprotein 4	2	0.2

Table 2. Proteins from the skeletal muscle DRMs with altered expression *Dhcr7*$^{T93M/T93M}$ mice.

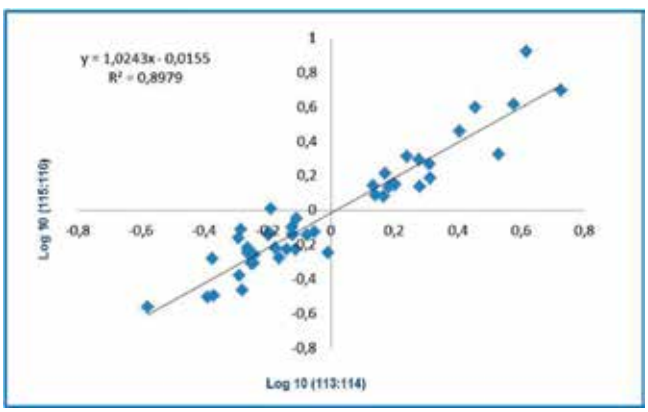

Figure 4. Comparison of the individual ratio values found for 38 proteins of DRMs extracted from skeletal muscle, which exhibited distinct levels of expression on hypomorphic *Dhcr7*$^{T93M/T93M}$ mice and wt-BL6 controls.

Proteins with altered expression corresponded to a number of biological processes. We found alterations affecting several membrane transporters. Even though no results from skeletal muscle studies of SLOS are available, membrane trafficking abnormalities, in other cells harboring inborn errors of cholesterol biosynthesis, had already been reported. For example, in cultivated human skin fibroblasts from SLOS patients the membrane fluidity is altered, calcium permeability is augmented whereas folate uptake and membrane-bound Na^+/K^+ ATPase activity are markedly decreased [33]. In agreement with these data, we now report decreased expression of phospholemman, a small plasma transmembrane protein that acts as a channel or channel regulator and modulates Na^+/K^+ ATPase activity [34, 35]. Further, we detected significantly decreased expression of another integral membrane glycoprotein associated with DRMs, the fatty acid translocase (FAT also called Cd36 or platelet glycoprotein 4) on

sarcolemma of SLOS mice, responsible for the uptake of long chain fatty acids [36–39]. Upregulated transporters include mitochondrial phosphate carrier protein (which transports inorganic phosphate into the mitochondrial matrix, essential for the aerobic synthesis of ATP) and ADP/ATP translocases 1 and 2 (that catalyzes the exchange of cytoplasmic ADP with mitochondrial ATP across the mitochondrial inner membrane).

These abnormalities of mitochondrial transporters may be indicative of more generalized defect in mitochondrial function and ATP production. Decreased expression of subunits corresponding to complexes I, III and IV of the oxidative phosphorylation (OXPHOS) system was observed, while four ATP-synthase subunits showed increased expression (ATP synthase mitochondrial subunits alpha, beta, delta and b1). Despite their mitochondrial origin, these proteins should not be considered as contaminants of DRMs preparations. Several biochemistry and proteomic studies had already shown the presence of complex I and ATP-synthase subunits in DRMs, [40, 41] and Poston showed that Triton X-114-resistant DRMs are also present in mitochondria and contain proteins that facilitate ATP production and export from this organelle [23], compatible with the increasing evidence of the presence of raft-like microdomains in mitochondria [21, 42].

Ca^{2+} is one of the most important signaling compounds involved in various cellular processes being intracellular Ca^{2+} levels tightly regulated by specialized proteins in the plasma membrane, sarcoplasmic reticulum (SR) and mitochondria [43]. Recent studies suggest that membrane rafts are involved in coordinating the protein interactions required for proper Ca^{2+} exchange between the MAM and mitochondria [23]. In the present study, we found increased expression of four membrane proteins related with Ca^{2+} homeostasis namely: annexin A2, sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (SERCA1), calsequestrin-1 (CASQ1) and stromal interaction molecule 1 (STIM1).

Annexin A2 is a calcium-regulated membrane-binding protein, which holds two calcium ions. It has been proposed that it could play a key role in many processes including, (1) endocytosis and (2) exocytosis, (3) ion channel conductance, (4) link of F-actin cytoskeleton to the plasma membrane, (5) membrane organization, (6) formation of membrane cholesterol-rich microdomains and (7) regulation of cellular redox [44–46]. By LC-MS/MS, we found that annexin A2 was six times more abundant in DRMs obtained from *Dhcr7*$^{T93M/T93M}$ mice than in wt-BL6 and then confirmed such difference by immunocytochemistry (**Figure 5**).

It is possible that the presence of 7DHC in SLOS mice membranes drives a higher annexin A2 incorporation in microdomains since 7DHC may promote microdomain formation [47]. An alternate hypothesis would be that increased annexin A2 expression is related to its role in the regulation of redox potential. In fact, several data suggest an increase of oxidative stress in SLOS: (1) over a dozen of oxysterols have been produced from 7DHC by free radical oxidation in solution [48], (2) the oxysterol mixture derived from 7DHC free radical oxidation is biologically active and leads to morphological changes in Neuro2a cells treated with these oxysterols [49], (3) the synthesis of 3β,5α-dihydroxycholest-7-en-6-one (DHCEO), an oxysterol recently identified as a biomarker of 7DHC oxidation (in fibroblasts from SLOS patients and brain tissue), was found to be inhibited by an antioxidant compound in SLOS fibroblasts [47], (4) retinas from a SLOS rat model contain high levels of lipid hydroperoxides [50], (5) 7DHC

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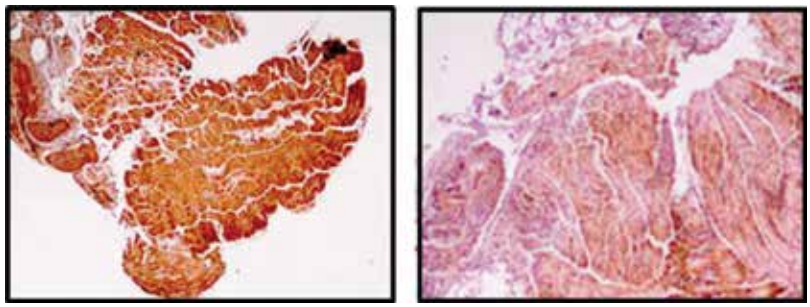

Figure 5. Immunohistochemical stain for annexin A2 (×4). Skeletal muscle samples of *Dhcr7*$^{93M/93M}$ (left) and wt-BL6 (right). The *Dhcr7*$^{93M/93M}$ (SLOS) sample shows a clear stronger coloration.

peroxidation is a major source of oxysterols observed in cells [51] and (6) we previously identified a significant increase of the antioxidant enzyme superoxide dismutase mitochondrial in cultivated human SLOS fibroblasts. Considering these facts, we can hypothesize that there is an alteration of redox state of the cells in SLOS and annexin 2 is overexpressed as a part of cell strategy to compensate such a situation and protect cells' biological compounds from peroxidation.

Another Ca^{2+} binding protein found upregulated in SLOS was SERCA1 an intracellular pump located in the SR of muscle cells, which catalyzes the hydrolysis of ATP coupled with the translocation of Ca^{2+} from the cytosol to the SR lumen, thus contributing to calcium sequestration involved in muscular excitation/contraction process. Another protein from this group is CASQ1, the major Ca^{2+}-binding protein in the skeletal muscle SR. CASQ1 acts as an internal calcium store in muscle. The release of calcium bound to this protein through a calcium release channel triggers muscle contraction. Finally, stromal interaction molecule is a transmembrane protein essential for the activation of store-operated Ca^{2+} entry (SOCE), a major Ca^{2+} influx mechanism.

We also found integrin beta-1, overexpressed in affected mice. This microdomain-associated protein belongs to the integrin family which incorporates heterodimeric transmembrane proteins that function as major receptors for extracellular matrix proteins [52]. Integrin beta-1, plays a role in the maintenance of the cytoarchitecture of mature muscle as well as in the functional integrity of the muscle cells and is present at the neuromuscular junctions in skeletal muscle ones [53]. One wonders if its overexpression could be one of the factors which protect skeletal muscle from severe dysfunction in SLOS in opposition to other organs.

Ordered domains are formed when actin filaments attach to the plasma membrane [54]. Kwik and collaborators found changes in the organization and activity of actin and actin-modifying proteins after cholesterol depletion, and Gangully and Chattopadhyay demonstrated that cholesterol depletion mimics the effect of cytoskeletal destabilization [55, 56]. Differences in myofilaments and cytoskeleton proteins were also suspected in our study.

Furthermore, some of the proteins identified were not so far, according to the available bibliography, associated with lipid-rafts or MAMs; nevertheless, they are involved in intracellular

physical connections like cytoskeleton-associated protein 4 (CKAP4) which is a transmembrane protein that further to its function as receptor also links endoplasmic reticulum (ER) to the cytoskeleton [57]. It is predictable that a membrane compact microdomain be involved in such function, in order to provide further support to the anchor. Such hypothesis is sustained by the fact that CKAP4 is a reversibly palmitoylated protein [58], and it is well described that palmitoylation of cytoplasmic proteins regulates the interaction of these soluble proteins with specific membranes or membrane domains. It is possible that palmitoylation controls the conformation of transmembrane segments, to modify the affinity of a membrane protein for specific membrane domains and to control protein–protein interactions [59].

Finally one should consider that both lipids and proteins for microdomains constructs are synthesized in the ER/Golgi before transport to the plasma membrane and, indeed, those proteins can be in a detergent-resistant, cholesterol-dependent state while residing there or in vesicle trafficking [60].

4. Conclusion

Our purpose was to contribute to the biological characterization of this *Dhcr7*$^{T93M/T93M}$ hypomorphic mouse model and identify differences that could help to go deeper in the understanding of SLOS physiopathology.

Proteomic analysis of DRMs of skeletal muscular samples clearly show differences between SLOS and wild-type mice, concerning proteins linked to membranes. The employed methodology allowed us to identify further alterations related with calcium homeostasis and membrane trafficking associated with SLOS and detected, for the first time, changes in mitochondrial energy metabolism in this mouse model.

To the best of our knowledge, this is the first research study focusing on the skeletal muscle of SLOS. The differential protein expression profile identified will open the way to comparative studies with more severally affected disease mice models as well as to similar approaches in human cells which may be helpful to uncover cellular mechanisms related to SLOS.

Acknowledgements

The authors are thankful to Prof Forbes D. Porter and Doctor Christopher A. Wassif from NIH who kindly provided the genetically modified mice model of SLOS used in this investigation. FDP and CAW are supported by the intramural research program of 30 the Eunice Kennedy Shriver National Institute of Child Health and Human Development.

Requimte received finnantial support from the European Union (FEDER funds POCI/01/0145/FEDER/007265) and National Funds (FCT/MEC, Fundação para a Ciência e Tecnologia and Ministério da Educação e Ciência) under the Partnership Agreement PT2020 UID/QUI/50006/2013.

This work received financial support from FCT/MEC through national funds and co-financed by FEDER, under the Partnership Agreement PT2020 (UID/MULTI/04378/2013 – POCI/01/0145/FERDER/007728).

Conflict of interest

The authors declare that there are no conflicts of interest.

Abbreviations

Cd36	cluster differentiation 36
BHT	butylhydroxytoluene; 2,6-bis(tert-butyl)-4-methylphenol
BSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
CASQ1	calsequestrin-1
α-CHCA	α-cyano-4-hydroxycinnamic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
D	Day
7DHC	7-dehydrocholesterol
DHCEO	3β,5α-dihydroxycholest-7-en-6-one
7-DHCR	7-dehydrocholesterol redutase
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
iTRAQ	isobaric tagging reagents
DRMs	detergent-resistant membranes
FAT	fatty acid translocase
GTP	guanine triphosphate
LCFAs	long-chain fatty acids
MMTS	S-methyl-methanethiosulfonate
mt-DNA	mitochondrial DNA

OXPHOS	oxidative phosphorylation
PBS	phosphate buffered saline
PIP2	phosphatidylinositol-4,5-biphosphate
SLOS	Smith-Lemli-Opitz syndrome
Dhcr7$^{T93M/T93M}$	Smith-Lemli-Opitz syndrome mouse, homozygous for T93M mutation
SR	sarcoplasmic reticulum
STIM1	stromal interaction molecule 1
TEAB	bicarbonate salt of triethylamine
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
TFA	trifluoroacetic acid
UC	ultra-centrifuge
VDAC	voltage dependent anion-selective channels
wt-BL6	wild-type C57/BL6

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Edited by Madan L. Nagpal

"What is this cholesterol?" In this book entitled *Cholesterol- Good, Bad, and the Heart*," now you got the answers given by the experts in the field. Moreover, you can explore more by reading the references/citations given in the articles of each chapter. It is still an emerging field and lot more is being discovered. You will be amazed how much knowledge is already there in this book on cholesterol. You will realize that cholesterol is an essential and extremely important building block of cell membranes and thus serves vital functions in the body. I believe this book will be incredibly powerful and useful in teaching to give new perspectives on cholesterol.

Published in London, UK

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ISBN 978-1-83881-688-9