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REVIEW

Cholesterol: from feeding to gene regulation

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Abstract We present here a brief description of the path that cholesterol covers from its intestinal absorption to its effects exerted on gene regulation. In particular, the relationship between cholesterol and the protein complexes involved in the intricate gene regulation mechanism implicated in cholesterol homeostasis will be discussed. In addition, a new target role for the pharmacological interventions of one of these factors, the insulin-induced gene (Insig) protein, will be introduced.

Keywords Cholesterol · HMG-CoA reductase · Insig · LDLr · SCAP · SREBPs

Abbreviations

HMG-CoAR	3-Hydroxy-3-methylglutaryl coenzyme A reductase
HMG-CoAS	3-Hydroxy-3-methylglutaryl coenzyme A synthase
PGDH	6-Phosphogluconate dehydrogenase
DHCR	7-Dehydrocholesterol reductase
ACC	Acetyl-CoA carboxylase
ACAT	Acyl-CoA:cholesterol acyltransferase
aa	Amino acid
APP	Amyloid peptide
Apo	Apoprotein
ABC	ATP binding cassette
bHLH-Lzip	Basic-helix–loop–helix-leucine zipper
CMs	Chylomicrons
Cyp7α	Cholesterol 7α hydroxylase
CETP	Cholesteryl ester transfer protein
CBP	CREB binding protein
ADD1	Determination and differentiation factor 1
ERC	Endocytic recycling compartment
ER	Endoplasmic reticulum
FPP	Farnesyl diphosphate
FAS	Fatty acid synthase
GPP	Geranylgeranyl pyrophosphate synthase
GPAT	Glycerol-3-phosphate acyltransferase
G6PD	Glucose-6-phosphate dehydrogenase
HDL	High-density lipoprotein
Insig	Insulin-induced gene
CY51	Lanosterol 14α-demethylase
LPL	Lipoprotein lipase
LXR	Liver X receptor
LDL	Low-density lipoprotein
LDLr	Low-density lipoprotein receptors
MAPK	Mitogen-activated protein kinase
ALLN	*N*-Acetyl-L-leucyl-L-leucyl-norleucinal
NPC1L1	Nieman-Pick C1-like protein1
nSREBP	Nuclear SREBP
PI(3)-kinase	Phosphatidyl inositol (3) kinase
PM	Plasma membrane
PKB	Protein kinase B
PKCk	Protein kinase Ck
SR-B1	Scavenger receptor class B type 1
SCAP	SREBP cleavage activating protein
S1P	Site 1 protease
S2P	Site 2 protease
SUMO–1	Small ubiquitin-related modifier-1
SCD	Stearoyl-CoA desaturase
SSD	Sterol sensing domain
SRE	Sterol regulatory element

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SREBPs	Sterol regulatory binding proteins
S_f	Svedberg flotation
TG	Triglyceride
VLDL	Very low-density lipoprotein
DRIP	Vitamin D receptor-integrating protein
WAT	White adipose tissue

Introduction

Cholesterol (Fig. 1) plays several structural and metabolic roles that are vital to human biology. Although cholesterol spreads along the entire plasma membrane of the cell, where it modulates fluidity, it also concentrates in specialized sphingolipid-rich domains called rafts and caveolae [95]. In addition, cholesterol is a substrate for steroid hormones [93]. Too much cholesterol in cells, however, can have pathological consequences. This is particularly true for cells of the artery wall, where accumulation of cholesterol initiates atherosclerotic cardiovascular disease [132]. Therefore, the body relies on a complex homeostatic network to modulate the availability of cholesterol for tissues. This network operates on both the cellular level and within the plasma compartment. Cholesterol is both synthesized by cells and taken in with food intake. The liver is the principal site for cholesterol homeostasis maintenance [19], carried out in many mechanisms, such as biosynthesis, via 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR, E.C. 1.1.1.34) activity, uptake through low-density lipoprotein receptors (LDLr), lipoprotein release in the blood, storage by esterification, degradation and conversion into bile acids [130].

The path from cholesterol intestinal absorption to the cholesterol dependent gene regulation will be described in the following.

Intestinal cholesterol absorption

Intestinal cholesterol absorption is most accurately defined as the transfer of intraluminal cholesterol into intestinal or thoracic duct lymph. Absorption needs to be distinguished from "cholesterol uptake", which refers to entry of cholesterol into intestinal absorptive cells.

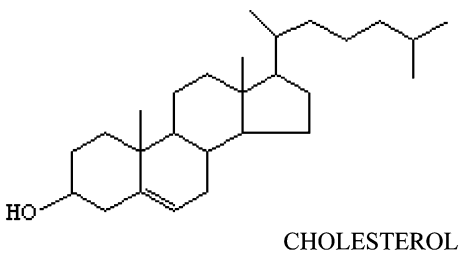

Fig. 1 Cholesterol structure

Cholesterol enters the lumen of the small intestine via three sources: diet, bile and intestinal epithelial sloughing. The average daily cholesterol intake in the Western diet is approximately 300–500 mg. Bile provides 800–1,200 mg cholesterol per day to the intraluminal pool. The turnover of intestinal mucosa epithelium establishes a third source of intraluminal cholesterol, which is estimated to contribute 300 mg cholesterol per day. The main sites of absorption are the duodenum and proximal jejunum [59].

Cholesterol absorption begins in the stomach when diet constituents are mixed with lingual and gastric enzymes. The stomach also regulates the delivery of gastric chyme to the duodenum where it is mixed with bile and pancreatic juice. This process continues within the lumen of the small intestine. Some of the products derived from the lipolytic enzyme activity, including cholesterol, are nearly insoluble in a pure aqueous system and are therefore dependent on the solubilizing properties of bile salt solution [41, 106, 125, 126]. Bile salts are biological amphipathic detergents and can spontaneously form aggregates micelles. Although simple micelles are able to dissolve lipids, cholesterol is only sparingly soluble in bile salt solutions. The addition of phospholipid or monoacylglyceride to bile salt solution strikingly augments cholesterol solubility by forming mixed micelles [23, 122].

Before cholesterol molecules in the small intestinal lumen can interact with a cholesterol transporter for uptake and subsequent transport across the brush border of the enterocyte, micelles must pass through a diffusion barrier that is located at the intestinal lumen-membrane interface, which may alter the kinetics of cholesterol absorption. This barrier includes an unstirred water layer and surface mucous coat that is important for the presence of mucins that are extensively bound to cholesterol prior to transfer into enterocytes [125].

During the absorption of cholesterol, there is a little increase in the cholesterol content of the small intestinal wall, indicating that cholesterol can be rapidly processed and exported from the enterocytes into intestinal lymph [115, 125]. After entering the enterocytes, approximately half of the cholesterol molecules move to the endoplasmic reticulum (ER) where cholesterol is esterified by acyl-CoA:cholesterol acyltransferase (ACAT) before incorporation into nascent chylomicron (CM) particles. It is interesting to note that all cholesterol moving from the intestinal lumen into enterocytes is unesterified; in contrast, cholesterol secreted into intestinal lymph following a cholesterol-rich meal is approximately 70–80% esterified. Therefore, the cholesterol esterifying activity of enterocyte appears to be an important regulator of intestinal cholesterol absorption. Because cholesterol absorption is a

multistep process, any factor that changes the transportation of cholesterol from the intestinal lumen to the lymph could influence intestinal cholesterol absorption efficiency. There are a lot of dietary, pharmacologic, biliary, cellular and luminal factors capable of influencing intestinal cholesterol absorption. For example the increase of cholesterol intake by diet does not change intestinal cholesterol absorption, while the increase of fiber, plant sterols, fish oil or ezetimibe by diet reduces intestinal cholesterol absorption [17, 50, 82, 91, 97, 108, 119]. Among the proteins indicated as intestinal cholesterol transporters, it has been recently demonstrated that NPC1L1 (Nieman-Pick C1-like protein1), that is expressed predominantly in the gastrointestinal tract with peak expression in the proximal jejunum, is the most involved. In fact *NPC1L1*-deficient mice exhibit a 70% reduction in intestinal cholesterol absorption [3]. Among cellular factors, ACAT2 and/or HMG-CoAR activity modifications could influence cholesterol intestinal absorption [16, 37, 83, 121].

Some authors observed that intestinal cholesterol absorption efficiency increases markedly during ageing [24, 116, 117, 120, 124]. On the contrary, Bosner et al. [11] showed that, the percent cholesterol absorption was significantly higher in African–Americans than in other racial groups, but that was independent of age and sex. Other studies have shown gender differences in cholesterol absorption efficiency [116, 124], which suggests that ageing and female sex hormones could also modify cholesterol absorption. It has been found that ageing significantly increases secretion rates of biliary lipids (bile salt, cholesterol and phospholipid), and cholesterol content of bile, as well as sizes and hydrophobicity indices of the bile salt pool [24, 117, 120, 124, 127]. These biliary factors could together exert a major influence on increased efficiency of intestinal cholesterol absorption with age in both sexes [124]. That estrogen significantly increases output of biliary bile salt, cholesterol and phospholipids as well as bile salt-dependent bile-flow rates have also been observed. These biliary factors markedly augment intestinal cholesterol absorption in animals and humans, especially those being exposed to high levels of estrogen, though the molecular mechanisms underlying remain unclear [38, 128], and so these issues are till now very debated.

Cholesterol export from enterocyte to the intestinal lumen requires the expression of transporters of the ATP binding cassette (ABC) superfamily (well reviewed in [89]). ABC are the major components of the reverse cholesterol pathway and therefore crucial for the efflux of excess cellular cholesterol. ABC transporters, and in particular ABCG5 and ABCG8 represent apical sterol efflux of cholesterol from enterocytes back to into the intestinal lumen (Fig. 2) [59].

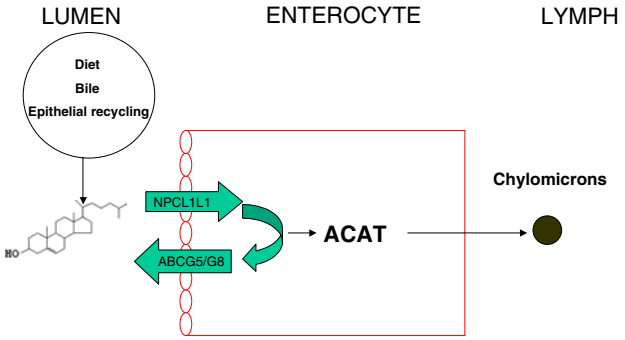

Fig. 2 Cholesterol intestinal absorption is a multi-step process. Here, there is a simplified picture illustrating that intestinal cholesterol derives from diet, bile and epithelial cells recycling. Cholesterol enters the enterocyte though a newly identified sterol influx transporter located at the apical membrane of the cell, the Niemann-Pick C1-like 1 protein (NPC1L1). ATP binding cassette G5/G8 (ABCG5/G8) promotes the efflux of cholesterol from the enterocyte into intestinal lumen for excretion. The combined regulatory effects of NPC1L1 and ABCG5/G8 may play a critical role in modulating cholesterol amount that reach the lymph. Once cholesterol is in the enterocyte, a lot of processes intervene to produce chylomicrons. Among them the cholesterol esterification is probably the most important

Formation of intestinal chylomicrons and very low-density lipoproteins

Despite the wealth of information gathered over the last 30 years about intestinal lipid absorption, the mechanism of intracellular assembly, modification and secretion of lipoproteins from the small intestinal epithelial cells is not fully understood. This is partly due to the complexity of the processes involved and the lack of good experimental models to study the various steps. Intestinal cell culture systems (e.g. Caco-2 cells) have been used extensively to study the formation and the secretion of lipoproteins [49, 54, 55, 74, 113] and the genetic expression and posttranslational modification of apolipoproteins [75]. During fasting, very low-density lipoproteins (VLDLs) are the only lipoproteins produced by the small intestine [87, 88, 114]. Following a meal, the small intestine produces predominantly CMs [114, 135]. CM particles are much bigger than VLDLs and therefore carry significantly more Triglycerides (TGs) than VLDLs. Currently, the separation of intestinal CMs and VLDLs is based on operational criteria. Lipoproteins that have a Svedberg flotation (S_f) rate exceeding 400 are classified as CMs; those with a S_f rate of 20–400 are defined as VLDLs [63]. Numerous studies support the idea that intestinal CMs and VLDLs represent lipoprotein produced by two separate pathways. Okner and Manning [87] demonstrated that the intraduodenal infusion of palmitate causes a marked increase in VLDLs transport, but VLDL output remains unchanged when oleate and linoleate are infused. In contrast CMs output is markedly increased when oleate and

linoleate are infused. Further evidence in support of the theory of separate biosynthetic pathways for lipoprotein is provided by Vahouny et al. [118], who demonstrated that puromycin had no significant effect on the incorporation of radioactive leucine in VLDL peptides in male rats. In contrast, however, the incorporation of radioactive leucine into CM peptides was markedly inhibited.

From enterocytes to limph and liver

CMs are secreted to lymph for delivery into the bloodstream. CMs deliver TGs to peripheral tissues via vascular lipoprotein lipase (LPL) mediated hydrolysis, and their cholesterol-enriched remnants are taken up by the hepatocytes. In the liver, cholesterol is channeled to bile acid synthesis or is incorporated again into the VLDLs that are delivered into systemic circulation. The proteins associated with the lipoproteins and apoproteins, serve as lipid acceptors, enzyme cofactors or ligands for receptor-mediated cellular uptake. Each lipoprotein particle has distinct properties and distinct destinations. In particular, VLDL particles are also assembled in the liver and distribute TGs and cholesterol to the peripheral cells. They become LDL after partial depletion in TGs has occurred, due to the vascular LPL activity. LDL is considered as the bad cholesterol compartment, due to their cholesterol delivery to peripheral tissues, in contrast to high density lipoprotein (HDL), which contain apoA1 and apoA2 proteins that serve as cholesterol acceptors and which efficiently catch cholesterol from peripheral tissues. VLDLs form the primary source of cholesterol for peripheral tissues, mainly via LDL receptor (LDLr) expression, which is a main regulatory step for adjusting the import of cholesterol [133]. Cholesterol export from cells requires both the expression ABC superfamily and the extracellular presence of apolipoproteins as free cholesterol acceptors.

At the level of the whole organism, if cholesterol is in excess, its conversion into bile acids increases, allowing its elimination in the faeces, which is the only way of excreting cholesterol. This excretion is further regulated by the enteropathic cycle of bile acids (i.e., excretion in the gut lumen, followed by the absorption in the ileum and entry into the liver), which is in part aimed at saving the complex and energy-costly molecules such as cholesterol and its bile acid derivatives. In contrast, if the cholesterol supply is low, de novo synthesis takes place mainly in the liver (this part will be discussed later).

Cholesterol cellular trafficking

Cellular uptake of LDL particles via the LDLr is a classical example of receptor-mediated endocytosis [2, 12]. There are four general compartments in the endocytic pathway, defined by different protein and lipid compositions: (1) early endosomes, (2) the endocytic recycling compartment (ERC), (3) late endosomes and (4) lysosomes. Although the LDLr road in this pathway is well described, the fate of LDL-derived cholesterol is subjected to much investigations.

LDL bound to cell surface LDLr is internalised via clathrin-coated pits, and these vesicles shed their coats and fuse with early endosomes. The lower pH in early endosomes promotes the dissociation of LDL from LDLr. The LDLr and other recycling proteins then localize to early endosomal tubular extensions, which bud off vesicles that fuse with the plasma membrane (PM). In the same manner, some amount of early endosomal membrane free cholesterol, from both LDL and endocytosed PM, may also sort the ERC. Hydrolysis of LDL cholesterol esters to free cholesterol is widely thought to occur in the late endosomes and lysosomes, but the acid lipase enzyme was recently localized to an earlier acidic compartment, shows that LDL cholesterol ester-derived free cholesterol may be generated soon after endocytosis [109]. The nonrecycled contents of early endosomes proceed to late endosomes by a process, which perhaps involves vesicular transport or the transformation of early to late endosomes. Late endosomes fuse with the Golgi-derived vesicles containing hydrolytic enzymes and then mature into lysosomes. It is unclear where cholesterol normally leaves the endosomal pathway and how it effluxes and redistributes to other sites. LDL-derived cholesterol leaves late endosomes to reach other compartments like ER and PM. Cholesterol is thought to follow at least two pathways from endosomes to ER. The major pathway involves the PM as an intermediate; this was demonstrated by experiments in which cyclodextrin-mediated cholesterol extraction (mainly from the PM) inhibits esterification of LDL-derived cholesterol by 70%. A minor pathway that bypasses the PM is suggested by the 30% of esterification non inhibited by cyclodextrin [81].

It appears clear that changes in LDLr synthesis, trafficking or membrane exposition can contribute to modify cellular and blood cholesterol content. These processes are also directly or indirectly modulated by hormones such as estrogens, thyroid hormones and insulin [20, 68, 80, 92]. During ageing, an increase of LDLr mRNA and protein has been reported, but a decrease of its membrane exposition has been observed. This contributes to the age-related hypercholesterolemia and cardiovascular disorders [10, 92].

Interestingly, Robinet et al. [98] have recently demonstrated an important role of dynamin in cholesterol delivery to ER; in fact they demonstrated that dynamin inactivation led to the accumulation of free cholesterol within the late endosomal/lysosomal compartment resulting in insufficient

delivery of regulatory cholesterol to the ER where the transcriptional control of sterol-sensitive genes occurs.

Cholesterol gene regulation

To monitor levels of membrane sterols, cells employ two membrane-embedded proteins of the ER: SCAP (SREBP Cleavage Activating Enzyme) and HMG-CoAR. These proteins share a polytopic intramembrane sequence called the Sterol Sensing Domain (SSD). This domain is found in several proteins that are involved in cholesterol homeostasis [25]. Through this domain, sterols cause SCAP and HMG-CoAR to bind Insulin Induced Gene (Insig), newly characterized membrane proteins of the ER. Upon binding SCAP and HMG-CoAR, Insigs stand at the crossroads between the transcriptional and post-transcriptional regulatory mechanism that assure cholesterol homeostasis [34]. A simplified picture of the protein complex involved cholesterol gene regulation and homeostasis is depicted in Fig. 3.

SREBPs

The sterol regulatory binding proteins (SREBPs) are basic-helix–loop–helix-leucine zipper (bHLH-LZip) transcription factors synthesized as 1150 amino acid (aa) inactive precursors bound to the membranes of the ER [47]. There are three major SREBP isoforms encoded by two genes [13]: SREBP-1a and 1c produced from a single gene (*SREBF-1*) located on human chromosome 17p11.2 [48] and SREBP-2 from a separate gene located on human chromosome 22q13 [69]. These important proteins regulate the expression of more than 30 genes involved in cholesterol, fatty acid, TG, and phospholipid metabolism [13, 43]. Each SREBP precursor is organized in three domains: (a) a NH_2-terminal domain of about 480 aa that contains the transactivation domain, a region rich in serine and proline and the bHLH-LZip region for DNA binding and dimerization; (b) two hydrophobic transmembrane spanning segments interrupted by a short loop of about 30 aa that projects into the lumen of the ER; and (c) a ≈ 590 aa COOH-terminal segment regulatory domain. Upon activation, the ER anchored SREBP precursor undergoes a sequential two-step cleavage process to release the NH_2-terminal active domain, designated as the nuclear form of SREBP (nSREBP) (this part will be discussed later) [78, 79]. Once translocated into the nucleus, nSREBPs promote the expression of many genes involved in cholesterologenesis and lipogenesis.

SREBP-1 and SREBP-2 proteins share 47% of homology. SREBP-1a and 1c transcripts are produced through use of the alternative transcription start site and differ in their first exon (exon 1a and 1c). SREBP-1a is a more potent transcriptional activator than SREBP-1c due to its longer NH_2-terminal transactivation domain [104]. However, SREBP-1c is the predominant isoform expressed in most of mice and humans tissues, with especially high levels in the liver, white adipose tissue (WAT), skeletal muscle, adrenal gland and brain. In contrast, SREBP-1a is highly expressed in cell lines and tissues with a high capacity for cell proliferation, such as spleen and intestine [105]. Unlike other bHLH-LZip transcription factors, which contain a well conserved arginine residue in their basic domain, SREBPs have a tyrosine residue, that allows

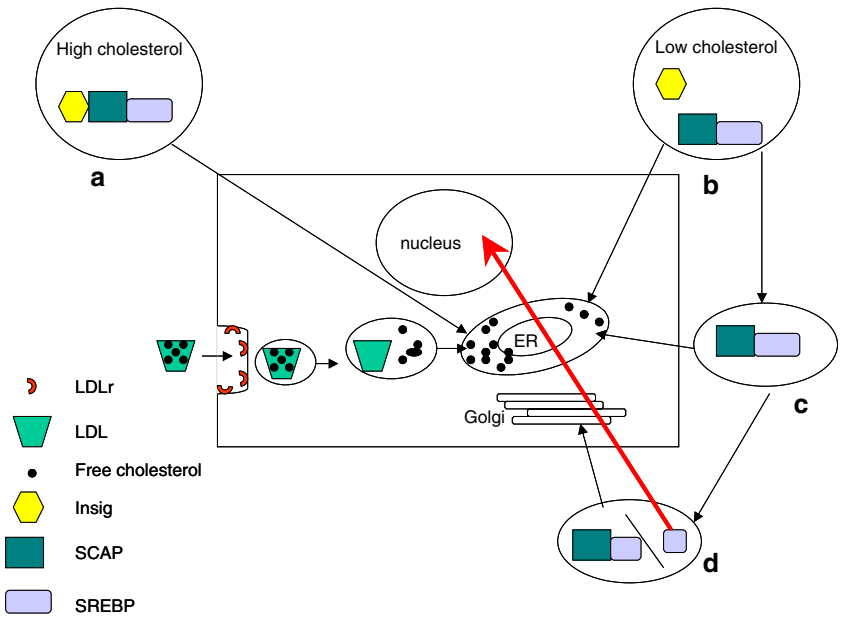

Fig. 3 LDL pathway and cholesterol influence on Insig/SCAP/SREBP fate. *a* When cellular cholesterol levels are high or normal, sterol regulatory element binding protein (SREBP), SREBP cleavage activating protein (SCAP) and insulin-induced gene (Insig) form a macromolecular complex in the endoplasmic reticulum (ER). *b*, *c* Low levels of cholesterol in ER allow Insig to escape from the complex and permit to SCAP/SREBP to escort into Golgi apparatus. *d* In Golgi apparatus, SREBP is cleaved and mature SREBP is released into cytoplasm from where migrates into nucleus

them to bind on both E-boxes (5′-CANNTG-3′, where N represents any base), like all bHLH proteins, but also sterol regulatory element (SRE) sequences (5′-TCACNCCAC-3′) [57].

Under normal conditions, SREBP-1c mainly regulates the expression of ATP citrate lyase (producing acetyl-CoA) as well acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) for palmitate synthesis, demonstrating a selective induction of lipogenic genes [65, 90]. In addition, SREBP-1c regulates the long-chain fatty acyl elongase complex for the production of stearate from palmitate, stearoyl-CoA desaturase (SCD) for the production of oleate from stearate, and glycerol-3-phosphate acyl-transferase for TG and phospholipid synthesis [72, 73]. SREBP-2 is the predominant isoform affecting cholesterol homeostasis through the activation the regulation of the expression of 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoAS), HMG-CoAR, farnesyl diphosphate synthase, and squalene synthase. Both SREBP-1c and 2 regulate three genes, malic enzyme, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase. These are required for the generation of NADPH, which is necessary at multiple stages of the lipogenic pathway [43]. SREBP-1a is not the predominant in vivo. Overexpression of nSREBP-1a in mice liver [44, 103] markedly increases the expression of genes involved in cholesterol synthesis (for example HMG-CoAS, HMG-CoAR, squalene synthase) and fatty acid synthesis (for example ACC, FAS and SCD) and cause corresponding accumulation of both cholesterol and TG [45, 103]. A general picture of the genes regulated by SREBPs is depicted in Fig. 4.

SREBP transcription factors are regulated at three major levels: (1) proteolytic cleavage of SREBP precursors, (2) transcription, and (3) postraslational modification of nSREBPs. Despite the fact that there is some overlap of regulatory mechanisms across SREBP isoforms, significant differences do exist. In particular, whereas SREBP-1a and 2 appear to be primarily regulated at the level of precursor cleavage, evidence suggests that SREBP-1c is mainly regulated at the transcription level. The unique regulation and activation properties of each SREBP isoform facilitate the co-ordinate regulation of lipid metabolism; however, further studies are needed to understand the detailed regulation pathways that specifically regulate each SREBP isoform.

SREBPs activation by proteolytic cleavage

Following SREBP mRNA translation, SREBP precursors are retained in the ER membranes through a tight association with SCAP [85]. Under low cholesterol conditions, SCAP escorts the SREBP precursors from the ER to the Golgi apparatus where two functionally distinct proteases, site 1 protease (S1P) and site 2 protease (S2P), sequentially cleave the precursor protein releasing the nuclear SREBP (nSREBPs) in the cytoplasm [100, 129]. First, S1P cleaves within the hydrophilic luminal loop of the SREBP precursors [21, 26]. S2P then cuts the protein within the first transmembrane fragment [22]. The S1P and S2P proteases are not specific to SREBPs and are involved in other transmembrane protein cleavages such as the amyloid peptid (APP) or Notch [15]. SCAP is embedded in ER membranes through its NH_2-terminal domain, which is composed of eight transmembrane helices separated by hydrophilic loops [85]. Transmembrane helices 2–6 comprise the SSD [46, 84].

Brown and Goldstein [14] have demonstrated that SREBP processing can be controlled by the cellular sterol content (Fig. 3). In sterol-depleted cells, the SCAP/SREBP complex exits the ER in COPII-coated vesicles that bud from ER membranes [27, 86, 110]. SCAP mediates this exit by binding to COPII coat proteins through the general mechanism defined for yeast and mammalian membrane proteins that move from ER to Golgi [5, 6, 8].

When cells have abundant cholesterol, SCAP binds to Insig-1, stabilizing the protein and leading to an accumulation of a stable Insig-1/Scap/SREBP complex. The nuclear content of SREBP falls and thus Insig-1 mRNA levels decline. The stable Insig-1/SCAP/SREBP complex serves as a reservoir for SREBP. When cells are acutely deprived of sterols, SCAP/SREBP dissociates from Insig-1, whereupon the latter is ubiquitinated and degraded in proteasomes [35]. The free SCAP/SREBP complex binds COPII proteins and moves to the Golgi complex where the SREBP is processed to its nuclear form. The nSREBP activates the genes transactivating cholesterol biosynthesis enzymes and LDLr protein. At the same time, the nSREBP activates the gene for Insig-1. This increases the amount of Insig-1 synthesis, but the protein will be rapidly degraded unless sufficient cholesterol has accumulated to bind to SCAP, allowing SCAP to stabilize Insig-1. The block of SREBP processing thus, requires the convergence of two molecules: newly supplied cholesterol (either from synthesis or uptake) and newly synthesized Insig-1 [35]. The reason for this convergence is not yet fully clear. It likely relates to the fact that SREBPs regulate the mevalonate pathway, which produces vital isoprenoid end products in addition to cholesterol [33]. If cholesterol alone could block SREBP processing, and if cholesterol accumulated rapidly, the mevalonate pathway could be blocked before other isoprenoids have accumulated to sufficient levels. The requirement for concurrent Insig-1 synthesis might be a failsafe mechanism, which confirms that SREBP has entered the nucleus and has turned on all the genes of the mevalonate pathway.

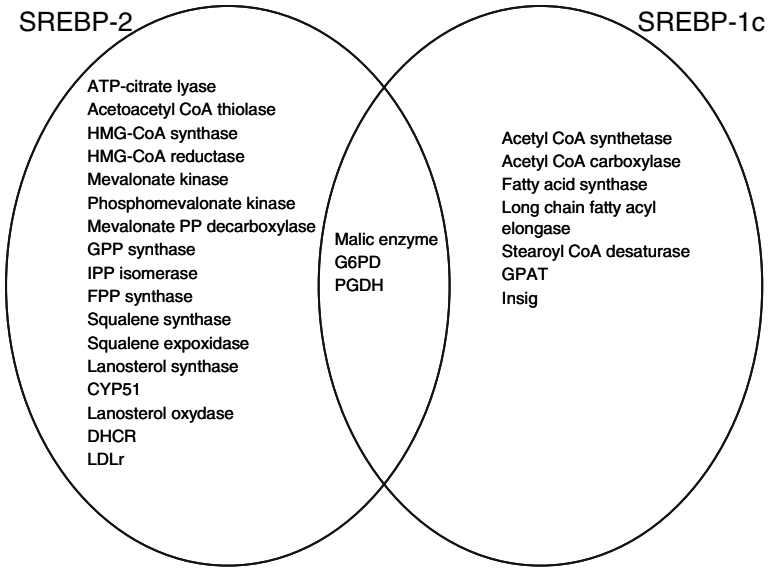

Fig. 4 Genes regulated by SREBPs. SREBP-1a is a potent activator of all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, fatty acids, and triglycerides. High-level transcriptional activation is dependent on exon 1a, which encodes a longer acidic transactivation segment than does the first exon of *SREBP-1c*. The roles of *SREBP-1c* and *SREBP-2* are more restricted than that of SREBP-1a. *SREBP-1c* preferentially enhances transcription of genes required for fatty acid synthesis but not cholesterol synthesis. Like SREBP-1a, *SREBP-2* has a long transcriptional activation domain, but it preferentially activates cholesterol synthesis. SREBP-1a and *SREBP-2* are the predominant isoforms of SREBP in most cultured cell lines, whereas *SREBP-1c* and *SREBP-2* predominate in the liver and most other intact tissues

However, this sterol-sensitive SREBP process does not apply to all three SREBP isoforms and other factors such as insulin seem to be involved in the cleavage. All these studies on SREBP process mechanism and control by sterols were done in cell lines that express SREBP-1a and SREBP-2 predominantly [105]. Interestingly, studies done in vivo have shown that sterol depletion does not regulate the cleavage of the SREBP-1c isoform [102]. In vivo, SREBP-1c is primarily regulated by changes in nutritional status, which has no effects on SREBP-2 expression [42, 56].

Studies in vivo and in vitro have demonstrated that SREBP-1c cleavage is under the control of insulin, in the absence of any variation in sterol concentration [36]. This suggests that Insig proteins could be responsible for the differential effects of insulin versus sterols on the cleavage of the SREBP-1c.

The Insig-1 mRNA was originally identified in 1991 as a transcript (designated CL-6) that increased in regenerating rat liver and in cultured rat H35 hepatoma cells after treatment with insulin [70]. Later, CL-6 was renamed Insig1 to denote "Insulin-induced gene" [94]. Insig-2 was discovered in 2002 through bioinformatics as a closely related isoform that resembled Insig-1 in its SCAP binding function but differed in its pattern of regulation [131]. Human Insig-1 and Insig-2 contain 277 and 225 amino acids, respectively. Both Insigs are extremely hydrophobic. Topology studies suggest that most of the protein consists of six transmembrane helices separated by short hydrophilic loops [28]. Both Insigs bind to cholesterol-loaded SCAP, and both retain the SCAP/SREBP complex in the ER [1, 131]. Insigs appear to enhance the response to cholesterol by supporting cholesterol binding to SCAP. Overexpressing Insig-1 or Insig-2 lowers the threshold for producing the conformational changes in SCAP, which are detectable by trypsin digestion of membrane preparations [1]. The most likely interpretation is that cholesterol dissociates readily from SCAP, and dissociation is retarded when cholesterol-loaded SCAP binds to Insigs, thereby blocking cholesterol into the Insig/SCAP/SREBP complex.

The major differences between Insig-1 and Insig-2 relate to the regulation of their expression [131]. Insig-1 is an obligatory SREBP target gene [43, 52, 64]. Thus, the Insig-1 mRNA is produced only when cleaved SREBP is present in the nucleus, and transcription falls dramatically when SREBP processing is blocked. In contrast, Insig-2 is expressed at a low, but constitutive level, at least in cultured cells. It is not regulated by SREBPs [131]. Insig-1 mRNA levels are regulated by cholesterol, as much as *Insig-1* transcription is dependent upon SREBPs,. Thus, Insig-1 mRNA rises when cells are sterol depleted, and falls when cholesterol accumulates. Remarkably, the level of Insig-1 protein varies oppositely to the level of its mRNA, owing to sterol-regulated degradation. When cells are deprived of sterols, the Insig-1 protein is rapidly ubiquitinated and degraded by proteasomes with a half-life

of less than 30 min [35]. When cholesterol is present, cholesterol-loaded SCAP binds to Insig-1, thereby stabilizing Insig-1 and preventing its ubiquitination. Thus, in sterol-overloaded cells, Insig-1 has a relatively long half-life (>2 h). This stabilization is totally dependent on SCAP. In contrast to Insig-1, Insig-2 has a much longer half-life [61], which is not regulated by sterols [35].

Regarding the proteins involved in this pathway, some modifications have been observed in different physiological states even if more studies have to be done. During ageing, for example, an increased nSREBP-2 due to a decreased Insigs has been observed [92]. The declined Insigs is due to the well known reduced cellular sensitivity to insulin [32, 66, 71]. These variations taken together could justify the increased transcription of LDLr above mentioned and modify the correct working of the cellular transcription and the enzymatic machinery triggering age-related hypercholesterolemia.

SREBP transcriptional control

Changes in nutritional status (fasting/refeeding) regulate SREBP-1c expression in liver [42], WAT [56] and skeletal muscle [9, 18]. Experiments in isolated adipocytes [56] and hepatocytes [30] showed that transcription of SREBP-1c is induced by insulin. This induction of SREBP-1c transcription leads to a parallel increase in expression of both the ER membrane-bound precursor and the nuclear form of the transcription factor [7]. In a typical negative regulatory fashion, the effects of insulin on SREBP-1c transcription are opposed by glucagon via cAMP [31]. The effects of insulin on SREBP-1c expression are mediated by a phosphatidyl inositol (3) kinase (PI(3)-kinase) dependent pathway [7, 29]. The downstream effector(s) remain unclear, with evidence suggesting that both PKB/Akt [29, 96] and PKCk [67] may be involved.

SREBP-1c transcription can also be induced by the activation of liver X receptor (LXR)α. LXRα is a hormone nuclear receptor with high hepatic expression that is activated by oxysterols (derivatives of cholesterol) [53, 62]. This transcription factor induces the expression of a range of genes involved in cholesterol efflux and clearance. The LXR dependent-genes involved in cholesterol efflux and clearance are: cholesterol 7α hydroxylase (CyP7α), the rate-limiting enzyme in the conversion of cholesterol to bile acids; cholesteryl ester transfer protein (CETP), that mediates transfer of cholesterol esters from HDL to triglycerides-rich lipoproteins; ATP binding cassette A1 (ABCA1) that mediates the active efflux of cholesterol from cells to apolipoproteins; ATP binding cassette G1 (ABCG1), that mediates the active efflux of cholesterol and phospholipids from cells to apolipoproteins; ATP binding cassette G4 (ABCG4), the cellular transmembrane transport of endogenous lipid substrates; ATP binding cassette G5/G8 (ABCG5/8), that has an important role in entero-hepatic sterol transport; apoprotein E (ApoE), that facilitates cholesterol efflux outside the entero-hepatic axis; LXRα(involved in its autoregulation and scavenger receptor class B type 1, (SR-B1) the HDL receptor involved in reverse cholesterol transport.

All the mentioned genes have an LXR response element, 5′-AGGTCANNNNAGGTCA-3′ [134], found in the promoter or are regulated by exposure to an LXR agonist (well reviewed in Steffensen and Gustaffson [107]). In addition, in vivo studies have identified a role for LXRα to induce SREBP-1c and lipogenic genes in liver. It is believed that LXRα acts as a cholesterol sensor. Consistent with its role, it has been proposed that LXRα induces SREBP-1c in order to generate FA needed for the formation of cholesterol esters, which buffer the free cholesterol concentration (Tontonoz and Mangelsford [112]). As above described, SREBP-1a and SREBP-2 transcription is induced under sterol-depletion conditions by Insig-mediated mechanism; however, for SREBP-1a this is only true in culture cells and not in the liver in vivo [105]. Transcription of SREBP-2 may be mediated by a feed-forward mechanism in which nSREBP-2 binds to a SRE in the promoter of the SREBP-2 gene to induce its own transcription [101]. A similar mechanism may also contribute to the transcriptional regulation of SREBP-1c gene [4].

Post-translational modifications of SREBPs

Inside the nucleus, the transcriptional activity of nSREBP is regulated by covalent modifications or by interaction with other proteins. The first evidence indicating that SREBPs are modified at a post-translational level came from a study performed by Kim et al. [56], which showed that insulin augments the transcriptional activity of nSREBP-1c overexpressed in 3T3-L1 adipocytes. Further studies (mainly performed in cell lines) have shown that insulin also stimulates the transcriptional activity of nSREBP-2 and nSREBP-1a through a mitogen-activated protein kinase (MAPK) pathway. The S-117 residue has been identified as the major phosphorylation site for MAPK in SREBP-1a [99]. S-432 and S-455 are described as the MAPK phosphorylation sites [58] in vitro and in vivo in SREBP-2. These phosphorylations do not modify DNA binding but enhance SREBP-2 transactivation capacity. A role for MAPK in the modification of SREBP-1c transcriptional activity remains controversial. Although the S-117 target of MAPK in SREBP-1a is also present in the SREBP-1c isoform, the use of inhibitors of the MAPK pathway in cultured hepatocytes do not antagonize the effect of insulin

on SREBP-1c target genes [51, 123], suggesting that SREBP-1c is not phosphorylated by MAPK in hepatocytes. Moreover PKB seems to be able to phosphorylate purified nSREBP-1c in vitro. Co-transfection experiments in cultured hepatocytes indicate that phosphorylation by insulin and PKB stimulate the transcriptional activity of nuclear SREBP-1c. It has been shown that in the nucleus, nSREBPs are modified by ubiquitination and rapidly degraded by the 26S proteasome [40]. In the presence of the proteasome inhibitors, *N*-acetyl-L-leucyl-L-leucyl-norleucinal (ALLN), and lactacystin, and nSREBPs are stabilized and expression of their target genes is enhanced. It has been further demonstrated that SREBP transcription factors are ubiquitinated and degraded through a transcription-dependent pathway involving the proteasome, which thereby terminates the transcriptional signal. These effects require both a functional transactivation and DNA binding domain in SREBPs [111]. SREBP-1 and SREBP-2 are also modified by the small ubiquitin-related modifier (SUMO)-1. SREBP-1a and SREBP-2 contain two and one sumoylation sites, respectively. Sumoylation of SREBPs does not affect ubiquitination and thus neither the stability of the proteins, but instead represses their transcriptional activities [39]. SREBP-1a and SREBP-2 associate through their NH_2-terminal domains with various transcriptional co-activators including p300, CBP and ARC/DRIP, but no interaction between SREBP-1c and co-activators has been demonstrated [76, 77]. It is important to note that most studies have been performed in cell lines in which SREBP-1c is not the predominant isoform expressed. Interaction of SREBP-1a and SREBP-2 with the transcriptional activator p300 results in acetylation of SREBPs. Acetylation of lysine residues in SREBP-1a and SREBP-2 enhances the stability of these transcription factors. In SREBP-1a, the acetylation binding site has been mapped to a specific lysine residue (K-333) in its DNA binding domain. This site was identified previously as an ubiquitination site and so acetylation stabilizes SREBP-1a by preventing ubiquitination on the same residue. In recent years, efforts have been made to identify proteins, which modify its transcriptional activity. Lee et al. [60] have shown that SREBP-1c interacts with Twist2, a protein expressed in fat and liver. Overexpression of Twist2 repressed the transcriptional activity of ADD1/SREBP-1c by inhibiting its binding to target promoters.

Conclusions and perspectives

A conclusion of such a story must be short.

It appears significant to underline the important feature of the transcriptional regulation of cholesterol homeostasis involved enzymes and its link with cholesterol itself.

Cholesterol homeostasis results from the network of a complex of processes including intestinal absorption, transport, cell trafficking and gene regulation, all which are committed to cholesterol homeostasis maintenance.

We hope that this review has been successful in showing the intense network between transcription factors (i.e., *SREBPs*) and the proteins able to regulate them, through which cholesterol homeostasis can be achieved. It appears clear that a little modification of one of the factors involved in cholesterol homeostasis maintenance could cause deep alterations and in turn diseases as observed, for example age-related hypercholesterolemia that is the main cause of cardiovascular disease. A lot of work has to be done in comprehending the relationship between hormonal modifications, their effect on transcription factors and cholesterol metabolism in different physiological and pathological conditions. More detailed studies will be required to define the specific roles of each Insig protein and to determine the metabolic consequences of their reciprocal regulation. Insigs, in fact, could represent a new target for pharmacological intervention to maintain blood cholesterol levels in the optimal range since they are required for feedback regulation of SREBP processing and HMG-CoA reductase degradation.

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