Intracellular Cholesterol Transport

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Abstract—Intracellular cholesterol transport is essential for the maintenance of cholesterol homeostasis. Many aspects of cholesterol metabolism are well-known, including its synthesis in the endoplasmic reticulum, its extracellular transport in plasma lipoproteins, its uptake by the low-density lipoprotein receptor, and its regulation of SREBP and LXR transcription factors. These fundamental pathways in cholesterol metabolism all rely on its proper intracellular distribution among subcellular organelles and the plasma membrane. Transport involving the ER and endosomes is essential for cholesterol synthesis, uptake, and esterification, whereas cholesterol catabolism by enzymes in mitochondria and ER generates steroids, bile acids, and oxysterols. Cholesterol is a highly hydrophobic lipid that requires specialized transport in the aqueous cytosol, involving either vesicles or nonvesicular mechanisms. The latter includes hydrophobic cavity transporters such as StAR-related lipid transfer (START) proteins. Molecular understanding of intracellular cholesterol trafficking has lagged somewhat behind other aspects of cholesterol metabolism, but recent advances have defined some transport pathways and candidate proteins. In this review, we discuss cholesterol transport among specific intracellular compartments, emphasizing the relevance of these pathways to cholesterol homeostasis.

Key Words: intracellular cholesterol transport ■ cholesterol metabolism ■ START proteins

Cholesterol is an essential component of mammalian cell membranes, but its excess is toxic and contributes to several diseases, notably atherosclerotic vascular disease. Understanding of cholesterol homeostasis and its fine regulation has developed in several stages. Early biochemical studies by Bloch et al elucidated the multi-enzyme pathway of cholesterol synthesis. Other studies described extracellular cholesterol transport by plasma lipoproteins, such as low- and high-density lipoproteins (LDL and HDL), and their effects on atherosclerosis. Brown and Goldstein et al showed that cellular cholesterol exerts negative feedback on cholesterol-regulatory enzymes and LDL receptors via sterol regulatory element-binding protein (SREBP) transcription factors. In recent years, focus has shifted to cellular cholesterol efflux and reverse transport, whereby cholesterol moves from peripheral cells to the liver for elimination. Oxysterol derivatives of cholesterol are ligands for liver X receptor (LXR) transcription factors, which stimulate ATP-binding cassette transporters (ABCA1, ABCG1, ABCG5/ABCG8) and other genes involved in reverse cholesterol transport.

Many known pathways in cholesterol metabolism require transport of this highly hydrophobic lipid among intracellular compartments. Most cellular cholesterol resides in the plasma membrane (PM), where it constitutes 35% to 45% of lipid molecules. To reach the PM and other compartments, cholesterol must exit the endoplasmic reticulum (ER), where it is synthesized, cytosolic lipid droplets, where cholesterol esters are stored, and endocytic compartments, where uptake occurs. Cholesterol transport is also essential for its effects on transcription: it must reach the ER to regulate SREBPs, and oxysterols must be generated in mitochondria and elsewhere to regulate LXRs. Understanding of intracellular transport has lagged behind other aspects of cholesterol metabolism, and this field represents a research frontier.

There are 2 general ways that cholesterol can move intracellularly, vesicular and nonvesicular. Cholesterol is present in the membranes of intracellular vesicles that shuttle among compartments. Vesicular traffic typically requires an intact cytoskeleton, the tracks along which vesicles move, and ATP, providing energy for motor proteins. Although some cholesterol transport pathways are vesicular, others persist when vesicles are blocked. Nonvesicular transport can be mediated by diffusible carrier proteins, which have hydrophobic cavities to bind cholesterol and transport it across the aqueous cytosol. An example is the steroidalogenic acute regulatory protein (StAR), the prototype for the StAR-related lipid transfer (START) gene family. StAR is a cholesterol transport protein that stimulates the mitochondrial conversion of cholesterol to steroids. Another form of nonvesicular transport may involve spontaneous desorption of cholesterol from one membrane and diffusion to another closely juxtaposed membrane, perhaps brought together at contact sites by specialized proteins.
There are marked asymmetries in cholesterol concentration among intracellular membranes, despite vesicular and nonvesicular transport that might be expected to equilibrate cholesterol distribution. Vesicles may have sorting mechanisms to exclude or incorporate cholesterol, and intracellular transfer proteins may have specificity in targeting. Specificity could arise from interactions with receptor proteins on target membranes. Alternatively, membranes may have intrinsic differences in ability to accept cholesterol, because cholesterol has highest affinity for membranes enriched in sphingolipids and saturated phospholipids. It is uncertain how intracellular gradients between compartments are formed and maintained, and how cholesterol moves with and against these gradients.

**ER Cholesterol Transport**
The ER is the crucial regulatory compartment in cholesterol homeostasis, despite being a cholesterol-poor organelle. The ER is the primary site of cholesterol synthesis and esterification, and recent data indicate that excess free cholesterol may exert its cytotoxic effects via the ER. The surface areas of the PM and ER are similar in many cells, yet much more cholesterol is in the PM. Methods to determine the cholesterol content of various cellular membranes are subject to technical limitations, but it is commonly cited that 65% to 80% of total cellular cholesterol is in PM, whereas only 0.1% to 2% is in ER. ER cholesterol levels can fluctuate widely: perturbations resulting in modest 50% changes in PM cholesterol result in large 10-fold changes in ER cholesterol. Transport between ER and PM is dynamic, because it has been estimated that the entire PM cholesterol-pool cycles to the ER and back with a half-time of 40 minutes.

**SREBPs Regulate Cholesterol Synthesis**
SREBP transcription factors are a homeostatic mechanism whereby cellular cholesterol levels exert negative feedback on cholesterol synthesis. There are 3 SREBP proteins: SREBP-2 primarily activates genes involved in cholesterol synthesis, whereas SREBP-1a and SREBP-1c have greater effects on genes involved in fatty acid synthesis. SREBPs are synthesized as transcriptionally inactive ER transmembrane proteins. When cholesterol is abundant, SREBPs remain in the ER associated with the escort protein SCAP (SREBP cleavage activating protein) and the ER retention protein Insig (Figure 1A). Low cholesterol causes a conformational change in the sterol-sensing domain of SCAP, dissociating Insig and allowing SREBP-SCAP to reach the Golgi. Two proteases in the Golgi release the active form of SREBP, which translocates to the nucleus to activate transcription of target genes. Cholesterol synthesis is also regulated posttranscriptionally: high cholesterol accelerates degradation of HMG CoA reductase (HMGK), the rate-limiting enzyme in cholesterol synthesis, by promoting association of its sterol-sensing domain with Insig. The final enzyme of cholesterol synthesis, 7-dehydrocholesterol reductase (DHCR7), also has a sterol-sensing domain and may be similarly regulated.

**Cholesterol Precursor Transport**
In the cholesterol synthetic pathway, cyclization of squalene generates lanosterol, the first sterol intermediate. Lanosterol generates lanosterol, the first sterol intermediate. Lanosterol has highest affinity for membranes enriched in sphingolipids and saturated phospholipids. It is uncertain how intracellular gradients between compartments are formed and maintained, and how cholesterol moves with and against these gradients.

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**Cholesterol Precursor Transport**
In the cholesterol synthetic pathway, cyclization of squalene generates lanosterol, the first sterol intermediate. Lanosterol is modified in 19 steps by 9 enzymes to generate cholesterol, resulting in a number of other precursor sterols, some of which have physiological functions. Like cholesterol, these precursors may also require intracellular transport. The ER is the primary site of sterol synthesis, but other compartments like peroxisomes and the PM may be involved. Some cholesterologenic enzymes localize to peroxisomes, and there is conflicting data on cholesterol synthesis in various models of peroxisome deficiency. In peroxisome-deficient fibroblasts from humans with Zellweger syndrome, different reports have indicated decreased or unchanged cholesterol synthesis rates. In mouse models lacking peroxisomal assembly (PEX) genes, cultured PEX5-deficient cells show wild-type cholesterol synthesis rates, whereas PEX2-deficient mice showed tissue-specific increases and decreases in cholesterol synthesis. Therefore, loss of peroxisomes fails to globally inhibit cholesterol synthesis, but in these models peroxisomal enzymes mislocalize to the cytosol, where their activity and regulation may be altered. The PEX2-deficient mice also showed reduced plasma and liver cholesterol levels, as well as dysregulation of various genes involved in cholesterol metabolism, suggesting that peroxisomes play some undefined role in cholesterol homeostasis. The PM may also play a role in cholesterol synthesis, because in some cell types precursor sterols reach the PM with cholesterol but at different rates. Precursors like zymosterol in the PM rapidly...
DHCR7 (Smith-Lemli-Opitz syndrome) or other postlanosterol malformations would be expected, as seen with deficiency of essential for cholesterol synthesis, then multiple congenital abnormalities are expected. SCP-2 knockout mice are viable, and the return to the ER for conversion to cholesterol. Precursor sterols may thus shuttle between ER, peroxisomes, and PM, or even different domains of the same compartment (Figure 1B). Three potential reasons for the compartmentalization of cholesterol synthesis have been proposed: (1) to ensure optimal cofactor concentrations; (2) to colocalize enzymes in functional units; or (3) to provide additional levels of regulation.

Soluble proteins are known to stimulate enzymatic steps of cholesterol synthesis in vitro, and sterol-carrier protein 2 (SCP-2) was purified as an activator of DHCR7 activity. SCP-2 can bind and transfer cholesterol and other sterols but has similar activity for many lipids including fatty acids, fatty acyl-CoA, and phospholipids, resulting in the name “non-specific lipid transfer protein.” Over the past 20 years, data have been generated suggesting some role for SCP-2 in nearly every intracellular cholesterol transport pathway, whereas recent reports have proposed myriad functions in the metabolism of phospholipids and fatty acids. It is difficult to discern which of these many potential functions are physiological. SCP-2 knockout mice are viable, and the primary defects in peroxisome function are explained by the absence of SCP-x, the peroxisomal precursor to SCP-2 that includes a thiolase enzymatic domain. If SCP-2 were essential for cholesterol synthesis, then multiple congenital malformations would be expected, as seen with deficiency of DHCR7 (Smith-Lemli-Opitz syndrome) or other postlanosterol enzymes. Subtle defects in hepatic cholesterol metabolism have been reported in SCP-2-deficient mice, but they may be secondary to major alterations in peroxisome function and fatty acid metabolism. In summary, postlanosterol cholesterol synthesis could require transport proteins for precursor sterols, but none has been definitively described.

### Cholesterol Transport From ER to PM

Nascent cholesterol must leave the ER to exert many of its functions, and it moves against a steep concentration gradient to reach the PM. Vesicular transport along the protein secretory pathway through the Golgi is one route from ER to PM. Consistent with such a vesicular mechanism, ATP depletion or low temperature inhibit rapid ER to PM cholesterol transport. However, inconsistent with vesicular transport, disruption of the cytoskeleton has no effect. Furthermore, Brefeldin A treatment, which causes Golgi disassembly and fusion with ER, blocks >90% of protein secretion but only decreases nascent cholesterol transport to PM by ~20% in the same cells. Therefore, vesicular transport through the Golgi may bring some nascent cholesterol to the PM, but it is not the major pathway (Figure 1C).

Although nontraditional vesicles are possible, cholesterol transport from ER to PM is likely nonvesicular. Sites of close physical membrane apposition between ER and PM could facilitate this transport. With or without such membrane contact sites, cytosolic transfer proteins are likely involved. Some data support roles for SCP-2 and caveolin. In cells lacking SCP-2 because of peroxisome-deficiency or SCP-2 antisense treatment, the initial appearance of nascent cholesterol at the PM after 10 minutes is decreased. However, there is no defect at 20 minutes, indicating that SCP-2 may affect rate but is not necessary for transport. Caveolin-1 is an acylated integral membrane protein essential for formation of caveolae, a subset of lipid raft membrane microdomains. Caveolin-2 is usually coexpressed with caveolin-1 and forms membrane hetero-oligomers with caveolin-1, whereas caveolin-3 replaces caveolin-1 in striated muscle cells. Caveolin-1 is also soluble in compartments including the cytosol, and it binds cholesterol and fatty acids. Cultured lymphocytes lacking caveolins exhibit relatively slow appearance of nascent cholesterol at the PM, but transfection of caveolin-1 increases the initial rate ~4-fold. However, like SCP-2, caveolin-1 is not necessary for this transport, because similar amounts of nascent cholesterol reach the PM in transfected and untransfected cells by 1 hour. Further studies have identified 2 distinct cytosolic complexes of caveolin-1 and various chaperone proteins, one containing nascent cholesterol and another cholesterol esters. It has been proposed that these caveolin-cholesterol-chaperone complexes represent novel intracellular lipid particles analogous to plasma lipoproteins. Adenovirus-mediated overexpression of caveolin-1 in mouse liver causes subtle changes in plasma HDL cholesterol, liver free cholesterol, and liver bile acid secretion. Despite these potential roles, caveolin-1 knockout mice, which also do not express caveolin-2 and lack caveolae in all nonmuscle cells, are viable with no clear defects in cholesterol metabolism reported to date. Therefore, neither of caveolae.
Enzymes have been cloned from macrophages and liver. The resulting cholesterol esters are stored along with triglycerides in the cores of cytosolic lipid droplets. The mechanism of droplet formation is uncertain, but they likely form as extensions from the ER. Neutral cholesterol ester hydrolase (nCEH) breaks down cholesterol esters in lipid droplets. The enzymes responsible for nCEH activity differ among cell types and are distinct from secreted pancreatic lipase and acid lipase in the endocytic pathway. In steroidogenic cells, the functional nCEH is hormone-sensitive lipase, which also hydrolyzes triglycerides. Other nCEH enzymes have been cloned from macrophages and liver.

The synthesis and hydrolysis of cholesterol esters by ACAT and nCEH, known as the cholesterol ester cycle, is thought to play a crucial role in macrophage foam cell formation and atherosclerosis. Esterification by ACAT may serve a protective role, because the esters are less cytotoxic than free cholesterol. However, only free cholesterol is available for cellular efflux, so mobilization of stored esters could also mobilize free cholesterol released from lipid droplets by nCEH may also protect against foam cell formation. The exact role of cholesterol esterification in lesional macrophages is unclear, because both ACAT and nCEH could be protective, perhaps at different stages of atherogenesis.

To complete the cholesterol ester cycle, the nCEH reaction products, free cholesterol and fatty acids, must leave the lipid droplet. Fatty acids are thought to equilibrate instantaneously with the cytosolic pool buffered by fatty acid binding proteins. It is unlikely that lipid droplets directly contact the ER, so free cholesterol transport to ER or other compartments is necessary. Endosomes may affect cytosolic lipid droplets, because Niemann Pick C (NPC) mutant cells, which accumulate cholesterol in endosomes as described later, also accumulate a cholesterol analog in enlarged lipid droplets. Because NPC cells show decreased cholesterol esterification by ACAT (see later), enlarged droplets would not be expected and could indicate fusion of smaller droplets or defective cholesterol efflux from droplets. Soluble transfer proteins could also mobilize free cholesterol released from lipid droplets, and both caveolin and StAR have been implicated. Caveolin coats cytosolic lipid droplets, where it may affect free cholesterol distribution to other intracellular sites. In adrenal cells, StAR interacts with the nCEH hormone-sensitive lipase, perhaps stimulating cholesterol ester hydrolysis and free cholesterol mobilization to mitochondria for steroidogenesis. In nonsteroidogenic cells, other cholesterol-binding proteins may mobilize free cholesterol from lipid droplets.

### Cholesterol Transport From PM to ER

While newly synthesized cholesterol leaves the ER, excess cellular cholesterol from other compartments returns to the ER for esterification. It is clear that cholesterol travels from PM to ER via a different route than nascent cholesterol leaving the ER, because many treatments can repress or stimulate one pathway independently of the other. Treatment of cells with sphingomyelinase (SMase), which digests the sphingolipid component of lipid raft microdomains, triggers release of PM cholesterol for esterification. The mechanism whereby this cholesterol moves from PM to ER is uncertain. SMase causes vesiculization of 15% to 30% of the PM, and these vesicles are unique in that they form in the absence of ATP. Without ATP they remain in the cell periphery, and ATP addition causes delivery of their contents to late endosomes and lysosomes. PM cholesterol could thus undergo vesicular transport to endosomes and then reach the ER for esterification. Consistent with a role for endosomes, NPC mutant cells, which accumulate cholesterol in endosomes, show defective SMase-induced cholesterol esterification. In addition to endosomes, retrograde vesicular transport through the Golgi apparatus has also been implicated in SMase-induced PM-to-ER transport. Other data, however, strongly suggest nonvesicular pathways of PM-to-ER cholesterol transport. SMase triggers cholesterol movement to ER even in the ATP-depleted condition when PM-derived vesicles remain in the cell periphery. SMase-induced cholesterol esterification is also unaffected by other inhibitors of classic vesicular traffic. The nonvesicular pathway could proceed directly from PM to ER, or perhaps from the unique SMase-induced peripheral vesicles to the ER. Thus, PM cholesterol is thought to follow at least 2 paths to the ER: (1) a vesicular route via endosomes and/or Golgi; and (2) a nonvesicular alternative route (Figure 1E).

The mutant Chinese hamster ovary (CHO) cell line 3-6 has an isolated transport defect from PM to ER, with preserved transport of LDL-derived endosomal cholesterol. In contrast, Nrel-4 mutant CHO cells show defective transport of both PM and endosomal cholesterol to the ER. The mutated gene in 3-6 cells has not been cloned, whereas Nrel-4 cells are unable to synthesize plasmalogens, modified phospholipids of unknown function. Further studies of these cells could shed light on molecular mechanisms of cholesterol transport to the ER for esterification.

### The ER and Oxysterols

The oxysterols 25- and 27-hydroxycholesterol are potent repressors of SREBP processing and activators of cholesterol esterification, suggesting that they act directly on SCAP and ACAT, respectively. However, recent data indicate that SCAP senses cholesterol itself, as it undergoes a conformational change in response to cholesterol but not these oxysterols. Likewise, cholesterol itself was shown to be the most potent allosteric activator of ACAT in vitro. Therefore, oxysterols may affect the ER regulatory compartment directly by triggering intracellular cholesterol transport to the ER by an unknown mechanism.

ER cholesterol and cholesterol synthesis may also generate oxysterols that activate the LXR transcription factors. A partial LXR agonist, 25-hydroxycholesterol, is generated by cholesterol 25-hydroxylase, a non-P450 transmembrane protein localized primarily in the ER. A strong LXR agonist, 24(S),25-epoxycholesterol, is produced by a diversion in the postlanosterol cholesterol biosynthetic pathway. Experi-
ments in hepatoma cells are consistent with such an LXR agonist derived from cholesterol synthesis. In these cells, despite abundant exogenous lipoprotein-derived cholesterol, inhibition of endogenous cholesterol synthesis by HMGR inhibition decreases expression of an LXR element reporter or an LXR-target gene, and this is relieved by addition of the HMGR reaction product mevalonate.61,62 Other important oxysterols are also generated in the ER: the initial and rate-limiting enzyme in classic bile acid synthesis, cholesterol 7α-hydroxylase (Cyp7A1), resides in the ER (Figure 1F).63 The oxysterol binding protein (OSBP) binds 25-hydroxycholesterol and other oxysterols, but its function is unknown.64 OSBP overexpression decreases ACAT activity and increases cholesterol synthesis,65 suggesting an effect on the ER regulatory pool, either by sequestration of oxysterols or by some undefined regulatory mechanism. OSBP is not an ER protein, because it normally localizes to the cytoplasm but translocates to Golgi on oxysterol treatment.66 OSBP may function in cholesterol metabolism, because its Golgi localization and phosphorylation state are altered by experimental treatments affecting ER and cellular cholesterol.67–69

Excess ER Free Cholesterol and ER Stress

The feedback mechanisms described allow the ER to handle excess cholesterol by decreased synthesis and increased esterification. The ER may also accommodate some excess free cholesterol via its large surface area and ability to synthesize phospholipids, thus maintaining an acceptable cholesterol-to-phospholipid ratio.46,70 When cultured mouse macrophages are loaded with free cholesterol, via treatment with acetylated LDL and an ACAT inhibitor, the phospholipid biosynthetic response is overwhelmed and apoptosis ensues.46 The relevance of this apoptotic pathway in human atherosclerosis remains uncertain, because cytotoxicity is not observed in similarly treated cultured human macrophages.71 In mouse macrophages, it was initially proposed that excess PM cholesterol triggers this apoptotic response,72 but a recent report strongly implicates ER cholesterol.73 Cholesterol-loaded mouse macrophages trigger the unfolded protein response (UPR),7 in which ER stress signals are transduced to the nucleus to affect gene transcription.73 The UPR is classically activated by unfolded ER proteins, and the mechanism whereby excess free cholesterol activates the UPR is uncertain. It is thought that cholesterol-induced dysfunction of ER calcium pumps affects calcium-dependent chaperones and consequently ER protein folding.7 UPR-induced genes generally either preserve ER protein secretory function or trigger apoptosis if stress is too severe, but some UPR target genes are involved in lipid metabolism and other processes.74 We have shown that the putative cholesterol-transfer START protein StarD5 is also transcriptionally activated by ER stressors (R.S. and J.B., unpublished data). The link between cholesterol and ER stress-mediated apoptosis in macrophages opens a new field of atherosclerosis research and underscores the importance of cholesterol transport pathways to and from the ER.

Endosomal Cholesterol Transport

Cellular uptake of LDL particles via the LDL receptor is a classic example of receptor-mediated endocytosis.75,76 There are 4 general compartments in the endocytic pathway, defined by different protein and lipid compositions: (1) early or sorting endosomes; (2) the endocytic recycling compartment (ERC) or recycling endosomes; (3) late endosomes; and (4) lysosomes. Although the itinerary of the LDL receptor in this pathway is well-described, the fate of LDL-derived cholesterol is the subject of much investigation.

Cholesterol Transport in Early and Recycling Endosomes

LDL bound to cell surface LDL receptors is internalized via clathrin-coated pits, and these vesicles shed their coats and fuse with early endosomes (Figure 2A). The lower pH in early endosomes promotes dissociation of LDL from LDL receptors. The LDL receptors and other recycling proteins then localize to early endosomal tubular extensions, which bud off of vesicles that fuse with the ERC.75 In the same manner, some amount of early endosomal membrane free cholesterol, from both LDL and endocyctosed PM, may also sort to the ERC. Hydrolysis of LDL cholesterol esters to free cholesterol is widely thought to occur in late endosomes and lysosomes, but the acid lipase enzyme was recently localized to an earlier acidic compartment (Figure 2B), so LDL cholesterol ester-derived free cholesterol may be generated soon after endocytosis.77 Vesicles from the ERC return LDL receptors and other recycling proteins and lipids to the PM. The ERC is a
cholesterol-rich compartment,78,79 and the major non-PM intracellular sterol storage organelle in CHO cells.80 Cholesterol is transported from ERC to PM via the same vesicles that carry recycling proteins.81,82 Consistent with this observation, cells overexpressing Rab11, a GTPase that inhibits ERC-to-PM vesicular transport, accumulate cholesterol along with recycling proteins in enlarged ERC organelles.81 Cholesterol also traffics in the opposite direction from PM to ERC, and this is considered nonvesicular because it is rapid and ATP-independent (Figure 2C).80 Similar rapid nonvesicular sterol transport has been observed in polarized HepG2 hepatoma cells, involving the apical membrane and recycling compartment.82,83 The mechanism of PM to ERC nonvesicular cholesterol transport remains speculative, but lipid transfer proteins may be involved.

**Cholesterol Transport in Late Endosomes and Lysosomes**

The nonrecycled contents of early endosomes proceed to late endosomes, by a process perhaps involving vesicular transport or the evolution of early to late endosomes.75 Late endosomes fuse with 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.

The fatal neurodegenerative disease NPC is an autosomal-recessive lipid storage disorder characterized by intracellular free cholesterol accumulation. This cholesterol appears in hybrid storage bodies that have characteristics of late endosomes and lysosomes.84 Late endosomes are normally dynamic structures, but they become static, enlarged, and cholesterol-rich in NPC cells.85 Whereas LDL is the primary source of this intracellular cholesterol, newly synthesized cholesterol also accumulates at a slower rate.86,87 This may reflect the ER-derived PM cholesterol that is endocytosed but does not sort to the ERC, instead reaching late endosomes for accumulation.

There has been controversy regarding the pathway of LDL-derived cholesterol to late endosomes. Two groups used cyclodextrin extraction, a method thought to remove only PM cholesterol, to show that internalized LDL cholesterol ester returns to the PM as free cholesterol at the same rate in wild-type and NPC mutant cells. These surprising data were interpreted to mean that early endosomal LDL cholesterol traffics back to the PM before reaching late endosomes.88,89 However, recent experiments showed acid lipase activity in early endosomes and that prolonged cyclodextrin treatment can extract free cholesterol from this compartment. Thus, it is likely that endocytosed cholesterol proceeds via the traditional pathway from early to late endosomes not involving the PM (Figure 2D).55,77

Causative genes have been cloned for 2 NPC complementation groups. NPC1 is a late endosomal membrane protein with 13 predicted transmembrane segments, including a sterol sensing domain like those found in SCAP, HMGR, DHCR7, and Patched.5 NPC1 is also homologous to bacterial transmembrane molecular pumps, and NPC1 expression in *Escherichia coli* increases fatty acid uptake, although not cholesterol, in this assay.90 Despite this negative result, NPC1 could function as a cholesterol pump, or it could affect localization of another lipid with cholesterol following passively. NPC2 is a soluble cholesterol-binding protein in the lumen of late endosomes and lysosomes.91,92 Because late endosomes have internal membranes, NPC2 could transfer cholesterol from these to the limiting membrane, where cholesterol efflux must occur.5

Most current models of NPC involve defective or slowed cholesterol efflux from late endosomes, but the precise defect is unknown. Cholesterol and sphingolipids have high affinity for one another and are the 2 main components of lipid raft microdomains, so accumulation of one can cause trapping and accumulation of the other.93 In yeast, activating mutations in NPC1-related gene 1 (NCR1), which can functionally substitute for NPC1 in mammalian cells, affect sphingolipid but not sterol metabolism.94 This supports a primordial function for NPC1 in sphingolipid trafficking. Indeed, NPC1-deficient neurons store extensive sphingolipids more than cholesterol, and inability to synthesize certain sphingolipids results in absent or decreased cholesterol accumulation.95 This may reflect specialized lipid metabolism in the brain, where the endocytic pathway may be more involved in degradation of membrane components than LDL uptake. However, recent work has shown neuronal cholesterol accumulation in early postnatal NPC1-deficient mice, suggesting a primary role for cholesterol even in brain.96 Alternatively, rather than causing a specific lipid transport defect, NPC mutations could generally inhibit late endosomes. Consistent with this hypothesis, overexpression of Rab7 or Rab9, GTPases involved in late endosome function, corrects both cholesterol and sphingolipid accumulation in NPC cells.97

Despite increased cholesterol content, NPC cells have repressed ACAT activity, high LDL receptor activity, and elevated cholesterol synthesis, all of which suggest low cholesterol in the ER regulatory pool. However, recent data indicate that NPC cells generate up to 90% less 25- and 27-hydroxycholesterol in response to LDL and thus fail to repress SREBPs and activate LXRαs.98 This suggests that endosomal cholesterol does not reach the sites of oxysterol generation, resulting in altered transcriptional regulation of cholesterol homeostasis contributing to the NPC phenotype. Consistent with this hypothesis, oxysterol treatment can markedly reduce cholesterol accumulation in NPC cells.99,100 However, because cholesterol trafficking and oxysterol generation clearly influence each other reciprocally, it is difficult to discern whether decreased oxysterol levels are a cause or consequence of altered trafficking.

In addition to NPC1 and NPC2, other proteins are likely involved in cholesterol transport from endosomes (Figure 2E). CHO mutant M87 cells have an NPC-like phenotype despite wild-type NPC1 and NPC2.101 An NPC1-like gene (NPC1L1) has been cloned100 and recently implicated in intestinal cholesterol absorption, although its exact subcellular location remains unclear. The OSBP-related protein (ORP) family has 12 members in humans,64 and 2 have been implicated in endosomal cholesterol metabolism. ORP4-S associates with intermediate filaments and when overexpressed decreases esterification of LDL-derived cholesterol by ~40%.102 ORP1-L localizes to the surface of late endosomes, is induced ~100-fold on differentiation of monocytes
to macrophages, and when overexpressed enhances LXR reporter activity. Finally, the cholesterol-binding START protein MLN64/StarD3 has a membrane-spanning domain causing localization in late endosomal membranes. In cultured cells, overexpression of a mutant truncated MNL64 lacking the START domain, or of the homologue MENTHO that naturally lacks a START domain, results in an NPC-like phenotype of endosomal cholesterol accumulation. Because MLN64 colocalizes with NPC1, late endosomal cholesterol could move sequentially from luminal NPC2, through NPC1, to the cytosolic START domain of MLN64, then to a soluble acceptor. However, MLN64 is unnecessary for this process, because recently described MLN64 START domain-deficient mice are viable and fertile with no NPC-like neurological defect and no major alterations in sterol metabolism.

The NPC phenotype can also be reproduced by treatment of normal cells with steroids like progesterone or with hydrophobic amines (class II amphiphiles) like U18666A. The mechanism of U18666A action is unknown, although U18R mutant CHO cells are resistant to it, and a putative membrane protein-binding site has been described but not identified. The lipid-lowering drug GW707 was originally described as a SCAP ligand, but recent data show that it has SCAP-independent effects on cholesterol trafficking similar to U18666A. Further studies of these drugs and their targets, as well as NPC1, NPC2, MLN64, and other candidate genes, should further elucidate cholesterol efflux from endosomes. The potential connection of this efflux pathway to atherogenesis also merits further study. In a recent study using the ApoE-deficient atherosclerotic mouse model, animals heterozygous for NPC1 deficiency showed altered lesion morphology with smaller acellular regions and less apoptosis.

**Cholesterol Transport From Endosomes to Other Compartments**

In normal non-NPC cells, LDL-derived cholesterol leaves late endosomes to reach other compartments like ER and PM. Cholesterol is thought to follow at least 2 pathways from endosomes to ER. The major pathway involves the PM as an intermediate, because cyclodextrin-mediated cholesterol extraction (presumably 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 not inhibited by cyclodextrin.

The major PM-dependent cholesterol transport pathway from endosomes to ER has been divided into 2 steps, the proximal endosome-to-PM and the distal PM-to-ER (Figure 2F). One step of this pathway involves the Golgi, because treatment of cells with Brefeldin A causes all LDL-derived cholesterol to bypass the PM to reach ACAT. Brefeldin A blocks vesicles moving anterograde (ER to Golgi) but not retrograde (Golgi to ER), resulting in a merged Golgi/ER. If the Golgi were involved in only the distal step (hypothetical PM-to-Golgi-to-ER), then endocytosed cholesterol would still reach PM in Brefeldin A-treated cells. Therefore, the Golgi must play a role in the proximal step (endosome-to-Golgi-to-PM). Brefeldin A treatment may divert cholesterol to the merged Golgi/ER, thus bypassing the PM and the distal PM-to-ER step. Alternatively, Brefeldin A may block the entire PM-dependent pathway, causing all cholesterol to follow the poorly defined PM bypass route, which is normally the minor pathway (Figure 2G). Given these multiple cholesterol transport pathways (with multiple steps) from late endosomes to other compartments like ER, there is potential for both vesicular and nonvesicular mechanisms. SCP-2 was considered a nonvesicular candidate, but peroxisome-deficient cells lacking SCP-2 have normal trafficking of LDL-derived cholesterol to the ER and PM.

In the PM-dependent major pathway for LDL-derived cholesterol, one might imagine the distal PM-to-ER pathway to be the same as the SMase-induced PM-to-ER transport discussed, but 3 lines of evidence show they are distinct. First, ATP depletion or vesicle blockers inhibit the distal pathway but not the SMase pathway. Second, mutant 3 to 6 CHO cells show normal esterification of LDL-derived cholesterol despite defective esterification in response to SMase. Third, low doses of U18666A inhibit esterification of LDL-derived cholesterol, but not proximal endosome-to-PM transport or SMase-induced PM-to-ER transport. The fact that the PM-to-ER pathways induced by LDL or by SMase are distinct suggests multiple pools of PM cholesterol.

Studies are beginning to address the role of the ERC in distribution of endosomal cholesterol to other sites. Some cholesterol transport to ER proceeds via ERC: Rab11-mediated ERC cholesterol accumulation decreases ACAT activity ~40%, both in the presence and absence of LDL, but does not affect esterification of cholesterol delivered directly to the PM. ERC cholesterol appears independent of the NPC late endosomal pathway, because Rab11 overexpression causes ERC cholesterol accumulation even in NPC1-deficient or U18666A-treated cells. Furthermore, late endosomal cholesterol efflux appears independent of the ERC, because NPC1-deficient cells transfected with NPC1 clear cholesterol from late endosomes even when cholesterol is trapped in the ERC because of Rab11. Many more experiments will be necessary to further dissect the role of the ERC.

**Mitochondrial Cholesterol Transport**

Mitochondria are considered cholesterol-poor organelles, with the outer mitochondrial membrane containing more cholesterol than the inner. Two important cholesterol-metabolizing P450 enzymes reside on the matrix side of the inner mitochondrial membrane, the P450 side chain cleavage system (P450scc/Cyp11A1) and sterol 27-hydroxylase (Cyp27). Expressed only in steroid hormone-producing cells, P450scc converts cholesterol to pregnenolone, which is modified by other enzymes to generate steroid hormones. The widely expressed Cyp27 converts cholesterol to 27-hydroxysterol, the most abundant oxysterol in plasma. This important oxysterol serves at least 4 functions, as (1) the first intermediate in the alternative pathway of bile acid synthesis from cholesterol; (2) a more soluble transport form of cholesterol in plasma; (3) a potent repressor of SREBP processing; and (4) a partial LXR agonist. Some data have supported 27-hydroxycholesterol as the key LXR ligand in macrophages, so cholesterol transport to mitochondrial
Cyp27 may play a key role in reverse cholesterol transport and atherosclerosis.

**Sources of Mitochondrial Cholesterol**

The source of cholesterol for steroidogenesis or oxysterol generation in mitochondria is unclear and may vary in different cell types or conditions. HDL cholesterol esters, taken up by scavenger receptor B1 (SRB1) and hydrolyzed extralysosomally, appear to be the predominant substrate for adrenocortical steroidogenesis.119,120 LDL cholesterol via the endosomal pathway may also be used, because late endosomal membranes have been shown to transiently contact mitochondria.105 Furthermore, NPC mutant or U18666A-treated cells show decreased use of LDL-derived cholesterol in the production of steroids121 or 27-hydroxycholesterol.111 However, NPC mutant cells are capable of using cholesterol from other sources, because no defect in steroidogenesis has been observed in NPC1-deficient humans or mice. PM cholesterol may be another source, because steroidalogenic stimulation of MA-10 mouse Leydig tumor cells causes increased internalization of PM cholesterol to an endosomal compartment.122 Cytosolic lipid droplets may also provide free cholesterol to mitochondria.49 Therefore, cholesterol from multiple sources ultimately reaches mitochondria, and it is unclear whether any one compartment is the immediate proximal source.

**StAR and Peripheral-Type Benzodiazepine Receptor**

Steroidogenic stimuli activate expression of the cholesterol transfer protein StAR, and StAR mutations cause lipid congenital adrenal hyperplasia with defective steroidogenesis.4 Although there is controversy about the exact site of action of StAR, most data support the outer mitochondrial membrane.123 Like P450scc, StAR expression is limited to steroidogenic cells, so more widely expressed START proteins could deliver cholesterol to mitochondria in other tissues. Cholesterol must then reach the P450 enzymes on the inner membrane, and a candidate for this transport step is the transmembrane peripheral-type benzodiazepine receptor (PBR). PBR is expressed widely, located in outer mitochondrial membranes, and concentrated at contact sites with inner membranes.124 Many lines of experimental evidence have implicated this protein in mitochondrial cholesterol transport: PBR gene disruption in Leydig cells blocks steroidogenesis, PBR binds cholesterol with high affinity, bacteria expressing PBR take-up cholesterol, and PBR ligands stimulate steroidogenesis and 27-hydroxycholesterol formation.124 The sequential action of a START protein and PBR could deliver cholesterol to mitochondria then to P450 enzymes on the inner membrane (Figure 3).

**Conclusions**

There are many complexities in intracellular cholesterol transport. Because pools of intracellular cholesterol are interconnected, trafficking between 2 compartments may be direct or may involve intermediate compartments. For a given transport pathway, different vesicular or nonvesicular mechanisms may act redundantly or sequentially, making their relative contributions difficult to discern. Even within 1 compartment, there may be distinct pools of cholesterol with different kinetics of mobilization to various pathways.

**Figure 3.** Mitochondrial cholesterol transport. The source of mitochondrial cholesterol is uncertain, and multiple subcellular compartments may contribute. The outer mitochondrial membrane (OMM) is represented by a thicker line, whereas the inner membrane (IMM) is thinner and the matrix is shaded. A, In steroidogenic cells, the P450 side chain cleavage system (P450scc/Cyp11A1) resides on the matrix side of the IMM. The steroidogenic acute regulatory protein (STAR) stimulates P450scc-mediated conversion of cholesterol to pregnenolone. STAR is a cholesterol transfer protein ultimately imported into mitochondria, but it is thought to function at the OMM. The peripheral-type benzodiazepine receptor (PBR) resides in the OMM and may mediate cholesterol transfer to the IMM. B, In many cell types, including nonsteroidogenic cells, cholesterol 27-hydroxylase (Cyp27) resides on the matrix side of the OMM, where it generates the important oxysterol 27-hydroxycholesterol. PBR is widely expressed, but other STAR-related lipid transfer (START) proteins are likely to play the role that STAR plays in steroidogenic cells.

Nonvesicular transport mechanisms are clearly important for intracellular cholesterol homeostasis. In the past, nonspecific transporters like SCP-2 were proposed, but more specific intracellular lipid transfer proteins have emerged and are likely the physiological transporters. The prototypical START protein StAR is the best example to date, because StAR mutations cause a specific phenotype in intracellular cholesterol distribution (lipid congenital adrenal hyperplasia).4 Definitive studies have yet to address the lipid-binding promiscuity of all 16 mammalian START domains,125 but they appear relatively specific. The phosphatidylcholine transfer protein (PCTP/StarD2) is highly selective for that phospholipid but not other phospholipids or sterols,126 whereas GPBP/CERT/StarD11 extracts ceramide from membranes with no activity toward sphingosine, sphingomyelin, phosphatidylcholine, or cholesterol.127 StAR can transfer cholesterol and the plant sterol β-sitosterol, but not phosphatidylcholine.128 Among START domain proteins, MLN64/StarD3, StarD4, StarD5, and StarD6 are most related to StAR.129 StarD4 is an SREBP2 target gene, so it and the other putative sterol-binding START domains are strong candidates for roles in intracellular cholesterol homeostasis.125

Cholesterol plays many well-described roles within the cell, but how cholesterol moves to and from key organelles to perform these roles is not as well-known. This review described pathways of intracellular cholesterol transport essential for normal metabolism. Transport involving the PM, ER, and endosomes—perhaps with the ERC and Golgi as intermediates—is essential for cholesterol synthesis, uptake, and esterification. Cholesterol catabolism by enzymes in mitochondria or ER generates steroids, bile acids, and oxysterols, including LXR ligands. Although beyond the scope of this review, intracellular transport pathways must also deliver cholesterol to the distinct apical and basolateral aspects of polarized cells, and to transmembrane proteins that mediate cellular efflux, such as ABCA1 and ABCG5/ABCG8.130
Finally, dysfunctional intracellular cholesterol transport is thought to contribute to atherosclerosis, cholelithiasis, and Alzheimer disease. The emergence of more sophisticated cell biological methods, as well as new candidate proteins, should lead to a greater understanding of intracellular cholesterol transport and its role in normal physiology and disease states.

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