CHOLESTEROL TRANSFER BY NPC2 PROTEIN AND CYCLODEXTRINS IN NIEMANN PICK TYPE C DISEASE

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ABSTRACT OF THE DISSERTATION

Cholesterol transfer by NPC2 protein and cyclodextrins in Niemann Pick type C disease

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The cholesterol storage disorder Niemann-Pick type C (NPC) disease is caused by mutations in either of two lysosomal proteins, NPC1 or NPC2. NPC2 is a 16kDa soluble protein that binds cholesterol. Previous work showed that NPC2 rapidly transports cholesterol to/from vesicles via direct interaction with membranes. In the present site-directed mutagenesis studies, results suggest that the NPC2 surface may have two membrane interacting domains necessary for its cholesterol transport properties. Using a light scattering assay, it was found that NPC2 promotes vesicle-vesicle interactions, supporting the hypothesis for two membrane interaction domains. Results from kinetic sterol transfer assays also indicate that lysobisphosphatidic acid (LBPA), found uniquely in endo/lysosomes, dramatically enhances cholesterol transfer rates by wt NPC2. Sterol transfer and protein-lipid binding studies of NPC2 mutant proteins indicate that NPC2 directly interacts with LBPA, and suggest that the 'hydrophobic knob' region of NPC2 is the LBPA-sensitive domain.

Cyclodextrins (CD) have been shown to reduce symptoms and extend lifespan in murine models of NPC disease. In the present studies the mechanism of sterol transport by CD was investigated. Results indicate that CD directly interacts with membranes to transfer sterol,

similar to NPC2. Absolute transfer rates were slower for CD than NPC2, however, and unlike NPC2, LBPA did not enhance transfer rates by CD. CD also promoted vesicle-vesicle interactions, similar to NPC2. Thus, the efficacy observed in NPC disease models is likely the result of CD enhancement of cholesterol transport between membranes, with rapid sterol transfer occurring during CD−membrane interactions. Unfortunately, continuous administration of CD is required for therapeutic benefit, due to rapid renal elimination. We tested a library of novel CD polyrotaxanes (PRTx), which should have increased circulation time in plasma, in *npc2*$^{-/-}$ fibroblasts. These polymers have environmentally sensitive endcaps, theoretically allowing for controlled release of CD monomers at high concentrations within the endo/lysosomal compartment. Indeed, the PRTx compounds rapidly reduced accumulated intracellular cholesterol in *npc2*$^{-/-}$ fibroblasts, suggesting that they may prove useful in therapeutic application to NPC disease.

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Chapter 4 of this dissertation, entitled "Cholesterol transfer by cyclodextrin is similar but not identical to NPC2", was originally published in *Biochemistry* in July 2011. The studies presented in Chapter 4 represent an equal contribution from myself and a post-doctoral fellow, Dr. Zhi Xu, who worked under Dr. Judith Storch. Likewise, Dr. Xu and I contributed equally to the composition of the manuscript, which was edited by me and Dr. Storch.

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> McCauliff LA, Xu Z, Storch J. Sterol transfer between cyclodextrin and membranes: Similar but not identical mechanism to NPC2-mediated cholesterol transfer. *Biochemistry*. 2011;50(34):7341-7349. Copyright 2011 American Chemical Society.

Chapter 5 of this dissertation, entitled "Cyclodextrin polymers are effective in vitro as an NPC therapy", presents published data collected through collaboration with Dr. David Thompson at Purdue University. Chris Collins and Yawo Mondjinou, pre-doctoral members of the Thompson group, performed the primary synthesis and characterization of the compounds presented in the chapter. Chris and Yawo also originally wrote the sections of the manuscripts that directly pertain to the work they performed. Experiments pertaining to the *in vitro* cellular assays presented in the chapter were solely conducted by myself, as was the writing of the sections in the manuscripts describing this work. Editing of the complete manuscripts was equally conducted by myself, Chris, Yawo, Dr. Thompson and Dr. Storch.

Only the data generated by the Thompson group that was deemed necessary for understanding of the work I performed, in addition to the significance and novelty of the study, were included in this dissertation.

The original articles have been adapted with permission from:

Collins CJ, McCauliff LA, Hyun SH, Zhang Z, Paul LN, Kulkarni A, et al. Synthesis, characterization, and evaluation of pluronic-based beta-cyclodextrin polyrotaxanes for mobilization of accumulated cholesterol from niemann-pick type C fibroblasts. *Biochemistry*. 2013;52:3242-53. Copyright 2013 American Chemical Society.

Mondjinou YA, McCauliff LA, Kulkarni A, Paul L, Hyun SH, Zhang Z, et al. Synthesis of 2-hydroxypropyl-beta-cyclodextrin/pluronic-based polyrotaxanes via heterogeneous reaction as potential niemann-pick type C therapeutics. *Biomacromolecules*. 2013;[Epub ahead of print]:doi:10.1021/bm400922a. Copyright 2013 American Chemical Society.

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List of Abbreviations

BBB	Blood brain barrier
BCD	β-cyclodextrin
BMP	Bis-monoacylglycerol phosphate (also known as LBPA, lyso-bisphosphatidic acid)
CD	Cyclodextrin
CE	Cholesteryl esters
CF	Carboxyfluorescein
DHE	Dehydroergosterol
EPC	Egg phosphatidyl choline
ER	Endoplasmic reticulum
FFAT	Diphenylalanine in an acidic tract motif
FRET	Fluorescence resonance energy transfer
HDL	High density lipoprotein
HPCD	2-hydroxypropyl-β-cyclodextrin
IDL	Intermediate density lipoprotein
LBPA	Lyso-bisphosphatidic acid (also known as BMP, bis-monoacylglycerol phosphate)
LDL	Low density lipoprotein
LDLr	Low density lipoprotein receptor
LE/LY	Late endosome/lysosome
LSD	Lysosomal storage disease
LUV	Large unilamellar vesicle
LXR	Liver X receptor
M6PR	Mannose 6 phosphate receptor
MBCD	Methyl-β-cyclodextrin
MCS	Membrane contact site
NBD	Nitrobenzoxadiozole
NTD	N-terminal domain
ORP	Oxysterol binding protein related protein
PE	Phosphatidyl ethanolamine
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)
PPG	Poly(propylene glycol)
PPO	Poly(ethylene oxide)
PRTx	Polyrotaxane
NPC	Niemann Pick C
NPC1	Niemann Pick C 1 protein
NPC2	Niemann Pick C 2 protein
SRE	Sterol regulatory element
SREBP	Sterol regulatory element binding protein
StAR	Steroidogenic acute regulatory protein
STARD	StAR-related lipid transfer (START) domain
SUV	Small unilamellar vesicle
TNB	Trinitrobenzene
TNBS	2,4,6-Trinitrobenzenesulfonic acid

VAP	Vacuole associated protein
VLDL	Very low density lipoprotein
WT	Wild type

Chapter 1.

Introduction and Review of the Literature

Introduction

Cholesterol is an important molecule that is necessary for normal cellular function. It is a precursor for numerous biologically active molecules, including steroid hormones, oxysterols and bile acids, and it plays a major role in membrane structure and function. The processes involved in cellular cholesterol homeostasis, including synthesis, intestinal absorption, esterification and metabolism, are controlled by transcriptional and post-transcriptional feedback mechanisms (1-3). Disturbances in these highly regulated homeostatic responses have the potential to be pathological. Aberrant accumulation of cholesterol in arterial endothelium, for instance, is a hallmark of atherosclerosis (4, 5), while increased concentrations of cholesterol in bile may lead to formation of gallstones (6). Conversely, lack of sufficient cholesterol levels can also be detrimental. During embryonic development, for example, insufficient fetal cholesterol synthesis has the potential to cause numerous congenital birth defects, such as structural brain malformations and microcephaly, as well as neonatal mortality (7).

Much is currently known about cholesterol metabolism, including its synthesis, extracellular transport in plasma lipoproteins and uptake of cholesterol rich low density lipoproteins (LDL) by the LDL receptor, yet questions remain regarding its intracellular trafficking. Studies have clearly demonstrated that cellular uptake of LDL, via receptor-mediated endocytosis (8), is followed by fusion of the endosomal and lysosomal compartments, where the cholesteryl ester core of LDL is hydrolyzed to unesterified cholesterol and free fatty acids (4). Free cholesterol is then rapidly trafficked from the late endosomal/lysosomal (LE/LY) compartment, primarily to the cell membrane and endoplasmic reticulum (ER). Within the ER, unesterified cholesterol

induces homeostatic responses to regulate the cellular cholesterol pool, including the regulation of LDL receptor expression and de novo cholesterol synthesis (9, 10).

Cholesterol is extremely hydrophobic, and transport of this molecule has been shown to occur via vesicular and protein mediated mechanisms (11). The processes involved in the egress of LDL-derived cholesterol from the endosomal/lysosomal system, however, remain unclear. The lysosomal cholesterol storage disorder, Niemann Pick C (NPC) disease, has provided insight into this critical step of intracellular cholesterol transport, through identification of two endo/lysosomal proteins, NPC1 and NPC2, whose function is necessary for preservation of normal cholesterol transport out of the LE/LY compartment. Questions regarding the mechanism(s) by which these proteins mediate cholesterol transport, however, still remain. Given the critical nature of this process in the maintenance of cellular and whole-body cholesterol homeostasis, elucidation of the mechanism(s) by which cholesterol is transported out of the LE/LY compartment is of great fundamental importance.

Intracellular cholesterol trafficking

Cells obtain cholesterol either from exogenous sources, i.e. circulating lipoproteins, or through endogenous biosynthesis. The processes involved in cholesterol synthesis are tightly regulated by plasma and cellular cholesterol levels, and occur in all nucleated cells. Qualitatively, however, the primary sites of cholesterol synthesis are the liver and the intestine (1, 12). Thus, most cells meet cholesterol requirements through uptake of circulating lipoproteins. The liver packages cholesterol, along with triglyceride, into very low density lipoproteins (VLDL) for delivery to extra-hepatic cells. The cholesterol present in these lipoproteins includes that which was synthesized by hepatocytes, as well as diet-derived cholesterol that was delivered to the liver by chylomicron remnants (1). VLDL delivers fatty acids to adipose and muscle via lipoprotein lipase, resulting in the conversion of these lipoproteins to cholesterol rich intermediate density lipoproteins (IDL). Some IDL, roughly 40 to 60%, will be recycled back to the liver (5, 13). The IDL remaining in circulation is converted to low density lipoprotein (LDL) through the actions of hepatic lipase, which catabolizes additional triacylglycerol at the surface of hepatocytes (4, 13). Conversion of VLDL particles to IDL and LDL also involves transfer of cholesteryl esters from circulating high density lipoproteins (HDL), via cholesteryl ester transfer protein, in exchange for triacylglycerol (4, 13). The cholesterol present in LDL particles represents 70 to 75% of the total cholesterol in plasma (5, 13), yet is less than 10% of the body's total cholesterol pool (5).

Cellular uptake of cholesterol rich LDL occurs when the lipoprotein binds to LDL receptors on the surface of cells. Within clathrin coated pits, the LDL receptor complex, along with other receptors and proteins, lipids and solutes, undergoes invagination (4, 8). The endocytosed

contents are delivered to mildly acidic early endosomes, where they are efficiently sorted for either recycling or degradation. This sorting process includes the uncoupling of LDL from the LDL-receptor, and rapid segregation of the two components (14). Tubules within this compartment form recycling endosomes, which carry the segregated LDL receptors, among other contents, back to the plasma membrane. On the contrary, contents destined for degradation, including LDL-derived cholesterol and down regulated receptors, are collected within large vesicles forming on early endosome membranes. These membrane invaginations are contained within the newly formed early endosome vesicles, giving these intermediary compartments a hallmark multivesicular appearance (15).

Nearly half of the cholesterol present in the endosomal system is identified within the multivesicular bodies/endosomal carrier vesicles (16), which detach from early endosomes for transport to late endosomes and lysosomes. Furthermore, most of the cholesterol present within these acidic (pH ~5.5) (17) endosomal carrier vesicles localizes to the internal membranes (16). It is not clear whether this cholesterol is in an esterified or unesterified form, as little is actually known regarding the composition of endosomal carrier vesicle membranes. Acid lipase is commonly believed to be a lysosomal enzyme, which would suggest that that cholesterol present in multivesicular bodies is primarily in an esterified form. However, Chang and colleagues (18) demonstrated that this “lysosomal” enzyme is in fact localized to an acidic compartment between early and late endosomes. Thus, it is likely that acid lipase may actually function within multivesicular bodies to hydrolyze the cholesteryl ester core of LDL to free, unesterified cholesterol, before reaching the late endosomal and lysosomal compartments.

Following detachment from peripheral early endosomes, endosomal carrier vesicles are transported over long intracellular distances, in a microtubule- and motor-dependent fashion, towards perinuclear late endosomes (14, 17, 19). Utilizing well-defined mechanisms, the multivesicular bodies then selectively dock and fuse with late endosomes (14).

Similar to multivesicular bodies, late endosomes contain a complex network of internal membranes. In contrast to multivesicular bodies, however, the cholesterol content of internal late endosomal membranes is quite low, due to a rapid decrease in late endosomal cholesterol content immediately following the fusion event (16). This transition mirrors the unique late endosomal/lysosomal (LE/LY) phospholipid, lyso-bisphosphatidic acid (LBPA), which is present at high concentrations in the internal membranes of these later (16, 20-23), but not earlier, compartments (16, 23). Besides accumulating large amounts of this unique LE/LY anionic phospholipid, the internal membranes of late endosomes also undergo a high degree of remodeling, which LBPA itself may be involved in (14, 15, 21). LBPA may additionally play a role in directing transport of materials through the endo/lysosomal system. The mannose 6-phosphate receptor (M6PR), for instance, delivers lysosomal enzymes to the endolysosomal system and is subsequently recycled back to the trans-Golgi network. Within the LE/LY, M6PR has been found to localize in LBPA-rich inner membranes. Interestingly, loss of M6PR expression leads to an increase in LBPA content in internal membranes of late endosomes (24). Conversely, antibodies against LBPA lead to the ablation of M6PR transport (21), in addition to the accumulation of cholesterol (22). Thus, late endosomal membranes rich in LBPA likely play a role in transport through the endosomal system.

Within late endosomes, some of the contents present in the intraluminal vesicles are able to "escape" lysosomal degradation by utilizing a mechanism known as back-fusion. This process involves fusion of specific intra-endosomal vesicles with the limiting membrane, for transport back to the plasma membrane or other regions of the cell. Studies have demonstrated that anthrax toxin (17, 25, 26) and vesicular stomatitis virus (17, 27) exploit this particular mechanism in order to reach the cytoplasm. Some inner-membrane contents are also excreted from late endosomes within exosome-like particles (17). Proteins and lipids destined for degradation, however, remain within the late endosome, though the mechanism(s) involved in this late endosomal sorting have yet to be fully elucidated.

Vesicles containing acid hydrolases are released from the trans-Golgi network and fuse with late endosomes. Following acid-induced dissociation of the membrane-bound M6RP receptors, these enzymes are released into the lumen of late endosomes. Having thus accrued a full complement of lysosomal acid hydrolases necessary for degradation of the proteins, lipids, DNA and RNA remaining within the late endosome, this compartment matures into a lysosome. The boundary between late endosomes and lysosomes, however, is not entirely clear. This is primarily because LE/LY hybrid organelles contain components from each independent compartment (14, 28), in addition to the fact that a considerable amount of degradation occurs in the LE/LY, not lysosomes (28). Nevertheless, cholesterol must then leave this LE/LY compartment in order to induce the homeostatic responses that regulate flux of the total cholesterol pool. Indeed, rapid transport of this metabolite does occur, though the mechanisms involved in this process of egress are incompletely understood. However, investigations into the lysosomal cholesterol storage disorder, Niemann Pick type C (NPC) disease, have afforded some

insight. Specifically, identification of the genes that underlie NPC disease has focused attention on the role of two lysosomal proteins in this trafficking process. NPC1 is a polytopic thirteen-transmembrane domain protein, with a putative sterol sensing domain, that resides within the limiting LE/LY membrane, while NPC2 is a small, intralysosomal protein. Both proteins have been shown to bind cholesterol, and studies have further demonstrated that NPC2 can traffic cholesterol between membranes *in vitro* (29, 30). Additionally, due to the observation that deficiencies in either NPC1 or NPC2 result in similar cholesterol accumulation and clinical phenotypes, it has been hypothesized that the two proteins may function cooperatively in normal LE/LY cholesterol efflux.

LDL-derived cholesterol leaving the LE/LY compartment is primarily transported to the plasma membrane and the ER, where it can be reesterified. Studies have indicated that post-lysosomal transport of LDL-derived cholesterol to the plasma membrane likely occurs in a Golgi-dependent manner (31). This movement may occur via formation of cholesterol-rich caveolae, which have been shown to cycle between the plasma membrane and the Golgi complex (32). Transport to the ER is also dependent upon the Golgi, yet it is currently unclear whether LE/LY cholesterol moves directly to the ER, or must first pass through the plasma membrane. Regardless of its pathway from the LE/LY, once within the ER free cholesterol induces an array of homeostatic responses to regulate the cellular cholesterol pool, including regulation of LDL receptor expression as well as the expression of enzymes involved in de novo cholesterol synthesis (9, 10).

Autophagy

While the endocytic pathway is integral in the degradation of extracellular and plasma membrane based molecules, it is also utilized in a separate cellular pathway for removal of intracellular components, such as proteins and organelles. This particular lysosomal degradation pathway, known as autophagy, can be triggered by a number of extracellular signals including stress and nutrient starvation (33). The process commences when an autophagosome forms around a portion of the cytoplasm, which is facilitated by cup shaped structures known as a phagophores or pre-autophagosomes. Autophagosomes contain undigested cytoplasmic components, yet none of the enzymes necessary for their break down (34). Thus, these newly formed vesicles must deliver their contents to the lysosome for degradation; a process that is facilitated by fusion of autophagosomes with endosomal pathway compartments. Although autophagosomes may interact with any endosomal vesicle to form an amphisome, studies have indicated that the primary endocytic compartment for autophagosome fusion is multivesicular bodies (34). While this particular fusion event represents entrance of the autophagic pathway into the endosomal pathway, the amphisome is morphologically distinguishable from other endocytic structures, and is thus considered to be an independent prelysosomal vesicle (34, 35). The amphisome can then mature into an autolysosome, similar to late endosome to lysosome maturation, where the contents of the initially formed autophagosome can be degraded. Autophagosomes are also able to fuse directly with lysosomes for induction of degradation.

Endogenous cholesterol

Apart from cells of the liver and intestine, the majority of a cell's cholesterol requirement is met through endocytosis of exogenous, LDL-derived cholesterol. As previously mentioned, however, all nucleated cells synthesize cholesterol. The process of *de novo* cholesterol synthesis occurs within the ER, and is tightly regulated by existing cellular cholesterol levels. Genes involved in cholesterol synthesis exhibit a sterol regulatory element (SRE) sequence in the promoter region of their gene sequence, where the transcription factor known as SRE binding protein (SREBP) binds (1, 4). There are three known mammalian isoforms of SREBPs, varying in their tissue distribution and target gene selectivity; SREBP-2 specifically activates genes involved in endogenous cholesterol synthesis (1). When low levels of intracellular cholesterol are sensed by the cell, SREBP-2, normally located on the ER membrane, is cleaved and its N-terminal domain is trafficked to the nucleus where it binds to SREs, activating transcription of genes for enzymes involved in cholesterol biosynthesis (1, 4). Liver X receptors (LXRs), in addition to the SREBPs, also contribute to the regulation of cholesterol biosynthesis. These nuclear receptors are activated by a number of sterol metabolites and specifically activate the transcription of genes involved in cellular cholesterol efflux. Thus, when high levels of cholesterol are sensed within the cell, LXRs, which form heterodimers with retinoid X receptors, activate the transcription of genes that encode for proteins involved in cellular cholesterol efflux (1).

When cholesterol synthesis is stimulated in the ER, the newly synthesized sterol must leave the ER in order to perform many of its functions. The primary destination is the plasma membrane, where most cellular free cholesterol is found (4, 36, 37). While exogenous cholesterol is trafficked from the endo/lysosomal system to the plasma membrane in a Golgi-mediated

manner, only a small fraction of *de novo* synthesized cholesterol utilizes this mode of vesicular transport. Instead, studies have indicated that most of the cholesterol synthesized within the ER moves to the plasma membrane in a Golgi-independent fashion (38, 39). It is likely that this trafficking occurs by nonvesicular, protein-mediated pathways (36) , though the proteins involved have yet to be identified.

Figure 1-1.

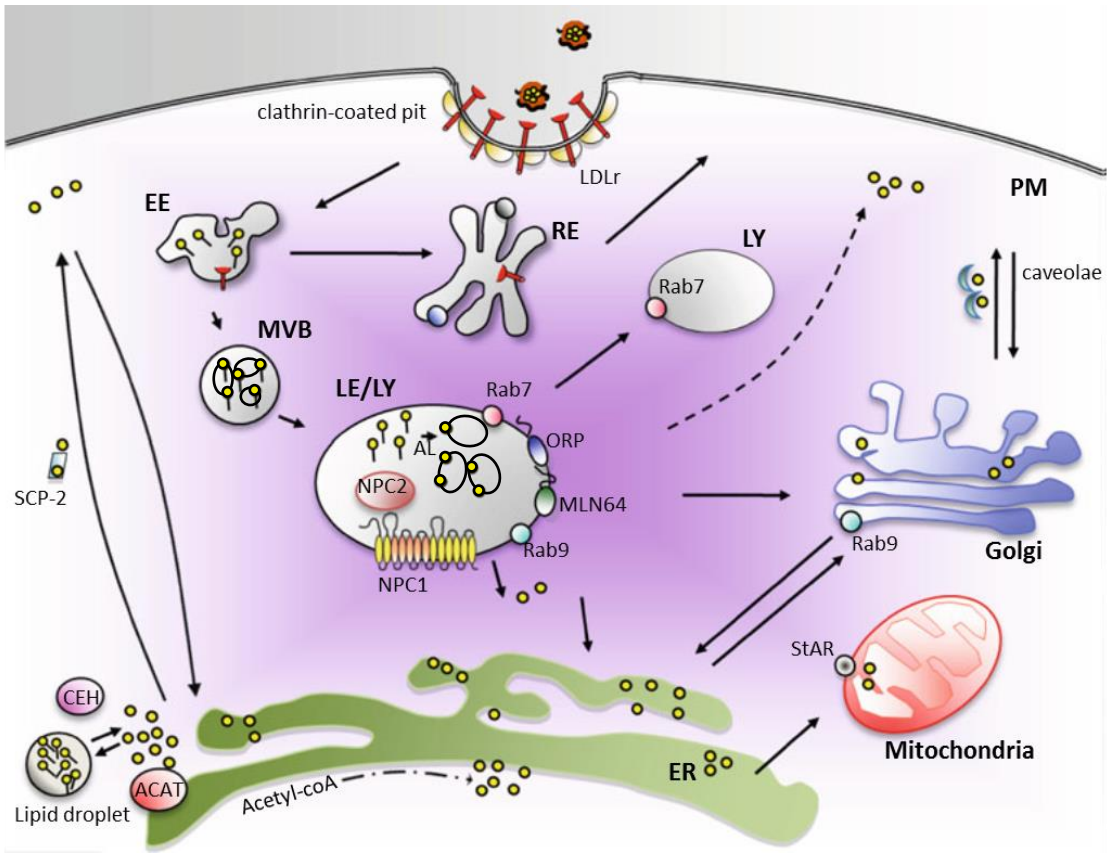


Figure 1-1. Intracellular cholesterol trafficking.
Mechanisms of intracellular trafficking for endogenous and exogenous cholesterol sources are summarized. Exogenous cholesterol, in the form of circulating LDL, is taken up by cells via LDL-receptor mediated endocytosis. Engulfed LDL is delivered to, and progresses through, the endo/lysosomal system, where acid lipase hydrolyses LDL-derived cholesterol esters to free cholesterol (yellow circles). Free cholesterol is transported out of the late endosome/lysosome (LE/LY) via the actions of at least two lysosomal proteins, NPC1 and NPC2, and is subsequently trafficked to either the plasma membrane or ER in a primarily Golgi-dependent manner. Cells also synthesize cholesterol endogenously in the ER. Endogenous cholesterol is also trafficked to the PM, mainly utilizing a distinct, Golgi independent pathway. *LDLr, LDL receptor; EE, early endosome; MVB, multivesicular body; LE/LY, late endosome/lysosome; RE, recycling endosome; LY, lysosome; ER, endoplasmic reticulum; PM, plasma membrane.* Image adapted with permission from Goedeke and Fernandez-Hernando Regulation of cholesterol homeostasis *Cell Mol Life Sci* 2012;69(6). Copyright 2012 Springer.

Lysosomal storage disorders

Lysosomal storage disorders (LSD) include approximately fifty rare, genetic diseases that result in the disruption of lysosomal homeostasis. These inborn errors of metabolism have been estimated to occur at a collective frequency of approximately 1 in 5,000 live births (40), though this figure may underestimate actual incidence due to undiagnosed and/or misdiagnosed cases. Common to all LSDs are genetic mutations that result in either deficient enzyme activity or loss of protein function, with examples listed in **Table 1-1**. Most LSDs are caused by defects in either specific lysosomal enzymes, required for the catabolism of complex molecules, or membrane transport proteins that are responsible for egress of end products from the lysosomal compartment. Causation can also be linked to defects in non-enzymatic soluble or transmembrane proteins, believed to be essential for lysosomal pH regulation, trafficking and/or sorting or macromolecules, vesicle fusion and so on (41). Regardless of the mechanism, these defects consequently lead to the progressive, aberrant accumulation of specific substrates within endo/lysosomes (40, 42).

LSDs are most frequently grouped together based on the major substrate found to accumulate within the LE/LY. These broad categories consist of the lipidoses, the sphingolipidoses, the gangliosidoses, the mucopolysaccaridoses, the glycoproteinoses, the mucolipidoses and others that do not fit under the above categories (43). Classification of disorders is not always straight forward however, as more than one substrate typically accumulates (**Table 1-1**). Complex storage patterns commonly arise in LSDs following excessive accumulation of primary substrates within LE/LYs. Aberrant levels of these macromolecules or catabolic products inhibit other, normally functioning endo/lysosomal proteins, resulting in the accumulation of secondary

Table 1-1.

Disease	Defective protein	Primary substrate(s) stored
Defects in specific lysosomal enzymes		
Mucopolysaccharidoses (MPS)		
MPS I (Hurler, Scheie, Herler/Scheie)	α-Iduronidase	Dermatan sulphate, heparan sulphate
MPS II (Hunter)	Iduronate-2-sulphatase	Dermatan sulphate, heparan sulphate
MPS IIIA (Sanfilippo)	Heparin-N-sulphatase	Heparan sulphate
MPS IVA (Morquio-A)	Galactose-6-sulphatase	Keratan sulphate
Sphingolipidoses		
Tay Sachs (GM2-gangliosidosis)	β-hexosaminidase A	GM2-ganglioside, globoside, oligosaccharides, glycolipids
Sandhoff (GM2-gangliosidosis)	β-hexosaminidase A & B	GM2-ganglioside,,oligosaccharides
Fabry	α-Galactosidase A	Globotriaosylceramide
Gaucher	β-Glucosidase	Glucosylceramide
Farber	Ceramidase	Ceramide
Niemann Pick A and B	Sphingomyelinase	Sphingomyelin
MLD	Arylsulfatase A	Sulfatides
Oligosaccharidoses and glycoproteinoses		
Aspartylglucosaminuria	Aspartylglucosaminidase	Aspartylglucosamine
Schindler disease	α-Galactosidase B	Glycopeptides, oligosaccharides
Glycogenosis		
Pompe	α-Glucosidase	Glycogen
Lipidosis		
Wolman	Acid lipase	Cholesterol esters
Defects in post-translational modification of lysosomal proteins		
Mucopolysaccharidoses (MPS)		
Multiple sulfatase deficiency	Formylglycine-generating enzyme	Dermatan sulphate, heparan sulfate
Mucolipidoses		
Mucolipidosis type II (I-Cell)	N-acetylglucosamiyl-1-phosphotransferase	Carbohydrates, lipids, proteins
Mucolipidosis type IIIA (pseudo-Hurler)	N-acetylglucosamiyl-1-phosphotransferase	Carbohydrates, lipids, proteins
Defects in structural lysosomal membrane proteins, protective proteins, transporters and trafficking		
Lipidosis		
Niemann-Pick C	NPC1 or NPC2	Cholesterol and sphingolipids
Monosaccharide, amino acids and monomers		
Cystinosis	Cystinosin	Cystine
Danon disease	LAMP-2	Glycogen and other autophagic components
Infantile sialic acid storage disorder (ISSD)	Sialic acid transporter	Sialic acid, glucuronic acid
Mucolipidoses		
Mucolipidosis IV	Mucolipin-I	Mucopolysaccharides and lipids

Table 1-1. Lysosomal storage disorders. Examples of lysosomal storage disorders, organized according to the nature of the protein defect, are listed. Information gathered from Ballabio and Gieselmann *Biochim Biophys Acta* 2009;1793(4), Wraith *Semin Neonatl* 2002;7(1) and Wraith *Dev Med Child Neurol* 2001;43(9)

substrates (41, 44). Thus, while the aforementioned classification system is clinically useful and generally accepted, from a pathophysiological or biochemical standpoint it is less so; secondary storage compounds may affect pathogenesis, and substrate accumulation is not entirely systematic.

Various compartments of the endosomal-autophagic-lysosomal system are also found to accumulate in cells, based on substrate storage patterns. Primary storage substrates typically result in the accumulation of lysosomes, in some cases lipid rich multilamellar LE/LYs, and autolysosomes (44, 45). As secondary substrates begin to accumulate, stress on the endo/lysosomal system may lead to the additional buildup of autophagosomes, multivesicular bodies, and late endosomes (44, 45). Beyond the effects on the endosomal-autophagic-lysosomal system, sequestration of primary and secondary substrates has the potential to impact other organelles such as the ER, mitochondria, and Golgi, as well as general function of the cell (44).

Clinical presentation of LSDs, like other metabolic diseases, varies considerably. The range in rate of disease progression between individuals with the same disorder is also broad, as is the spectrum of affected tissues. Most individuals with LSDs are asymptomatic at birth, however, and exhibit normal early development. Although age of onset does vary considerably between and among diseases, it is typically during infancy and early childhood that initial signs of the condition become apparent. Neurodegeneration, marked by retardation in developmental progress, may become apparent in diseases where the central nervous system is affected (43, 46). With other LSDs, physical changes such as organomegaly or dysmorphic facial appearance may be the first sign (43). Some individuals have also been found to display relatively normal

progress through childhood and adolescence, only presenting with symptoms of the disease in adulthood (43, 46).

A number of available tests are utilized for diagnosing individuals with lysosomal storage disorders. Urine samples are often collected in suspected cases of mucopolysaccharidoses and glycoproteinoses, where analyses of glycosaminoglycan or oligosaccharide excretion patterns are used as an initial screen (43, 46). Other suspected disorders may instead require screening via an electrocardiogram, as in Pompe disease, or histological analysis, as in Ceroid lipofuscinosis (CLN) or NPC disease (46). Given the general nature of these analyses, screening tests are typically followed by specific enzyme assays to ensure accurate diagnoses, though, as discussed below, enzymatic assays are not used for the diagnosis of NPC disease. In cases where simple screening assays are not available, more specific assays are the first step towards diagnosis. For many disorders, whole blood cell or plasma enzyme assays are utilized, which are fairly non-invasive approaches. Unfortunately, false negatives are obtained at times from these biochemical analyses. More invasive procedures, such as an organ biopsy for histological analyses, may thus be necessary for these particular patients where an LSD is strongly suspected (46). Regardless of the disease, demonstration of lysosomal sequestration via electron microscopy is considered the quintessential method for diagnosis (46).

Variability in the clinical features of LSDs presents clinicians with a number of obstacles pertaining to the development of a systemic therapeutic approach. Improving quality of life through supportive care, however, is integral in the management of all LSDs. Until fairly recently, in fact, general palliative care measures were the only available therapeutic options for

most patients. Within the last two decades, much headway has been made in therapeutic development for this set of rare metabolic diseases. Enzyme replacement therapy, substrate reduction therapy, pharmacological chaperone therapy and gene therapy have become available to patients with many of the LSDs. Enzyme replacement therapy is based on observations that many lysosomal enzymes are secreted and subsequently sequestered by lysosomes, via the mannose-6-phosphate receptor-mediated pathway, in distant tissues (47). Thus, specific lysosomal enzymes are able to be administered via the bloodstream in order to correct for the particular LSD enzyme deficiency. Proof of concept was shown in patients with type I Gaucher disease; administration of modified β-glucocerebrosidase effectively ameliorated hepatosplenomegaly and skeletal damage while improving anemia and thrombocytopenia, consequently becoming the standard treatment for these patients (48-50). More recently, enzyme replacement therapy has been shown to be effective for individuals with Fabry disease, Pompe disease and mucopolysaccharidoses type I, II and IV, though degree of efficacy has been shown to vary significantly (50). One substantial disadvantage of enzyme replacement therapy, however, is the non-homogenous distribution of the administered enzyme among tissues. Most notably, due to the nature of the blood brain barrier (BBB), little of the administered enzyme is able to reach cells within the brain (48, 51). Hence, LSDs with appreciable effects on the CNS show less benefit with this particular therapy.

In contrast to enzyme replacement therapy, which focuses on removing stored materials from the lysosome, substrate reduction therapy can be used to limit influx of these materials into the endo/lysosomal pathway. This is done by partially inhibiting biosynthesis of the stored metabolite through the use of small molecules. The primary advantage to this approach,

especially compared to enzyme replacement therapy, is the ability to design molecules that might bypass the BBB for effective treatment within the CNS. A prime example of substrate reduction therapy is the inhibition of ceramide glucosyltransferase, a key enzyme in the biosynthesis of glycosphingolipid, for treatment of type I Gaucher disease (49, 51). This particular example provided proof of principle for substrate reduction therapy, and the approach has since been investigated in a number of other LSDs including Tay-Sachs, Sandhoff and NPC disease.

Gene therapy, which focuses on the replacement of mutated genes with normal copies, is another option that has been investigated for use in LSDs. One advantage to this therapy is that even small increases in the levels of target gene expression can have significant therapeutic benefit for patients (48). Additionally, since single genes are found to be defective in LSDs, this is an attractive approach to treatment of these disorders (48, 52). The physiologically ubiquitous nature of this group of disorders would typically preclude a gene therapy approach. However, as in enzyme replacement therapy, existence of the mannose-6-phosphate receptor pathway provides a mechanism to overcome the multi-organ target issue (52). Adenoviruses, adeno-associated viruses and multiple retroviruses, including lentiviruses and recombinant herpesviruses, have all been used as vectors for accomplishing *in vivo* gene transfer in a number of LSD animal models (52). Treatment efficacy varied significantly between these studies, however, and observation of adverse effects in a number of treated animals challenges the safety of viral vectors. Hence, although gene therapy is considered to be a promising treatment for many LSDs, it is a risky option presenting a number of obstacles that have yet to be overcome before considering this a viable option for patients.

Mutations in a large number of the genes that lead to LSDs may be quite subtle, and have little to no effect on the synthesis of the gene product. In fact, these mutant proteins may be functionally capable, yet their alterations have effects on the stability of the folded protein. Lack of proper folding, and the subsequent problems associated with processing and trafficking to the endo/lysosomal system, results in the accumulation of these misfolded proteins in the ER and Golgi. Small molecule pharmacological chaperones can selectively bind to these misfolded proteins and effectively stabilize their three dimensional structures. Once stabilized, trafficking of the protein through the Golgi to the lysosome is restored (53, 54). Once in the lysosome, the chaperone dissociates allowing for function of the protein, as long as the mutation does not disrupt any domains required for normal activity. In some disorders, such as Fabry and Pompe disease, low level restoration of enzyme activity via pharmacological chaperone therapy has been sufficient for attenuation of disease symptoms (54).

Niemann Pick C disease

The term 'Niemann-Pick disease' was coined in the 1920s based on research conducted by Albert Niemann and Ludwig Pick, and is used to identify a group of heterogeneous, autosomal recessive, lipid storage disorders. Clinical and biochemical analyses of several patients, performed by Crocker and Farber in 1958, revealed wide variability in the age of onset, clinical presentation, and sphingomyelin storage patterns (55). This work led to Crocker's proposal for classification of these disorders into four subgroups, types A-D (56). Patients with type A and B exhibited marked accumulation of sphingomyelin in extraneural organs, with type A individuals also exhibiting severe, early CNS deterioration. These two Niemann Pick disorders were eventually linked to a deficiency in acid sphingomyelinase (57, 58), leading types A and B to be recognized as sphingolipidoses. Individuals with types C and D are clinically indistinguishable, yet type D patients are separated based on their homogenous Acadian ancestry. Both type C and D exhibited milder organomegaly than type A, neurological involvement that was only moderately acute, and, most notably, milder visceral sphingomyelin storage than types A or B (55, 56). Additionally, neither type C nor D could be linked to deficiencies in lysosomal sphingomyelinase (58), suggesting that these two types of disorders are metabolically distinct from A and B. Indeed, studies conducted by Peter Pentchev and colleagues led to the reclassification of Niemann Pick type C (NPC) from a sphingomyelin to a cholesterol storage disorder (59, 60). Subsequent work demonstrated that the observed sequestration of unesterified cholesterol in the endolysosomal system was specifically due to deficiencies in the intracellular transport of endocytosed cholesterol (59, 61-63).

Today, inclusive of individuals formerly classified as type D, NPC continues to be described as a lysosomal cholesterol trafficking disorder. Accordingly, it is characterized by the aberrant accumulation of cholesterol and other glycolipids in the LE/LY of cells. The trafficking defect is ubiquitous, yet lipid storage patterns do vary between organs. For instance, a two- to five-fold increase in unesterified cholesterol and sphingomyelin content, in addition to moderate accumulation of LBPA and glycolipids, has been observed in the liver and spleen of NPC patients (64). Conversely, the cholesterol and sphingomyelin content of the whole brain does not evidently increase (65-68); aberrant cholesterol accumulation *is* observed in neuronal cell bodies (65, 69), but a decrease in the cholesterol content of distal axons (65) likely accounts for the absence of change in the whole brain. Instead, significant accumulations of glycosphingolipids, namely gangliosides GM2 and GM3, glucosylceramide and lactosylceramide have been reported in the NPC brain (64, 68, 70).

NPC disease is classically a neurovisceral condition with patients commonly displaying hepatosplenomegaly and progressive neurodegeneration (60, 71). Similar to what is seen in other LSDs, however, the clinical presentation is quite heterogeneous, with age of onset ranging from birth to late adulthood. The lifespan of NPC individuals also varies significantly, though most patients prematurely pass away between the ages of 10 and 25 (72-74). Fatality in these patients can be attributed to the neurological disease, which is primarily manifested through cerebellar ataxia, dysphagia, dysarthria and progressive dementia. Dystonia, cataplexy and seizures are also commonly exhibited, and in late-onset patients psychiatric disturbances are noted as occurring frequently (75). Systemic disease, involving the liver, spleen and sometimes lung, always precedes the onset of neurological symptoms, yet is usually mild and well tolerated

by individuals beyond the perinatal period. In fact, visceral involvement has been found to be either absent or minimal in approximately 15% of early-onset and nearly half of all adult-onset patients (75).

The disease has been estimated to affect approximately 1 in 150,000 to 1 in 100,000 individuals (64, 75). Similar to other LSDs, true prevalence is difficult to assess due to insufficiencies in clinical awareness and the difficulties associated with disease testing. Defects in either of the genes encoding for two lysosomal proteins have been shown to account for all disease cases; mutations in the NPC1 gene account for approximately 95% of NPC cases (76), while mutations in NPC2 are responsible for the remaining 5% (77). While the accumulation of cholesterol has been associated with impairment in normal LE/LY efflux, the pathways for trafficking of lysosomal cholesterol and the specific roles of NPC1 and NPC2 remain unknown.

NPC2

NPC2 is a small, 132 residue intralysosomal protein. It was previously identified as the cholesterol-binding protein present in mammalian epididymal fluid, termed HE1 (78). Crystallization of apo bovine NPC2 revealed a loosely packed hydrophobic core, originally suggested to be an incipient, internal ligand pocket (79). Studies of holo NPC2 bound to cholesterol sulfate have demonstrated accommodation of this steroid via expansion of the pocket in a 1:1 stoichiometric ratio of ligand:protein (80). Work completed by Cheruku et al (29) as well as Friedland et al (79) have further demonstrated a significant increase in binding of cholesterol analogs by NPC2 at acidic pH, consistent with the hypothesis that NPC2 binds cholesterol in the LE/LY compartment.

While the functional implications of cholesterol binding by NPC2 have yet to be understood, it can be hypothesized that this property is necessary for egress of cholesterol from the LE/LY, as NPC patients exhibit accumulation of free cholesterol in this compartment. Evidence supporting this theory stems from research showing effective clearance of cholesterol from NPC fibroblasts upon addition of purified NPC2 protein to media (81). Given partitioning of cholesterol to internal membranes of multivesicular bodies and LE/LYs (16, 23), it has further been hypothesized that NPC2 may function as a cholesterol transport protein (29, 30), moving cholesterol from inner-LE/LY membranes to the limiting lysosomal membrane, where cholesterol must pass for delivery to the ER or plasma membrane. The inability for three NPC2 mutants identified by Ko et al as being able to bind cholesterol normally, but unable to reverse the sterol accumulation in *npc2*$^{-/-}$ fibroblasts (81) , supports this idea.

There are two basic mechanisms by which a hydrophobic ligand, such as cholesterol, can be transferred from a binding protein to a membrane. The first mechanism involves complete desorption of lipid from the donor protein, followed by diffusion of the ligand through aqueous media and final incorporation of lipid into the acceptor membrane. The rate-limiting step in this mechanism of aqueous diffusion is the release of ligand from protein. Thus no change in transfer rates are anticipated as membrane concentration changes since the rate of ligand dissociation from the protein is independent of the acceptor (82, 83). The second mechanism requires direct interaction between the protein and membrane, with rates of lipid transfer determined, in part, by the number of collisions that occur between donor and acceptor. In this case, the rate of lipid transfer is expected to increase as acceptor membrane concentration rises due to an increase in the number of protein-membrane collisions (82, 83)

Studies analyzing NPC2 as an intracellular sterol transport protein have not only revealed the ability for this protein to transfer cholesterol to and from model membranes, but have further shown significant increases in intermembrane transfer rates of dehydroergosterol (DHE), a fluorescent cholesterol analog, in the presence of NPC2 (29, 30). Kinetic analyses have demonstrated that transfer of cholesterol occurs via direct interaction of NPC2 with donor and acceptor membranes, and that rates of cholesterol transfer are dramatically increased by physiological relevant parameters such as acidic pH and presence of the unique LE/LY phospholipid LBPA (30). Further insight into the potential role of NPC2 in normal LE/LY cholesterol efflux has come from studies demonstrating the inability for certain NPC2 mutants with normal binding affinity to reverse cholesterol accumulation in *npc2*$^{-/-}$ fibroblasts (81).

NPC1

In contrast to NPC2, mature NPC1 is a large, 1252 residue polytopic glycoprotein that resides in the limiting LE/LY membrane. It is predicted to have thirteen transmembrane domains and a putative sterol sensing domain with homology to the sterol sensing domain of two key regulators of cholesterol homeostasis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and sterol regulatory element binding protein cleavage-activating protein (SCAP) (84). Formed by a cluster of five membrane-spanning sequences, this domain appears necessary for normal function of NPC1 as mutations in this region lead to inactivation of the protein (85). The exact role of the sterol sensing domain in NPC1, however, is currently unknown. NPC1 also includes two motifs that have the potential to mediate protein multimerization. These include a cysteine rich loop with a ring-finger motif, located on the third luminal domain of the protein, and a leucine-zipper motif on a highly conserved domain on the luminal facing amino terminus

(76). Importantly, NPC1 has also been shown to bind cholesterol in its N-terminal domain (NTD) (86-88), and has recently been shown to bind oxysterol-derivatives in a different, as yet unidentified, ligand binding site (89). Regardless of the identification of these motifs, all of which may be important for NPC1 mediated efflux of lysosomal lipids, the mechanism by which NPC1 transfers cholesterol out of the LE/LY remains unknown.

Studies in NPC1 and NPC2 disease models have indicated that both proteins are necessary for normal trafficking of LDL-derived cholesterol out of the endolysosomal system (64, 73, 75). Studies have further indicated that mutations in either NPC1 or NPC2 result in similar cellular and clinical phenotypes (64, 75), suggesting that these proteins may function cooperatively. Supporting this hypothesis, and certainly a prerequisite for direct interactions between NPC1 and NPC2, substantial or partial overlap in the subcellular localization of the proteins has been described (90-92). Another finding supporting direct transfer of cholesterol between the proteins is the opposing orientation of cholesterol in the binding pockets of NPC2 and the NTD of NPC1. Specifically, crystallographic studies have shown that NPC2 incorporates the isooctyl side chain of cholesterol into its binding pocket, leaving the 3β-hydroxyl portion of the molecule exposed (79, 80), whereas the NTD of NPC1 binds the 3β-hydroxyl region of cholesterol, leaving the isooctyl side chain partially exposed (86). Thus it has been proposed that cholesterol can transfer directly from the binding pocket of NPC2 to the binding pocket of the NPC1-NTD, without altering its orientation. NPC2 has recently been shown to directly interact with the second luminal domain of NPC1, however this domain does not bind cholesterol, and sterol transfer between the proteins was not observed (93). Conversely, Infante et al demonstrated that equilibrium distribution of cholesterol between the soluble NTD of NPC1 and lipid bilayers

was increased in the presence of NPC2 (87). Based on this observation, it was suggested that cholesterol may be transferred directly between the two proteins (87). Subsequent studies from this group indicated that the observed transfer of sterol was dependent upon three residues on the surface of NPC2 (88). Computational studies also identified a number of NPC1 and NPC2 surface residues, including those indicated by Wang et al, that would likely play a role in the interaction between the proteins in the instance direct transfer of cholesterol occurs (94). These findings led to the proposal that normal LE/LY cholesterol trafficking involves direct 'handoff' of the sterol from NPC2 to the NTD of NPC1, though protein-protein interactions have yet to be directly demonstrated (88). Several attempts have been made using classical approaches for detection of interactions between NPC2 and the NPC1, yet none have thus far been successful (88)(M.Scott and P.Lobel personal communication).

Therapeutic approaches to Niemann Pick C disease

Like many other LSDs, therapeutic approaches for NPC have been aimed at alleviating the symptoms caused by progression of the disease, and improving quality of life. To date, management of NPC disease is still primarily symptomatic (75). Anticholinergic agents, for instance, can be administered for improvement of dystonia and tremor in some patients (73, 75). Additionally, antiepileptic drugs have the potential to at least partially control seizures until patients develop fairly advanced stages of the disorder (73, 75, 95). Non pharmacological approaches such as physiotherapy, used for management of spasticity and prevention of contractures, can also be beneficial (73, 75). Advances have been made in the development of therapies for NPC disease over the last several years, yet early and accurate diagnosis of patients remains an obstacle for the efficient treatment of NPC disease.

Miglustat

Progressive neurodegeneration observed in patients with NPC disease is most often fatal, and is therefore a primary target for therapy. Although cholesterol does accumulate in neuronal cell bodies, the whole brain content of cholesterol does not actually increase in patients. Instead, gangliosides and other glycosphingolipids are found to accumulate excessively in the NPC brain (64), and contribute to at least some of the neuropathological features of the disease. In fact, the abnormalities observed in NPC disease closely resemble the neuropathalogical features observed in primary gangliosidoses (69, 96, 97). Rationale was thus provided for trials of substrate reduction therapy using an inhibitor of glucosylceramide synthase. The chosen iminosugar, known as miglustat or N-butyl-deoxynojirimycin (NB-DNJ), had originally been approved for treatment of patients with Gaucher disease.

Initial studies pertaining to the therapeutic efficacy of miglustat in NPC were conducted with murine and feline models of the disease. It was found that administration of the inhibitor led to reductions in neural ganglioside accumulation, cellular pathology and clinical neurological progression; less than 60% of treated mice were characterized as having a clinical phenotype by the time all untreated animals had died from the disease. A 25% increase in the longevity of treated mice was observed, though 80% of the animals in this group did eventually develop the clinical NPC phenotype (98). Controlled clinical trials were subsequently conducted in neurologically symptomatic NPC patients. Individuals receiving miglustat therapy showed stabilization, and in some cases slight improvement, in several clinically relevant endpoints such as progressive dysphagia, auditory acuity, ambulation and, most notably, horizontal saccadic eye movement (HSEM) velocity, which can be correlated with disease progression (99). Subsequent long term studies in children, juveniles and adults with NPC revealed stabilization of some neurological parameters of NPC disease progression in more than 70% of patients that had received miglustat therapy for at least one year (54, 100).

In January 2009, based on the success of this substrate reduction therapy in NPC disease, miglustat (Zavesca®) received FDA approval for treatment of progressive neurodegeneration in both pediatric and adult patients (75). It is not recommended for use in patients with systemic disease only, however, as the inhibitor is not expected to have an effect on the systemic manifestations of the disease. Adverse effects of miglustat treatment are mostly mild in nature, including diarrhea, flatulence, weight loss and tremor, and clinicians are recommended to treat NPC patients at the first sign of neurological disease.

Cyclodextrin

Cyclodextrins (CD) are natural macrocyclic oligosaccharides produced by the enzymatic cyclization of glucopyranoside units linked by α-1,4-connections. α-, β-, and γ-CD, contain 6, 7 and 8 glucopyranose units, respectively, though CDs can be generated with more than 15. These molecules possess a toroidal topology with a hydrophobic internal cavity, allowing binding and solubilization of small hydrophobic molecules such as cholesterol. β-cyclodextrin (BCD) and its derivatives have garnered the most attention due to their widespread use in the pharmaceutical and food industry, given their low toxicity and price, where they are used as solubilizing agents, permeability enhancers, and active ingredient stabilizers (101, 102). Additionally, they have historically been used in experimental applications to either enrich or deplete plasma membrane cholesterol (103), and are used clinically as a vehicle for drug delivery (104).

CD was originally identified as a potential therapy for NPC disease when it was used as a vehicle to deliver potential therapeutic compounds to murine models of the disease. Much to their surprise, the researchers noted that the CD vehicle alone appeared to have substantial benefit on ameliorating manifestations of the disease (105, 106). Indeed, Liu et al. demonstrated that the administration of a single dose of a BCD derivate, hydroxypropyl-β-CD (HPCD), at 7 days of age to *npc1*$^{-/-}$ mice resulted in the rapid release of cholesterol accumulation from the LE/LY compartment, as monitored by cholesterol esterification in the ER and the restoration of sterol-dependent regulation of SREBP2 and LXR-mediated target gene expression (106). Remarkably, the CD-treated mice also showed diminished neuropathology and >40 % extension of lifespan

over untreated controls (107). Similar benefits were found in studies using chronic administration of HPCD in both NPC1 and NPC2 deficient mice (105). Interestingly, two other lipid storage diseases, characterized by primary accumulation of gangliosides (GM1 gangliosidosis) or mucopolysaccharides (MPS IIIA) and secondary cholesterol accumulation, were not ameliorated by CD treatment (105).

The molecular mechanisms by which CD leads to the rapid restoration of normal post-LE/LY cholesterol transport are beginning to be understood, and current models suggest that CD enters the endocytic pathway in order to promote egress of sequestered LE/LY cholesterol to the ER. Indeed, Rosenbaum et al. showed that CDs were functioning within the LE/LY compartment following fluid phase pinocytosis, rather than acting at the plasma membrane level (108). Decreases in the mRNA levels of SREBP2 and its target genes, and an opposing increase in LXR target genes in the brains and livers of NPC1-deficient mice treated with CD, (107) further support this model. Additionally, studies have demonstrated that *in vitro* ACAT-mediated cholesterol esterification increases in cultured NPC1-decificient mouse fibroblasts following CD administration, indicating that cholesterol is being mobilized to the ER in the presence of CD (109). An alternative hypothesis for CD action in NPC disease is extraction of cholesterol from the plasma membrane, depleting cellular cholesterol levels and in turn likely promoting an increase in cholesterol biosynthesis. Results obtained from the aforementioned studies indicate cholesterol synthesis is overall decreased in NPC disease models treated with CD, arguing against a model of cholesterol extraction from the plasma membrane. Based on the effectiveness of CD in treatment of NPC1-deficient mice, it was suggested that the CD may be functioning by delivering acid lipase-derived cholesterol to NPC2, thereby substituting for the

defective NPC1 (107). While specific interactions between CD and NPC2 have not been reported, interactions of CD with a number of other proteins have been observed (110), making NPC2-CD interactions a plausible hypothesis.

Cyclodextrin polymers

Cholesterol accumulation has been observed to decrease in murine models of NPC disease following administration of CD, however intracellular sequestration of cholesterol was unfortunately found to resume in these animals shortly after the CD treatment was terminated (107, 111, 112). Studies have thus demonstrated that while BCD derivatives have the potential to overcome deficient trafficking of cholesterol in NPC disease, prolonged effects of the treatment are seemingly dependent upon continual administration (112, 113). Indeed, Liu et al reported that 6 hours following BCD administration in *npc1*$^{-/-}$ mice, only 8% of the dose remained in animals while only 3% was detected at 24 hours post-administration (107). Similar results were found in normal mice administered BCD or HPCD, where 80% of the dose was detected in urine after 5 hours (114). Frinjlink et al. also showed that approximately 10% of a single intravenous dose of either BCD or HPCD was still detected in plasma of rats 30 minutes following administration. Recovery of nearly the entire administered dose in urine, unaltered, within a 24 hour period suggests rapid glomerular filtration of the compounds (115).

It may be possible to overcome the issues facing current CD treatments by increasing the efficiency of CD delivery and improving the pharmacokinetics and biodistribution of the administered dose. One potential method for achieving these goals would be to deliver CD via a high molecular weight vehicle. This type of BCD formulation would offer increased circulation

times in blood, as size would allow bypass of glomerular filtration, potentially allowing for delivery of a more efficacious dose of monomeric CD to the LE/LY. These changes to current methods of administering CD could represent a beneficial new treatment for NPC disease patients.

Polyrotaxanes (PRTx) are supramolecular assemblies that have been of considerable interest in biomaterials applications such drug and gene delivery and hydrogel formation (116-120). These complexes are typically prepared by noncovalently threading macrocycles, such as CD, onto a polymer core. The addition of bulky endcaps to the termini of the polymer core, which prevent dethreading of the macrocycles, produce a polyrotaxane (**Figure 1-2**). Specificity can be engineered into a PRTx through the modification of these endcaps. The use of endcaps that are bioresponsive towards changes in pH (121) or specific enzyme activity (122, 123), for instance, allow for control over when and how the PRTx degrades to release its macrocyclic cargo. Cyclodextrin-based PRTx derivatives have been synthesized using many polymer cores including polyesters (124), polyamides (125), poly(ethylene glycol) (PEG) (126-128), polypropylene glycol (PPG) (129) and many di- and tri- block copolymers (130). These compounds have not been tested for therapeutic efficacy in a model of NPC disease, however.

In summary, while many of the processes involved in intracellular cholesterol metabolism have been elucidated, several questions still remain, particularly those pertaining to the mechanisms of cholesterol transport within the endolysosomal system and the manner by which cholesterol leaves the LE/LY compartment. Identification of the two proteins, NPC1 and NPC2, exhibiting deficiencies in the LE/LY cholesterol storage disorder, NPC disease, initially shed some light on

Figure 1-2

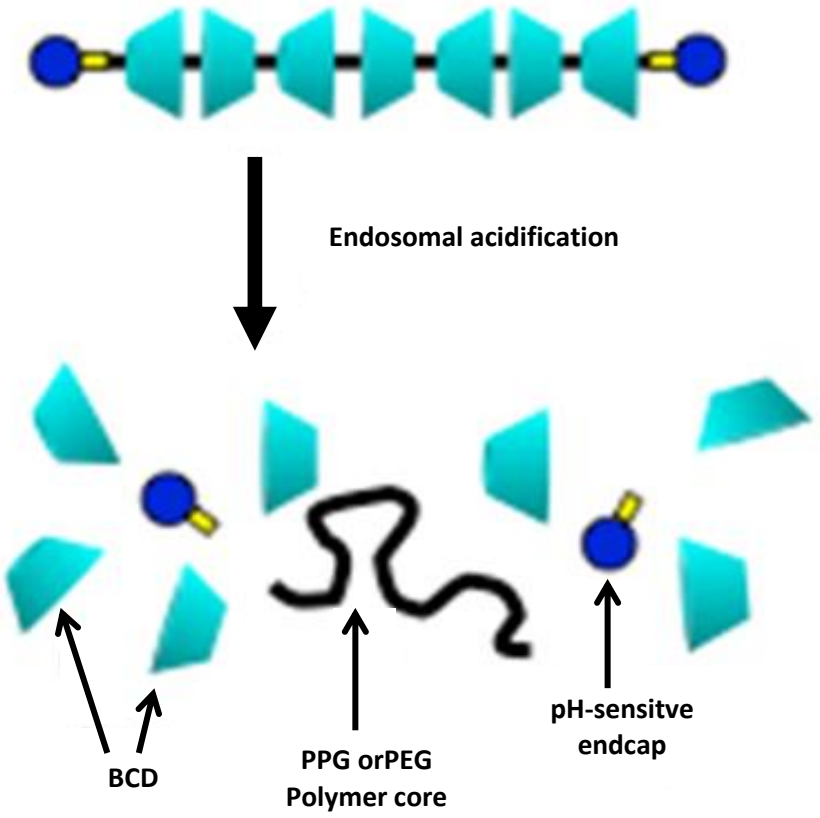


Figure 1-2. Schematic representation of a BCD polyrotaxane. Construction of CD polyrotaxanes involves threading of CD units onto a polymer core. CD monomers are held on the polypropylene glycol (PPG) or polyethylene glycol (PEG) core by the addition of bulky endcaps. Specific endcaps are used in the construction of these polymers to confer specificity to the dethreading process. Acid labile endcaps, for instance, will be cleaved when the PRTx enters an environment with a low pH, allowing for dethreading of the CD monomers.

this subject. The mechanisms by which NPC1 and NPC2 direct egress of cholesterol out of the LE/LY compartment, however, remain unknown. Studies demonstrated the ability for NPC2 to bind cholesterol (79) and, subsequently, that this binding was necessary but not sufficient for reversal of cholesterol accumulation in an NPC2-deficient cell model (81). This suggested that this small, soluble LE/LY protein may function in the normal transport of LE/LY cholesterol. Indeed, studies focused on examining NPC2 as a cholesterol transport protein demonstrated the ability for NPC2 to transfer sterol between membranes (29, 80), supporting a role for NPC2 in normal LE/LY cholesterol trafficking. The structural basis for this sterol transfer function by NPC2 remains unknown, however, as does its relationship with NPC1 in directing normal efflux of cholesterol from the LE/LY.

In the absence of therapies based on a molecular level understanding of NPC1 and NPC2 function, current therapies for NPC disease have primarily focused on substrate reduction. Cyclodextrins have proved useful in this regard, effectively ameliorating aberrant cholesterol accumulation and pathological manifestations of the disease in animal models, additionally extending life span. The mechanism(s) of CD mediated LE/LY cholesterol egress have not been determined, however, and the therapy exhibits some shortcomings. Most notably, continuous administration of CD therapy is required to sustain positive effects of the therapeutic in animal models as well as NPC patients, likely due to rapid renal filtration. In an effort to overcome the drawbacks of the current therapy, David Thompson's group at Purdue University synthesized a library of novel CD polymers, designed to theoretically allow for increased circulation times in plasma and the delivery of CD to the LE/LY compartment at high concentrations.

Based on the issues presented, the work included in this dissertation focuses on determining the mechanisms by which NPC2 and CD transfer cholesterol out of LE/LY compartment, and also focuses on the therapeutic potential of a group of novel CD polymers. The studies present an effort to further our understanding of the processes involved in LE/LY cholesterol efflux, which will allow for further development of effective therapies for NPC disease. Thus, the specific aims are as follows.

Specific Aims

Lysosomal storage disorders, affecting nearly 40,000 Americans, are characterized by the accumulation of harmful levels of macromolecules in the late endosomal/lysosomal compartment (LE/LY) of cells. Over time this leads to permanent tissue damage, particularly in the brain, peripheral nervous system, liver, spleen, and bone marrow, and is often fatal. Niemann Pick Type C (NPC) disease is specifically characterized by the accumulation of LDL-derived cholesterol as well as other lipids in the LE/LY, a result of deficiency in either of two lysosomal proteins, NPC1 or NPC2. The mechanisms by which NPC1 and NPC2 direct normal efflux of cholesterol from LE/LYs remain unknown, although the similarities in cellular and clinical phenotypes of NPC1 disease and NPC2 disease suggest they act in a common pathway. Studies from our laboratory have indicated that NPC2 acts to transfer cholesterol out of the LE/LY compartment via direct interaction with internal LE/LY membranes that contain lysobisphosphatidic acid (LBPA), a phospholipid that is uniquely enriched in internal lysosomal membranes.

For patients lacking NPC1 or NPC2, effort has focused on so-called substrate reduction therapy. The sterol binding hydrocarbon 2-hydroxypropyl-β-cyclodextrin (HPCD) has recently been shown to effectively reduce cellular cholesterol accumulation in both *npc1*$^{-/-}$ and *npc2*$^{-/-}$ mice, ameliorating neurological and other disease manifestations and extending lifespan. Although approved by the FDA for compassionate use in a number of NPC patients, the therapeutic potential of CD is limited, likely due to rapid renal elimination. This has driven development of CD polymers with the expectation that such compounds may reveal greater efficacy.

The ultimate goal of this work is to understand how to enhance and/or complement NPC functions in affected patients. The work focuses on both the fundamental mechanisms of NPC protein actions, and on the utility of novel therapeutics to bypass these proteins and ameliorate sterol accumulation in a cellular model of NPC disease.

The specific aims of this project are to:

AIM 1. Establish the structure-function relationships for NPC2 in cholesterol transport. Using kinetic assays developed in the laboratory for the wild type protein, NPC2 was shown to interact directly with membranes to extract and/or deliver cholesterol, with dramatically increased sterol transport rates in the presence of LBPA (29, 30). Preliminary studies using site-directed mutagenesis of NPC2 suggest that more than one domain on the surface of NPC2 is necessary for cholesterol transport activity. It was therefore hypothesized that NPC2 acts at membrane contact sites, functioning as a "bridge" between inner lysosomal membranes and the limiting lysosomal membrane, to clear cholesterol from LE/LYs. Studies in this aim will use a series of fluorescence-based kinetic assays, as well as membrane-membrane interaction assays, to determine the structural basis of NPC2 function in cholesterol transport and its apparent sensitivity to LBPA.

AIM 2. Determine the kinetic mechanisms and regulation of cholesterol transport by cyclodextrins. Intravenous administration of the sterol-binding hydrocarbon 2-hydroxypropyl-β-cyclodextrin (CD) effectively reduces cellular cholesterol accumulation and increases lifespan of NPC1 deficient mice (105-107, 111). The mechanism(s) underlying these effects, however, are

not understood. Utilizing the methods developed for analyzing the rate and mechanism of cholesterol transfer by NPC2, rates of cholesterol movement from CD to membranes, from membranes to CD, and the intermembrane transfer of sterol in the absence and presence of CD will be quantified. Additionally, the potentially efficacy of CD in NPC2 disease will be evaluated by analyzing the amelioration of cholesterol accumulation in an NPC2 deficient cell line treated with the compound.

AIM 3. Examine the utility of novel polymeric CDs in reducing sterol accumulation in NPC deficient cells. CD shows promise in the treatment of NPC deficiency, however its rapid renal clearance diminishes therapeutic efficacy. A novel group of CD polymers, expected to have longer circulation times, has been synthesized by the Thompson laboratory at Purdue; monomeric CDs are released from the polymer only under acid pH conditions, thus it is likely that CD monomers will be liberated in the LE/LY. In the proposed studies, these novel CD polymers will be examined for effective cholesterol transport in *npc2*$^{-/-}$ cells, a necessary step prior to further development of effective in vivo therapies for NPC disease and, perhaps, other lysosomal storage disorders.

Chapter 2

Site directed mutagenesis of NPC2 reveals sterol transport domains

Abstract

The cholesterol storage disorder Niemann-Pick type C (NPC) disease is caused by defects in either of two genes that encode for the late endosomal/lysosomal (LE/LY) proteins NPC1 and NPC2. NPC2 is a 16kDa soluble protein that binds cholesterol in a 1:1 stoichiometry. NPC2 may also function as a cholesterol transport protein, transferring cholesterol between membranes by a mechanism that involves protein-membrane interactions, as demonstrated in our previous work (29, 30). To determine the structural basis of NPC2 function in cholesterol trafficking, a series of point mutations were generated across the surface of NPC2. Mutants were analyzed for their ability to transfer cholesterol or the fluorescent sterol analog, dehydroergosterol, in a set of fluorescence-based transfer assays, as well as for their ability to promote the egress of accumulated intracellular cholesterol from *npc2*$^{-/-}$ fibroblasts. Several NPC2 mutants were found to have deficient sterol transport properties in the transfer assays. Notably, these mutants were also unable to reverse cholesterol accumulation in *npc2*$^{-/-}$ fibroblasts. The mutations mapped to two distinct regions on the surface of the protein, suggesting that NPC2 can potentially bind to two membranes simultaneously. Using a light scattering assay, we previously demonstrated that wild type NPC2 causes vesicle-vesicle interactions (131). Here we show that point mutations causing defective sterol transfer kinetics and the inability to promote cholesterol efflux from NPC2-deficient cells also abrogate vesicle-vesicle interactions. Thus, we hypothesize that NPC2 traffics cholesterol, at least in part, by acting at membrane-membrane contact sites within the multilamellar interior of late endosomes and lysosomes, thereby promoting rapid cholesterol transfer between closely appositioned membranes and, ultimately, effecting cholesterol egress from the LE/LY compartment.

Introduction

Niemann Pick Type C (NPC) disease is a lysosomal lipid storage disorder specifically characterized by the accumulation of unesterified cholesterol and glycolipids in the late endosome/lysosome (LE/LY) compartment. Over time, permanent damage to cells and tissues occur as a result of this excessive lipid storage, particularly in the brain, peripheral nervous system, liver, spleen and bone marrow (60, 71). The accumulation of cholesterol in this rare, autosomal recessive disorder is due to mutations in either of two lysosomal proteins, NPC1 or NPC2, which result in defective cholesterol trafficking. The mechanisms by which NPC1 and NPC2 lead to cellular cholesterol egress are only beginning to be understood at the molecular level.

NPC1 is a polytopic transmembrane protein residing in the limiting lysosomal membrane. It contains a transmembrane sterol-sensing domain, binds cholesterol in its soluble N-terminal domain (86-88), and has recently been shown to bind oxysterol-derivatives in a different, as yet unidentified, ligand binding site (89). NPC2, in contrast, is a small, 132 residue soluble intralysosomal protein. It has also been shown to bind cholesterol, with 1:1 stoichiometry (79, 80), and we have shown *in vitro* that it rapidly catalyzes cholesterol transfer between membranes via direct protein-membrane interactions (29, 30). Notably, sterol transport rates by NPC2 are dramatically enhanced by the unique LE/LY phospholipid lysobisphosphatidic acid (LBPA) (30), which is enriched in intralysosomal membranes (16, 23).

NPC2 deficient cells and mouse models accumulate free cholesterol in LE/LY's, highlighting the essential role of NPC2 function in normal cholesterol egress from this compartment. While the

structural basis for the cholesterol transport function of the protein remains largely unknown, one particular report has been informative in this regard. Prior to the availability of the NPC2 tertiary structure, Ko et al. used site-directed mutagenesis of evolutionarily conserved residues and made the important observation that NPC2 point mutants unable to bind cholesterol could not reverse cholesterol accumulation in *npc2*$^{-/-}$ fibroblasts, thus demonstrating the functional requirement for cholesterol binding by NPC2 for the first time (81). Interestingly, they also described three point mutations in NPC2 that, despite normal cholesterol binding affinity, were nevertheless ineffective in *npc2*$^{-/-}$ cell cholesterol clearance (81). We hypothesized that these three residues were likely important in cholesterol transport, and the tertiary structure of NPC2 later revealed that all three residues were on the protein surface (79). More recently, it has been suggested that NPC2 directly transfers cholesterol to the luminal N-terminal domain (NTD) of NPC1 (86, 88, 93). Thus, the final step in cholesterol egress from the LE/LY compartment may involve sterol transfer between the two proteins, although direct interactions between NPC2 and the NPC1 NTD have not as yet been demonstrated (88).

In the present studies we show that the three mutants identified by Ko et al as being able to bind cholesterol normally but unable to 'rescue' the cholesterol accumulation phenotype of NPC2-deficient cells (81), are indeed markedly ineffective in transferring cholesterol between membranes. Several other point mutations on the surface of NPC2 also result in deficient cholesterol transfer properties, and, interestingly, appear to form two separate domains on the surface of NPC2. This observation suggests the potential ability for NPC2 to cause membrane-membrane interactions, and to perhaps form bridges between membranes, as occurs at so-called membrane contact sites (MCS) (132-134). It is thus possible that NPC2 stimulates rapid

transfer of cholesterol at zones of close apposition between membranes, as exist in the interior of multivesicular endo/lysosomes (14), and perhaps between these inner membranes and the limiting lysosomal membrane.

Material and Methods

Materials.

Cholesterol was obtained from Sigma (St. Louis, MO). Egg phosphatidylcholine (EPC), dehydroergosterol (DHE), dansyl phosphatidylethanolamine (dansyl-PE), nitrobenzoxadiozole phosphatidylcholine (NBD-PC) and Lissamine Rhodamine phosphatidylethanolamine (Liss Rhodamine PE) were from Avanti (Alabaster, AL). Filipin III was obtained from Fisher (Pittsburg, PA). Human fibroblast cells from an apparently healthy donor (GM03652), and from an NPC2 patient (GM18455) were from Coriell Institute of Medical Research (Camden, NJ).

Generation and purification of NPC2 mutants.

Point mutations were generated with the Stratagene QuikChange Site Directed Mutagenesis Kit, using a myc 6xHis-tagged murine NPC2 plasmid generously provided by Dr. Matt Scott (81). Wild type and mutant myc 6xHis-tagged NPC2 proteins were purified from transfected CHO KI cells as previously described (30, 81), using a 10 kDa cutoff flow filtration membrane to initially concentrate conditioned media (Millipore, Bedford, MA).

Cholesterol binding by NPC2.

NPC2 has two tryptophan residues at positions 109 and 122, and binding of cholesterol by the protein results in quenching of the endogenous tryptophan fluorescence. Thus, cholesterol binding by wt and mutant NPC2s was determined by incubating the proteins with increasing concentrations of cholesterol and monitoring tryptophan quenching. A 50 mM stock solution of cholesterol was prepared in dimethylsulfoxide (DMSO). Proteins in citrate buffer (20mM sodium citrate, 150mM NaCl pH 5.0) were incubated with increasing concentrations of

cholesterol for 20 minutes at 25°C. The final concentration of DMSO was ≤1% (v/v). Equilibrium binding constants were determined by a three parameter hyperbolic decay fit of the data using Sigma Plot software (San Jose, CA).

Membrane vesicle preparation.

Small unilamellar vesicles (SUV) were prepared by sonication and ultracentrifugation as previously described (29, 30, 83). Vesicles were maintained at temperatures above the phase transition temperatures of all constituent lipids. Standard vesicles were composed of 100 mol % EPC. 25 mol% DHE and 3 mol% dansyl-PE were substituted for EPC in the donor and acceptor vesicles, respectively, used in the intermembrane sterol transfer assays. All vesicles were prepared in citrate buffer (20mM sodium citrate, 150mM NaCl pH 5.0). For the preparation of large unilamellar vesicles (LUV), EPC dissolved in chloroform was dried under nitrogen for 1 hour to create a lipid film, and resuspended in citrate buffer, forming multilamellar structures. The lipid suspension was placed alternately on dry ice and in a water bath above 37°C for 7 freeze-thaw cycles, followed by extrusion through a 100 nm pore membrane (Avestin, Ottawa, Ontario, Canada) using a mini-extruder (Avestin, Ottawa, Ontario, Canada) for 11 passes. The final phospholipid concentration of all vesicles was determined by quantification of inorganic phosphate (83).

Cholesterol transfer from NPC2 to membranes.

The endogenous tryptophan fluorescence of NPC2 was used to monitor cholesterol transfer from NPC2 to membranes, as previously described, using a stopped-flow mixing chamber interfaced with a Spectrofluorometer DX18-MV or SX20 (Applied Photophysics Ltd., UK) (29, 30).

Conditions to ensure the absence of photobleaching were established before each experiment. Data were analyzed with the software provided with the Applied Photophysics stopped flow spectrofluorometer, and the cholesterol transfer rates were obtained by exponential fitting of the curves. All curves were fit well by a single exponential function.

Intermembrane transfer of sterol.

As described by Xu et al (30), the fluorescent cholesterol analog DHE is used in donor membranes and its fluorescence resonance energy transfer (FRET) partner, dansyl-PE, in acceptor membranes. Intermembrane transfer of DHE is measured by DHE quenching or the sensitized emission of dansyl fluorescence, and is examined in the absence and presence of wt or mutant NPC2 protein (30). Conditions to ensure the absence of photobleaching were established before each experiment, and data were analyzed as described above.

Clearance of cholesterol from npc2$^{-/-}$ fibroblasts by wt and mutant NPC2s

Wild type and *npc2*$^{-/-}$ fibroblasts, plated onto 8-well tissue culture slides (Nalgene) at a density of approximately 6,000 cells per well, are administered a single dose of purified wt or mutant NPC2 protein to culture media 24-36 hours after plating. The final concentration of protein is 0.4 nM. Cells are fixed and stained with 0.05mg/ml filipin 3 days following treatment, at which time cells are imaged on a Nikon Eclipse E800 epifluorescence microscope and the filipin stain area is quantified with the accompanying NIS-Elements software (Nikon Inc), as previously described (30, 131).

Membrane aggregation and fusion

LUVs are mixed with wt or mutant NPC2 proteins, or bovine serum albumin (BSA), and absorbance at 350nm is used to monitor membrane-membrane interaction (aggregation), as described by Schulz et al (135). Membrane fusion is assessed in two ways; mixing of vesicle contents and mixing of membrane lipids. Monitoring fusion by mixing of contents is done as described previously (136) using 100mol% EPC LUVs filled with > 100mM 5-carboxyfluorescein (5-CF), at which point the 5-CF is self-quenched. Release of self-quenching (i.e., increase in fluorescence) is monitored upon mixing of 50 μM 5-CF-filled EPC LUVs with 50 μM citrate buffer-filled EPC LUVs in the presence of 1μM wt NPC2, using λex 493/λem 520nm. Membrane fusion is also determined using resonance energy transfer between NBD and rhodamine, as developed by Struck et al (137). Briefly, 50 μM EPC SUVs containing 1 mol% NBD-PC are mixed with 50μM EPC SUVs containing 1 mol% Liss Rhodamine PE, in the absence or presence of 1μM wt NPC2. NBD is excited at 460nm and the decrease in NBD fluorescence (λ534) or the sensitized emission of rhodamine (λ571) upon mixing of the two vesicle populations, indicative of membrane fusion, is monitored over time. All experiments are conducted in citrate buffer at 25°C. For each membrane fusion assay, conditions are established to verify the absence of photobleaching.

Results

Several mutants have deficient sterol transfer properties

All NPC2 point mutants generated bound cholesterol similar to wt protein (**Table 2-1**) with dissociation constants in the sub micromolar range. Having verified that the point mutations had little or no effect on the cholesterol binding properties of the protein, the ability for each mutant to transport sterol to membranes or between membranes was assessed using the above-described kinetic transfer assays and EPC vesicles. As shown in **Table 2-2**, the K97A, E99A and K6A mutations had little or no effect on the sterol transfer rates by the protein in the two independent assays. The K16A and E70A mutations reduced sterol transfer rates by nearly half of wt values in both a protein to membrane and an intermembrane transfer assay, indicating that they may play some role in cholesterol trafficking by NPC2. The most significant attenuations in sterol transfer by NPC2, however, were observed with the remaining mutations shown in **Table 2-2**, which showed at least a 70% relative reduction in transfer rates. I62D, V64A and D113A had the most dramatic effect of all the mutations, with essentially undetectable cholesterol transfer.

Sterol transfer kinetics are reflective of cellular events

The kinetic data obtained from the model membrane sterol transfer studies suggest that several point mutations on the surface of NPC2 result in defective cholesterol trafficking properties by the protein. To determine whether these results reflect NPC2 function at the cellular level, *npc2*$^{-/-}$ fibroblasts were incubated with purified wt or mutant NPC2 proteins, and the effects on cellular cholesterol accumulation was observed via filipin staining. Following a three day

Table 2-1

	Kd, Cholesterol
wt NPC2	0.37 μM
K97A	0.27 μM
E99A	0.48 μM
K6A	0.30 μM
K16A	0.36 μM
E70A	0.30 μM
K32A	0.19 μM
N39A	0.63 μM
K115A	0.33 μM
D85A	0.60 μM
K75A	0.27 μM
D72A	0.89 μM
E108A	0.29 μM
H31A	0.41 μM
Q29A	0.13 μM
D113A	0.31 μM
I62D	0.32 μM
V64A	0.31 μM

Table 1-1. Cholesterol binding affinity by wt and mutant NPC2 proteins. The dissociation constants (Kd) for cholesterol were determined by tryptophan quenching using 5 μM protein as described in Materials and Methods. A standard error of 15 to 20% of the reported binding constant was calculated for all mutants.

Table 2-2

	NPC2 to membranes	Intermembrane
wt NPC2	1.00	1.00
K97A	0.93 ± 0.038	0.70 ± 0.050
E99A	0.90 ± 0.048	0.75 ± 0.040
K6A	1.1 ± 0.027	0.56 ± 0.029
K16A	0.54 ± 0.012	0.40 ± 0.021
E70A	0.35 ± 0.019	0.43 ± 0.022
E108A	0.14 ± 0.003	N.D.
H31A	0.13 ± 0.007	N.D.
Q29A	0.10 ± 0.006	N.D.
K32A	<0.01	0.32 ± 0.017
N39A	<0.01	0.26 ± 0.014
K115A	<0.01	0.22 ± 0.008
D85A	<0.01	0.21 ± 0.012
K75A	<0.01	0.21 ± 0.011
D72A	<0.01	0.17 ± 0.009
D113A	< 0.01	N.D.
I62D	< 0.01	N.D.
V64A	< 0.01	N.D.

Table 1-2. Relative *in vitro* sterol transfer rates of NPC2 mutant proteins. Cholesterol transfer from NPC2 proteins to membranes, or DHE transfer from donor to acceptor membranes in the presence of NPC2 was determined as described in Materials and Methods. Kinetic data are presented relative to wt NPC2 transfer rates. The absolute rate of cholesterol transfer from wt NPC2 to membranes was 0.0367 ± 0.0031 s^{-1}, while intermembrane transfer rates of DHE in the presence of wt NPC2 was 0.0094 ± 0.0006 s^{-1}. N.D., not determined.

incubation with wt NPC2, cholesterol accumulation in the *npc2*$^{-/-}$ fibroblasts was essentially reversed; the protein promoted efflux of greater than 90 % of the cholesterol as compared to untreated controls (**Figure 2-1**). E99A and K97A, two mutants that had little effect on sterol transfer rates in the kinetic studies, were also found to stimulated cholesterol efflux from the *npc2*$^{-/-}$ fibroblasts; incubation with the mutants reduced filipin stain in the fibroblasts to nearly 15% of untreated controls. In contrast to the effects observed with wt, E99A and K97A NPC2, E108A, H31A, Q29A, N39A, K115A, D85A, K75A, D72A, D113A, I62D and V64A were all found to be ineffective at reversing the cholesterol accumulation phenotype (**Figure 2-1**). Thus, the inability of these eleven mutants to reverse the cholesterol accumulation in *npc2*$^{-/-}$ fibroblasts is consistent with results from the sterol transfer assays, where the mutants exhibited considerably attenuated rates of cholesterol transfer (**Table 2-2**). Similarly, mutants exhibiting a moderate decline in relative sterol transfer rates (E70A and K16A) reduced filipin staining, by approximately 60 to 70% (**Figure 2-1**).

Results from the sterol transfer assays and the cellular cholesterol efflux studies allowed for the identification of several residues on the surface of NPC2 that appear necessary for the cholesterol transport properties of the protein. These affected residues form two separate large domains on the surface of NPC2. One of these domains is at the “top” of the protein, including E108, D113, K115, D85, D72, K75 and N39, while the second domain lies at the “base” of the protein, encompassing I62, V64, Q29, K32 and H31 (**Figure 2-2**). Considering that NPC2 directly interacts with membranes as a mechanism for transferring cholesterol, the presence of two opposing cholesterol transport domains on the surface of NPC2 suggests that the protein may be able to interact with greater than one membrane simultaneously. To test this hypothesis, a

Figure 2-1

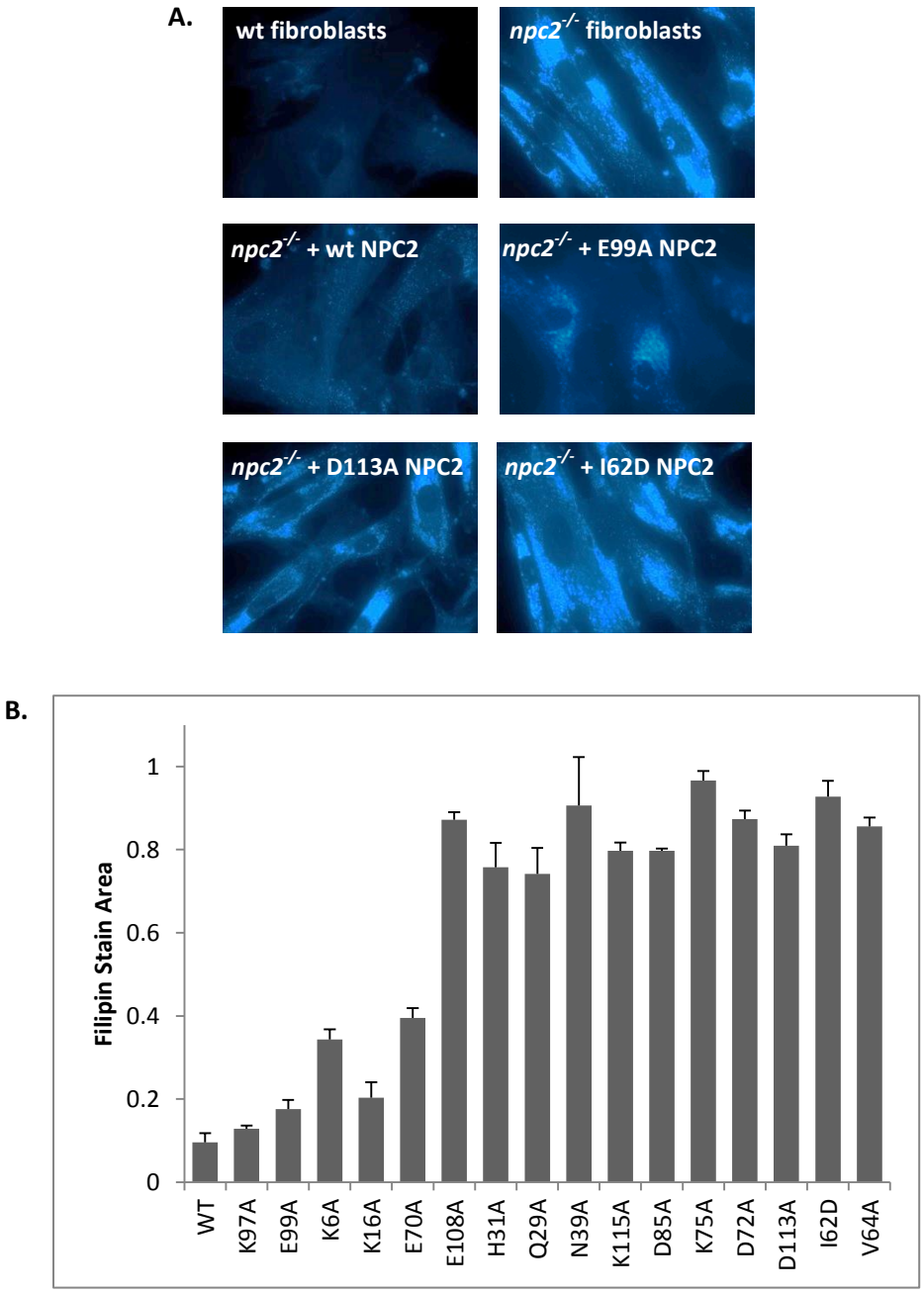


Figure 2-1. Effect of wt and mutant NPC2s on filipin accumulation in *npc2*$^{-/-}$ fibroblasts.
Human NPC2 deficient fibroblasts were incubated with 0.4nm purified wt or mutant NPC2 protein and were fixed and stained with filipin. (A) Representative images of *npc2*$^{-/-}$ fibroblasts incubated with wt or mutant NPC2s. (B) Filipin accumulation was determined as the ratio of filipin stain area to total cell area. Results are expressed relative to control untreated cells, and are represented as mean ± SE.

Figure 2-2

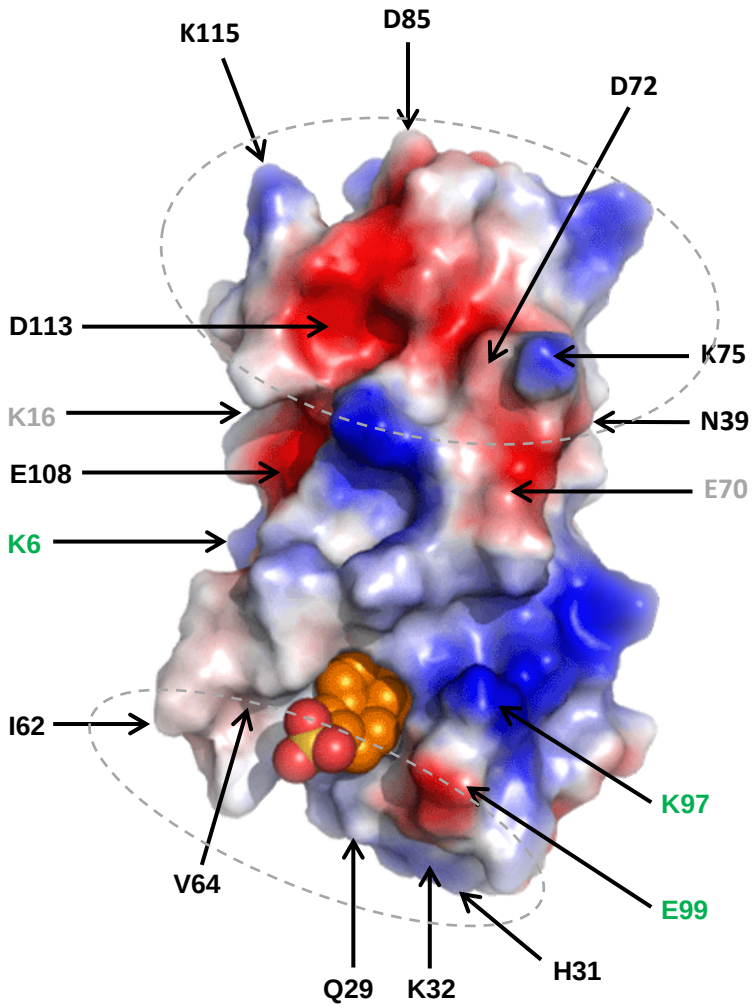


Figure 2-2. Cholesterol transport domains on the surface of NPC2. Mutated residues with little or no effect on sterol transfer rates are shown in **green** on the electrostatic charge model of NPC2. Those resulting in moderate to substantial attenuation of sterol transfer rates are indicated in gray and **black**, respectively. Results from the kinetic sterol transfer assays indicate the presence of at least two cholesterol transport domains on the surface of NPC2; one at the "base" of the protein and the other at the "top", shown encircled by the dashed lines.

simple light scattering assay that monitors membrane-membrane interactions was conducted.

Two membrane interactive domains on the surface of NPC2

As shown in **Figure 2-3**, when EPC LUVs are mixed with wt NPC2 an increase in A350 is observed over time. These results indicate occurrence of membrane-membrane interactions. Two mutants found to have cholesterol transport properties similar to wt, K6A and K97A, also cause an increase in light scattering upon addition to EPC LUVs. In contrast to these results, no change in A350 is observed when bovine serum albumin (BSA), a protein that binds cholesterol but does not cause membrane-membrane interactions (131, 135), is mixed with vesicles. Similarly, mutants with deficient cholesterol transfer properties (D85A, K75A, V64A, I62D and Q29A) do not cause changes in A350, indicating that they are unable to promote membrane-membrane interactions.

NPC2 promotes membrane aggregation, not fusion

Although the above turbidity assay allows for identification of membrane-membrane interactions precipitated by NPC2, it cannot distinguish between vesicle aggregation and fusion. Thus, in order to determine whether NPC2 promotes membrane fusion, an assay which monitors the mixing of vesicle contents was performed. Mixing of 5-CF filled LUVs with buffer filled EPC LUVs, in the presence of wt NPC2, resulted in a small increase in the relative fluorescence of 5-CF (**Figure 2-4A**), indicating a possible membrane fusion event. The relative fluorescence of 5-CF continued to increase up to 15 minutes after being mixed, although the degree of 5-CF dequenching was low compared to that caused by a cell lysis buffer (**Figure 2-4A**). Conversely, upon mixing of the two vesicle populations in the presence of NPC2 using a

Figure 2-3

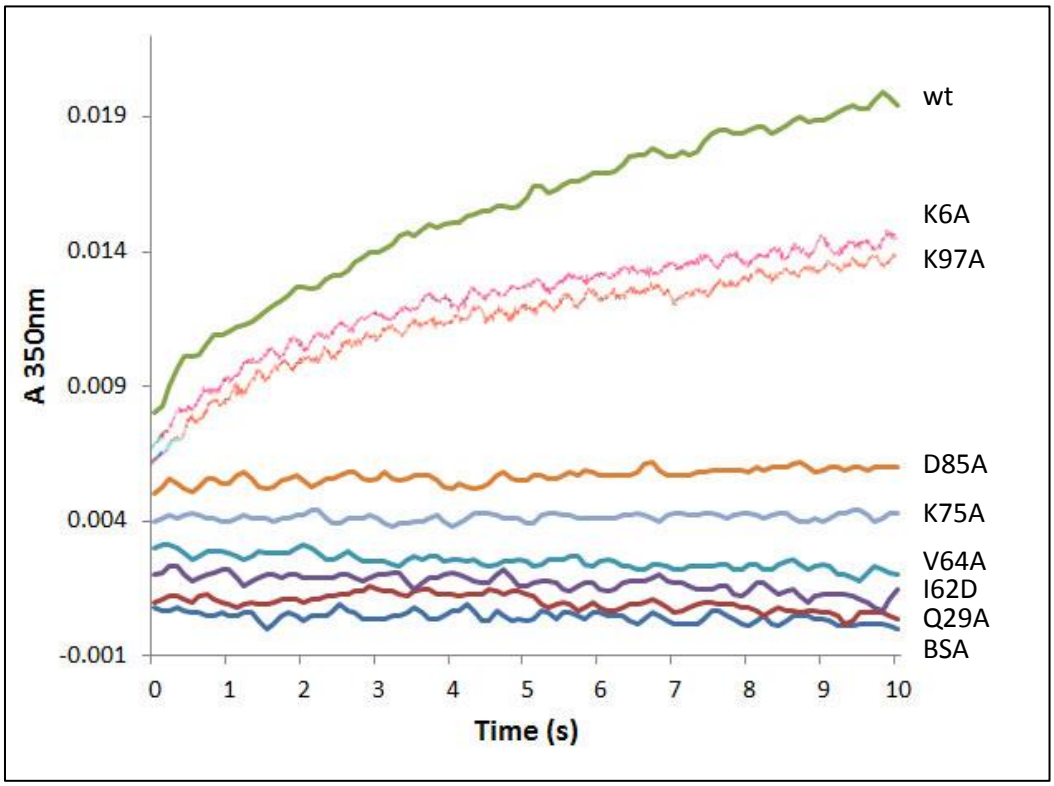


Figure 2-3. Promotion of membrane-membrane interactions by NPC2 proteins. Time dependent changes in the absorbance of 50 µM LUVs mixed with 1 µM of bovine serum albumin (BSA), wt or mutants NPC2 proteins were observed at 350nm using an SX20 stopped-flow spectrofluorometer.

stopped flow spectrofluorometer, a moderate decrease in 5-CF emission was observed, possibly due to photobleaching of the CF signal (Figure 2-4A, inset). Considering these conflicting results, in addition to the possibility that the relatively slow increase in fluorescence may reflect leakage due to interactions by NPC2, a second membrane fusion assay involving mixing of NBD- and rhodamine-labeled membrane phospholipids was undertaken. Upon mixing of the two vesicle populations in the presence of wt NPC2, no appreciable changes in the emission of either fluorophore is observed over a period of 12 minutes (**Figure 2-4B**), indicating the absence of membrane fusion. Additionally, no changes in the relative fluorescence of NBD is observed over a period of 100s on an SX20 stopped flow spectrofluorometer (**Figure 2-4B, inset**), again suggesting that membrane fusion is not occurring. Taken together, these results suggest that NPC2 interacts with membranes to the extent that it may cause some leakage, but that it does not promote membrane fusion.

Figure 2-4

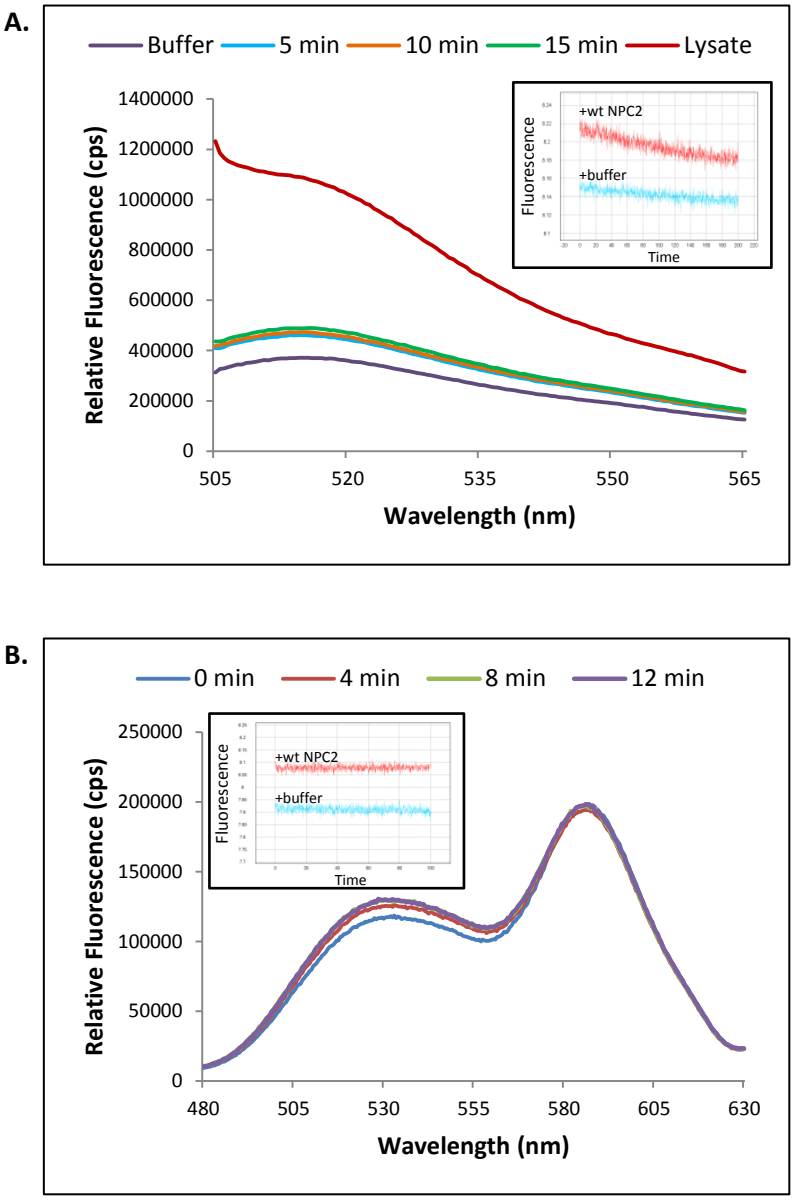


Figure 2-4. Wt NPC2 does not promote membrane fusion. The ability for NPC2 to cause membrane fusion was tested by (A) mixing 5-CF filled vesicles with buffer filled vesicles in the presence of wt NPC2 or cell lysate buffer. 5-CF fluorescence was also monitored over time on an SLM fluorometer or stopped flow spectrofluorometer (inset). The ability of wt NPC2 to cause membrane fusion was also investigated by (B) mixing NBD-PE vesicles with Liss-Rhodamine-PE vesicles in the presence of wt NPC2. Changes in the fluorescence of either FRET partner was monitored over time on an SLM fluorometer. Change in NBD fluorescence was also monitored using an SX20 stopped flow spectrofluorometer (inset).

Discussion

The NPC2 mutagenesis results for sterol transfer kinetics and *npc2*$^{-/-}$ cell rescue suggest the presence of two separate cholesterol transport domains on the surface of NPC2, necessary for normal functioning of the protein. We therefore hypothesize that NPC2 may catalyze cholesterol transfer at zones of close apposition between membranes, as exist in the multilamellar interior of the LE/LY compartment. The ability of NPC2 to promote membrane interactions *in vitro*, further supports the idea that NPC2 may bridge membranes at membrane contact sites, which have been proposed to be important for rapid lipid transfer between different organelles (133, 135). Within the LE/LY compartment, two potential zones at which NPC2 could stimulate rapid transfer of cholesterol between membranes exist. The first is between inner-lysosomal membranes, where cholesterol likely partitions (**Figure 2-5**) (16, 23). This would allow NPC2 to transfer cholesterol from more interior membranes towards the limiting lysosomal membrane, which the sterol must ultimately cross in order to maintain cellular cholesterol homeostasis. As noted above, we previously showed that NPC2 sterol transfer rates are markedly increased by the addition of LBPA to membranes. LBPA has been shown to be enriched in inner lysosomal membranes (23). Interestingly, our recent studies have indicated that NPC2 may directly interact with this unique LE/LY phospholipid (Chapter 3). Additionally, it has been suggested that cholesterol may colocalize with LBPA in these membranes (138), where it would be readily available for extraction by an NPC2 protein that is transiently bound to LBPA. Indeed, functionally cooperativity between NPC2 and LBPA in LE/LY cholesterol efflux has been suggested by studies demonstrating the development of an NPC-like cholesterol accumulation phenotype upon treatment of healthy cells with an anti-LBPA antibody

(22). We have also shown that the significant enhancement of NPC2 sterol transfer rates by LBPA is abrogated in the presence of the anti-LBPA antibody (30).

Membrane contacts sites may also be formed between the inner lysosomal membranes and the limiting lysosomal membrane, allowing for the deposition of cholesterol by NPC2 into the inner leaflet of the limiting lysosomal membrane (**Figure 2-5**). Again, these contact sites may involve direct interactions between NPC2 and LBPA within inner lysosomal membranes. Regarding the limiting lysosomal membrane, NPC2 could form a membrane contact site by simply interacting with headgroups of the membrane phospholipids. Another possibility is that it may interact with the second luminal domain of NPC1, which has been shown to bind holo-NPC2 (93), or with the N-terminal domain of NPC1, as suggested by Wang et al (88).

NPC2 is a small protein, with its cholesterol binding pocket located near one of the membrane interactive domains on the surface of the protein. It is thus unlikely that a single NPC2 protein would be able to directly transfer cholesterol between membranes at contact sites while maintaining a stable bridge. Indeed, in order for the cholesterol binding pocket to come in contact with an acceptor membrane, NPC2 would need to turn, resulting in loss of a stable membrane contact site. It is possible, however, that one molecule of NPC2 functions to stabilize the membrane contact site while a second protein functions to rapidly transfer cholesterol between the closely apposed membranes. It can also be hypothesized that stabilization of membrane contact sites by NPC2 lowers the activation energy for desorption of cholesterol from the donor membrane, allowing for diffusion of cholesterol through the aqueous media to the acceptor membrane at rates that may exceed spontaneous transfer. Neither of these

mechanisms has been demonstrated for transport proteins functioning at fully elucidated membrane contact sites, however, thus further investigation into the mechanisms by which NPC2 may function at these inner-LE/LY contact sites is warranted.

Although the mechanism by which NPC1 transfers cholesterol out of the LE/LY was not directly investigated in the present study, the roles of both NPC2 and NPC1 must be considered in elucidating the path of normal cholesterol efflux from LE/LYs. Currently, little is known regarding the molecular basis of NPC1 function in cholesterol efflux from the LE/LY. Studies have demonstrated, however, that its NTD, which projects into the lysosomal lumen, can bind cholesterol (86-88). Additionally, the equilibrium distribution of cholesterol between the soluble NTD of NPC1 and liposomes was shown to be increased in the presence of holo-NPC2 (87), which is able to transfer cholesterol to the NPC1 NTD (88). Based on these results, it has been hypothesized that normal trafficking of cholesterol in the LE/LY compartment involves a "handoff" of cholesterol from NPC2 to NPC1 NTD (88), though direct interactions between NPC2 and NPC1 NTD have yet to be demonstrated. Alternatively, our cholesterol transfer results suggest that NPC2 may deliver cholesterol to the limiting lysosomal membrane. Transfer may occur following diffusion of NPC2 through the lysosomal lumen and direct interaction with the limiting lysosomal membrane, or could potentially occur at membrane contact sites (**Figure 2-5**). Once the cholesterol is deposited in the limiting membrane, it can diffuse laterally in the plane of the membrane to NPC1, which could then facilitate egress of cholesterol from the LE/LY compartment by an unknown mechanism (**Figure 2-5**). Based on recent evidence from the Karten group, however, it is also possible that the egress of cholesterol from this compartment could occur in an NPC1-independent manner. Briefly, Kennedy et al showed that NPC2 mutants

unable to transfer cholesterol to NPC1 were still able to promote the efflux of LE/LY cholesterol to the mitochondria (139). Thus, transfer of cholesterol across the limiting lysosomal membrane could occur directly following deposition of the sterol into the inner leaflet by NPC2, without interaction with NPC1 (**Figure 2-5**). The fact that Kennedy et al further demonstrated that the transfer of LE/LY cholesterol to mitochondria occurred in a non-vesicular manner (139) suggests that efflux of LE/LY cholesterol may involve either a cytosolic cholesterol transport proteins or perhaps the formation of membrane contact sites between the limiting lysosomal membrane and other organelles, such as mitochondria or the endoplasmic reticulum.

One issue that arises in considering NPC2 transferring cholesterol directly to the limiting lysosomal membrane is the presence of the lysosomal membrane glycocalyx, which extends into the lumen of the organelle. Based on the structure and dimensions of this glycoprotein coat, we must consider the possibility that, spatially, NPC2 may not be able to interact directly with the limiting membrane. We have previously shown, however, that inclusion of 25 mol% of glycolipids, including lactosyl ceramide and gangliosides, into model membranes did not decrease the cholesterol transfer rates from NPC2 to membranes. In fact, inclusion of 25 mol% GM3 into EPC membranes actually resulted in a 3-fold increase in cholesterol transfer rates by NPC2 (30). Moreover, several studies have indicated direct interactions between proteins and the endothelial glycocalyx (140, 141). Thus, direct interaction between NPC2 and the glycocalyx in the LE/LY is possible.

The interaction of holo-NPC2 with the limiting LE/LY membrane may result in lowering of the activation energy for desorption of cholesterol from NPC2, similar to the mechanism used by

Figure 2-5

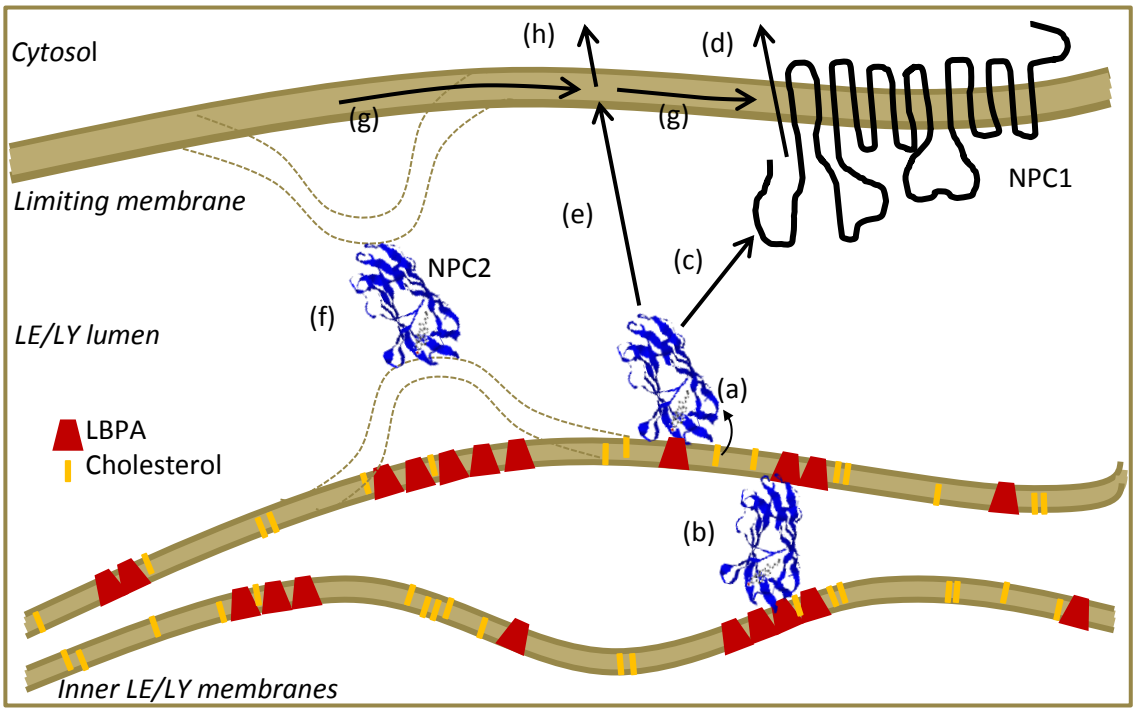


Figure 2-5. Hypothesis for cholesterol transport in the LE/LY. Free cholesterol is liberated by acid lipase and largely partitions to LBPA-rich inner LE/LY membranes. (a) NPC2 extracts cholesterol from these membranes via direct interaction, potentially by (b) facilitating sterol transfer at 'membrane contact sites' between inner LE/LY membrane lamellae, which may involve some NPC2-LBPA interaction. NPC2 may then (c) transfer cholesterol directly to the N-terminal domain of NPC1, which then (d) facilitates cholesterol efflux from the LE/LY by an unknown mechanism. It is also possible that NPC2 may (e) deliver cholesterol directly to the limiting (outer) LE/LY membrane, in part, potentially, at membrane contact sites between the inner and outer LE/LY membranes (f). Cholesterol in the limiting LE/LY membrane may access NPC1 via (g) rapid lateral diffusion in the plane of the membrane, or might also (h) egress in an NPC1-independent manner.

sterol carrier protein 2 (SCP2) (142). Cholesterol released into the aqueous phase would then diffuse through the lysosomal glycocalyx layer to the limiting lysosomal membrane. Interestingly, the lysosomal glycocalyx layer has been determined to be only approximately 8nm thick (143). Such a mechanism has been demonstrated for the movement of membrane cholesterol into lipoprotein particles (HDL), whose 70-120Å diameters render them too large to traverse the glycocalyx for direct interaction with membranes (144-146). We have demonstrated, as has Ko et al., that the off rate of cholesterol from NPC2 into the aqueous phase is quite slow (29, 81, 131) while transfer of sterol from NPC2 to membranes occurs rapidly (29, 30). While the rate of cholesterol transfer from NPC2 to model membranes was unaffected by a high concentration of glycolipids in the membranes (30), the effect of a highly glycosylated biological membrane, such as the limiting lysosomal membrane, on the rate of transfer by NPC2 has not been determined.

Studies that have identified MCSs between the endoplasmic reticulum and other organelles, where hydrophobic species may rapidly move through an aqueous phase, indicate distances between organellar membranes of approximately 10nm. The 8nm thickness of the lysosomal glycocalyx, which contrasts sharply with cell membrane glycocalyces of several hundred nanometers (143), may allow NPC2 to bring inner lysosomal membranes within a distance reported for other MCS. This may especially be so if NPC2 directly interacts with membrane protein oligosaccharides.

While further investigation is necessary to determine the mechanisms by which NPC2, and ultimately NPC1, transfers cholesterol out of the LE/LY, the present studies indicate that NPC2

acts to traffic cholesterol between membranes. We hypothesize that NPC2 functions, at least in part, at membrane contact sites within the multilamellar interior of late endosomes and lysosomes, thereby promoting rapid cholesterol transfer between closely appositioned membranes. Ultimately, sterol transport by NPC2 leads to the egress of cholesterol from the LE/LY, which may and/or may not depend upon the presence and cholesterol transport properties of NPC1.

Chapter 3

NPC2 directly interacts with LBPA to transfer cholesterol in the late endosome/lysosome (LE/LY)

Abstract

Niemann Pick C disease is characterized by the accumulation of cholesterol and other glycolipids within the late endosomal/lysosomal (LE/LY) compartment of cells. The disease is caused by defects in either of two LE/LY proteins, NPC1 or NPC2, which are necessary for the normal egress of cholesterol from this compartment. The mechanism(s) by which these proteins direct LE/LY cholesterol trafficking, however, remain mostly unknown. NPC2 is a small, soluble lysosomal protein that binds cholesterol and has been shown to transfer cholesterol between membranes via protein-membrane interactions (29, 30). The unique LE/LY phospholipid, lysobisphosphatidic acid (LBPA), dramatically enhances sterol transfer rates by NPC2 (29, 30), suggesting a relationship between NPC2 and LBPA in LE/LY cholesterol transport. In the current studies we found that inclusion of LBPA into model membranes is able to restore the cholesterol transfer properties of several NPC2 proteins with surface point mutations that otherwise exhibit deficient sterol transfer rates to zwitterionic egg phosphatidylcholine (EPC) vesicles. Interestingly, however, deficient sterol transfer rates by two of the mutants were unaffected by the presence of LBPA, leading to the hypothesis that LBPA may enhance NPC2 sterol transfer rates by directly interacting with the protein. Indeed, results from protein-lipid binding analyses demonstrate that NPC2 directly binds to LBPA. Furthermore, NPC2 mutants whose sterol transfer rates were enhanced by LBPA were found to bind LBPA similar to wt protein, while the two mutants with transfer rates unaffected by LBPA exhibit decreased binding to the phospholipid. Thus, it is likely that LBPA aids in NPC2-mediated LE/LY cholesterol transport by directly interacting with a specific domain on the surface of the protein. NPC2 has also been shown to promote vesicle-vesicle interactions (131), suggesting the protein may function to transport cholesterol, in part, at membrane contact sites within the LE/LY. In additional studies

we found that LBPA dramatically enhances the rate at which the NPC2-mediated vesicle-vesicle interactions occur. These results suggest that the relationship between NPC2 and LBPA in cholesterol transport may involve the formation of membrane contact sites within the LE/LY compartment.

Introduction

Cholesterol is acquired by most cells via receptor mediated endocytosis of low density lipoproteins (LDL), which are sequentially transferred from early endosomes to late endosomes and lysosomes for degradation (8). Within the endosomal system, free cholesterol is liberated by the actions of lysosomal acid lipase (LAL) on LDL-derived cholesterol esters (8, 18). Within normal, healthy cells, the cholesterol is then transferred out of the late endosomal/lysosomal (LE/LY) compartment to the plasma membrane and endoplasmic reticulum, where it exerts its homeostatic effects on cellular cholesterol metabolism.

Following LDL internalization, molecules destined for degradation, including down-regulated receptors and cholesterol, are collected into large vesicles forming on early endosome membranes. Membrane invaginations are contained within the vesicles, giving these intermediary compartments a hallmark multivesicular appearance (15). Cholesterol preferentially distributes to the internal membranes of these multivesicular bodies (16, 147-149). Following fusion with the LE and LY compartments, however, the cholesterol content in these membranes rapidly decreases (16), likely due to normal processes of egress. This pattern mirrors the unique LE/LY phospholipid, lysobisphosphatidic acid (LBPA), which is present at high concentrations in the internal membranes of LE/LYs (16, 20-23), but not earlier compartments (16, 23).

A relationship between cholesterol and LBPA has been implicated in Niemann Pick type C (NPC) disease, where deficiencies in either NPC1 or NPC2 proteins result in the aberrant accumulation of cholesterol in the LE/LY. Specifically, studies have shown that LBPA accumulates parallel to

cholesterol in tissues of NPC patients and animal models (105, 150, 151). Additionally, treatment of cells with anti-LBPA antibodies has been shown to result in cholesterol accumulation in LEs, similar to NPC disease (14, 22, 152). Interestingly, Chevallier et al demonstrated that supplementation of exogenous LBPA to NPC1-deficient fibroblasts reversed the cholesterol accumulation phenotype (153), thus presenting evidence to specifically implicate LBPA in the normal mechanism of LE/LY cholesterol efflux.

The soluble cholesterol binding protein, NPC2, has been shown to extract and deliver cholesterol to membranes *in vitro*, and enhances rates of sterol transfer between membranes (29, 30). Sterol transfer, facilitated via direct interaction of NPC2 with membranes (29, 30), occurred more rapidly when charged phospholipids were incorporated into model membranes, suggesting the electrostatic interactions may be involved in the mechanism of sterol transfer between NPC2 and membranes. Interestingly, the most pronounced increase in sterol transfer rates (up to 200 fold) by NPC2 were observed by incorporation of LBPA in the membranes (30), suggesting a functional role for LBPA in normal cholesterol trafficking by NPC2. Support for this hypothesis was obtained when the effects of LBPA on NPC2 sterol transfer rates were eliminated following pre-incubation of the vesicles with an anti-LBPA antibody (30).

Recent studies in our laboratory have identified a number of residues on the surface of NPC2 that when mutated, result in deficient rates of cholesterol transfer to and from phosphatidylcholine membranes. These residues form at least two putative membrane interactive domains on the surface of the protein and appear necessary for the cholesterol transport properties of NPC2. In the present studies we report that the presence of LBPA in

vesicles restores ‘normal’ sterol transport properties of some, but not all of these mutants, suggesting the existence of a cooperative mechanism between NPC2 and LBPA in normal cholesterol trafficking within the LE/LY compartment.

Materials and Methods

Materials

Cholesterol was obtained from Sigma (St. Louis, MO). Egg phosphatidylcholine (EPC), oleoyl-lysobisphosphatidic acid (LBPA), and LBPA Snoopers were from Avanti (Alabaster, AL). The 6x-His-tagged murine NPC2 plasmid was generously provided by Dr. Matt Scott (Stanford University).

Generation and purification of NPC2 mutants.

Point mutations were generated with Stratagene QuikChange Site Directed Mutagenesis Kit, using a myc 6xHis-tagged murine NPC2 plasmid generously provided by Dr. Matt Scott (81). Wild type and mutant myc 6xHis-tagged NPC2 proteins were purified from transfected CHO KI cells as previously described (30, 81), using a 10 kDa cutoff flow filtration membrane to initially concentrate conditioned media (Millipore, Bedford, MA).

Membrane vesicle preparation.

Small unilamellar vesicles (SUV) were prepared by sonication and ultracentrifugation as previously described (29, 30, 83). Vesicles were maintained at temperatures above the phase transition temperatures of all constituent lipids. Standard vesicles were composed of 100 mol % egg phosphatidylcholine (EPC). 25 mol% LBPA was substituted for EPC in the donor vesicles where indicated. All vesicles were prepared in citrate buffer (20mM sodium citrate, 150mM NaCl pH 5.0).

For the preparation of large unilamellar vesicles (LUV), phospholipids dissolved in chloroform were dried under nitrogen for 1 hour to create a lipid film, and resuspended in citrate buffer, forming multilamellar structures. The lipid suspension was placed alternately on dry ice and in a water bath above 37°C for 7 freeze-thaw cycles, followed by extrusion through a 100 nm pore membrane (Avestin, Ottawa, Ontario, Canada) using a mini-extruder (Avestin, Ottawa, Ontario, Canada) for 11 passes. The final phospholipid concentration of all vesicles was determined by quantification of inorganic phosphate (83).

Cholesterol transfer from NPC2 to membranes.

The endogenous tryptophan fluorescence of NPC2 was used to monitor cholesterol transfer from NPC2 to membranes, as previously described, using a stopped-flow mixing chamber interfaced with a Spectrofluorometer SX20 (Applied Photophysics Ltd., UK) (29, 30). Conditions to ensure the absence of photobleaching were established before each experiment. Data were analyzed with the software provided with the Applied Photophysics stopped flow spectrofluorometer, and the cholesterol transfer rates were obtained by exponential fitting of the curves. All curves were fit well by a single exponential function.

Vesicle-vesicle interaction

Vesicle-vesicle interactions were determined as described by Schulz et al (135). Briefly, 100μM LUVs containing either 100% EPC or 25 mol% LBPA were mixed with increasing concentrations of wt NPC2 protein or a control protein which also binds cholesterol, bovine serum albumin (BSA). Following an incubation period of 15 minutes, absolute absorbance of the samples at 350nm was determined using a spectrometer (Hitachi). The rates of vesicle-vesicle interactions

in the absence and presence of NPC2 were determined by mixing 1μM wt NPC2 with 450μM 100 mol% EPC or 25 mol% LBPA/EPC LUVs at time = 0, and monitoring changes in A350nm over time using a stopped-flow mixing chamber interfaced with a Spectrofluorometer SX20 (Applied Photophysics Ltd., UK).

Protein-lipid binding studies

LBPA Snoopers (Avanti), containing 1 μg spots of pure LBPA isomers, were blocked with tris buffered saline (TBS) + 3% BSA, followed by a one hour incubation with 5 μg of the indicated NPC2 protein in TBS + 3% BSA, at a final concentration of 0.5 μg/ml protein. Protein solutions were removed and the Snoopers were washed with TBS. NPC2 bound to LBPA isomers was detected by incubating the Snoopers with rabbit anti-c-myc-tag pAb antibody (GenScript) at a concentration of 0.5μg/ml in TBS + 3% BSA for one hour at room temperature. Following removal of the primary antibody, the strips were washed with TBS and incubated with anti-rabbit IgG HRP-linked antibodies (GE Healthcare) at a 1:20,000 dilution in TBS + 3% BSA. After a one hour incubation with the secondary antibody, the Snoopers were washed with TBS + 0.05% Tween and developed with ECL reagents (GE Healthcare).

Results

LBPA enhances sterol transport rates by NPC2

Transfer of 2.5μM cholesterol from 2.5μM wt NPC2 to 250μM 100mol% EPC or 25mol% LBPA/EPC SUVs was monitored over time in order to determine the effect of LBPA on sterol transfer rates by NPC2. **Figure 3-1** shows that dequenching of NPC2 tryptophan fluorescence, which is used as an indicator of cholesterol transfer, occurs much more rapidly when the holo-protein is mixed with SUVs containing LBPA. Single exponential fitting of the curves indicate a rate of cholesterol transfer from wt NPC2 to LBPA SUVs of 0.301 ± 0.006 s^{-1}, nearly 10 fold faster than rates of transfer to EPC SUVs (0.038 ± 0.005 s^{-1}). These results are in agreement with those reported by Xu et al, who initially described the effect of LBPA on sterol transfer rates by NPC2.

LBPA enhances rates of vesicle-vesicle interactions promoted by wt NPC2

NPC2 has been shown to promote vesicle-vesicle interactions *in vitro* (131), and we have hypothesized that NPC2 utilizes such a mechanism for rapid transport of cholesterol between membranes. In order to determine whether LBPA has an effect on the ability of NPC2 to promote these interactions, a simple light scattering assay was performed. As shown in **Figure 3-2B**, when increasing amounts of wt NPC2 were incubated with membranes containing 25 mol% LBPA, a concentration dependent increase in light scattering was observed, indicated by an increase in A350nm, signifying the occurrence of vesicle-vesicle interactions. LBPA has been reported to exhibit pH-dependent fusogenic properties (23, 153-156) , however, and could thus cause vesicle-vesicle interactions in the absence of NPC2. In order to rule out this possibility, the assay was repeated in the presence of a control protein (BSA) that does not cause vesicle-vesicle interactions (**Figure 3-2A**). **Figure 3-2B** reveals the absence of a change in light scattering

Figure 3-1

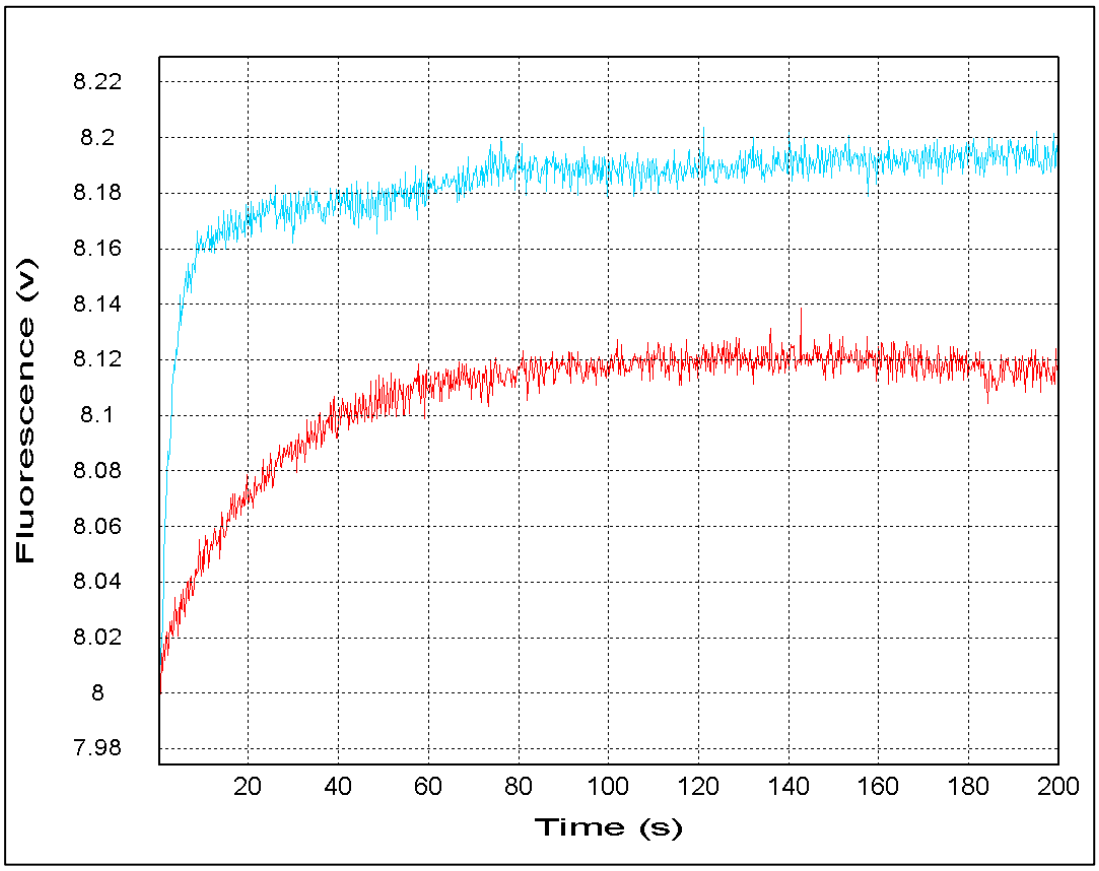


Figure 3-1. LBPA dramatically enhances cholesterol transfer rates by NPC2. The tryptophan emission of 2.5 µM holo wt NPC2 increases over time when mixed with 250 µM EPC SUVs (**red** trace) or membranes containing 25 mol% LBPA (**blue** trace), suggesting transfer of cholesterol is occurring from NPC2 to membranes. When LBPA was incorporated into membranes, the sterol transfer rate from wt NPC2 to membranes (0.301 ± 0.006 s^{-1}) was found to be approximately 10-fold greater than the rate of transfer to EPC vesicles (0.038 ± 0.005 s^{-1}).

when LBPA LUVs are incubated with increasing concentrations of BSA, demonstrating that the effects of NPC2 on promoting vesicle-vesicle interactions are independent of the purported fusogenic properties of LBPA.

To further determine if LBPA has an effect on the rate by which NPC2 stimulates these interactions, wt NPC2 protein was mixed with EPC or LBPA LUVs in a stopped-flow mixing chamber, and changes in light scattering were monitored over time. The results in **Figure 3-2C** show that the change of A350nm when 1μM wt NPC2 was mixed with 450μM LBPA LUVs (18.42 ± 2.16s^{-1}) occurred at rates that were nearly 30 fold faster than with EPC LUVs (0.64 ± 0.11s^{-1}). Thus, similar to the effect of LBPA on rates of sterol transfer by NPC2, the unique LE/LY phospholipid enhances the rate at which NPC2 promotes vesicle-vesicle interactions.

LBPA restores normal cholesterol transport rates for some NPC2 mutants

Ongoing mutagenesis studies in our laboratory have indicated the presence of at least two domains on the surface of NPC2 that are necessary for its cholesterol transport properties. Initial *in vitro* cholesterol transfer assays with NPC2 proteins expressing a point mutation within these domains demonstrated their inability to transfer sterol to, from and between zwitterionic EPC vesicles (Chapter 2). In order to determine whether LBPA has any effect on the sterol transfer activity of these mutants, 25 mol% LBPA was included in acceptor membranes and transfer of cholesterol from NPC2 to these membranes was monitored over time. Interestingly, inclusion of LBPA in the acceptor vesicles resulted in sterol transfer rates similar to wt NPC2 for the Q29A, H31A, E108A and D113A mutants; these mutants were markedly defective in cholesterol transfer to zwitterionic EPC membranes (**Table 3.1**). Conversely, LBPA had no effect

Figure 3-2

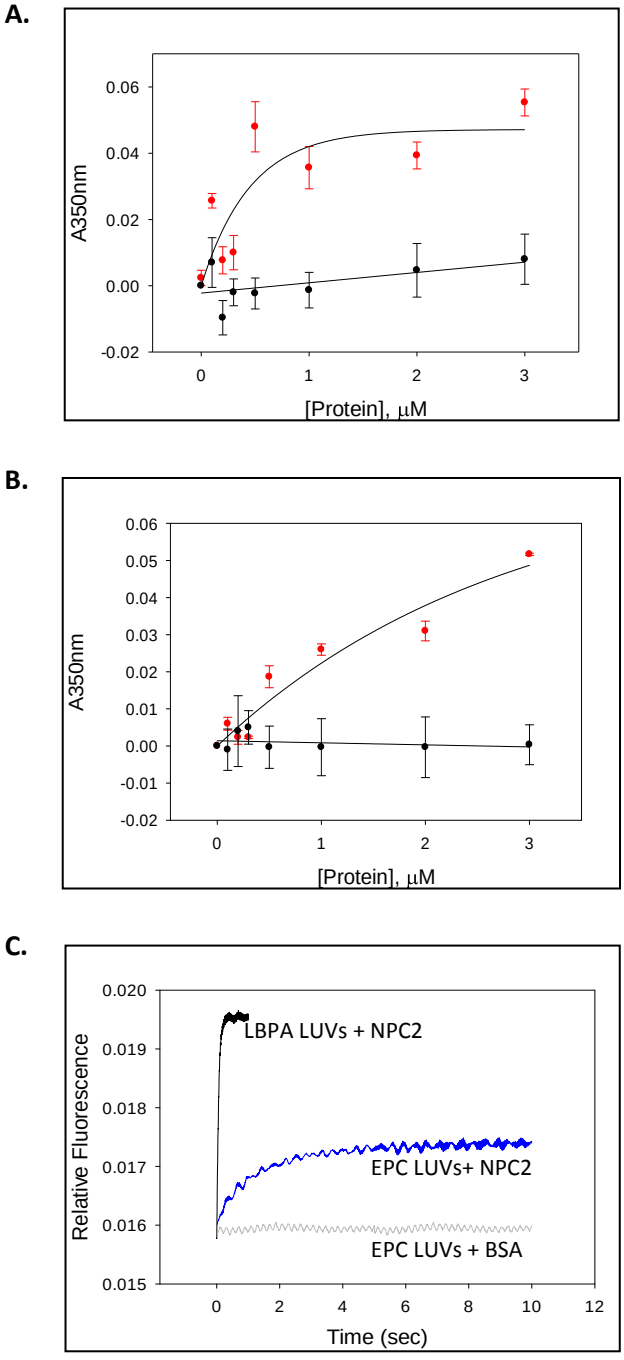


Figure 3-2. LBPA enhances the rate of vesicle-vesicle interactions by NPC2. Changes in the absorbance at 350nm was monitored for (A) EPC LUVs and (B) LUVs containing 25 mol% LBPA incubated with increasing concentrations of wt NPC2 (red) or BSA (black) as an indication of membrane-membrane interactions (C) Changes in absorbance at 350nm was monitored over time on a stopped flow fluorometer following mixing of 100 mol% EPC vesicles with BSA (gray), wt NPC2 (blue) or 25 mol% LBPA/EPC vesicles with wt NPC2 (black).

Table 3-1

	100% EPC SUV		25% LBPA/EPC SUV	
	Absolute rate	**Relative rate**	**Absolute rate**	**Relative rate**
wt NPC2	0.038 ± 0.001 s^{-1}	1.00	0.301 ± 0.006 s^{-1}	1.00
Q29A	0.004 ± 0.002 s^{-1}	0.10 ± 0.01	0.269 ± 0.009 s^{-1}	0.89 ± 0.05
H31A	0.005 ± 0.006 s^{-1}	0.13 ± 0.01	0.212 ± 0.017 s^{-1}	0.70 ± 0.04
E108A	0.004 ± 0.001 s^{-1}	0.14 ± 0.01	0.308 ± 0.017 s^{-1}	1.03 ± 0.06
D113A	< 0.001 s^{-1}	< 0.01	0.274 ± 0.013 s^{-1}	0.91 ± 0.06
I62D	< 0.001 s^{-1}	< 0.01	< 0.001 s^{-1}	< 0.01
V64A	< 0.001 s^{-1}	< 0.01	< 0.001 s^{-1}	< 0.01

Table 3-1. LBPA increases cholesterol transfer rates for some, but not all mutants, similar to wt NPC2. Transfer of cholesterol from 2.5 µM wt or mutant NPC2 to either 100% EPC of 25% LBPA/EPC vesicles was monitored on an SX20 Stopped Flow Spectrofluorometer. Transfer of cholesterol from protein to EPC membranes revealed several mutants that exhibit deficient sterol transfer rates relative to wt NPC2. Incorporation of LBPA into membranes, however, restored "normal" cholesterol transfer rates in some, but not all of the mutants.

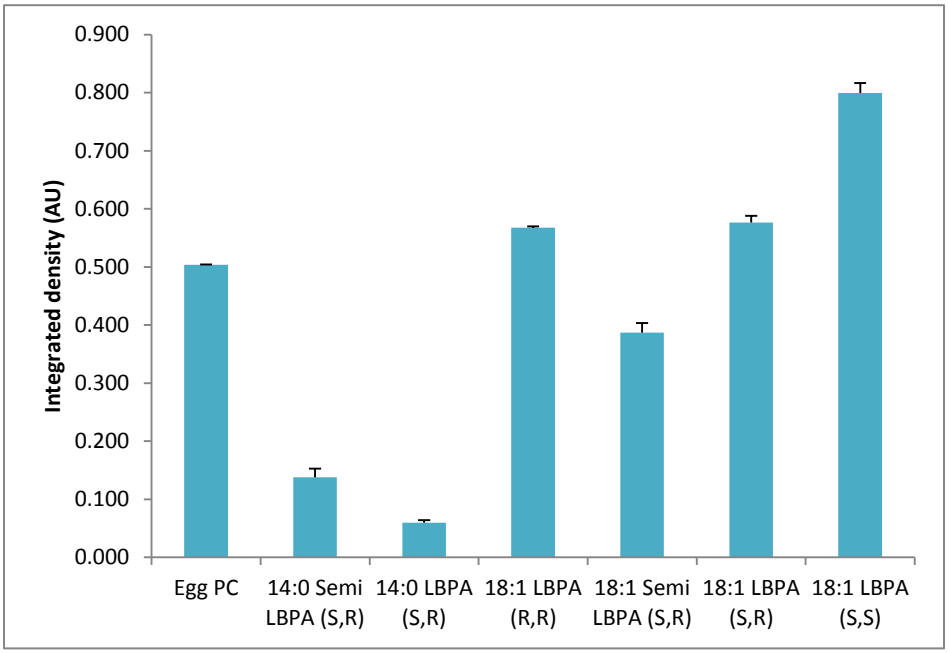
on the sterol trafficking properties of the I62D and V64A mutants, which exhibited cholesterol transfer rates of less than 0.001 s^{-1} regardless of membrane phospholipid composition. These results support a role for LBPA in NPC2 mediated cholesterol trafficking within the LE/LY, and further suggest that hydrophobic I62 and V64 may be key residues on NPC2 that are involved in this cooperative relationship.

Presence of an LBPA binding domain on the surface of NPC2

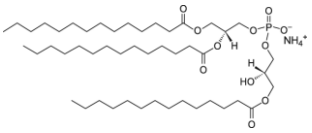
Significant enhancements in the rates of sterol transfer by NPC2 in the presence of LBPA suggest that LBPA may play a role in LE/LY cholesterol trafficking. The ability for LBPA to essentially reverse cholesterol trafficking deficiencies in a set of NPC2 mutants further suggests that LBPA may be directly facilitating transfer of cholesterol between NPC2 and membranes. Absence of an observed effect on the cholesterol transfer properties of the I62D and V64A mutants, each of which significantly alters the hydrophobic nature of the surrounding surface region of the protein, leads to the hypothesis that NPC2 may be directly interacting with LBPA, and these two residues may be involved in this functional association. In order to determine whether the relationship between LBPA and NPC2 in cholesterol trafficking indeed involves direct interactions, protein-lipid binding assays were conducted. Custom LBPA Snoopers containing 1µg blots each of various LBPA isomers were prepared by Avanti Polar Lipids. These strips were incubated with wt NPC2 protein, and binding was assessed via an integrated density analysis of the antibody probed strip. The results shown in **Figure 3-3** demonstrate that wt NPC2 does bind LBPA, with greater affinity towards isomers with oleoyl (C18:1), as opposed to myristoyl (C14:0), fatty acyl chains. Interestingly, wt NPC2 exhibited the greatest degree of binding to the *S,S* 18:1

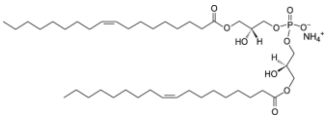
Figure 3-3

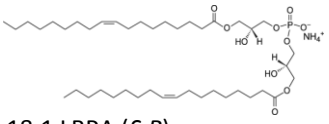
A.

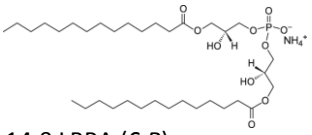


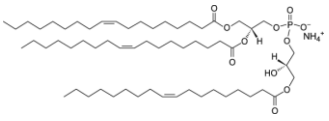
B.


14:0 Semi LBPA (*S,R*)


18:1 LBPA (*R,R*)


18:1 LBPA (*S,R*)


14:0 LBPA (*S,R*)


18:1 Semi LBPA (*S,R*)

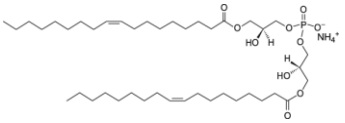

18:1 LBPA (*S,S*)

Figure 3-3. NPC2 binds to LBPA isomers. (A) Wt NPC2 protein was incubated with strips containing LBPA isomers. LBPA-bound protein was detected with anti-c-myc antibody as described in Materials and Methods, and degree of binding is represented by the integrated density of the blots. (B) Structures of the LBPA isomers.

Figure 3-4

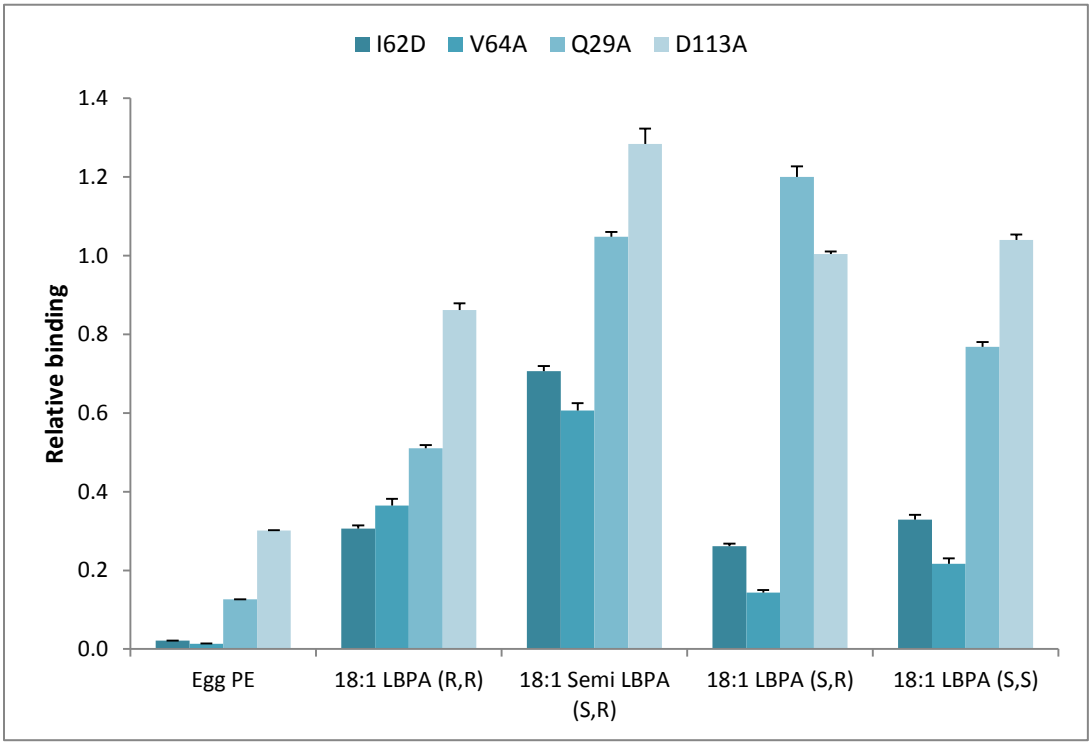


Figure 3-4. Two residues on NPC2 are necessary for binding to LBPA. Mutant NPC2 proteins were incubated with strips containing LBPA isomers. LBPA-bound protein was detected with anti-c-myc antibody as described in Materials and Methods. Binding of the NPC2 mutants to the LBPA isomers is determined as the integrated density of the blots relative to wt NPC2.

LBPA, which possess not only the favored stereoconfiguration in mammals (23, 157, 158), but also the preferred fatty acyl chains in most cell types studied thus far (23, 159).

To examine whether the binding of NPC2 to LBPA involves the I62 and V64 residues, NPC2 mutants were also analyzed for their ability to bind LBPA. The I62D and V64A mutants bound the C18:1 *R,R* isomer to nearly 60% less than wt, and the *S,S* and *S,R* isomers at approximately 20% of wt (**Figure 3-4**). These two mutants exhibited greater affinity towards the 18:1 Semi LBPA species, which has three oleoyl acyl chains (**Figure 3-3B**), with binding of approximately 70% of wt NPC2. Two NPC2 mutants that were found to have cholesterol transfer rates similar to wt NPC2 in the presence of LBPA were also analyzed for their ability to bind to the phospholipids. In contrast to the I62D and V64A mutants, the Q29A and D113A mutants bound to the Hemi BMP, *S*,*R* and *S*,*S* isomers similar to the wt protein. Binding of Q29A to the *R,R* isomer was only about half of wt NPC2 however, while the D113A mutant was found to bind this isomer similar to the wt protein. These results suggest that the mechanism by which LBPA stimulates rates of cholesterol transfer by NPC2 involves direct interaction of the protein with the membrane phospholipid. These results further suggest that NPC2 possesses an LBPA binding domain on its surface, inclusive of the hydrophobic I62 and V64 residues.

Discussion

The results presented in this study suggest that LBPA plays an integral role in LE/LY cholesterol transport by enhancing NPC2 mediated cholesterol trafficking. We specifically propose that NPC2 binds to LBPA at cholesterol rich inner LE/LY membranes, and this direct interaction between protein and phospholipid facilitates rapid transfer of cholesterol from the membranes to NPC2. We further speculate that, given the ability for NPC2 to promote vesicle-vesicle interactions, LBPA is involved in the formation of potential NPC2-mediated membrane contact sites, which could exist between closely apposed inner LE/LY membranes or between inner and the limiting LE/LY membrane. Thus, by acting in concert with LBPA, NPC2 acts to facilitate the egress of cholesterol from the LE/LY compartment.

In healthy cells, the decrease in inner LE/LY membrane cholesterol content and concurrent enrichment with LBPA may be reflective of the efficient role LBPA plays in normal LE/LY cholesterol efflux. If this mechanism involves direct interactions between LBPA and NPC2, the rate limiting step in this particular process of cholesterol transport is the number of interactions between NPC2 and LBPA. While the concentration of LE/LY LBPA has been shown to increase in parallel with cholesterol and other lipids in NPC disease (105, 150, 151), it is possible that LBPA levels nevertheless remain at concentrations that are too low to provide support for transport of the elevated cholesterol levels by NPC2. Indeed, the suggestion that LBPA may be limiting in NPC disease has been supported by studies demonstrating that supplementation of exogenous LBPA to NPC1-deficient fibroblasts reverses the cholesterol accumulation phenotype (153). Since NPC2 remains present and active in NPC1 disease, it is therefore possible that the supplemental increase in LBPA concentration aids in restoring near normal cholesterol transfer

rates by NPC2. These results additionally support the existence of an NPC1-independent mechanism of LE/LY cholesterol egress, which may be dependent upon NPC2 function. LBPA supplementation studies in NPC2-deficient cell models are currently underway in collaboration with the Gruenfeld laboratory (Genevea, Switzerland) in an effort to address this specific hypothesis. We predict that, unlike the case with NPC1-deficient cells (153), supplementation of LBPA may not be able to 'rescue' the cholesterol accumulation in NPC2-deficient cells.

The quantitative nature by which LBPA enhances sterol transfer rates by NPC2 further implies a precise mechanism for this phospholipid in normal LE/LY cholesterol efflux. Specifically, Xu et al showed that rates of cholesterol transfer from NPC2 to EPC membranes were increased approximately 10 fold when 25 mol% LBPA was incorporated into the donor vesicles (30), which was confirmed in the present study (**Table 3-1**). By contrast, when transfer of cholesterol *from* membranes *to* NPC2 was monitored, the presence of 25 mol% LBPA in donor vesicles resulted in a nearly 200-fold increase in sterol transfer rates by the protein. Considering the molar ratio between protein and vesicles remained constant in these studies, the results suggest that the preferred directionality of cholesterol transfer between membranes containing LBPA and NPC2 is *from* the membranes *to* NPC2. Given the sequestration of cholesterol within inner LE/LY membranes in NPC disease, the physiologically relevant direction of cholesterol movement within this compartment is *from* LBPA-containing inner lysosomal membranes *to*, and eventually across, the limiting lysosomal membrane. Taken together with the data shown by Xu et al, this would suggest that LBPA potentially functions in normal LE/LY sterol trafficking by facilitating efficient transport of cholesterol from inner LE/LY membranes to NPC2.

LBPA is described as having an inverted cone-like structure, which affects its spontaneous curvature and allows the phospholipid to potentially form convex monolayers (160). It is therefore possible that this phospholipid plays an important role in the structure of inner-LE/LY membranes, perhaps promoting binding of proteins to the membrane surface. While the fatty acid composition of LBPA varies, all cell types studied thus far show elevated concentrations of oleic acid (C18:1) and enrichment in poly unsaturated fatty acids (23, 159, 161). It has been suggested that the predominant LBPA isoform in healthy mammalian cells is likely a 2,2'-dioleoyl species (23, 159). The presence of unsaturated fatty acyl chains would promote the cone-like shape of LBPA. Biophysical studies focused on the behavior of dioleoyl-LBPA in membranes, however, have demonstrated that physiological concentrations of this particular isoform have little effect on the physical properties of phosphatidylcholine (PC) membranes (162). Moreover, LBPA was not found to form microdomains as has been suggested (21, 23, 163), leading to the conclusion that the phospholipid does not function by recruiting soluble proteins to LBPA 'rafts' (162). In the present study we demonstrated the ability of LBPA to restore normal sterol transfer rates in some, but not all mutants. If the primary effect of LBPA on sterol transfer by NPC2 involved the influence of this phospholipid on membrane properties, we might expect to see similar effects of LBPA on all mutants, which is not the case. We also showed that NPC2 binds directly to LBPA and that efficient binding is dependent upon at least two surface residues on NPC2, which also appear necessary for the effects of LBPA on sterol transfer. Therefore, we propose that enhancements in NPC2 sterol transfer rates are not likely a general effect of LBPA on membrane structure. Rather, our results suggest a specific interaction of the "hydrophobic knob" domain of NPC2 with LBPA-rich membranes, and we propose that these interactions are important for efficient egress of cholesterol from the LE/LY.

As previously noted, LBPA has also been found to incorporate docosahexaenoic acid (C22:6) in a number of different cells types (161, 164-166). While the effects of these LBPA variants on the biophysical properties of membranes have not been determined, studies have shown that docosahexaenoic acid does play a role in cholesterol transport (167-169). Interestingly, Storch and colleagues showed that the fatty acyl chain composition of LBPA indeed had an effect on *in vitro* sterol transfer rates by NPC2. In particular, rates of cholesterol transfer from NPC2 protein to membranes containing dimyristoyl (14:0) LBPA were consistently around 60% slower than to vesicles containing dioleoyl LBPA (30). Further, LBPA has been found to remodel its fatty acid content in NPC disease, and a decrease in docosahexaenoic acid has been observed (20, 170). It is thus possible that alterations in the fatty acyl chains of LBPA in NPC disease leads to a reduction in cholesterol efflux from the LE/LY. Whether these changes in LBPA fatty acid composition are more likely to result in unfavorable modifications to membrane structure or diminishment in NPC2 interactions is as yet not known.

In summary, our results suggest that LBPA plays a role in LE/LY cholesterol trafficking by NPC2 in part by enhancing sterol transfer rates through direct interactions with the protein. Interestingly, and in support of our hypothesis, LBPA also plays a role in a number of other lysosomal processes, including transport of non-degradable proteins, such as the mannose-6-phosphate receptor (M6PR), through the endo/lysosomal system (21). Additionally, the activator-stimulated hydrolysis of gangliosides GM1 and GM2 has been shown to be dependent upon LBPA (171, 172), as is the hydrolysis of ceramide by acid ceramidase (173). Thus, the dynamic role LBPA plays in maintaining LE/LY homeostasis must be considered in future studies

aimed at determining the mechanisms by which this unique phospholipid aids in directing normal LE/LY cholesterol efflux.

Chapter 4

Cholesterol transfer by cyclodextrin is similar but not identical to NPC2

Abstract

Niemann−Pick C disease is an inherited disorder in which cholesterol and other lipids accumulate in the late endosomal/lysosomal compartment. Recently, cyclodextrins (CD) have been shown to reduce symptoms and extend lifespan in animal models of the disease. In the present studies we examined the mechanism of sterol transport by CD using in vitro model systems and fluorescence spectroscopy and NPC2-deficient fibroblasts. We demonstrate that cholesterol transport from the lysosomal cholesterol-binding protein NPC2 to CD occurs via aqueous diffusional transfer and is very slow; the rate-limiting step appears to be dissociation of cholesterol from NPC2, suggesting that specific interactions between NPC2 and CD do not occur. In contrast, the transfer rate of the fluorescent cholesterol analogue dehydroergosterol (DHE) from CD to phospholipid membranes is very rapid and is directly proportional to the acceptor membrane concentration, as is DHE transfer from membranes to CD. Moreover, CD dramatically increases the rate of sterol transfer between membranes, with rates that can approach those mediated by NPC2. The results suggest that sterol transfer from CD to membranes occurs by a collisional transfer mechanism involving direct interaction of CD with membranes, similar to that shown previously for NPC2. For CD, however, absolute rates are slower compared to NPC2 for a given concentration, and the lysosomal phospholipid lysobisphosphatidic acid (LBPA) does not stimulate rates of sterol transfer between membranes and CD. As expected from the apparent absence of interaction between CD and NPC2, the addition of CD to NPC2-deficient fibroblasts rapidly rescued the cholesterol accumulation phenotype. Thus, the recent observations of CD efficacy in mouse models of NPC disease are likely the result of CD enhancement of cholesterol transport between membranes, with rapid sterol transfer occurring during CD−membrane interactions.

Introduction

Cholesterol accumulation in the late endosomal/lysosomal (LE/LY) compartment is the cellular hallmark of the autosomal recessive disease Niemann–Pick C. In healthy cells, LDL-derived cholesterol is efficiently cleared and moves primarily to the plasma membrane and the endoplasmic reticulum (ER). In cells lacking wild type expression of either Niemann–Pick C 1 (NPC1) or Niemann–Pick C2 (NPC2) protein, the post-LE/LY transport and metabolism of cholesterol is blocked. The resulting physiological consequences, almost always including neurodegeneration, are thought to arise secondary to the specific absence of normal postlysosomal cholesterol metabolism and the effects of general lysosomal dysfunction arising from the buildup of cholesterol and other lipids in the LE/LY. Effective therapeutics that can restore cholesterol egress are under active investigation.

Cyclodextrins (CD) are cyclic oligosaccharides shaped like hollow truncated cones. The exterior of the cone is hydrophilic and the interior hydrophobic, imparting the ability to bind small hydrophobic molecules such as cholesterol in the interior, thereby solubilizing them in aqueous media (174-176). Cyclodextrins can have over 15 glucopyranose units per ring; derivatives of β-cyclodextrin, containing seven units, are most widely used in pharmaceuticals because of their high affinity for hydrophobic compounds, low toxicity, and price (101, 102). β-Cyclodextrin (BCD), methyl-β-cyclodextrin (MBCD), and 2-hydroxypropyl-β-cyclodextrin (HPCD) are the most commonly used β-cyclodextrin derivatives, with relative cholesterol affinities as MBCD > HPCD > BCD (103, 145). HPCD was shown to have lower toxicity compared with MBCD and to have greater specificity for cholesterol and triacylglycerol (101, 177).

In mouse models of NPC disease, CD was used as a vehicle to deliver potential therapeutic compounds to the animals, and it was noted that vehicle alone appeared to have substantial benefit (106, 178). Indeed, Liu et al. demonstrated that the administration of a single dose of HPCD at 7 days of age to *npc1*$^{-/-}$ mice resulted in the rapid release of cholesterol accumulation from the LE/LY compartment, as monitored by cholesterol esterification in the ER and the restoration of sterol-dependent regulation of SREBP2 and LXR-mediated target gene expression (106). Remarkably, the CD-treated mice also showed diminished neuropathology and >40% extension of lifespan over untreated controls (107). Similar benefits were found in studies using chronic administration of HPCD (105). Interestingly, two other lipid storage diseases, characterized by primary accumulation of gangliosides (GM1 gangliosidosis) or mucopolysaccharides (MPS IIIA) as well as secondary cholesterol accumulation, were not ameliorated by CD treatment (105).

The molecular mechanisms by which CD leads to the rapid restoration of normal post-LE/LY cholesterol transport are beginning to be understood. Rosenbaum et al. recently showed that CDs were functioning within the LE/LY compartment following fluid phase pinocytosis, rather than acting at the plasma membrane level (108). On the basis of the effectiveness of CD in treatment of NPC1-deficient mice, it was suggested that the CD may be functioning by delivering acid lipase-derived cholesterol to NPC2, thereby substituting for the defective NPC1 (107). While specific interactions between CD and NPC2 have not been reported, interactions of CD with a number of other proteins have been observed (110), making NPC2–CD interactions a plausible hypothesis.

We have shown that NPC2 catalyzes the rapid transfer of cholesterol to and from phospholipid membranes and that the mechanism of transfer involves direct protein–membrane interactions, with sterol transfer rates highest when membranes contain the LE/LY-specific lipid lysobisphosphatidic acid (LBPA, also known as bis-monoacylglycerol phosphate, BMP) (29, 30). Since the LDL-derived free cholesterol that accumulates in NPC disease is likely to be present largely in the internal membrane network of the LE/LY compartment, we considered that CD could also be acting by extracting membrane-bound cholesterol in the process of cellular rescue as well as, perhaps, delivering cholesterol to the limiting lysosomal membrane. While CD has long been used to manipulate plasma membrane cholesterol levels in cultured cells (103, 179), its mechanism of action is not fully understood (110). Thus, to explore the underlying mechanism by which CD decreases cholesterol accumulation in NPC1 deficiency, and the potential role of NPC2 in the CD-mediated amelioration of cholesterol accumulation in NPC1 cells, the present studies used kinetic approaches and spectroscopy to examine the mechanism of sterol transfer between CD and NPC2, the rates and mechanism of sterol transfer between phospholipid membranes and CD, and the effects of CD on intermembrane sterol transfer. The dose- and time-dependent effects of HPCD on cholesterol accumulation in *npc2*$^{-/-}$ fibroblasts were also examined.

The results do not provide evidence for a specific interaction of CD with NPC2, implying that the beneficial effects of CD in NPC1 disease are independent of NPC2. Nevertheless, CD functions to accelerate the rate of cholesterol transport from membranes, potentially behaving as a mimic for NPC2. At high concentrations, CD can generate sterol transfer rates that are on the order of those obtained with lower levels of NPC2. Similar to NPC2, the mechanism of CD action in

cholesterol transfer between membranes appears to be collisional, involving direct interactions of CD with membranes. However, the rates of sterol transfer between CD and membranes are independent of membrane LBPA, in contrast to the marked effects of this lipid on increasing sterol transfer rates between NPC2 and membranes. Finally, the results also show that CD can rescue the cholesterol accumulation phenotype of *npc2*$^{-/-}$ cells, indicating that the actions of CD are independent of NPC2, in agreement with the kinetic studies.

Materials and Methods

Materials

Cholesterol, dehydroergosterol (DHE), β-cyclodextrin (BCD), methyl-β-cyclodextrin (MBCD), and 2-hydroxypropel-β-cyclodextrin (HPCD) were obtained from Sigma (St. Louis, MO). Egg phosphatidylcholine (EPC), oleoyl lysobisphosphatidic acid (LBPA, also termed bis-monoacylglycerol phosphate or BMP), and dansyl phosphatidylethanolamine (Dansyl-PE) were from Avanti Polar Lipids (Alabaster, AL). Filipin was from Fisher (Pittsburgh, PA). Human fibroblast cells (GM03652) from an apparently healthy donor and from an NPC2 patient (GM18455) were from Coriell Institute of Medical Research (Camden, NJ).

Preparation of Cyclodextrin–Sterol Complexes

Cyclodextrin–sterol complexes were prepared as described by Hao et al (148). Solutions of 1 mg sterols in 1 mL of chloroform: methanol 1:1 (v:v) were dried under a gentle stream of nitrogen. 30 mg of CD was dissolved in 2.5 mL of citrate buffer (20mM sodium citrate, 150mM NaCl pH 5.0) and added to the sterol. The tube was vortexed to bring the dried sterol off the wall of the tube, followed by sonication in a water bath sonicator until clear. This solution was then placed in a shaking incubator at 37 °C overnight and was kept at room temperature and filtered through a 0.45 μM syringe filter (Millipore, Bedford, MA) immediately before use.

Purification of Human NPC2 Protein

Human NPC2 (hNPC2) protein was purified from transfected Chinese Hamster Ovary cell media as previously described (29) using a 10 kDa cutoff flow filtration membrane to concentrate media (Millipore, Bedford, MA).

Membrane Vesicle Preparation

Small unilamellar vesicles were prepared by sonication and ultracentrifugation as described (83). Vesicles were kept at temperatures above the phase transition temperatures of all constituent lipids. The standard vesicles were composed of 100 mol % egg phosphatidylcholine (EPC); however, for several experiments various other phospholipids were substituted for a portion on the EPC, as indicated. For intermembrane sterol transfer experiments, 25 mol % DHE and 3 mol % dansyl-PE were incorporated into donor and acceptor vesicles, respectively, by substituting for EPC. Vesicles were prepared in 20 mM sodium citrate, 150 mM NaCl pH 5.0 buffer. For the preparation of large unilamellar vesicles (LUV), lipids were dissolved in chloroform, and the desired composition was dried under nitrogen for 1 h to form a lipid film. Buffer was added to resuspend the lipids, forming multilamellar structures. The suspension was placed alternately in dry ice and a 55 °C water bath for seven freeze–thaw cycles, followed by extrusion through a 100 nm pore membranes (Avestin, Ottowa, Ontario, Canada) using a mini-extruder (Avestin, Ottowa, Ontario, Canada) for at least 11 passes. The final phospholipid concentration of all vesicles was determined by quantification of phosphate (83).

Cholesterol Transfer from hNPC2 to CD

The endogenous tryptophan fluorescence of hNPC2 was used to monitor cholesterol transfer from hNPC2 to BCD, MBCD, and HPCD. As described previously, the NPC2 tryptophan signal is quenched by cholesterol binding, therefore the transfer of cholesterol from hNPC2 to cyclodextrin can be monitored by the dequenching of the hNPC2 tryptophan fluorescence (29). For all transfer assays, CD was mixed with hNPC2 using a stopped-flow mixing chamber

interfaced with a spectrofluorometer SX20 (Applied Photophysics Ltd., UK), and the time-dependent change in tryptophan emission was used to obtain the transfer rates. The excitation wavelength was 280 nm, and emission was monitored using a 299 nm long pass filter. Transfer was monitored at 25 °C, and controls to ensure the absence of photobleaching were performed before each experiment. Data were analyzed using software provided with the Applied Photophysics stopped flow instrument, and the cholesterol transfer rates were obtained by exponential fitting of the curves, all of which were well fit by a single-exponential function. For each experimental condition, at least five replicates were done, and the averages ± SE for three or more experiments are reported.

Sterol Transfer between CD and Membranes

Fluorescence resonance energy transfer (FRET) between DHE and dansyl-PE was used to study the transfer of DHE, a fluorescent analogue of cholesterol (180). To examine the rate of DHE transfer from HPCD to membranes, HPCD/DHE was used as a donor complex, and vesicles containing dansyl-PE were used as acceptors. The fluorescence emission of DHE at 370 nm overlaps with the dansyl excitation spectrum; thus, when DHE is transferred to acceptor membranes containing dansyl-PE, its emission is quenched while the sensitized emission of the dansyl moiety at 510 nm is increased. The transfer of DHE from HPCD to acceptor membranes is therefore monitored directly by the decrease in DHE fluorescence or the increase in dansyl fluorescence over time. The DHE excitation wavelength was set between 300 and 323 nm so as to eliminate photobleaching, and emission was monitored using a 520 nm long pass filter to monitor the dansyl fluorescence or a 370 nm narrow band filter for monitoring DHE emission. Identical rates were obtained using either method. The same FRET pair was used to study DHE

transfer from membranes to HPCD. Donor vesicles contained both DHE and dansyl-PE. Upon addition of HPCD, the rate of DHE transfer from SUV containing dansyl-PE to acceptor HPCD can be monitored by the decrease in dansyl emission.

Intermembrane Transfer of DHE

The intermembrane transfer of DHE was also monitored using FRET between DHE and dansyl-PE, as described previously (30). Briefly, donor vesicles containing DHE were mixed with acceptor vesicles containing dansyl-PE, in the presence or absence of increasing concentrations of HPCD.

Membrane Aggregation Assay

Large unilamellar vesicles (LUV) at a concentration of 50 μM phospholipid were mixed with different concentrations of either hNPC2, HPCD, or bovine serum albumin (BSA), and absorbance at 350 nm (light scattering) was used to monitor membrane aggregation, as described by Schulz et al (135).

Effect of CD on npc2$^{-/-}$ Fibroblasts

Human fibroblasts from healthy (WT) and NPC2 patients were seeded on coverslips at a density of 6×10^4 cells, in 6-well tissue culture dishes, in Eagle's Minimum Essential Medium with Earle's salts and nonessential amino acids +15% FBS at 37 °C with 5% CO_2. Varying amounts of HPCD or MBCD were administered to cells 1 day after plating and allowed to incubate for varying time points, as indicated. Cells were then fixed and stained with filipin as described by Cadigan et al (181). Briefly, cells were washed with PBS, fixed in situ using 10% buffered formalin, washed

again with PBS, and stained with 50 ug/mL filipin in PBS. Images were taken on a Nikon Eclipse E800 epifluorescence microscope (Nikon Inc.) equipped with OpenLab version 12.2.5 software (PerkinElmer) using a DAPI filter set and light settings to ensure absence of photobleaching. Filipin accumulation in cells was determined using OpenLab selection tools and calculated as a ratio of filipin area to cell area. The results in CD-treated cells were normalized to filipin accumulation in untreated *npc2*$^{-/-}$ fibroblasts and are expressed as mean ± SE.

Results

Partition of Sterol between Phospholipid Membranes and Cyclodextrin

Prior to undertaking kinetics experiments, it was necessary to obtain the relative partition of sterol between HPCD and membrane vesicles, so as to ensure unidirectional transfer kinetics, as described previously (30, 82, 182). The partition of DHE between HPCD and membranes was determined utilizing the FRET between DHE and the dansyl moiety of dansyl-PE, as described in Materials and Methods. DHE partition between HPCD and membranes is obtained by plotting DHE distribution (equilibrium fraction of DHE in the HPCD) vs [HPCD]/[SUV], with the partition coefficient for DHE between HPCD and SUV obtained from the slope (182):

$$\frac{[\mathrm{DHE}]_{\mathrm{CD}}}{[\mathrm{DHE}]\mathrm{SUV}} = \left(\frac{1}{K_{\mathrm{CD}}^{\mathrm{SUV}}}\right) \times \frac{[\mathrm{HPCD}]}{[\mathrm{SUV}]}$$

$K_{\mathrm{CD}}^{\mathrm{SUV}}$ is the partition coefficient of DHE between the membranes and HPCD. From the results in **Figure 4-1**, the relative partition of DHE between model membranes and HPCD was determined to be 9.8 ± 1.0 in favor of the phospholipid membranes. Using 1-palmitoyl, 2-oleoyl PC LUV and MBCD with filtration separation methods, Niu and Litman reported a partition coefficient of 6.7 ± 0.5 (182). Considering that MBCD was found to have a greater efficiency in accepting cholesterol than HPCD (103, 145), the somewhat higher partition coefficient obtained in the present study is reasonable.

DHE Transfer from hNPC2 to CD

It was suggested that the amelioration of NPC symptoms in NPC1-deficient mice might be due to a specific interaction between NPC2 and CD. Therefore, we examined the rate and mechanism of sterol transport from hNPC2 to CD. **Figure 4-2A** shows that the transfer of DHE from NPC2 to HPCD is about 0.002 s^{-1}, considerably slower than rates of sterol transfer from NPC2 to

Figure 4-1

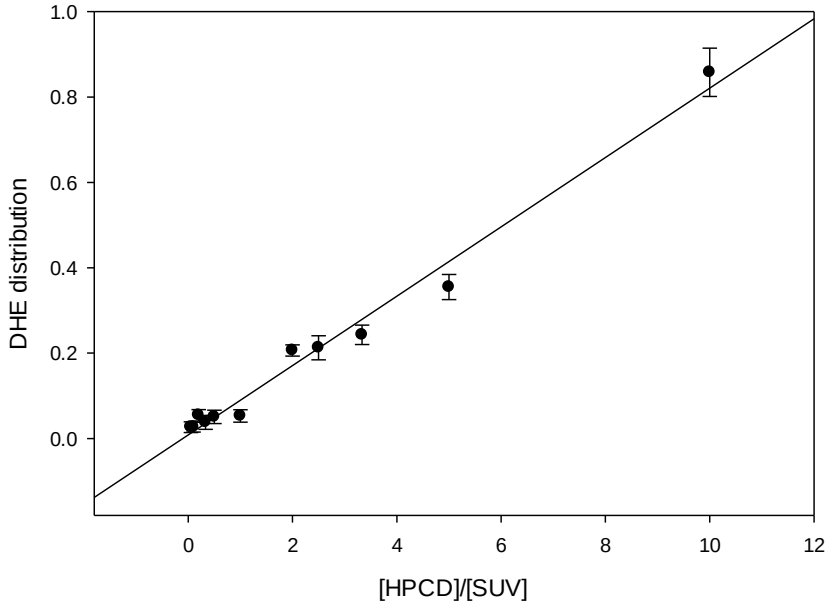


Figure 4-1. Determination of DHE partition between HPCD and phospholipid membranes. 0–200 µM EPC/dansyl-PE (97:3 mol %) SUV were added to 10 µM HPCD/DHE complex, and the distribution of DHE between HPCD and membranes was calculated from the DHE fluorescence, as described in Materials and Methods. Excitation was at 300 nm, using a slit of 1/16 nm. The calculated Kp was 9.8 ± 1.0. Results shown are an average of three separate experiments.

phospholipid membranes, which can range up to 10 s^{-1} (29, 30). Moreover, the acceptor CD concentration has no effect on the DHE transfer rate from hNPC2, strongly suggesting that transfer was occurring by a spontaneous aqueous diffusion mechanism. Similar results were found for the transfer of DHE from hNPC2 to MBCD and BCD (**Figure 4-2B**,**C**). Indeed, the DHE transfer rates from NPC2 to the three different CDs are very similar to each other, supporting the hypothesis that desorption from hNPC2 to the aqueous phase is the rate-determining step in DHE transfer from hNPC2 to CDs and arguing against an NPC2–CD interaction.

DHE Transfer from Phospholipid Membranes to CD and from CD to Membranes

For CD to deplete the cholesterol accumulation of NPC deficient fibroblasts, it must remove cholesterol from the late endosome/lysosome compartment, which contains varying levels of inner LE/LY membranes. Our previous studies suggested that NPC2 protein could rapidly transfer cholesterol from inner LE/LY membranes, particularly those enriched in the unique lysosomal phospholipid LBPA, to the limiting membrane of the organelle, via a collisional mechanism in which NPC2 interacts directly with the membranes (29, 30). We therefore examined the transfer of DHE from model phospholipid membranes to CD. **Figure 4-3** shows the rates of DHE transfer from DHE-containing SUVs to increasing concentrations of HPCD. Transfer rates were found to increase with acceptor HPCD concentration. Inclusion of LBPA in the donor membranes had no effect on rates of DHE transfer to HPCD (**Figure 4-3B**).

We also examined DHE transfer from HPCD to increasing concentrations of membranes. **Figure 4-4A** shows the results obtained when a constant concentration of HPCD/DHE complex was

Figure 4-2

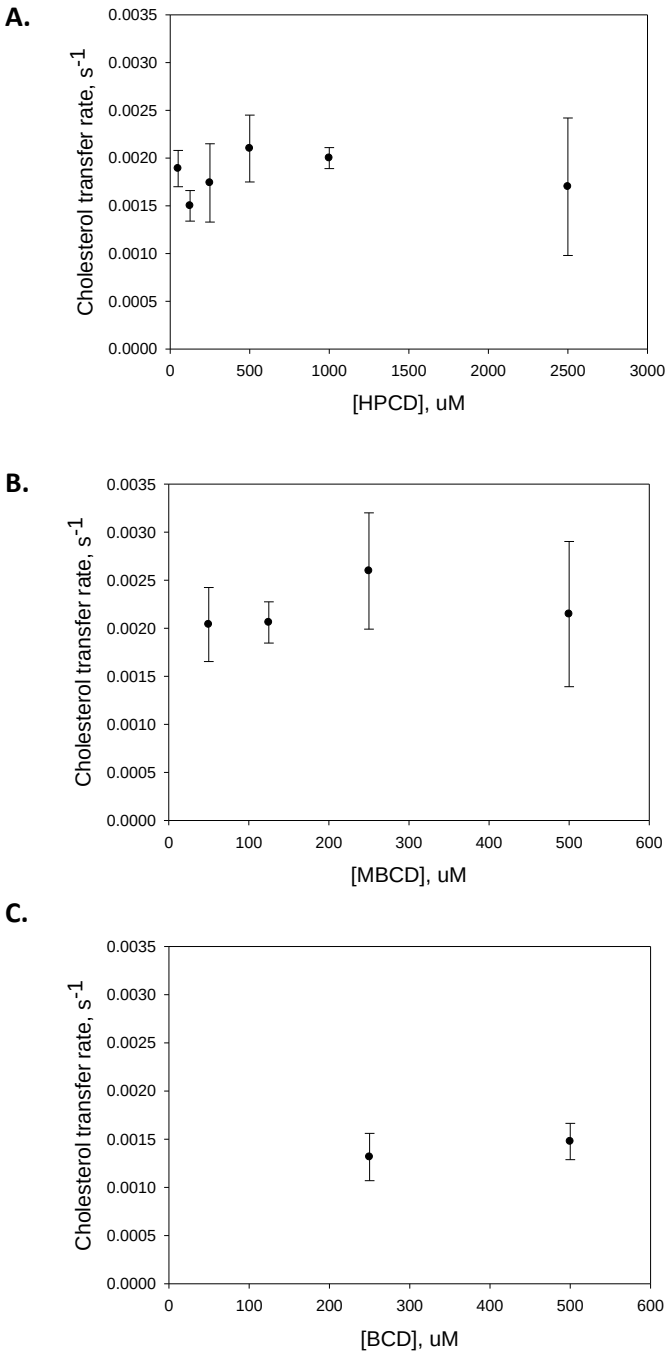


Figure 4-2. Transfer of cholesterol from hNPC2 to cyclodextrin. Transfer rates of cholesterol from 1 µM hNPC2 to 50-250 µM (A) HPCD, (B), MBCD, and (C) BCD. All experiments were at 25°C and pH 5.0, as described in Materials and Methods.

Figure 4-3

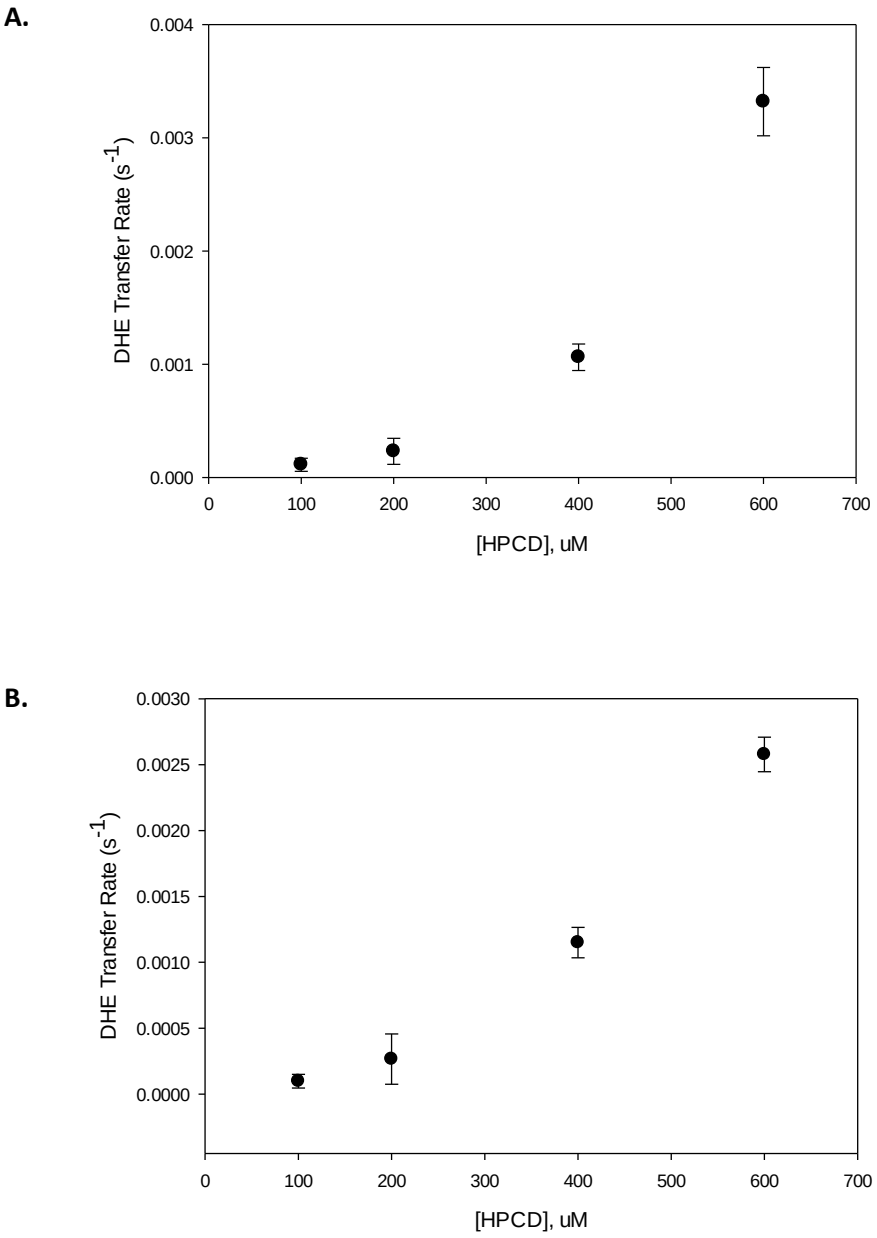


Figure 4-3. **Transfer of DHE from phospholipid membranes to HPCD.** Transfer rates of DHE from 10 µM (A) DHE:dansyl-PE:EPC (25:3:72mol %) or (B) DHE:dansyl-PE:LBPA:EPC (25:3:25:47 mol %) donor SUVs to increasing concentrations of HPCD. All experiments were conducted at 25 °C and pH 5.0 using an excitation wavelength of 300nm and a 520 nm long pass filter. Data are presented as mean ± SE (n = 3).

mixed with increasing concentrations of zwitterionic EPC SUVs. An increase in transfer rate with increasing EPC SUV concentration was observed, and virtually identical rates of DHE transfer were found when LBPA was incorporated into the membranes (**Figure 4-4B**). The results for LBPA-containing membranes are in marked contrast to what we observed for NPC2, where LBPA in the membranes led to order of magnitude increases in sterol transfer rates (29, 30).

Intermembrane Transfer

The spontaneous intermembrane transfer rate of DHE is very slow (30). However, we showed that addition of NPC2 enhances the intermembrane transfer rate of sterols by 40 to almost 300-fold (30), providing a mechanism by which NPC2 could function to prevent cholesterol accumulation in lysosomes. **Figure 4-5A** shows that the addition of HPCD can also increase the DHE intermembrane transfer rate, from about 0.0003 s^{-1} in the absence of CD to 0.0075 s^{-1} in the presence of 25 μM HPCD. In comparison to NPC2, where addition of 1 μM NPC2 resulted in a 40-fold increase in DHE intermembrane transfer rate for EPC membrane and a 280-fold increase for LBPA membranes, HPCD appears to require a higher concentration (25 μM) to obtain 25-fold increase, and LBPA incorporation into the membranes does not enhance the effect of cyclodextrin (**Figure 4-5B**). Indeed, sterol transfer rates from CD to LBPA containing membranes were slightly depressed compared to rates of transfer to the same concentration of EPC membranes. The intermembrane transfer rates of DHE are proportional to the increase in HPCD concentration, with possible saturation occurring at higher levels, further suggesting that sterol transfer occurs during CD–membrane interactions.

Figure 4-4

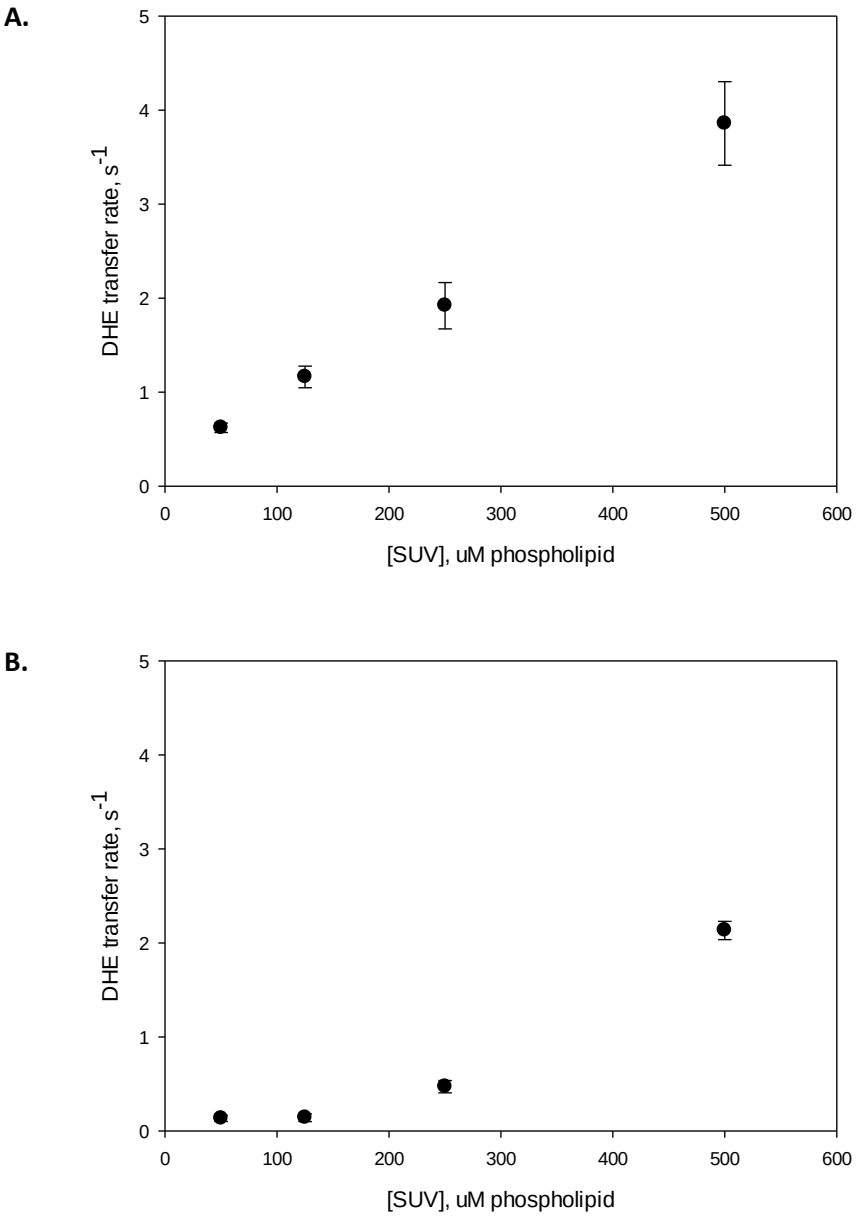


Figure 4-4. Transfer of DHE from HPCD to phospholipid membranes. Transfer rates of DHE from 5 µM HPCD/DHE(8:1) complex to acceptor membranes containing A) 3 mol % Dansyl-PE and 97 mol % EPC, or B) 3 mol % Dansyl-PE, 72 mol % EPC, and 25 mol % LBPA. All experiments were conducted at 25°C and pH 5.0 using an excitation wavelength of 300nm and a 520nm long pass filter. Data are presented as mean ± SE (n=3).

Figure 4-5

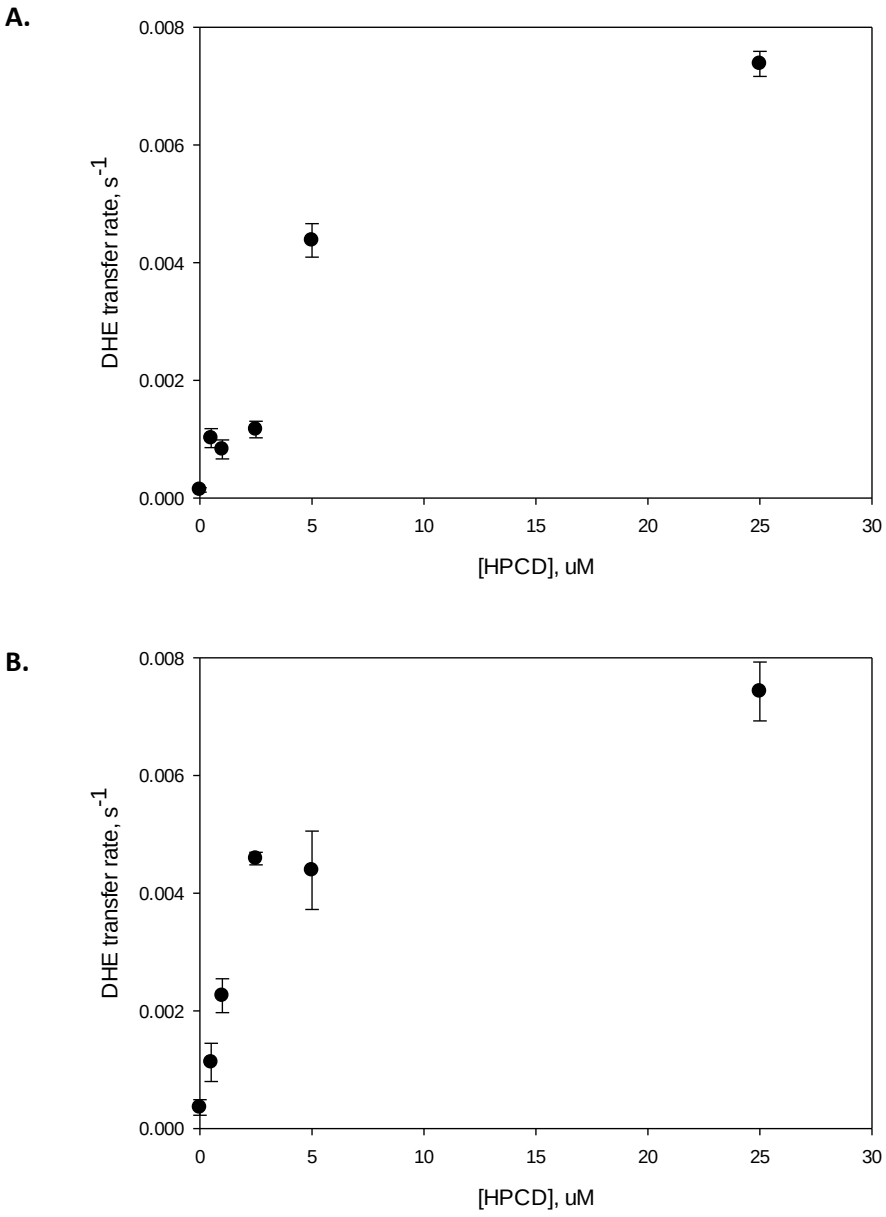


Figure 4-5. Effect of cyclodextrin on DHE intermembrane transfer. Transfer rates of DHE from 50 µM donor SUV composed of A) DHE:EPC (25:75 mol %), or B) DHE:LBPA:EPC (25:25:50 mol %), to 250 µM acceptor SUV (EPC:Dansyl-PE = 97:3 mol %) in the presence of 0 to 50 µM HPCD. All experiments were conducted at 25°C and pH 5.0, using an excitation wavelength of 300nm and a 520nm long pass filter. Data presented as mean ± SE (n=3).

Rescue of Cholesterol Accumulation in NPC2$^{-/-}$ Fibroblasts by CD

The kinetic data obtained from the model membrane studies suggest that CD can traffic cholesterol between donor and acceptor membranes via direct interaction, in a manner similar to NPC2 (30). To compare the effects of CD with those of NPC2 at the cellular level, we incubated *npc2*$^{-/-}$ fibroblasts with varying concentrations of CD and monitored the reduction in cellular cholesterol accumulation over time. The results shown in **Figure 4-6A,B** demonstrate that CD causes a dose-dependent decrease in sterol accumulation in NPC2-deficient cells, with 100 μM HPCD or MBCD equivalently decreasing filipin staining to levels achieved with 50 nM hNPC2. Moreover, the cholesterol clearance from *npc2*$^{-/-}$ fibroblasts treated with HPCD occurs quite rapidly. Incubation with 100 μM HPCD resulted in near complete correction of the NPC phenotype by 24 h, with 60% of accumulated cholesterol cleared after 1 h. A smaller dose of HPCD was also able to rapidly remove cholesterol from cells, with a half-time of 3 h; however, this concentration of CD was unable to remove more than about 70% of accumulated cholesterol relative to untreated cells (**Figure 4-6C,D**).

NPC2 and CD Cause Phospholipid Membrane Aggregation

In ongoing studies of NPC2 structure–function relationships, we found that NPC2 may interact with more than one membrane simultaneously [Xu, McCauliff, and Storch, unpublished observation], as reported for the yeast sterol binding protein Osh4p and others (135). To determine whether CD can act in a similar manner, we mixed varying concentrations of HPCD with 50 μM large unilamellar EPC vesicles and observed the changes in light scattering over time; 600 Å LUVs refract light to a small extent, but if they aggregate or fuse, the large size is easily detected by light scattering as increased absorbance at 350 nm. **Figure 4-7** shows that,

similar to hNPC2, HPCD, but not the control protein albumin, causes an increase in the A350 of EPC LUVs over time, indicating that membrane–membrane interactions are occurring.

Figure 4-6

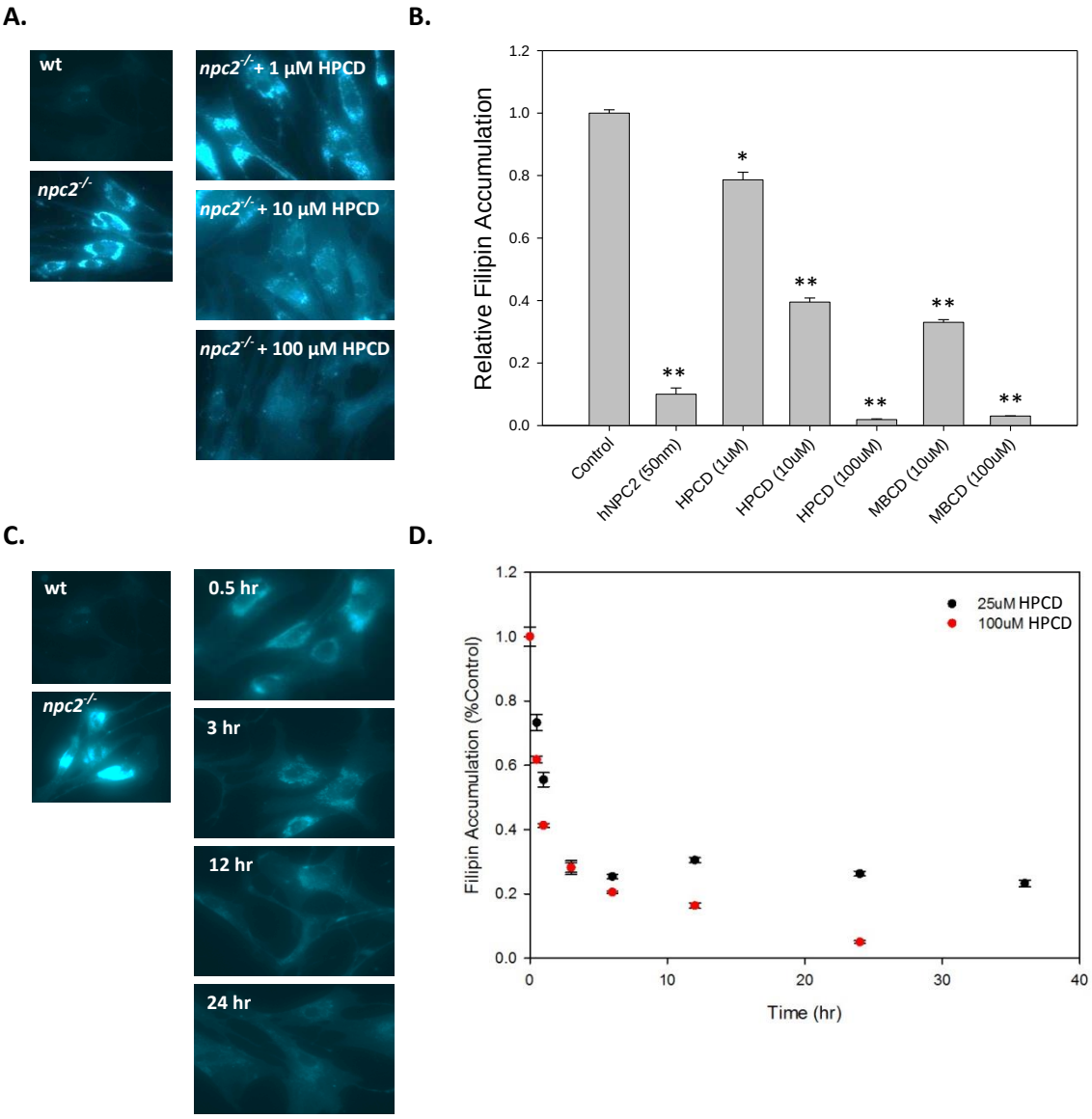


Figure 4-6. Filipin accumulation in NPC2 deficient fibroblasts treated with cyclodextrin. Human NPC2 deficient fibroblasts were incubated with CD and were fixed and stained with filipin at varying time points. Filipin accumulation was determined as the ratio of filipin stain area to total cell area. Results are expressed relative to control untreated cells, and are represented as mean ± SE. *$p < 0.01$; **$p < 0.001$. (A) Representative images of *npc2*$^{-/-}$ fibroblasts incubated with increasing concentrations of HPCD for 3 days. (B) Relative filipin accumulation in *npc2*$^{-/-}$ cells treated with varying concentrations of HPCD, MBCD or 50 nM hNPC2 for three days. (C) Representative images of filipin depletion over time in *npc2*$^{-/-}$ fibroblasts treated with 100 µM HPCD. (D) Depletion of the relative filipin accumulation in *npc2*$^{-/-}$ fibroblasts treated with 25 or 100 µM HPCD.

Figure 4-7

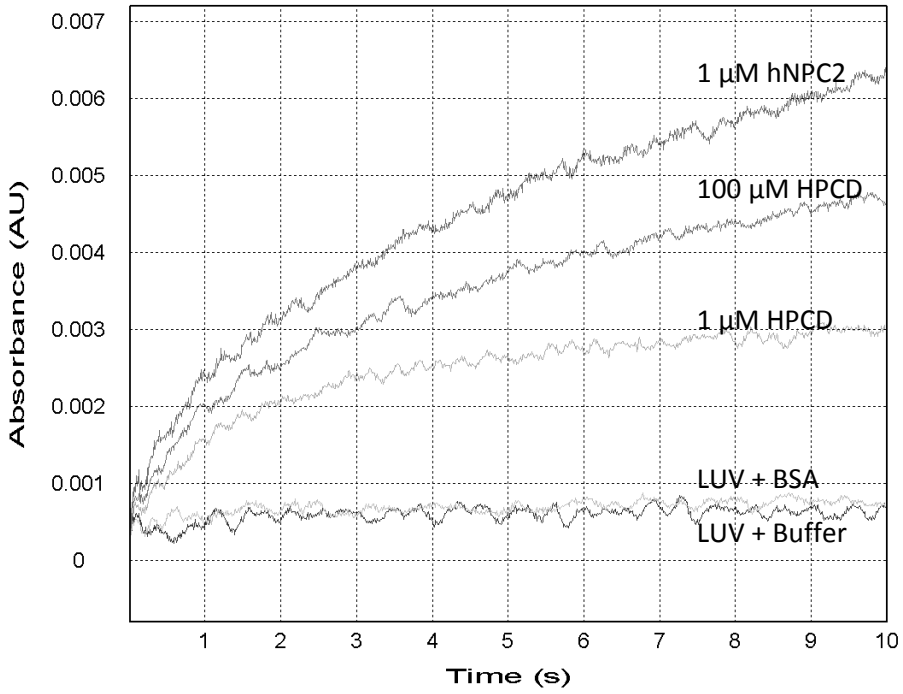


Figure 4-7. NPC2 and CD promote membrane-membrane interactions. Time dependent changes in the absorbance of 50 µM LUVs mixed with 1 µM of bovine serum albumin (BSA), hNPC2 and 1 or 100 µM HPCD were observed at 350nm using an SX20 stopped-flow spectrofluorometer.

Discussion

Deficiencies in either of the lysosomal proteins NPC1 or NPC2 lead to marked accumulation of LDL-derived cholesterol and glycolipids in the LE/LY compartment, the cellular hallmark of Niemann Pick Type C disease. CD was shown to reverse LE/LY accumulation of cholesterol in NPC1-deficient mice, resulting in lifespan elongation and delays in the onset of pathological symptoms (106, 111, 178). It was suggested that a possible mechanism of action of CD was via specific interaction with NPC2 (107). The present results do not support interactions between NPC2 and cyclodextrins. The absolute rates of cholesterol transfer observed in these stopped-flow kinetics analyses, 0.002 s^{-1}, are comparable to the calculated rate of cholesterol dissociation from NPC2 of 0.003 s^{-1}, obtained from equilibrium binding studies (81). Thus, the results strongly suggest that sterol transfer by CD occurs independently of NPC2. Not surprisingly, then, it has now been shown that CD is equally effective in the treatment of mice deficient in NPC2 as in NPC1-deficient mice (105), which would not be the case if the mechanism of action of CD involved a specific interaction with NPC2.

On the basis of the promising results in NPC-deficient mice, 2-hydroxypropyl-β-cyclodextrin has been proposed as an experimental therapy for human NPC disease (183). At present, its mechanism of action is only beginning to be understood. As noted earlier, it has recently been shown that extracellular CD is internalized into the endocytic vesicle system, indicating that it is functioning within the lumen of the LE/LY (108). It was also recently proposed that CD may stimulate relocation of cholesterol laden LE/LYs to the plasma membrane followed by exocytosis (184). The ability of CD to solubilize cholesterol and to both deliver and extract cholesterol to and from cellular membranes (103, 145, 179) led us to hypothesize an NPC2-like mechanism of

action, whereby CD may extract LDL-derived cholesterol from internal lysosomal membranes and deliver it either to the limiting lysosomal membrane or, potentially, to NPC1, for egress from the lysosome.

We tested this hypothesis by quantifying the absolute rates of cholesterol transfer to and from model phospholipid membranes and CD as well as examining the effects of CD on sterol transfer between membranes using stopped flow mixing and fluorescence spectroscopy. These experimental approaches are based on those used for many years to examine the transfer of small hydrophobic molecules between membranes and/or lipid-binding proteins or other carriers (82, 83, 185-188). By varying either CD or membrane concentration in these assays, we can distinguish between two possible sterol transfer mechanisms: aqueous diffusion or collisional transfer. Increasing the concentration of either acceptor membranes or CD increases the theoretical number of collisions between CD and membranes; therefore, if direct interactions between CD and membranes occur, the rate of DHE transfer will increase in proportion to the frequency of collision. In contrast, if transfer involves diffusion through an aqueous medium, the rate of DHE transfer from donor to acceptor would remain constant since desorption of DHE from the donor species would represent the limiting step of transfer (82, 83, 185-188). The results show that CD rapidly extracts and delivers DHE from/to membranes and that rates are directly dependent upon the concentration of both CD and membranes. We further observed that CD accelerates the transfer of DHE between donor and acceptor membranes, again at rates that are dependent upon CD concentration. These results strongly suggest that sterol transfer occurs during direct collisional interactions between CD and phospholipid membranes.

The rates of cholesterol transfer by NPC2 were found to dramatically increase with membranes containing the LE/LY-specific phospholipid LBPA (29, 30, 189). In distinct contrast to NPC2, no effects of this unique lysosomal phospholipid on sterol transfer rates between membranes and CD were observed. Thus, while CD may traffic cholesterol in a manner similar to NPC2, it exhibits no apparent specificity toward lysosomal membrane phospholipids. It is also worth noting that, in addition to the absence of LBPA effects, sterol transfer by CD is considerably slower than transfer by NPC2. For example, 100-fold greater concentrations of HPCD are necessary to yield similar rates of sterol transfer from donor membranes to NPC2 (30).

Several other lines of evidence using different experimental approaches have also suggested that CD is membrane-interactive. Yancey et al. found that the activation energy for cholesterol transfer from cells to CD was considerably lower than from cells to HDL particles and proposed that cholesterol transfer to CD proceeded directly from the bilayer into the CD, requiring much less energy than would desorption of cholesterol into an aqueous environment (145). Additionally, differential scanning calorimetry and atomic force microscopy studies have demonstrated directly that the β-CDs are membrane interactive (190, 191).

The mechanisms of sterol transfer from CD to membranes and from membranes to CD both appear to be collisional in nature; however, the slower transfer rates from vesicles to CD, as compared to rates from CD to membranes, suggest that the membrane-bound sterol is less efficiently transferred, and thus movement from membrane to CD is limiting; once the sterol is bound to CD, interactions with a bilayer lead to very rapid transfer. The intermembrane DHE

transfer rate is also slow, further indicating that the rate-determining step is the interaction of CD with the donor membrane and that subsequent delivery to the acceptor is very rapid.

While it had been proposed that the actions of CD in NPC1 deficiency might be due to its interaction with NPC2, the present kinetic studies predicted that CD should be able to function in the absence of NPC2. Thus, we examined the effectiveness of CD added to the medium of *npc2*$^{-/-}$ fibroblasts in clearing the LE/LY cholesterol accumulation and found that CD incubation resulted in sterol egress. In the course of these studies, similar effects of CD in *npc2*$^{-/-}$ cells were reported (108). It is therefore likely that CD is internalized by bulk-phase endocytosis (108), where it effectively removes cholesterol accumulation from the internal membrane lamellae of *npc2*$^{-/-}$ cells. A direct comparison of CD with NPC2 showed, in agreement with the model membrane studies, that CD is 50–200 percent less effective on a molar basis than NPC2 in clearing cellular cholesterol.

Our results demonstrate for the first time that CD can rapidly deliver and remove cholesterol from phospholipid membranes by a collisional mechanism that fundamentally resembles the mechanism by which NPC2 catalyzes cholesterol transport. Since the spontaneous transfer of cholesterol between membranes is exceedingly slow, this transfer mechanism may explain the observed efficacy of CD administration in models of NPC2 disease. The sterol transfer rates observed for both NPC2 and CD may also be related to their ability to promote membrane–membrane interactions. The interior of the LE/LY compartment has been shown to contain variable amounts of internal membranes as well as an outer limiting membrane (192); thus to effect the egress of cholesterol from the LE/LY interior, it may be necessary to transfer the sterol

between multiple membranes. We found that addition of NPC2 to vesicles caused a rapid increase in turbidity, indicative of membrane–membrane aggregation; HPCD displayed a similar membrane aggregation potential, whereas albumin had no effect. Recently, Abdul-Hammed et al. (193) used a steady-state vesicle pull down assay to demonstrate that NPC2 causes membrane aggregation. We hypothesize that in vivo NPC2 and its surrogate, CD, may catalyze cholesterol transfer at zones of close apposition of membranes, as might exist in the multilamellar interior of the LE/LY compartment. These so-called "membrane contact sites" have been proposed to be important for rapid lipid transfer between different organelles (133, 135). Interestingly, another sterol-binding protein, Osh4p in yeast, has also been shown to promote lipid transfer between membranes by inducing membrane–membrane interactions; several other Osh proteins were proposed to act similarly (135). Ongoing mutagenesis studies of NPC2 suggest the presence of two distinct membrane-interactive domains on the protein surface, indicating the potential for establishing membrane–membrane interactions.

It is less clear, at present, as to how CD is able to bypass NPC1. Using whole animal sterol turnover studies in NPC-deficient mice, Ramirez et al. have suggested that CD catalyzes the diffusion of cholesterol across the limiting lysosomal membrane via interaction with the sterol at the inner leaflet (194). The present results support this hypothesis. Thus, it is possible that by its membrane-interactive properties cholesterol-loaded CD in the lumen of the LE/LY could increase the level of cholesterol in the inner leaflet of the limiting lysosomal membrane, resulting in greater levels of transmembrane movement to the outer leaflet, even in the absence of NPC1.

Chapter 5

Cyclodextrin polymers are effective *in vitro* as an NPC therapy

The studies described in this chapter are based on a collaboration with Dr. David Thompson's group at Purdue University.

Abstract

Several lines of evidence suggest that β-cyclodextrin (BCD) derivatives initiate the efflux of accumulated, unesterified cholesterol from the late endosomal/lysosomal compartment in Niemann Pick C (NPC) disease models. Unfortunately, repeated injections or continuous infusions of current BCD therapies are required to sustain suppression of symptoms and prolong life. In an effort to improve the pharmacokinetics and increase the efficacy of CD treatment, thus presenting a more viable therapeutic for NPC disease, a library of BCD and 2-hydroxypropyl-β-CD (HPCD) polyrotaxanes (PRTx) was developed. These CD polymers were synthesized by threading either BCD or HPCD onto a variety of biocompatible poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol) (PEG-PPG-PEG) or poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymers, respectively. These compounds carry multiple copies of BCD or HPCD, as shown by various analytical procedures, and are retained on the copolymer strand using a sodium 2,4,6 trinitrobenzenesulfonate (TNBS) endcap. Free cyclodextrin contamination in the compounds was determined by two independent chromatographic approaches, and dethreading kinetics were also analyzed. Treatment of *npc2*$^{-/-}$ fibroblasts with the BCD and HPCD PRTx derivatives resulted in significant reductions of filipin staining, similar to that achieved with molar equivalents of monomeric CD. Colocalization of a carboxyfluorescein-labeled BCD PRTx with Lysotracker in *npc2*$^{-/-}$ cells demonstrate that the PRTx derivatives are likely entering the LE/LY compartment, where BCD and HPCD monomers dethread from the copolymer strand to promote efflux of the accumulated cholesterol. Thus, these Pluronics-based polyrotaxanes present a potential improvement to current CD therapies for NPC disease.

Introduction

Niemann-Pick Type C (NPC) disease is one of several lysosomal storage disorders specifically characterized by the accumulation of unesterified cholesterol and glycolipids in the late endosome/lysosome (LE/LY). This aberrant accumulation causes LE/LY dysfunction, ultimately leading to permanent cell and tissue damage (60, 71). Often, the defect manifests itself in the form of neurological, hepatic and/or pulmonary symptoms that increase in intensity over time (194). These can involve enlargement of organs, dysarthria, dysphagia, ataxia, and other neurological symptoms including epilepsy and dementia (77). NPC typically manifests in children and is ultimately fatal, even with current treatment regimens (66).

The underlying causes of NPC are genetic mutations in either of two lysosomal proteins; membrane bound NPC1 (95% of cases) or soluble NPC2 (5% of cases). These proteins are ubiquitously expressed and are localized to the LE/LY compartment (76, 77). In a normally functioning cell, NPC1 and NPC2 are responsible for the efflux of unesterified cholesterol from the LE/LY compartment into the cytosol and are generally thought to function in a common cholesterol efflux pathway (195). The dysfunction of either NPC protein causes aberrant sequestration of cholesterol in the LE/LY compartment (66). The accumulation of the cholesterol, in turn, causes lysosomal swelling, upregulation of the genes controlling cholesterol synthesis and lipoprotein uptake, and ultimately neurological effects including demyelination of brain cell axons and the death of Purkinje cells (107, 113, 196). Additionally, the onset of macrophage activity in affected tissues results in the release of pro-inflammatory cytokines in most organs (111-113, 197). The cumulative impact of these factors is cell death and presentation of the NPC disease state.

Since its demonstration by Camargo et al., an increasing body of evidence shows that derivatives of β-cyclodextrin (BCD), a macrocycle consisting of 7 glucose subunits, are able to initiate the efflux of accumulated cholesterol from the LE/LY compartment of NPC1 and NPC2 deficient cells (107, 108, 111-113, 131, 194, 196, 197). Dietschy and co-workers reported that subcutaneous injection of HPCD was able to mobilize cholesterol in the murine model of NPC1 disease, extending life span and improving hepatopathology and neuropathology in the treated animals (66, 105, 107, 112, 194, 196). Suppression of intrinsic cholesterol synthesis, activation of the target genes for the LXR nuclear receptors, and suppression of target genes for SREBP were observed in several organs with HPCD treatment. All of these factors contribute to rapid egress of sequestered cholesterol from sites of abnormal storage (107, 111, 197). When injected directly into the CNS, similar effects were seen in the brains of *npc1*$^{-/-}$ mice (113, 194), and continuous infusion with BCD derivatives was shown to prevent neurodegeneration (113). Continuous BCD infusion has also been shown to have similar positive effects in *npc2*$^{-/-}$ mice (105). At the cellular level, addition of BCD to either *npc1*$^{-/-}$ or *npc2*$^{-/-}$ patient fibroblasts has been shown to rescue the cholesterol accumulation phenotype of these cells (108, 131).

While reversal of cholesterol accumulation in mice was seen after administration of BCD, evidence indicated that the animals began reaccumulating cholesterol shortly after dosing was terminated (107, 111, 112). BCD derivatives, therefore, overcome cholesterol transport defects in NPC1 disease, but the alleviation is temporary such that their presence in the bloodstream is brief, likely due to their appreciable water solubility and relatively low molecular weight. Indeed, Liu et al reported that greater than 90% of an administered dose of HPCD was cleared

via glomerular filtration within 24 hours following injection (107). Hence, repeated or continuous infusions are needed for prolonged effects of CD (112, 113). By improving the pharmacokinetics and biodistribution of the dose, the efficacy of BCD derivatives may be improved, presenting a potentially more viable NPC therapeutic. This may be achieved through the design of long circulating, biocompatible macromolecules capable of delivering multiple copies of BCD to the LE/LY of NPC cells.

Polyrotaxanes (PRTx) are supramolecular assemblies that have been of considerable interest in biomaterials applications such as drug and gene delivery and hydrogel formation (116-120). Following the synthesis of α-CD:PEG polyrotaxanes (126, 198-200), efforts have focused on developing new generations of these complexes using different endcaps and/or cyclodextrin monomers (127, 201-207). PRTx architecture resembles a molecular necklace type of structure. Common characteristics include macrocyclic molecules noncovalently threaded onto a polymer chain with bulky caps at the polymer termini (198). The end-caps prevent macrocycle dethreading, and can be modified to respond toward changes in pH (121) or specific enzyme activity (122, 123). This allows for control over when, where and how the PRTx releases its macrocyclic cargo. Cyclodextrin-based PRTx derivatives have been synthesized using many polymer cores including polyesters (124), polyamides (125), poly(ethylene glycol) (PEG) (126-128), polypropylene glycol (PPG) (129), and many di- and triblock copolymers (130).

In this study, a library of PRTx derivatives has been synthesized using Pluronic block copolymers and BCD or HPCD as building blocks. Pluronic polymers have a PEG-PPG-PEG or PEO-PPO-PEO triblock copolymer architecture that differs in the relative lengths of the PEG/PEO and PPG/PPO

blocks. Molecular weights of the Pluronics used in this study range from 1900 to 12600 g/mol. These polymers were chosen because of their previous uses in pharmaceutical applications and their known biocompatibility (208). Our ultimate goal is to increase the efficiency of BCD or HPCD delivery into NPC cells by increasing the circulation time of the active CD, using biocompatible Pluronic-derived BCD PRTx, which release CD monomers at high concentration in the LE/LY compartment of cells.

In these studies, the capacity of these compounds to mobilize accumulated cholesterol in *npc2*$^{-/-}$ fibroblasts was evaluated by filipin staining, while PRTx localization to LE/LY compartments was assessed by monitoring fluorescent PRTx colocalization with Lysotracker Blue.

Materials and Methods

Materials

(1,1)-Carbonyldiimidazole (CDI) and 2,4,6-Trinitrobenzenesulfonic acid (TNBS) were purchased from Research Organics. Tris(2-aminoethyl)amine (TREN), β-cyclodextrin (BCD), Pluronic® triblock copolymers and 2,2'-(Ethylenedioxy)bis(ethylamine) were purchased from Sigma Aldrich. The four Pluronics used in this study were F127, F68, L64, and L35. All solvents were dried and distilled from an appropriate desiccant prior to use unless otherwise noted. Human fibroblasts cells from an apparently healthy donor (GM03652) and from an NPC2 patient (GM18455) were from Coriell Institute of Medical Research (Camden, NJ). Filipin and fluorescein were purchased from Sigma and Lysotracker Blue from Molecular Probes.

The following methods pertaining to the synthesis, purification and characterization of the BCD and HPCD:pluronic polyrotaxanes, in addition to the synthesis of the fluorescent BCD polyrotaxane, were conducted by Dr. David Thompson's group.

Synthesis and purification of BCD and HPCD:Pluronic Polyrotaxanes

The BCD and HPCD:Pluronic PRTx were synthesized by David Thompson's laboratory at Purdue University. Prior to use, all Pluronic precursors were initially dried by azeotropic distillation from benzene under a vacuum. The synthesis of TNBS capped BCD: and HPCD:Pluronic polyrotaxanes was then performed following the sequence of reactions shown in **Figure 5-1**. Variations between synthesis of BCD: and HPCD:Pluronic PRTx are indicated. Briefly, following a slightly modified procedure to that described by Li et al. (209), the precursors were initially modified with Tris(2-aminoethyl)amine (TREN). Dried TREN-pluronic intermediates were then added to

saturated aqueous solutions of BCD or HPCD in hexane. The suspensions were then vortexed, stirred and probe sonicated. Threading of the BCD units onto the TREN-terminated Pluronic PRTx was found to be quite slow and required agitation on a rocker plate for 14 days following sonication to promote threading. The reaction was much faster with HPCD in an organic solvent, requiring only 48 hours of constant stirring. TNBS was then added to the reaction, and the slurry was stirred or left on a rocking plate for 24 hours to allow for the endcapping reaction.

Purification of the trinitrobenzene(TNB)-endcapped CD:Pluronic polyrotaxanes

The BCD:Pluronic PRTx were collected from the reactions mixture via centrifugation and washed several times with water until the washes ran nearly clear. HPCD:Pluronic PRTx were collected by diethyl ether precipitation of products dissolved in methanol. BCD: and HPCD products were then dialyzed against H_2O, using tubing correlating to the estimated MW of the final product. Upon completion of dialysis, the purified products were frozen and lyophilized to yield yellow-orange powders. The PRTx were then analyzed by both reverse phase high-pressure liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) for free CD content. Samples containing >5% free BCD by RPLC were suspended in H_2O, vortexed, and collected by centrifugation. After three repetitions, the samples were lyophilized and reanalyzed for free BCD content. This process was repeated until the HILIC and/or RPLC analyses indicated that the free BCD content was ≤5%.

Synthesis of fluorescently labeled BCD:F127 polyrotaxane

Fluorescein modified BCD:F127 PRTx was synthesized by dissolution of BCD:F127 PRTx in dry DMSO before activation with (1,1)-Carbonyldiimidazole (CDI) and coupling to ethylene diamine.

Figure 5-1

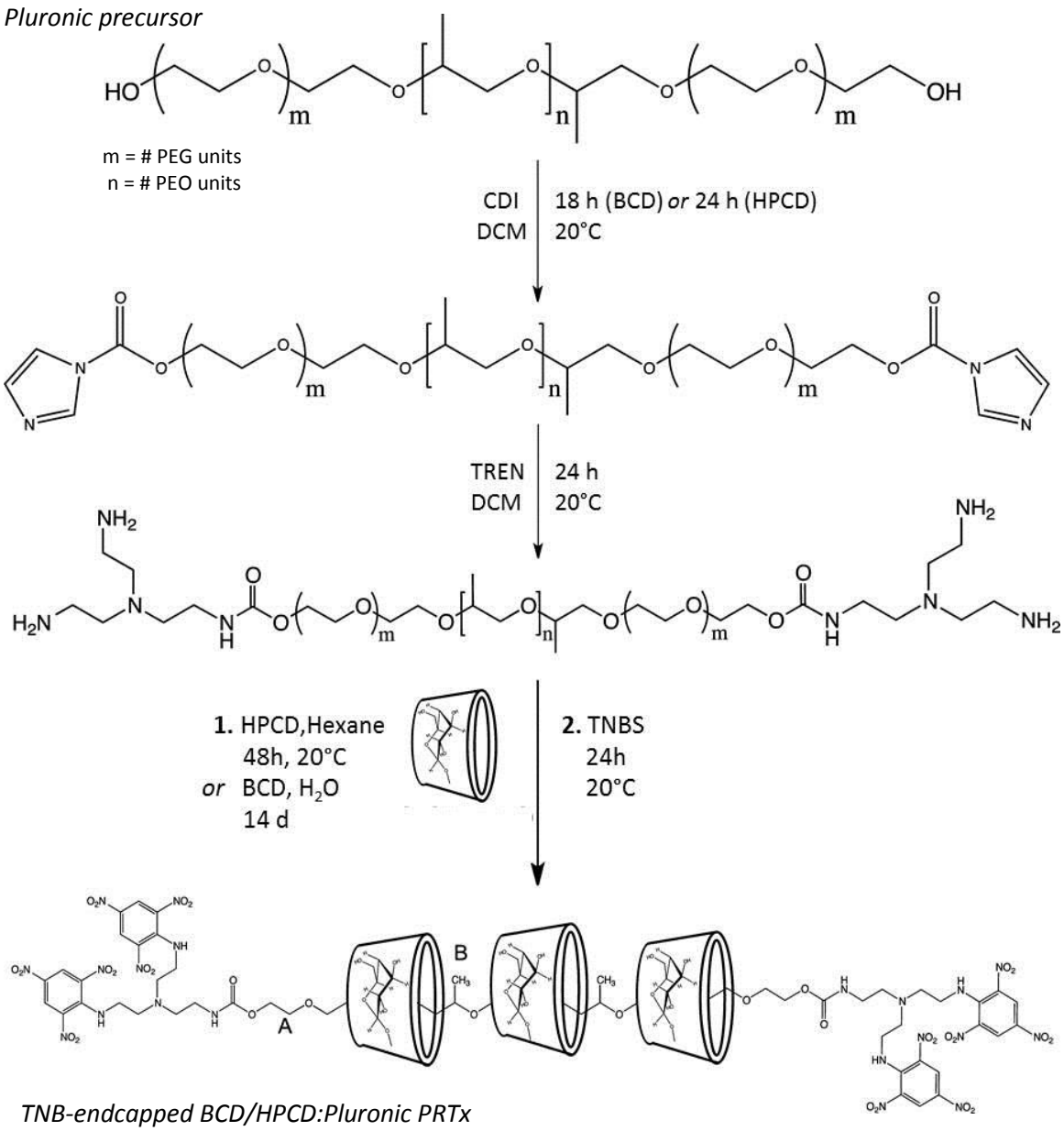


Figure 5-1. Synthesis of CD:Pluronic polyrotaxanes with TNB endcaps

A second activation with CDI, followed by fluorescein coupling, and precipitation twice in methanol yielded the fluorescein labeled PRTx derivative. The fluorescent PRTx was used without further purification.

Characterization of TNB-endcapped BCD: and HPCD:Pluronic polyrotaxanes

Two-dimensional nuclear overhouser effect spectroscopy (NOESY) was used to confirm successful threading of CD onto the Pluronic backbone. ^{1}H nuclear magnetic resonance (NMR) spectroscopy was used to determine the number of cyclodextrins threaded onto the Pluronic PEG:PPG:PEG chain, by comparing the integral intensities of the CD C_1-H and PPG CH_3 signals. The NMR based calculations of threading efficiency were corrected for free CD content in each sample, determined via gel permeation chromatographic (GPC) analysis. RPLC and HILIC techniques were also used to determine free CD content of the preparations. TNB endcapping was confirmed by ^{1}H NMR and UV-visible spectroscopy. GPC, analytical ultracentrifugation (AUC) and matrix assisted laser desorption/ionization mass spectrometry (MALDI) were used to determine the molecular mass of the products. MALDI was also used to determine heterogeneity of the PRTx products. Dethreading kinetics were studies by RPLC, ^{1}H NMR and UV-visible spectroscopy.

Clearance of cholesterol from npc2$^{-/-}$ fibroblasts by cyclodextrin polymers

To assess the therapeutic potential of the CD:Pluronic polyrotaxanes, human NPC2-deficient fibroblasts were treated with each of the compounds. Cells were seeded on eight-well tissue culture slides at a density of 6×10^3 cells, in Eagle's minimum essential medium with Earle's salts and nonessential amino acids +15% FBS and pen/strep at 37 °C with 5% CO_2. Sterile BCD: or

HPCD:Pluronic PRTx samples were solubilized in DMSO and diluted in cell culture media to a concentration yielding the equivalent of 25 µM free BCD or HPCD, respectively. The final DMSO concentration in all preparations was ≤0.001% (v/v). Thirty-six to 48 hours after plating, medium was removed from cells and the media containing each PRTx was added. Following administration of treatments, cells were fixed *in situ* at varying time points, as indicated, with 10% buffered formalin. Cells were then stained with 50 µg/ml filipin in PBS for 1 hour at room temperature. Slides were prepared with Prolong Gold antifade reagent (Molecular Probes) to ensure the absence of photobleaching. Images were acquired using a DAPI filter set on a Nikon Eclipse E800 epifluorescence microscope (Nikon Inc.), equipped with NIS-Elements BR 3.2 software (Nikon Inc.). The reduction in cholesterol accumulation was determined using NIS-Elements selection tools and calculated as a ratio of filipin area to cell area, as previously described (131). Results for all treatment conditions were normalized to filipin accumulation in untreated *npc2*$^{-/-}$ fibroblasts and are expressed as mean ± SE. No changes in cell number or viability (>95%), assessed by trypan blue exclusion, were found for any of the compounds under any of the treatment conditions.

Cyclodextrin Polyrotaxane Localization

Fluorescein labeled BCD:F127 PRTx was administered to *npc2*$^{-/-}$ fibroblasts, as described for the cholesterol clearance studies, approximately 24 h after plating. Cells were rinsed with PBS 0.5 h or 1 h after addition of the compound and incubated with 50 nM Lysotracker Blue (Life Technologies) in PBS for 10 min. Cells were rinsed again and fixed *in situ* with buffered formalin. Images were obtained using DAPI and B-2A filter sets for the fluorescent PRTx and Lysotracker, respectively.

Results

The results pertaining to the characterization and dethreading behavior of the BCD and HPCD:pluronic polyrotaxanes, in addition to the synthesis of the fluorescent BCD polyrotaxane, were conducted by Dr. David Thompson's group.

Characterization of TNB-endcapped BCD/HPCD:Pluronic PRTx

Several characteristics of the BCD: and HPCD:Pluronic PRTx products were analyzed, including presence of end-caps, CD threading efficiencies, molecular weights, and purity with respect to free CD content, using an array of techniques. Results of these analyses are reported in **Table 5-1** and **Table 5-2** for the BCD and HPCD:Pluronic PRTx, respectively.

The BCD and HPCD:Pluronic PRTx products were also analyzed for their solubility properties. All products proved to be poorly soluble in all solvents tested, except DMSO. Poor solubility is a well-known property of PEG-based PRTx compounds (210); poor solvent access to the threaded polymer core combined with high molecular weights, produce limited solubility of these derivatives (120). Pluronic-based BCD and HPCD PRTx were thus expected to have similar properties.

Dethreading behavior of Pluronic PRTx

Experiments were performed to characterize the dethreading behavior of the BCD and HPCD:Pluronic PRTx under acidic and reducing conditions in an attempt to mimic the LE/LY environment**.** Experiments in which BCD or HPCD:Pluronic PRTx were suspended in solutions at either pH 7.4 or 5.5 and then sampled at various time intervals showed no increase in free BCD

Table 5-1

PRTx	PEG:PPG	# CDs	% threading	% yield	% free CD	Avg MW (g/mol)
F127	200:65	15	71.4	32.9	4.4	31,400
F68	151:29	14	100	13.3	3.6	27,000
L35	26:30	12	92	29.1	2.4	12,100
L64	22:16	5	63	19.1	1.2	18,600

Table 5-1. Characteristics of BCD:Pluronic PRTx products.

Table 5-2

PRTx	HLB[a]	CAC(%)[a]	% free CD	# CDs	% threading	Avg MW (g/mol)
F127	22	0.004	3.3	7	22	24,500
F68	29	0.04	0.9	2	15	12,800
L35	19	1	2.3	4	44	8,500
L64	15	0.14	6.0	6	43	13,200
L81	2	0.0063	1.5	11	52	18,900

Table 5-2. Characteristics of HPCD:Pluronic PRTx products. The threading efficiency was calculated based on a presumed 1 HPCD:2 PO unit ratio. The number of HPCD molecules threaded onto the Pluronic core was determined by ^{1}H NMR integration. The free CD values (w/v) were determined by UPLC chromatography using HPCD as standard. Molecular weight was determined by NMR and MALDI with the average of the two methods reported. HLB: Hydrophilic-Lipophilic Balance, CAC: critical aggregation concentration. [a]values adapted from P. Laibinis et al (211).

or HPCD levels by RPLC analysis up to 24 h after dissolution (data not shown). This was in agreement with the design of the TNB-endcapped PRTx, which were intended to be stable under acidic conditions. Studies were also conducted to monitor changes in end-cap status upon exposure to acidic pH and 10 mM glutathione. ^{1}H NMR analysis of a short bis-amine PEG-NH-TNBS analogue showed no changes in the characteristic TNBS peaks after incubation with glutathione, indicating that the TNB nitro groups had not been reduced. Additionally, the UV/vis spectra of the TNB end-caps remained unchanged following incubation in glutathione at both acidic and neutral pH. These results suggest that dethreading of BCD or HPCD:Pluronic PRTx is not triggered by acid-mediated end-cap cleavage or glutathione reduction of the TNB end-cap, as summarized in **Figure 5-5**.

Prior to treating *npc2*$^{-/-}$ fibroblasts with the compounds, the possibility of dethreading occurring upon solubilization in DMSO and/or dilution in the cell culture media was investigated. ^{1}H NMR analysis of PRTx samples showed that incubation in DMSO/MEM or DMSO/PBS did not result in changes to threading of the samples when compared to PRTx controls (data not shown). BCD and HPCD:Pluronic PRTx should, therefore, be stable in culture before cellular encounter and internalization.

BCD Polyrotaxanes are effective at removing cholesterol from NPC2 deficient fibroblasts

Npc2$^{-/-}$ fibroblasts were incubated with each BCD:Pluronic PRTx derivative and reductions in cellular cholesterol accumulation were monitored over time to evaluate their impact on a cellular model of NPC disease. Initially it was hypothesized that this library of PRTx constructs, which was not designed to be either acid sensitive or reductively labile, would

stimulate little or no reduction in cholesterol accumulation. However, **Figure 5-2** shows that a single dose of any BCD:Pluronic PRTx, yielding 25 μM monomeric BCD, results in a reduction of approximately 60 to 75% in accumulated cholesterol levels by six hours, relative to untreated controls. Results were similar to monomeric BCD, which decreased cholesterol levels to approximately 30% of the level of untreated cells over the same time period. Interestingly, while each of the compounds induced rapid efflux of cholesterol, differences in the efficacy of the PRTxs were apparent. For example L35, carrying 5 BCDs, stimulated the lowest degree of cholesterol efflux from *npc2*$^{-/-}$ cells while L64, with 12 BCD monomers, was the most effective ($p < 0.05$).

HPCD Polyrotaxanes are also effective at promoting cholesterol efflux in npc2$^{-/-}$ *cells*

Like the BCD:Pluronic PRTx, the library of HPCD:Pluronic PRTx were not designed to allow for dethreading of the HPCD monomers in the acidic LE/LY compartment. However, cholesterol accumulation in *npc2*$^{-/-}$ fibroblasts treated with each HPCD:Pluronic PRTx, assessed via filipin staining, was significantly reduced over the six hour treatment period (**Figure 5-3**). Similar to equivalent molar amounts of the unmodified HPCD monomer, all HPCD:Pluronic PRTx tested resulted in a 60 to 80% reduction of filipin staining relative to untreated controls (**Figure 5-3B**).

Cyclodextrin polyrotaxanes work within the LE/LY compartment

Reductions in cholesterol accumulation following treatment of *npc2*$^{-/-}$ fibroblasts with the BCD: and HPCD:Pluronic PRTx suggest that the BCD and HPCD monomers are working within the LE/LY compartment to promote efflux of cholesterol. However, based on the cellular studies only it is not clear whether dethreading of the PRTx is occurring prior to BCD/HPCD cellular

Figure 5-2

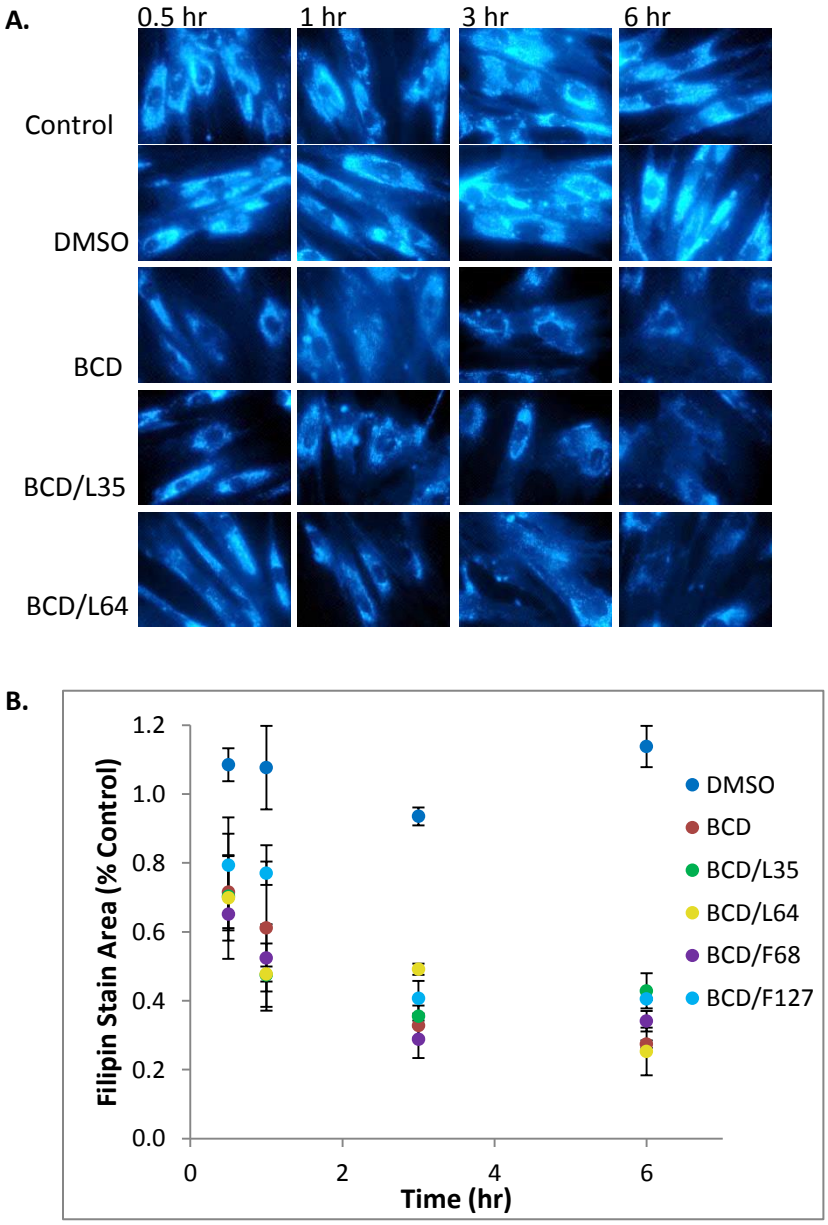


Figure 5-2. Filipin accumulation in NPC2-deficient fibroblasts treated with BCD:Pluronic PRTx. Human NPC2 deficient fibroblasts were incubated with 25 µM monomeric BCD or PRTx yielding 25 µM BCD and were fixed and stained with 0.05 mg/mL filipin at varying time points. (A) Representative images reveal stimulation of cholesterol efflux from *npc2*$^{-/-}$ fibroblasts by BCD and two PRTx compounds over time. (B) Filipin accumulation was determined as the ratio of filipin stain area to total cell area. Results are expressed relative to control untreated cells, and are represented as mean ± SE (n = 3).

Figure 5-3

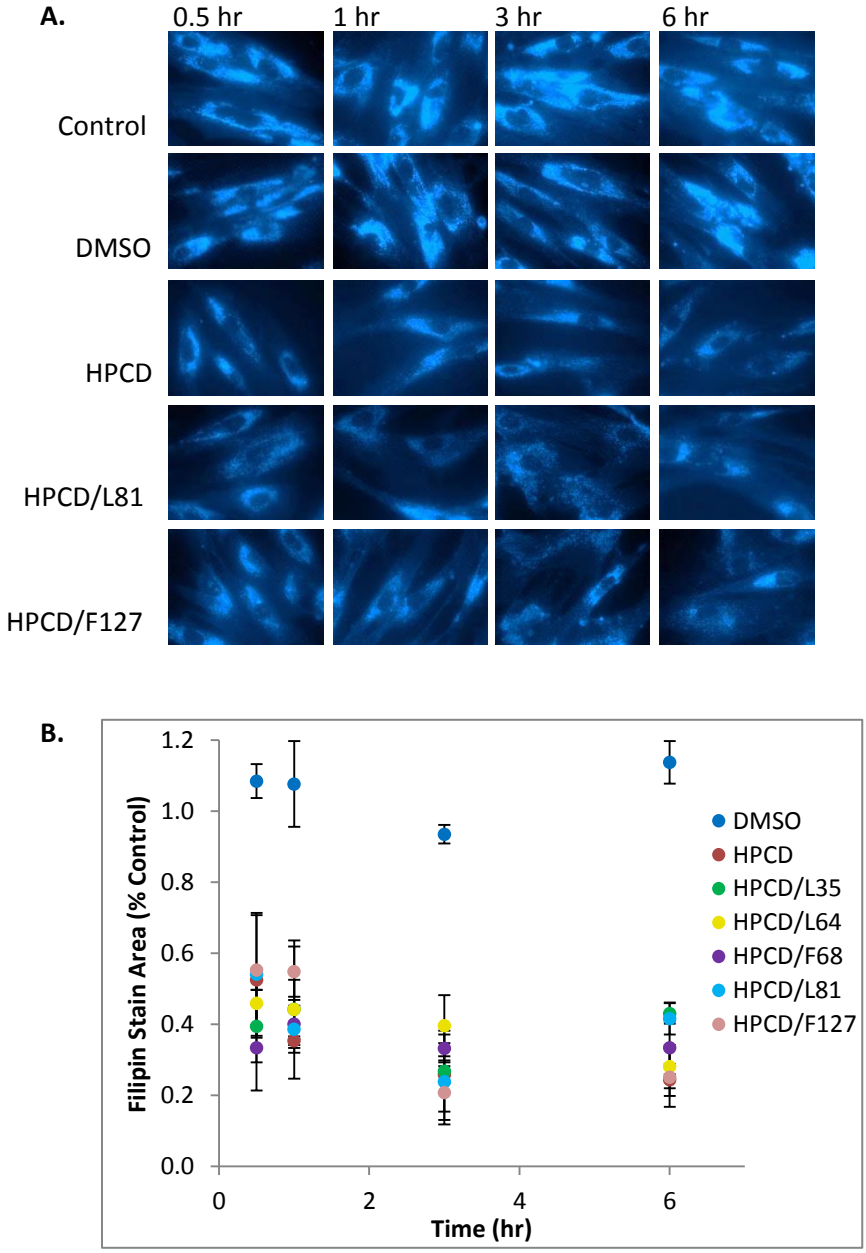


Figure 5-3. Filipin accumulation in NPC2-deficient fibroblasts treated with HPCD:Pluronic PRTx. Human NPC2 deficient fibroblasts were incubated with 25 µM monomeric HPCD or PRTx yielding 25 µM HPCD and were fixed and stained with 0.05 mg/mL filipin at varying time points. (A) Representative images reveal stimulation of cholesterol efflux from *npc2*$^{-/-}$ fibroblasts by HPCD and two PRTx compounds over time. (B) Filipin accumulation was determined as the ratio of filipin stain area to total cell area. Results are expressed relative to control untreated cells, and are represented as mean ± SE (n = 3).

uptake, e.g. in the media, or following uptake of the intact PRTx. Thus, in order to determine directly whether the BCD:Pluronic PRTx compounds were in fact entering the LE/LY compartment, a fluorescein labeled BCD:Pluronic PRTx, synthesized as described above, was used. Thirty minutes after addition of the compound to *npc2*$^{-/-}$ fibroblasts, fluorescein was observed to overlap entirely with Lysotracker Blue staining, indicating localization of the compound to the LE/LY (**Figure 5-4**).

Figure 5-4

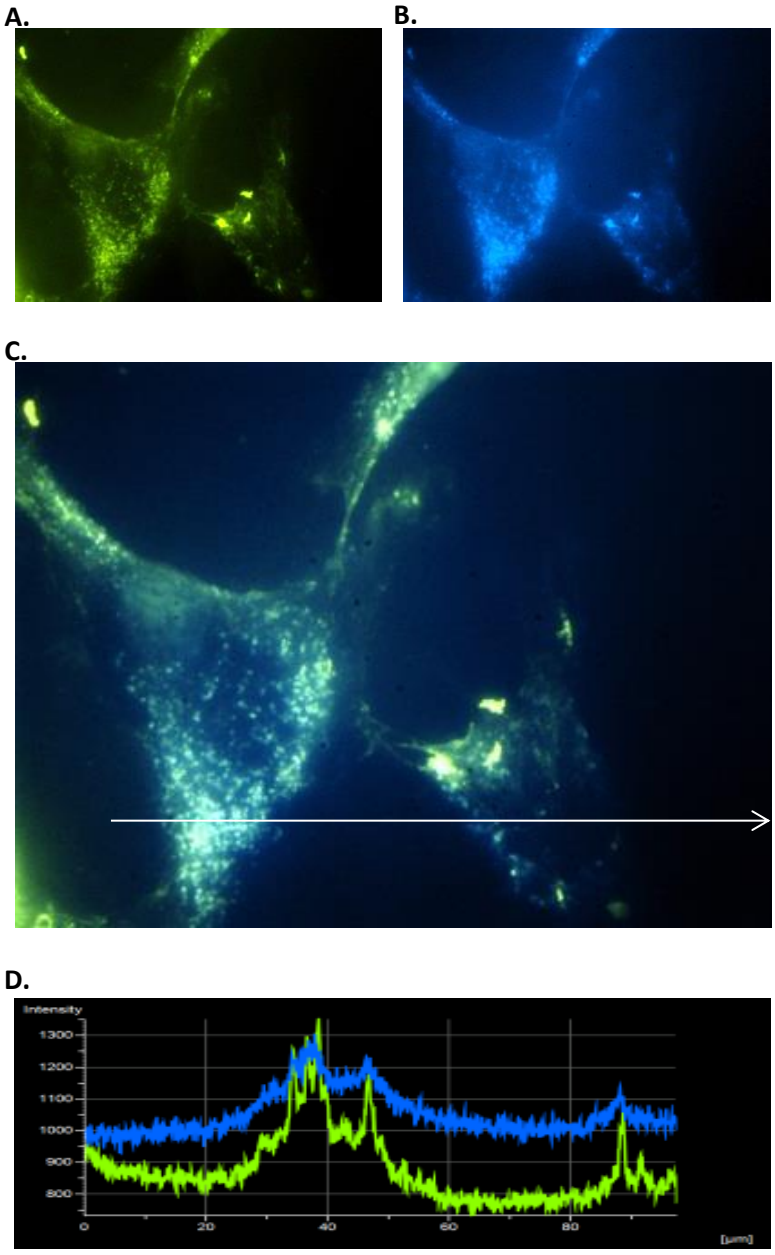


Figure 5-4. Fluorescein-labeled β-CD/F127 PRTx localizes to the LE/LY in npc2–/– fibroblasts. (A) Representative image of fluorescein localization in NPC2 deficient fibroblasts following a 1 h incubation period with labeled BCD:F127 PRTx. (B) Lysotracker Blue staining identifies LE/LY compartments. (C) Fluorescein fluorescence overlays with Lysotracker Blue, indicating presence of labeled BCD:F127 PRTx in the LE/LY compartment. (D) Intensity plot for each stain throughout an image section as indicated by the white arrow in panel (C). Overlap in the localization of peak intensities is shown.

Discussion

Current cyclodextrin therapies for NPC disease have shown to be effective at reducing cholesterol accumulation and extending life span in affected animals (105-107, 196). Unfortunately, alleviation of disease symptoms are not sustained without continuous administration of these therapies (112, 113). This observation can likely be attributed to the appreciable water solubility and relatively low molecular weight of BCD monomers, which allows for rapid renal filtration (114, 115). To address the therapeutic shortcomings of these current therapies, David Thompson's group at Purdue University successfully synthesized a family of BCD: and HPCD:Pluronic PRTx compounds whose high molecular weight should allow for increased circulation time in plasma. Additionally, the non-covalent association of CD with Pluronics-based polyrotaxanes allows for dethreading of the BCD and HPCD units from the polymer chain upon removal of the endcaps. This would specifically allow for delivery of BCD or HPCD monomers at high concentrations to the LE/LY compartment, where they may promote efflux of accumulated cholesterol.

Several characterization studies have provided evidence supporting PRTx formation. Notably, ^{1}H NMR analysis indicated successful endcapping of the Pluronic PRTx with TNBS, allowing for maintenance of 2 to 15 CD monomers on the Pluronic PEG:PPG:PEG chain (**Tables 1 & 2)**.

Analysis of the endcap cleavage and dethreading kinetics of the TNB-capped BCD and HPCD:Pluronic polyrotaxane complexes in a neutral environment (pH 7.4) yielded encouraging results pertaining to the stability of the PRTx under physiological conditions in the blood, prior to endocytosis. The compounds were also found to be quite stable in acidic conditions (pH 5.5),

suggesting that endcap cleavage from the CD:Pluronic PRTx within the LE/LY might be slow. Surprisingly, however, treatment of *npc2*$^{-/-}$ fibroblasts, with the TNB-capped BCD: or HPCD:Pluronic polyrotaxanes produced a substantial and rapid decrease in filipin staining similar to molar equivalents of monomeric BCD or HPCD, respectively (**Figures 5-2 and 5-3**). While the chemical basis of the dethreading mechanism remains unclear, results from the localization studies with the CF labeled BCD:Pluronic PRTx (**Figure 5-4**) suggest that the carbamate linkages used to attach the PRTx endcaps may have been cleaved by enzymatic activation within the LE/LY. Hydrolysis of carbamate linkages has been previously reported and has been used as a method for designing prodrugs (212-214). Thus, carbamate cleavage would allow for dethreading of the CD monomers at high concentration within the LE/LY (**Figure 5-5**), which would subsequently promote efflux of cholesterol from this compartment. Evidence for endcap cleavage and dethreading of CD within the LE/LY, following cellular uptake of the intact compounds, is further supported by the differences observed in relative cholesterol clearance by the various polyrotaxanes; BCD and HPCD:Pluronic PRTx carrying more molecules of CD were found to result in the greatest degrees of intracellular cholesterol clearance as assessed by filipin staining (**Figures 5-2B and 5-3B**).

In effect, if CD dethreading were occurring in the culture media prior to cellular uptake, approximately equal degrees of cholesterol clearance would instead be expected for all Pluronic PRTx compounds, since each treatment would yield 25 μM monomeric BCD in the media.

Taken together, these findings suggests that the polyrotaxanes were internalized to the LE/LY of npc2-/- cells, where carbamate cleavage of the TNB endcaps allowed for release of free CD

Figure 5-5.

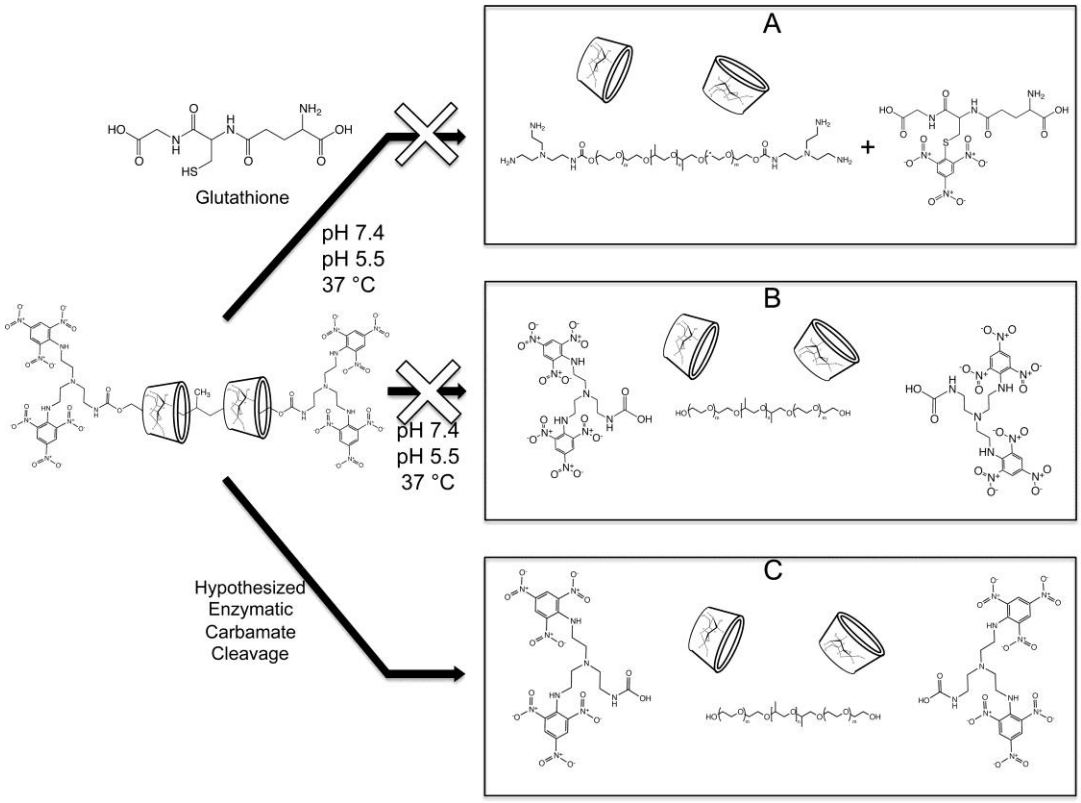


Figure 5-5. Hypothesized and experimentally tested mechanisms for PRTx dethreading. (A) Glutathione incubation with PRTx at acidic and neutral pH and (B) acid catalyzed dethreading at 37 °C showed no signs of dethreading. Hypothesized dethreading (C) by enzymatic cleavage of carbamate linkages yields free BCD in vitro.

(**Figure 5-6**). Work is currently in progress to directly determine the mechanism of PRTx dethreading and subsequent efflux of cholesterol in *npc2*$^{-/-}$ cells. Preliminary results also have demonstrated similar efficacy of the HPCD:Pluronic polyrotaxanes in *npc1*$^{-/-}$ cells. While this indicates that the dethreading mechanism is not unique to NPC2 disease, it also suggests that CD:Pluronic PRTx may be an equally effective therapy in NPC1 and NPC2 disease.

It is worth noting that the levels of CD monomers used in the present studies are within the range of effective concentrations shown by Peake and Vance to clear cholesterol from murine *npc1*$^{-/-}$ neurons and glial cells (215), as well as by us to clear cholesterol from human *npc2*$^{-/-}$ fibroblasts (131). Levels that were demonstrated to cause neuronal cell toxicity, e.g. 10 mM were not used in the present studies (215). In addition, although the fibroblasts were exposed to potentially cytotoxic TNB upon endcap release, no alterations in cell density or viability were observed in any of the cell samples.

Findings from the present studies suggest that these CD:Pluronic polyrotaxanes represent promising therapies for NPC disease. Thus, further investigation with regard to their safety, pharmacokinetics, biodistribution, and durability of response toward cholesterol efflux and/or synthesis suppression in an *in vivo* model of Niemann-Pick C disease is warranted.

Figure 5-6

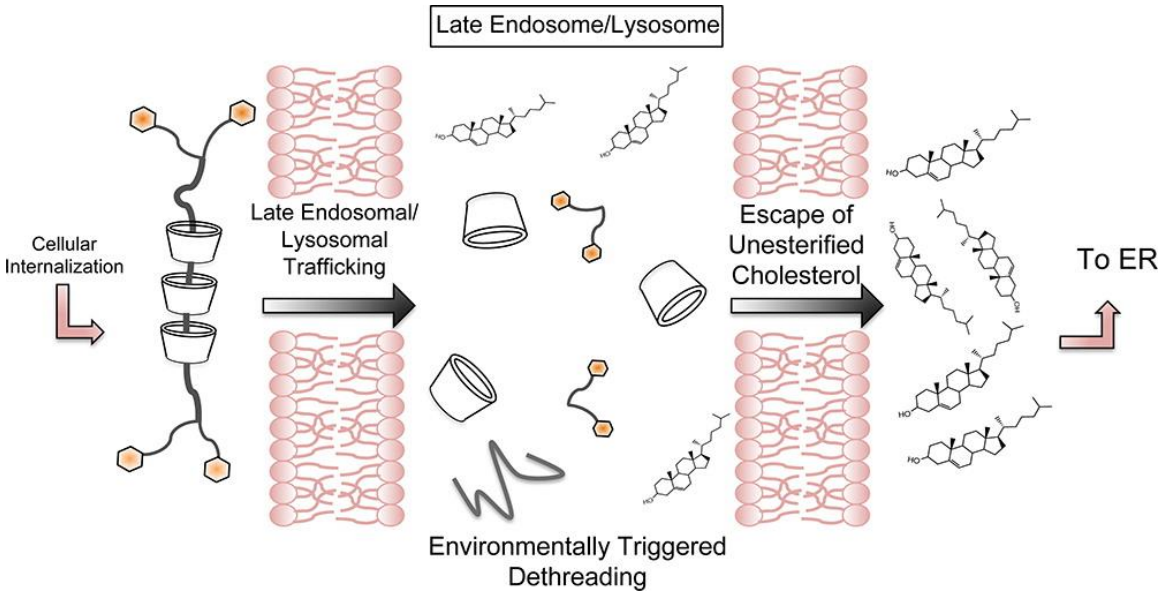


Figure 5-5. Hypothesis for the mechanism of CD:Pluronic PRTx action in NPC disease.

Chapter 6

General conclusions and future directions

All cellular metabolic processes are composed of a number of individual steps that must work in harmony in order to maintain cellular homeostasis and, in general, overall human health. Not all steps of these processes are considered equal, however, with some exhibiting greater degrees of regulation than others. The biosynthesis of cholesterol, for instance, is primarily controlled through regulation of one specific enzyme, HMG CoA reductase (1, 4). This particular enzyme catalyzes the irreversible formation of melavonate, and is the committed, rate limiting step of cellular cholesterol and non-steroid isoprenoid synthesis. There are more than twenty-five additional catalytic reactions required for successful synthesis of cholesterol, yet the enzymes involved in these steps are either not regulated or, as is the case with farnesyl diphosphate synthase and squalene synthase, are regulated to a lesser degree than HMG CoA reductase. It is therefore not surprising that this enzyme is the target of cholesterol lowering drugs, known as statins, for the prevention and treatment of cardiovascular disease. The physiological significance of this enzyme is further demonstrated in studies showing that disruption of the HMG CoA reductase enzyme results in early embryonic lethality (216). Thus, it would seem that physiology has identified perhaps the most critical steps in our biological processes that require the most stringent oversight. .

Another critical facet in the overall scheme of cholesterol metabolism, requiring stringent oversight, is trafficking of the sterol. For instance, atherosclerotic lesions have the potential to form along arterial walls when circulating LDL, rich in cholesterol, becomes elevated. Additionally, when intracellular cholesterol transport mechanisms are perturbed, as in Niemann Pick C (NPC) disease, the sterol accumulates within the late endosomal/lysosomal (LE/LY) compartment of cells, results in adverse physiological effects. Clinically considered to be a

progressive neurological disorder, this particular LSD is known to be caused by defects in either of two lysosomal proteins, NPC1 or NPC2, which are believed to be directly involved with LE/LY cholesterol egress. Research has begun to identify potential mechanisms utilized by these proteins for the normal egress of cholesterol from the LE/LY compartment, however many questions still remain. Similar to HMG CoA reductase, the apparent necessity of these two specific proteins in maintaining cellular cholesterol homeostasis, in addition to a general state of wellbeing, emphasize the need to elucidate the mechanisms by which these two proteins function in cholesterol transport.

Mechanism(s) of cholesterol transport by NPC2

The work presented in Chapters 2 and 3 demonstrates that the soluble lysosomal protein, NPC2, likely functions as a cholesterol transport protein, moving cholesterol between LE/LY membranes of healthy cells. Studies have indicated that the bulk of LDL derived cholesterol partitions to inner membranes of multivesicular bodies within the endo/lysosomal system (16, 147-149), thus the likely first step in cholesterol transport by NPC2 in the LE/LY compartment is extraction of cholesterol from these membranes (**Figure 6-1A**). Kinetic results from our *in vitro* sterol transfer assays suggest that LBPA, exclusively localized at inner LE/LY membranes (16, 20-23), is involved in this mechanism. Based on the results from the protein-lipid binding studies we hypothesize that NPC2 in fact binds to LBPA, and that this interaction facilitates rapid extraction of cholesterol from the inner LE/LY membranes. One question that arises from the present results, when considering the relationship between NPC2 and LBPA, is why aren't the NPC2 mutants whose rates of sterol transfer are restored in the presence of LBPA (Chapter 3), able to reverse sterol accumulation in an NPC2 deficient cell model where LBPA is present

(Chapter 2)? One potential answer to this question is that LBPA may be limiting in NPC disease, as has been suggested by studies demonstrating that ability of exogenous supplementation of LBPA to reverse the cholesterol accumulation phenotype in NPC1-deficient cells (153). LBPA levels have been shown to increase in some tissues in NPC disease, perhaps as a compensatory response to the aberrant accumulation of cholesterol, though the change is modest (105, 150, 151) and perhaps does not increase to the degree necessary for efficient LE/LY cholesterol efflux. One approach that may be taken in order to determine whether LBPA is necessary for NPC2-mediated LE/LY cholesterol efflux in NPC disease is to repeat the exogenous LBPA supplementation studies that were done using NPC1-deficient cells, in an NPC2-deficient cell model. If NPC2 is in fact driving the egress of cholesterol from the LE/LY compartment, and this transport is dependent upon LBPA, then exogenous LBPA should have no effect on the cholesterol accumulation phenotype in an NPC2-null model. If the intracellular cholesterol accumulation were observed to decrease in *npc2*$^{-/-}$ fibroblasts following administration of exogenous LBPA, then it may be concluded that an LBPA-dependent mechanism of LE/LY cholesterol efflux, independent of both NPC1 and NPC2, exists within this compartment. Collaborative studies are currently ongoing with Jean Gruenberg's group (Geneva Switzerland) to aid in addressing these questions.

Following extraction of cholesterol from inner LE/LY membranes, results from our sterol transport studies in Chapter 2 suggest that NPC2 is able to transport this cholesterol to the limiting membrane, which the sterol must traverse in order to reach the ER, for maintenance of cellular cholesterol homeostasis. The mechanism by which cholesterol actually exits the LE/LY compartment remains unknown, however. A number of proposals for this final step of

cholesterol egress have been suggested, including a direct handoff of cholesterol from NPC2 to the N-terminal domain (NTD) of NPC1 (**Figure 6-1B**). Brown and Goldstein's group, for instance, showed that *in vitro* cholesterol transfer from the cholesterol binding N-terminal domain (NTD) of NPC1 to liposomes was enhanced in the presence of NPC2 protein (87). In later studies, the group also demonstrated that cholesterol could be transferred *in vitro* from holo-NPC2 to the NPC1 NTD, and that this transfer was dependent upon three residues on the surface of NPC2 (88). While these two studies strongly suggest the presence of a direct cholesterol transfer mechanism between NPC2 and NPC1, neither demonstrated the existence of protein-protein interactions.

To fully elucidate the mechanism of LE/LY cholesterol transport and efflux by NPC1 and NPC2, it is necessary to determine whether these direct interactions do indeed exist. One approach that may be taken to address this question is to use sterol transfer kinetics, as employed to determine the mechanism of sterol transport between NPC2 and membranes (29, 30) as well as between NPC2 and cyclodextrin (Chapter 4). Briefly, by determining the rates of sterol transfer from a fixed concentration of holo-NPC2 to increasing concentrations of purified NPC1 NTD, it is possible to determine the mechanism of sterol transport between the two proteins. Indeed, as detailed previously, if protein-protein interactions are integral to the transfer of sterol from NPC2 to NPC1 NTD, the rate of transfer will increase as the concentration of NPC1 NTD increases, since the rate of lipid transfer for a collisional mechanism is directly proportional to the product of the donor and acceptor concentrations (82, 83). In contrast, the rate limiting step in a diffusional transfer mechanism is dissociation of the lipid from the donor, in this case NPC2, which is unaffected by the concentration of types of acceptor (82, 83). Thus, no change in

the rate of sterol transfer would be observed as the concentration of NPC1 NTD increases, if the two proteins do not in fact directly interact.

Another possible mechanism by which NPC2 may facilitate egress of cholesterol out of the LE/LY compartment is by directly depositing cholesterol into the inner leaflet of the limiting lysosomal membrane (**Figure 6-1B**), instead of directly delivering it to NPC1. Following this transfer, there are a couple of different ways in which the cholesterol may then be trafficked out of the compartment. The first involves lateral diffusion of the sterol in the plane of the limiting lysosomal membrane and subsequent binding and efflux by NPC1. Studies conducted in our laboratory, demonstrating that NPC2 can interact with membranes to directly and rapidly deliver cholesterol (29, 30), as well as by Sandhoff and colleagues, showing that NPC2 strongly interacts with membranes (193), lend support to this membrane-mediated mechanism. Additionally, the NPC2 mutants originally identified by Ko et al as being unable to reverse sterol accumulation in *npc2*$^{-/-}$ fibroblasts despite normal cholesterol binding (81), were not identified by Brown and Goldstein's group as being important for the supposed 'handoff' between NPC2 and NPC1 (88). Thus, other domains on the surface of NPC2 are likely essentially for some additional functionality beyond the putative transfer of cholesterol to NPC1.

The second manner in which cholesterol may exit the LE/LY compartment following deposition of the sterol into the limiting LE/LY membrane by NPC2, is via an NPC1-indpenedent pathway. Indeed, recent studies have indicated that transport of cholesterol from the LE/LY compartment to mitochondria is dependent upon NPC2, but is independent of NPC1 (139). The LBPA supplementation studies conducted by Gruenberg's group have also indicated the existence of

an NPC1-independent pathway of LE/LY cholesterol egress, though the dependence upon NPC2 was not determined (153). Other recent studies have further suggested that, in general, NPC2 and NPC1 function independently in the egress of lysosomal cargo (217). It is therefore possible that NPC2 utilizes two different mechanism of trafficking for egress of cholesterol from the LE/LY compartment, one that is dependent upon NPC1 and one that is NPC1-independent. It is possible that an NPC2 to membrane transfer mechanism underlies the NPC1-independent actions of NPC2 (139, 217), while cholesterol transport to the ER requires not only NPC2 but NPC1 as well (87, 88). Results from LBPA supplementation studies in an NPC2-deficient cell model, currently underway, will lend further insight into these potential mechanisms of NPC2 mediated LE/LY cholesterol efflux.

LE/LY cholesterol trafficking at membrane contact sites

In addition to trafficking cholesterol from inner membranes to the limiting membrane, our vesicle-vesicle interaction studies suggest that NPC2 may also function at membrane contact sites (MCS) within the LE/LY compartment to facilitate rapid transport of cholesterol. LE/LY architecture involves many zones of close apposition between membranes (14, 15, 17), allowing for these MCSs to form between inner lysosomal membranes, potentially by directly binding to LBPA, as well as between inner LE/LY membranes and the limiting lysosomal membrane (**Figure 6-1C**). Interestingly, many lipid transport proteins have been found to be targeted to MCSs, including the phosphatidylinositol transporter protein (PITP), the ceramide transporter (CERT) and several of the oxysterol-binding-protein related proteins (ORP) (218), indicating that this is a plausible mechanism for inner-lysosomal cholesterol transport by NPC2. The current data supporting this hypothesized mechanism of cholesterol transport by NPC2, however, was

Figure 6-1

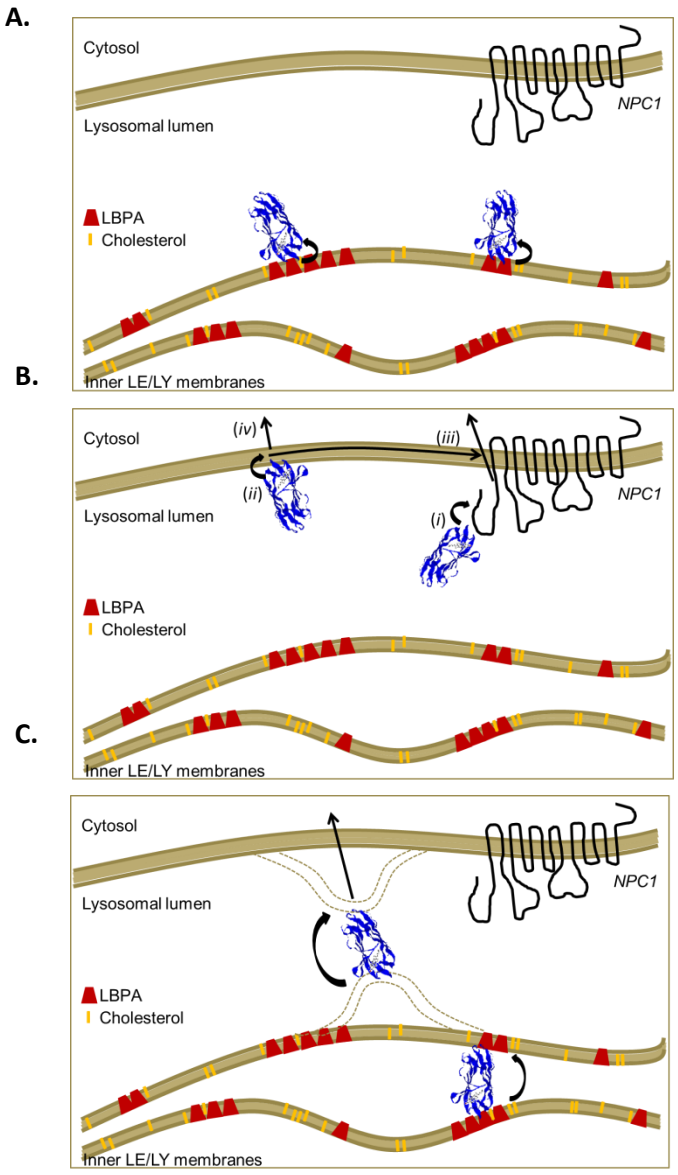


Figure 6-1. Possible mechanism(s) of LE/LY cholesterol transport by NPC2. (A) NPC2 directly interacts with LBPA-rich inner LE/LY membranes to extract cholesterol. This membrane to protein transfer may involve direct interactions between NPC2 and LBPA. (B) NPC2 transports cholesterol to the limiting LE/LY membrane, where it may (*i*) transfer the sterol directly to the NTD of NPC1, or (*ii*) deposit it into the limiting LE/LY membrane. The latter possibility may subsequently involve (*iii*) rapid lateral diffusion of cholesterol in the plane of the membrane to NPC1, which may facilitate efflux via an unknown mechanism. Conversely, egress of cholesterol may occur (*iv*) in an NPC1-independent manner. (C) NPC2 may also facilitate sterol transfer at membrane contact sites between inner LE/LY membrane lamellae and/or between the inner and outer LE/LY membranes.

obtained via *in vitro* analyses. It is thus essential to positively identify the formation of inner-LE/LY MCSs *in vivo*. The most basic methods for achieving this goal would likely include microscopic techniques. Fluorescence resonance energy transfer (FRET), for instance, in addition to real time imaging may allow us to observe the localization of NPC2 in spatial relation to inner-LE/LY membranes and the limiting LE/LY membrane. Based on the hypothesis that LBPA may be involved in the formation of the MCSs by NPC2, interaction studies, such as cross linking, could be employed to identify the LE/LY members integral to the formation of these cholesterol transfer sites. As well, immuno-electron microscopic localization of NPC2 and LBPA, using secondary antibodies with differently sized gold particles for the protein and the lipid, is a reasonable approach. It is also possible, based on the studies conducted by Deffeiu et al (93), that NPC2 binds to NPC1, possibly at its second luminal domain, as a means of forming membrane contact sites between inner- and the limiting-LE/LY membranes. Thus, fluorescent based crosslinking studies could also be conducted for microscopic analysis.

In addition to fully elucidating the mechanisms by which NPC2 and NPC1 function in the normal trafficking and efflux of LDL-derived cholesterol in the LE/LY compartment, future studies should be directed at determining the final step of cholesterol egress from the LE/LY compartment i.e. leaving the limiting membrane of the LE/LY. Current thought is that NPC1 mediates the transport of cholesterol across the limiting LE/LY membrane, however the function of this protein in cholesterol transport, including whether or not the protein binds cholesterol in its NTD and/or sterol sensing domain *in vivo*, is unknown. Elucidation of this mechanism has the potential to not only increase our understanding inner-lysosomal cholesterol trafficking by NPC2

and NPC1, i.e. must cholesterol be in the limiting membrane or bound to NPC1 for normal egress, but also aid in the development of therapies for NPC disease.

Inter-organelle membrane contact sites

We have hypothesized that NPC2 may function to rapidly transport cholesterol, in part, by functioning at membrane contact sites within the LE/LY compartment. Most of the MCSs that have been identified, however, occur not *within* a single organelle, but *between* two organelles, such as the ER and the plasma membrane. Thus, one mechanism of LE/LY cholesterol efflux that should be considered is via the formation of MCSs between the LE/LY compartment and other organelles, including the ER, plasma membrane and mitochondria. Studies have indeed demonstrated existence of ER-late endosome and ER-lysosome contact sites in yeast, though the function of these MCSs is not completely understood. Interestingly, however, ORPs like the yeast Osh4p sterol transport protein, involved in the formation of contact sites between the ER and vacuoles (yeast lysosome), have been shown to exchange sterols for phosphatidylinositol 4-phospate (PI_4P) between membranes (219), suggesting that Osh4p may transfer sterol between these two organelles. In mammalian cells, ORP1L has been shown to be involved in contact sites between the ER and LE/LYs, whose formation is controlled by the sensing of LE/LY cholesterol levels by the ORP1L protein (220). Additionally, Alpy et al recently reported that two proteins anchored to the LE/LY limiting membrane, STARD3 [steroidogenic acute regulatory protein (StAR) related lipid transfer (START) domain-3], also known as MLN64, and STARD3 N-terminal like (STARD3NL), also form membrane contact sites between the LE/LY and the ER (221). STARD3/MLN64 specifically binds cholesterol (222), and is thought to be involved in the intracellular trafficking of the sterol (223, 224). The role this protein plays in the post-LE/LY

transport of cholesterol to the ER remains unknown, however, and the necessity of STARD3/MLN64 in intracellular cholesterol trafficking *in vivo* has been challenged (225).

The formation of this MCS does suggest that lysosomal cargo is being transported to the ER, and it would be important to know whether, one, cholesterol is the substrate actively transported at the MCS and two, whether STARD3/MLN64 is indeed the transport protein necessary for this transfer. Future studies may also need to consider identifying other lipid transport proteins that may function at this MCS, which are necessary for normal intracellular trafficking of cholesterol from the LE/LY to the ER.

Lipid transport proteins that function at membrane contact sites often contain various combinations of surface domains that function to target the protein to specific cellular compartments. For instance, proteins expressing a conserved diphenylalanine-in-an-acidic-tract (FFAT) motif are targeted to the ER, where they bind directly to the integral membrane protein, vesicle-associated-membrane-protein-associated-protein (VAP) (134, 226, 227). Most of the ORPs identified in mammals contain this FFAT motif, as do some proteins containing a START domain, including the aforementioned STARD3/MLN64 and StAR (221, 227). As previously stated, STARD3/MLN64, a four-transmembrane domain protein located in the limiting membrane of LE/LYs, has recently been shown to be involved in the formation of membrane contact sites between the LE/LY and the ER. This protein has also been shown to be involved in intracellular trafficking of cholesterol from the LE/LY compartment to mitochondria (223, 224). Based on the fact that STARD3/MLN64 is anchored to the LE/LY compartment, it has been hypothesized that STARD3/MLN64-mediated cholesterol transfer to the mitochondria occurs at

membrane contact sites. Unfortunately, initial attempts at identifying the mitochondrial protein integral in the formation of these supposed MCSs have been unsuccessful (223).

The presence of this transfer mechanism is important in understanding the overall process of intracellular trafficking, however, and therefore warrants further investigation. Future studies may approach this problem by disrupting resident membrane-bound mitochondrial proteins and assessing the ability for healthy, NPC1-deficient and NPC2-deficient cell models to transport cholesterol. Studies could also focus on identifying specific domains on the cytosolic C-terminal of the MLN64 protein that may bind to a mitochondrial protein, using classical protein-protein interaction and mutagenesis techniques, especially since mitochondrial targeting motifs have yet to be identified. It is also possible that STARD3/MLN64, similar to NPC2, directly interacts with the outer mitochondrial membrane, and not a membrane protein, to facilitate transfer of cholesterol from the LE/LY, and future studies may need to address this possibility. Although STARD3/MLN64 has been the focus of this discussion, similar approaches may be taken to determine whether other lipid transport proteins, which are either present in the LE/LY limiting membrane or are targeted to this organelle, play a role in intracellular cholesterol transport through the formation of MCSs. Initially approaches at identifying these MCSs may employ an array of microscopic techniques, including fluorescence resonance energy transfer (FRET) and real time imaging, to look at the localization of LE/LYs in relation to the ER in cells. Furthermore, interaction studies, such as cross linking followed by co-immunoprecipitation, could be conducted as a means of detecting MCSs formed by suspected proteins.

Another possibility is that the final step of cholesterol egress form the LE/LY compartment may simply employ a soluble cytosolic lipid transport protein which binds cholesterol. This hypothetical protein could function by extracting cholesterol from the outer leaflet of the LE/LY limiting membrane, or could also receive cholesterol from NPC1. The later conjecture would of course be dependent upon the ability for native NPC1 to indeed bind and transport cholesterol across the limiting LE/LY membrane, which has not been concretely supported. Similar to what was proposed for detecting formation of MCSs, initial approaches at identifying this putative cytosolic lipid transport protein could involve a variety of imaging techniques such as real time FRET based analyses. Whatever the mechanism, elucidation of these processes will increase our understanding of LE/LY cholesterol transport by NPC2 and NPC1 and, furthermore, our understanding of the overall process of intracellular cholesterol trafficking.

This information could additionally aid in elucidating the mechanism(s) by which cyclodextrin, currently utilized therapeutically, promotes efflux of cholesterol from the LE/LY compartment. A more complete understanding of the actions of cyclodextrin in efflux of LE/LY cargo may allow for potential optimization of this mode of treatment in NPC disease as well as inform its possible use in other LSDs. Beyond aiding in primary generation of therapies for NPC disease, further insight into the mechanisms by which NPC2, NPC1 and, perhaps, other as yet unidentified proteins transport cholesterol within and out of the LE/LY compartment may be helpful in the development of therapies for other LSDs that display secondary accumulation of cholesterol, such as Farber disease. Recent studies have in fact indicated that the primary accumulation of ceramide, caused by deficiencies in acid ceramidase, can be reduced in a cellular model of Farber disease by targeting the secondary accumulation of cholesterol with CD treatment (228).

Indeed, therapies targeted at reducing secondary substrate accumulation can be highly useful in managing some pathophysiological symptoms, improving quality of life and extending life span. In NPC disease patients, for example, Miglustat is used to ameliorate some of the neuropathalogical features of the disease. This substrate reduction therapy, originally developed for treatment of patients with type 1 Gaucher disease, is used to reduce ganglioside levels via inhibition of glucosylceramide synthase, and does not target cholesterol. NPC disease may be characterized by the primary accumulation of cholesterol in the LE/LY compartment of cells, including neurons, but gangliosides and glycosphingolipids significantly accumulate in the brains of NPC patients (64) and contribute to the disease pathology. Thus, use of a non-cholesterol targeted therapy in NPC disease is warranted.

Cyclodextrin therapy for NPC disease

In developing therapies for rare diseases like LSDs, it is important for the scientific community to look towards agents that have received prior approval by the FDA for use in other conditions, with efficacy then established in an animal or cellular model of the rare disease. The process of gaining approval for use of these agents in a rare disease is streamlined compared to development of new therapeutic compounds because the former are considered candidates for repurposing and require only studies in determining formulation, effective dose and disease-specific toxicology. Cyclodextrin is one such example of a therapeutic that was able to be repurposed and is currently approved for use in NPC patients, via subcutaneous administration. Unfortunately the therapeutic efficacy of CD is limited due to its exceedingly short half-life in plasma (107, 113-115, 194), which prompted David Thompson's laboratory at Purdue University to develop a CD polymer construct with characteristics able to overcome the observed

deficiencies in the current treatment. The basic formulation of these so called pluronic polyrotaxanes (PRTx) have received prior approval by the FDA for use as drug delivery vehicles in humans, thus the newly developed β-cyclodextrin (BCD) PRTx compounds would face the same, limited requirements for gaining FDA approval for use in NPC patients as did the original CD treatment.

Our collaborative work with the Thompson group demonstrated the potential therapeutic efficacy of these novel CD compounds in a cell model of NPC disease. As shown in Chapter 5, administration of both libraries of the developed compounds to NPC2 deficient fibroblasts resulted in reversal of intracellular cholesterol accumulation at a rate similar to the monomeric BCD or HPCD. While these *in vitro* results are encouraging, what they do not address is whether the compounds are able to cross the blood-brain barrier (BBB), which is a critical issue given the primary neurodegenerative aspects of NPC disease. The ability for monomeric CD to pass the BBB has been controversial, though Dr. Daniel Ory has found in his studies that 0.1% of injected HPCD is able to cross this barrier, which is similar to amount reported for albumin (personal communication). The circulation times of the CD-based polyrotaxanes are predicted to be markedly increased *in vivo*, and if the extent to which these compounds cross the BBB is similar to monomeric HPCD, these compounds could be therapeutically beneficial in alleviating the neuropathology of NPC disease. Thus, the *in vivo* efficacy of the compounds must be demonstrated in models of NPC disease prior to gaining FDA approval for use in patients, which presents the next experimental stage of this project.

Glycosphingolipids, phospholipids and cholesterol are the most ubiquitous secondary substrates found to accumulate in LSDs, with variable storage patterns in the brain and visceral organs often exhibited in the same disease (41). An important issue that arises in many LSDs is the coordination cholesterol metabolism with the metabolism of sphingomyelin and glycolipids. Unesterified cholesterol accumulation, for instance, secondarily affects sphingomyelin metabolism (41, 229) while cholesterol homeostasis has conversely been shown to be adversely affected secondary to sphingolipids accumulation (230, 231). Thus, while the novel cyclodextrin polymers constructed by the Thompson group are primarily directed toward treatment of patients with NPC disease, the application of these polymers has the potential to be far reaching in the realm of LSDs, as many exhibit secondary cholesterol accumulation.

Concluding remarks

The studies presented here have shed much light on the processes involved in endo/lysosomal cholesterol trafficking, though much work is left to be done in order to fully understand the numerous steps involved in intracellular cholesterol transport. These studies have additionally provided insight into the mechanisms by which current cyclodextrin therapies ameliorate NPC disease progression, and have provided evidence for the therapeutic potential of a novel class of cyclodextrin polymers. Overall, this work has increased our understanding into how to enhance and/or complement NPC functions in affected patients, and may prove useful in the understanding of secondary cholesterol accumulation in other LSDs.

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