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Sterol Transfer between Cyclodextrin and Membranes: Similar but Not Identical Mechanism to NPC2-Mediated Cholesterol Transfer

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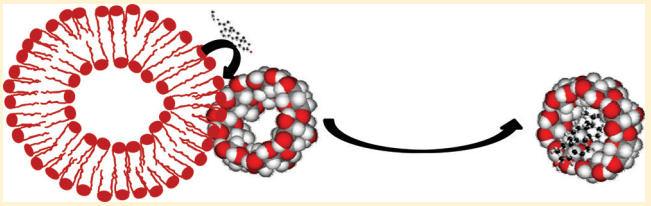
ABSTRACT: Niemann−Pick C disease is an inherited disorder in which cholesterol and other lipids accumulate in the late endosomal/lysosomal compartment. Recently, cyclodextrins (CD) have been shown to reduce symptoms and extend lifespan in animal models of the disease. In the present studies we examined 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.



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.[1−3] 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.[4,5] 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.[6,7] HPCD was shown to have lower toxicity compared with MBCD and to have greater specificity for cholesterol and triacylglycerol.[5,8]

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.[9,10] 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.[10] Remarkably, the CD-treated mice also showed diminished neuropathology and >40% extension of lifespan over untreated controls.[11] Similar benefits were found in studies using chronic administration of HPCD.[12] 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.[12]

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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.[13] 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.[11] While specific interactions between CD and NPC2 have not been reported, interactions of CD with a number of other proteins have been observed,[14] 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).[15,16] 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,[6,17] its mechanism of action is not fully understood.[14] 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 BMG), 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.[18] 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 buffer 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 protein was purified from transfected Chinese Hamster Ovary cell media as previously described,[15] using a 5 kDa cutoff flow filtration membrane to concentrate media (CDUF002 LC, Millipore, Bedford, MA).

Membrane Vesicle Preparation. Small unilamellar vesicles were prepared by sonication and ultracentrifugation as described.[19] 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.[19]

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.[15] For all transfer assays, CD was mixed with hNPC2 using a stopped-flow mixing chamber interfaced with a spectrofluorimeter 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.[20] 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.[16] 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 Schultz et al.[21]

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.[22] 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.[16,23,24] 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:[24]

$$[\mathrm{DHE}]_{\mathrm{CD}}/[\mathrm{DHE}]\mathrm{SUV} = (1/K_{\mathrm{CD}}^{\mathrm{SUV}})[\mathrm{HPCD}]/[\mathrm{SUV}]$$

$K_{\mathrm{CD}}^{\mathrm{SUV}}$ is the partition coefficient of DHE between the membranes and HPCD. From the results in Figure 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.[24] Considering that MBCD was found to have a greater efficiency in accepting cholesterol than HPCD,[6,7] the somewhat higher partition coefficient obtained in the present study is reasonable.

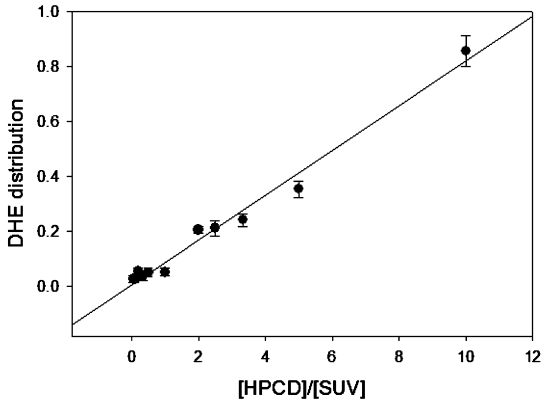

Figure 1. Determination of DHE partition between HPCD and phospholipid membranes. 0–200 μM EPC/dansyl-PE (97:3 mol %) SUV were added to 10 μM HPCD/DHE complex, and the distribution of DHE between HPCD and membranes was calculated from the DHE fluorescence, as described in Materials and Methods. Excitation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

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 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 phospholipid membranes, which can range up to 10 s^{-1}.[15,16] 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

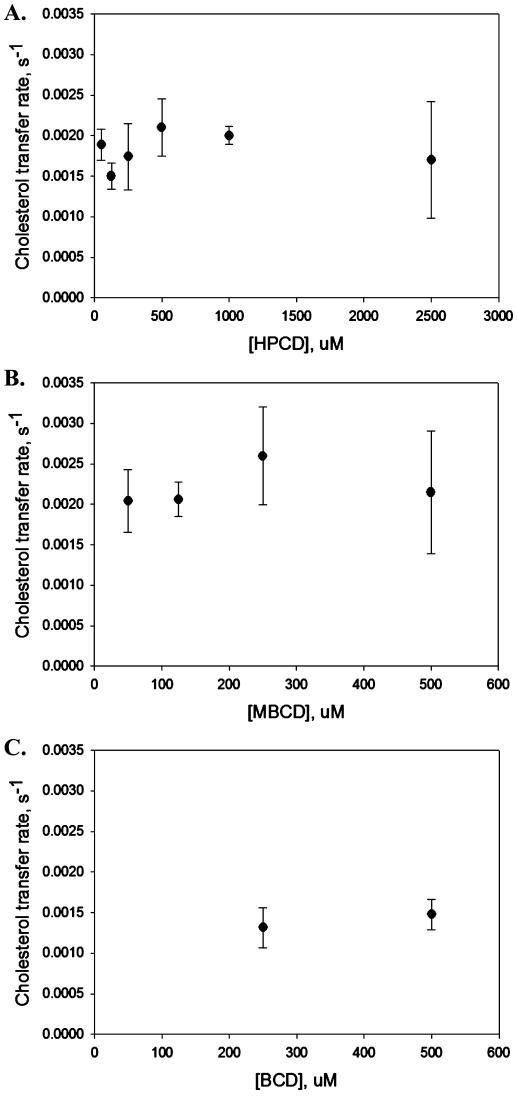

Figure 2. Transfer of cholesterol from hNPC2 to cyclodextrin. Transfer rates of cholesterol from 1 μM hNPC2 to 50−2500 μM (A) HPCD, (B) MBCD, and (C) BCD. All experiments were at 25 °C and pH 5.0, as described in Materials and Methods.

from hNPC2 to MBCD and BCD (Figure 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.[15,16] We therefore examined the transfer of DHE from model phospholipid membranes to CD. Figure 3 shows the rates of DHE transfer from DHE-containing SUVs to increasing concentrations of HPCD. Transfer rates were found

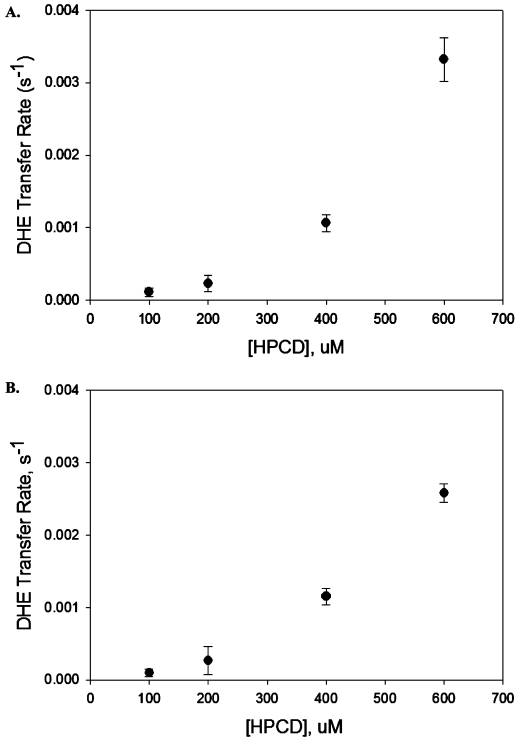

Figure 3. Transfer of DHE from phospholipid membranes to HPCD. Transfer rates of DHE from 10 μM (A) DHE:dansyl-PE:EPC (25:3:72 mol %) or (B) DHE:dansyl-PE:LBPA:EPC (25:3:25:47 mol %) donor SUVs to increasing concentrations of HPCD. All experiments were conducted at 25 °C at pH 5.0 using an excitation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

to increase proportionally with acceptor HPCD concentration. Inclusion of LBPA in the donor membranes had no effect on rates of DHE transfer to HPCD (Figure 3B).

We also examined DHE transfer from HPCD to increasing concentrations of membranes. Figure 4A shows the results obtained when a constant concentration of HPCD/DHE complex was mixed with increasing concentrations of zwitterionic EPC SUVs. A proportional increase in transfer rate with EPC SUV concentration was observed, and virtually identical rates of DHE transfer were found when LBPA was incorporated into the membranes (Figure 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.[15,16]

Intermembrane Transfer. The spontaneous intermembrane transfer rate of DHE is very slow.[16] However, we showed that addition of NPC2 enhances the intermembrane transfer rate of sterols by 40 to almost 300-fold,[16] providing a mechanism by which NPC2 could function to prevent cholesterol accumulation in lysosomes. Figure 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

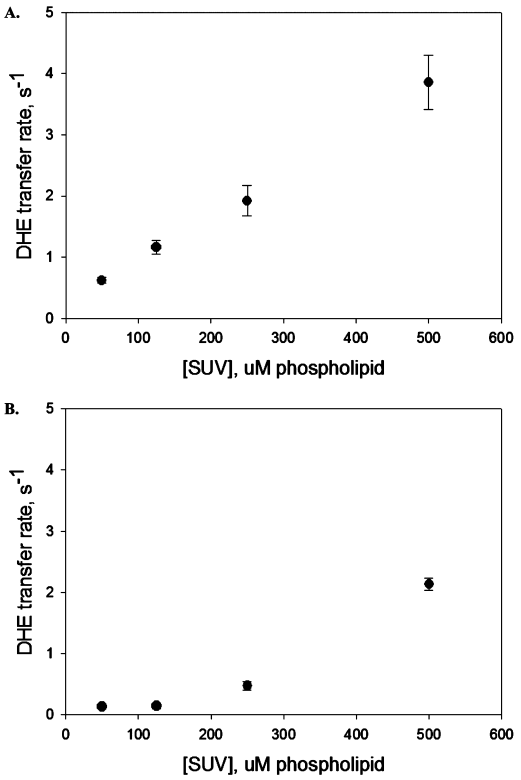

Figure 4. Transfer of DHE from HPCD to phospholipid membranes. Transfer rates of DHE from 5 μM HPCD/DHE(8:1) complex to acceptor membranes containing (A) 3 mol % dansyl-PE and 97 mol % EPC or (B) 3 mol % dansyl-PE, 72 mol % EPC, and 25 mol % LBPA. All experiments were conducted at 25 °C at pH 5.0 using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

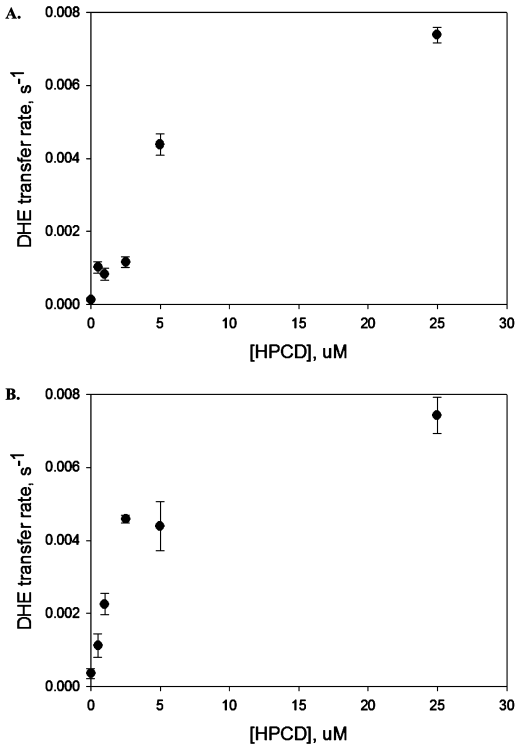

Figure 5. Effect of cyclodextrin on DHE intermembrane transfer. Transfer rates of DHE from 50 μM donor SUV composed of (A) DHE:EPC (25:75 mol %) or (B) DHE:LBPA:EPC (25:25:50 mol %) to 250 μM acceptor SUV (EPC:dansyl-PE = 97:3 mol %) in the presence of 0–25 μM HPCD. All experiments were conducted at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

(Figure 5B). 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.

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.[16] 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 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 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.[21] 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 of fuse, the large size is easily detected by light scattering as increased absorbance at 350 nm. Figure 7 shows that, similar to hNPC2, HPCD, but not the control protein albumin, causes an increase in the A_{350} of EPC LUVs over time, indicating that membrane–membrane interactions are occurring.

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.[9,10,25] It was suggested that a possible mechanism of action of CD was via specific interaction with NPC2.[11] 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.[26] Thus, the results strongly suggest

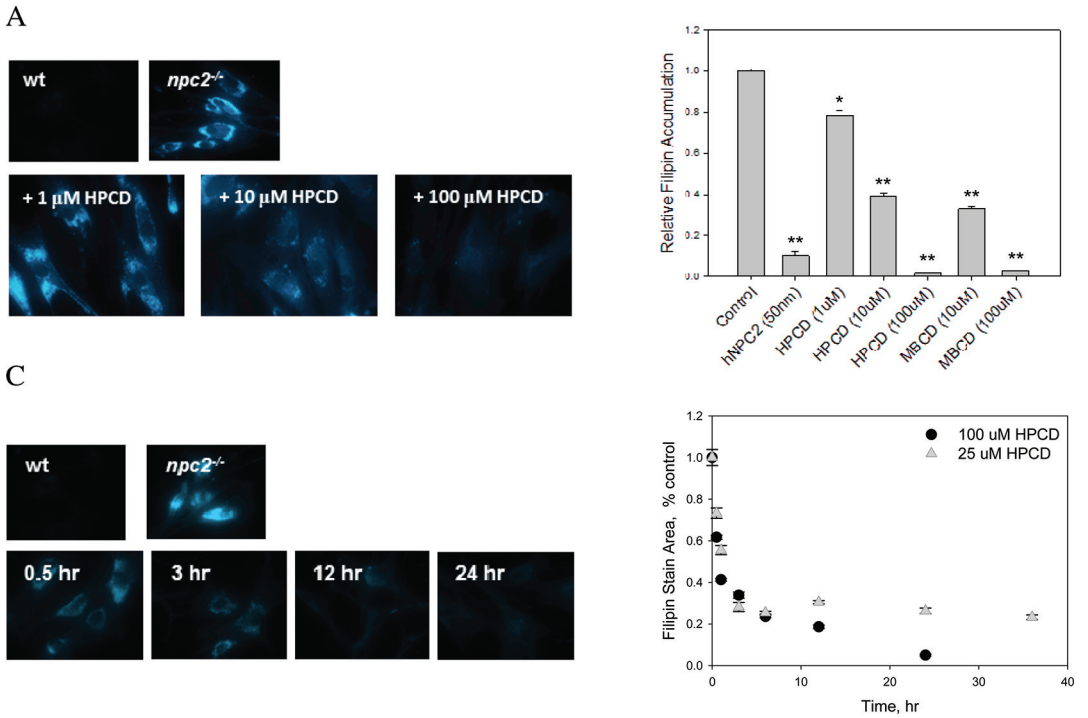

Figure 6. Filipin accumulation in NPC2-deficient fibroblasts treated with cyclodextrin. Human NPC2-deficient fibroblasts were incubated with cyclodextrin and were fixed and stained with 0.05 mg/mL filipin at varying time points. Filipin accumulation was determined as the ratio of filipin stain area to 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-deficient fibroblasts incubated with increasing concentrations of HPCD for 3 days. (B) Relative filipin accumulation in NPC2-deficient cells treated with varying concentrations of HPCD, MBCD, or 50 nm hNPC2 for 3 days. (C) Representative images of filipin depletion over time in NPC2-deficient fibroblasts treated with 100 μM HPCD. (D) Depletion of the relative filipin accumulation in NPC2-deficient fibroblasts treated with 25 or 100 μM HPCD.

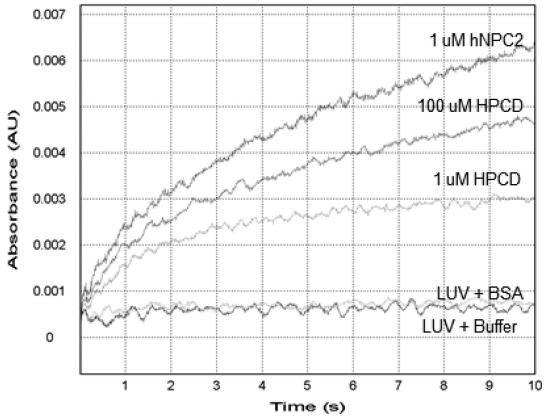

Figure 7. NPC2 and CD promote membrane−membrane interactions. Time-dependent changes in the absorbance of 50 μM LUVs mixed with 1 μM of bovine serum albumin (BSA), hNPC2, and 1 or 100 μM HPCD were observed at 350 nm using an SX20 stopped-flow spectrofluorimeter.

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,[12] 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.[27] 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.[13] It was also recently proposed that CD may stimulate relocation of cholesterol laden LE/LYs to the plasma membrane followed by exocytosis.[28] The ability of CD to solubilize cholesterol and to both deliver and extract cholesterol from cellular membranes[6,7,17] 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.[19,23,29−32] 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.[19,23,29−32] 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.[15,16,33] 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.[16]

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.[7] Additionally, differential scanning calorimetry and atomic force microscopy studies have demonstrated directly that the β-CDs are membrane interactive.[34,35]

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.[13] It is therefore likely that CD is internalized by bulk-phase endocytosis,[13] 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-fold 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;[36] 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.[37] used a steady-state vesicle pulldown 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.[21,38] 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.[21] 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.[a]

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.[39] 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.

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ABBREVIATIONS

NPC, Niemann−Pick Type C; CD, cyclodextrin; BCD, β-cyclodextrin; MBCD, methyl-β-cyclodextrin; HPCD, hydroxypropyl-β-cyclodextrin; LBPA, lysobisphosphatidic acid; LE/LY, late endosome/lysosome.

■ ADDITIONAL NOTE

[a]Xu, McCauliff, and Storch, unpublished observations.

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