The Transport of Low Density Lipoprotein-derived Cholesterol to the Plasma Membrane Is Defective in NPC1 Cells*

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Niemann-Pick disease type C (NPC) is characterized by lysosomal storage of cholesterol and gangliosides, which results from defects in intracellular lipid trafficking. Most studies of NPC1 have focused on its role in intracellular cholesterol movement. Our hypothesis is that NPC1 facilitates the egress of cholesterol from late endosomes, which are where active NPC1 is located. When NPC1 is defective, cholesterol does not exit late endosomes; instead, it is carried along to lysosomal storage bodies, where it accumulates. In this study, we addressed whether cholesterol is transported from endosomes to the plasma membrane before reaching NPC1-containing late endosomes. Our study was conducted in Chinese hamster ovary cell lines that display the classical NPC biochemical phenotype and belong to the NPC1 complementation group. We used three approaches to test whether low density lipoprotein (LDL)-derived cholesterol en route to NPC1-containing organelles passes through the plasma membrane. First, we used cyclodextrins to measure the arrival of LDL cholesterol at the plasma membrane and found that the arrival of LDL cholesterol in a cyclodextrin-accessible pool was significantly delayed in NPC1 cells. Second, the movement of LDL cholesterol to NPC1-containing late endosomes was assessed and found to be normal in Chinese hamster ovary mutant 3-6, which exhibits defective movement of plasma membrane cholesterol to intracellular membranes. Third, we examined the movement of plasma membrane cholesterol to the endoplasmic reticulum and found that this pathway is intact in NPC1 cells, i.e. it does not pass through NPC1-containing late endosomes. Our data suggest that in NPC1 cells LDL cholesterol traffics directly through endosomes to lysosomes, bypassing the plasma membrane, and is trapped there because of dysfunctional NPC1.

Niemann-Pick disease type C (NPC) is an autosomal recessive lysosomal storage disease that is caused by mutations in either the NPC1 or NPC2 genes and leads to progressive neurodegeneration and premature death (1). Fibroblasts from NPC1 patients exhibit massive storage of unesterified cholesterol and gangliosides in lysosomes, which results from defects in intracellular lipid trafficking (reviewed in Refs. 2 and 3). Most studies of NPC1 have focused on its role in intracellular cholesterol movement. In normal cells, low density lipoproteins (LDL) are internalized and transported through the endocytic pathway where the cholesteryl esters are hydrolyzed. The cholesterol that is released is transported to the plasma membrane and endoplasmic reticulum (4). Our working hypothesis has been that NPC1 facilitates the egress of free cholesterol from late endosomes, which are the location of functional NPC1 (5, 6). When NPC1 is defective, the free cholesterol does not exit the late endosomes; instead, it is carried along the endocytic pathway to lysosomal storage bodies where it accumulates along with mutant NPC1 protein.

In this study, we addressed whether cholesterol released by hydrolysis of LDL-derived cholesteryl linolate (CL) is transported to the plasma membrane in its transit from early endosomes to NPC1-containing late endosomes and lysosomes. Our work was prompted by two recent studies. First, Cruz et al. (7) reported that LDL cholesterol is transported to the plasma membrane independently of NPC1. On reaching the plasma membrane, LDL-derived cholesterol is proposed to be specifically reinternalized and delivered to a compartment that is functionally distinct from lysosomes and contains NPC1. Cholesterol is then mobilized to other cellular destinations such as the plasma membrane and endoplasmic reticulum. It is the movement of cholesterol from these storage vesicles that is proposed to be defective in NPC1 cells (7–9). Second, Lange et al. (9, 10) have reported that the rate of cholesterol movement from lysosomes to the plasma membrane in NPC cells is equal to or greater than that in normal cells.

Our study was conducted in Chinese hamster ovary (CHO) cell lines that display the classical NPC biochemical phenotype (11) and belong to the same complementation group as NPC1 fibroblasts and CT60, a CHO cell mutant isolated previously (12, 13). Mutations in the NPC1 gene were defined in two mutants from this complementation class.

We used three approaches to test our hypothesis that LDL-derived cholesterol en route to NPC1-containing late endosomes does not pass through the plasma membrane. First, we used cyclodextrins (CDs) to measure the arrival of LDL cholesterol at the plasma membrane and found that the arrival of LDL cholesterol in a CD-accessible pool was significantly delayed in NPC1 cells. Second, the movement of LDL cholesterol to late endosomes and lysosomes was assessed and found to be normal in CHO mutant 3-6, which exhibits defective movement of plasma membrane cholesterol to intracellular membranes (14). Third, we examined the movement of plasma membrane cholesterol to the endoplasmic reticulum and found that this pathway is intact in NPC1 cells, i.e. passage through NPC1-
containing organelles is not obligatory for this pathway. Together our data suggest that in NPC1 mutant cells LDL cholesterol traffic directly through endosomes to lysosomes, bypassing the plasma membrane, and becomes trapped there because of dysfunctional NPC1.

EXPERIMENTAL PROCEDURES

Materials—[2,6,7-3H]Cholesteryl linoleate (86 Ci/mmol), [1,2-3H]cholesterol (45 Ci/mmol), cholesterol [1-14C]oleate (59.5 mCi/mmol), and [a-33P]dCTP (3000 Ci/mmol) were obtained from PerkinElmer Life Sciences. Tissue culture reagents were from Mediatech Cellgro or Fisher. Other chemicals were from Sigma unless otherwise indicated. Fetal bovine serum and tissue culture–treated glass chamber slides were obtained from BD Biosciences.

2-Hydroxy-propyl-β-cyclodextrin (M, 1576) was obtained from Sigma, and a 100 mM stock was prepared in Ham’s F-12 medium. A 12.5 mM CD was prepared by diluting the stock in medium. A 25 mM CD that was 10% (v/v) lipoprotein-deficient newborn calf serum (H-5% LPDS). On day 0, CHO cells were seeded into 6-well plates (25,000 cells/well) in H-5% NCS. On day 1, cells were washed in balanced Hanks’ salt solution and refed H-5% NCS. Cells were transiently transfected at 60–70% confluence by using LipofectAMINE PLUS reagent (Invitrogen) with a total of 0.7 μg DNA/chamber of human NPC1 (generously supplied by Jill Morris and Peter Pentchev, National Institutes of Health) or no DNA, along with pEGFP (Clontech). After 3 h cells were refed H-5% LPDS and incubated for another 48 h. Additions of 50 μg/ml LDL were made, and cells were incubated for an additional 16–24 h. Filipin fluorescence microscopy was performed as described previously (14).

RESULTS

In normal mammalian cells, LDL is taken up and the choleseryl ester core is hydrolyzed in endocytic vesicles. The cholesterol that is released eventually reaches NPC1-containing organelles. What itinerary does cholesterol follow? Previous studies have concluded that LDL-derived cholesterol is transported to multivesicular late endosomes where active NPC1 resides (5) (Fig LA). NPC1-containing vesicles bud off of late endosomes, presumably carrying cholesterol and other cargo (6), move to the cell interior and cell periphery, and then return (20). In NPC1 cells, the movement of cholesterol out of late endosomes is greatly delayed (11, 21, 22). Thus, cholesterol and other lipids accumulate in lysosomes along with dysfunctional NPC1 protein (23). Some of the previous cholesterol transport studies relied on inefficient methods to assess the transit of LDL-derived cholesterol to the plasma membrane, such as spontaneous desorption of [3H]cholesterol from the plasma membrane and its entrapment in small unilamellar vesicles in the medium (11, 22). Other studies examined LDL cholesterol sequestration in lysosomes versus transport to the plasma membrane after long incubation periods with [3H]HCl-LDL and subcellular fractionation (11, 21, 22). All of these studies found delayed arrival of LDL cholesterol in the plasma membrane. Most recently, cholesterol movement to the plasma membrane has been quantified by using CD, which can stimulate cholesterol efflux from cells (24) and act as cholesterol shuttles (15, 25). By using CD that are complexed with varying levels of cholesterol, it is possible to manipulate the cholesterol content of cells ranging from net cholesterol enrichment to depletion (15, 26). Cruz et al. (7) assessed LDL cholesterol movement in NPC1 cells using short incubation times and CD to measure the arrival of [3H]cholesterol in the plasma membrane. Their results indicated that the pathway of LDL cholesterol transport was more complex than suspected previously, involving LDL cholesterol movement to the plasma membrane before arrival in NPC1-containing organelles (Fig. 1B).

Here we have evaluated this itinerary in CHO mutants 2-2 and CT60. Isolated in our laboratory and Chang's laboratory,
respectively, these NPC1-defective mutants display the classical NPC disease phenotype (11, 13). We compared the use of CD with CD/chol, which is 50% saturated with cholesterol, i.e. a shuttle system that samples the amount of cholesterol at the plasma membrane, presumably without disrupting the normal cholesterol trafficking pattern (15, 26).

Analysis of LDL Cholesterol Movement to the Plasma Membrane Using a Continuous Label Protocol—Wild-type CHO cells and mutants 2-2 and C760 were incubated for 10–120 min at 37 °C with [3H]CL-LDL. During the incubation, the [3H]CL-LDL will be internalized and hydrolyzed and [3H]cholesterol will be released. To measure [3H]cholesterol arrival in the plasma membrane, cells were briefly exposed to media containing CD/chol, which acts as a shuttle to sample cholesterol as it arrives at the plasma membrane (15, 26). Cell-associated [3H]cholesteryl linoleate and [3H]cholesterol and [3H]cholesterol that had desorbed to CD/chol were quantified (Fig. 2). We found that [3H]CL-LDL uptake was equivalent in all cells (Fig. 2A), and similar amounts of the [3H]cholesteryl linoleate were hydrolyzed at each time point (data not shown). No [3H]cholesterol appeared in media lacking CD/chol (data not shown). When cells were incubated for 10 min with [3H]CL-LDL and then exposed to media containing CD/chol for 10 min, we found no LDL-[3H]cholesterol in CD/chol-accessible pools. By 30 min of [3H]CL-LDL incubation, discernible LDL-derived [3H]cholesterol was found in the plasma membrane of wild-type cells but none in mutant 2-2 or C760 cells. After 30 min, we observed a time-dependent increase in CD/chol-accessible [3H]cholesterol in wild-type CHO cells; however, very little LDL-derived [3H]cholesterol became accessible to CD/chol in mutant 2-2 or C760 cells (Fig. 2, B and C). These data suggest that either LDL cholesterol does not move through the plasma membrane in NPC1 cells or that its appearance there is transient. Therefore, we next evaluated LDL-[3H]cholesterol transport following the protocol used by Cruz et al. (7).

Analysis of LDL Cholesterol Movement to the Plasma Membrane Using a Pulse-Chase Protocol—In this experiment, wild-type and mutant cells were pulsed for 1 h at 37 °C with [3H]CL-LDL and then incubated with either 25 mM CD/chol (as described above) or 12.5 mM CD alone (as used by Cruz et al. (7)). Cell-associated [3H]cholesteryl linoleate and [3H]cholesterol and [3H]cholesterol that had desorbed to CD/chol or CD were quantified (Fig. 3). All cells internalized equivalent amounts of [3H]CL-LDL during the 1-h pulse (data not shown). During the chase incubations, the [3H]cholesteryl linoleate was hydrolyzed to release free [3H]cholesterol (Fig. 3, A and C). In this experiment, less [3H]cholesteryl linoleate hydrolysis occurred in C760 than in CHO or 2-2 cells. However, the amount of LDL-derived [3H]cholesterol accessible to CD was calculated as a percentage of the available [3H]cholesterol. Under all conditions we found that LDL-derived [3H]cholesterol movement to the CD-accessible compartment was delayed by ~60 min in mutant 2-2 or C760 cells as compared with wild-type CHO cells (Fig. 3, B and D). After 60 min the rate at which LDL [3H]cholesterol appeared in a CD-accessible pool was equivalent in mutant and wild-type cells.

Analysis of LDL Cholesterol Movement in Mutant 3-6 Cells—A second strategy to test whether LDL cholesterol traffics through the plasma membrane en route to NPC1-containing organelles used a somatic cell mutant with impaired transport of plasma membrane cholesterol into the cell interior combined with pharmacological inhibition of NPC1. In such a cell model, LDL-derived cholesterol should not reach the NPC1-containing late endosomes if its transport pathway takes it through the plasma membrane, but the LDL cholesterol should accumulate internally if it passes through late
endosomes on its way to the plasma membrane. We created such a cell model by treating mutant 3-6 with either progesterone or hydrophobic amines. Mutant 3-6 is a CHO cell line that exhibits defective transport of cholesterol from the plasma membrane to the cell interior (14). Progesterone and hydrophobic amines such as U18666A and imipramine inhibit LDL cholesterol transport through NPC1—A third strategy to test this hypothesis is to determine whether plasma membrane cholesterol is transported through NPC1-containing organelles on the way to the endoplasmic reticulum. Cultured cells incubated with \[^3H\]cholesterol absorb the \[^3H\]cholesterol and distribute it according to their cellular cholesterol pools. With time, \[^3H\]cholesterol reaches acyl-CoA/cholesterol acyltransferase in the endoplasmic reticulum and is esterified. We incubated parental CHO cells and four mutant cell lines from the NPC1 complementation group with \[^3H\]cholesterol for various times. Fig. 5 shows a time-dependent incorporation of \[^3H\]cholesterol into \[^3H\]cholesterol esters in parental CHO cells and equivalent \[^3H\]cholesterol ester formation in each of the mutant cell lines. This result is not consistent with plasma membrane cholesterol being transported through NPC1-containing organelles to the cell interior.

**Sequence Analysis of Hamster NPC1 Mutants**—To confirm genetic evidence that our cholesterol transport-defective CHO cells are NPC1 mutants, we cloned and sequenced the NPC1 cDNA from our parental CHO-K1 cells and four independently isolated cell lines, mutants 2-2, 4-4, 10-3, and 1-2. The nucleotide and deduced amino acid sequence of NPC1 from our CHO-K1 cells was identical to that reported previously (7). The NPC1 mutations in the mutant cell lines were located by using a BLAST2 search after initial sequence analysis with AssemblyGNN and MacVector programs. Mutant 2-2 contains a base-pair insertion of C at nucleotide 1335, resulting in a frameshift and premature translational termination after 450 amino acids (Fig. 6). Mutant 4-4 contains a base insertion of C at nucleotide 1446, which results in a frameshift and premature translational termination after 652 amino acids (Fig. 6). Mutant 1-2 contains no apparent mutations in the NPC1 coding region. It is possible that mutations exist in the 3’- or 5’-untranslated regions. We were unable to obtain the complete sequence for mutant 10-3.

Northern blot analysis showed that parental and mutant cells express an NPC1 mRNA of comparable size. However, mRNA levels are reduced in mutant 2-2 and practically absent in mutant 10-3 (Fig. 7). The lack of NPC1 mRNA in mutant 10-3 may explain our difficulty in sequencing a cDNA.

**Transient Expression of Human NPC1**—A hallmark of NPC1 cells is the punctate filipin fluorescence, which denotes lysosomal cholesterol storage. To determine whether expression of the human NPC1 cDNA (phuNPC1) was able to correct the filipin-staining pattern in our mutants, we performed co-transfection experiments with phuNPC1 and pEGFP, which expresses the green fluorescent protein (GFP). Fig. 8 shows that filipin-stained parental CHO cells have diffuse fluorescence, whether they are untransfected, expressing pEGFP or phuNPC1 plus pEGFP. The expression of pEGFP alone in mutant cells had no effect on filipin fluorescence; however, when mutant cells were transfected with pEGFP plus phuNPC1, the GFP-positive cells showed correction of the filipin staining. These results support the complementation and sequence analyses of our NPC1-deficient mutants.

**DISCUSSION**

In this study, we have evaluated the fate of cholesterol released by LDL-cholesterol ester hydrolysis in the endocytic pathway. It is generally accepted that in normal cells LDL cholesterol is rapidly transported to the plasma membrane and other cellular membranes, whereas in NPC cells LDL cholesterol ends up sequestered in terminal storage compartments along with glycosphingolipids. What is disputed is whether NPC1 facilitates the initial transport of LDL cholesterol to the

**Basal Esterification of Plasma Membrane Cholesterol**

- The percentage of \[^3H\]cholesterol in lysosomes is rapidly transported to the plasma membrane and other cellular membranes, whereas in NPC cells LDL cholesterol ends up sequestered in terminal storage compartments along with glycosphingolipids. What is disputed is whether NPC1 facilitates the initial transport of LDL cholesterol to the plasma membrane.
plasma membrane, or whether LDL cholesterol is transported by other means to the plasma membrane and is then internalized to NPC1-containing organelles for further disposition (Fig. 1). The latter model calls for all plasma membrane cholesterol to be routed through the NPC1-containing organelles before arrival in other intracellular membranes (7).

We used three experimental approaches to evaluate these models in wild-type and NPC1-deficient CHO cell lines. First, CD/chol complexes were used to detect the arrival of LDL-derived [3H]cholesterol in the plasma membrane. We found that under all experimental conditions NPC1 mutants exhibited a distinct lag in the movement of LDL-derived [3H]cholesterol to the CD-accessible plasma membrane. We were unable to demonstrate any early transient appearance of LDL-[3H]cholesterol at the plasma membrane.

We found the same qualitative results using either CD or CD/chol as cholesterol acceptors in the media, although CD/chol consistently promoted more [3H]cholesterol efflux from cells than an equal concentration of CD alone. This finding is consistent with the notion of CD/chol as an effective cholesterol shuttle and suggests that cholesterol transfer to the plasma membrane stimulates the reciprocal cholesterol movement.

In the second approach, we tested specifically whether LDL-[3H]cholesterol passes through the plasma membrane before its arrival in the NPC1-containing late endosomes. We found that under all experimental conditions NPC1 mutants exhibited a distinct lag in the movement of LDL-derived [3H]cholesterol to the CD-accessible plasma membrane. We were unable to demonstrate any early transient appearance of LDL-[3H]cholesterol at the plasma membrane.

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In the second approach, we tested specifically whether LDL-[3H]cholesterol passes through the plasma membrane before its arrival in the NPC1-containing late endosomes. We used mutant 3-6, which exhibits defective transport of plasma membrane cholesterol to internal membranes (14). A classical genetic approach to this problem would be to perform epistasis analysis, in which an NPC1/3-6 double mutant would be evaluated. If LDL cholesterol passed through NPC1-containing organelles before arrival in the plasma membrane, then an NPC1/3-6 mutant would show lysosomal accumulation of cholesterol. However, if LDL cholesterol were transported to the plasma membrane and then to the NPC1-containing organelles, then an NPC1/3-6 double mutant would show no intracellular storage of cholesterol. An NPC1/3-6 double mutant is not available; however, we created an equivalent cell model using pharmacological agents that cause an NPC1 phenotype. We found that when 3-6 cells were treated with either progesterone or imipramine, incubation with LDL led to punctate filipin fluorescence that was indistinguishable from the classical NPC phenotype. This result is consistent with LDL cholesterol reaching the NPC1-containing late endosomes before reaching the plasma membrane.

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In the third approach, we evaluated whether plasma membrane cholesterol passes through NPC1-containing late endosomes on route to the endoplasmic reticulum. Lange et al. (30) have estimated that in cultured cells the entire plasma membrane cholesterol pool cycles between the plasma membrane and endoplasmic reticulum with a half-time of 40 min; thus, delayed kinetics in cholesterol exiting the NPC1 organelles should be readily apparent. We found identical basal [3H]cholesterol esterification in wild-type and NPC1-deficient cells. These results are consistent with our previous finding that imipramine has no effect on plasma membrane to endoplasmic reticulum cholesterol movement at concentrations that cause the NPC1 phenotype (4). Together they indicate that the plasma membrane to endoplasmic reticulum cholesterol transport pathway is intact and that the NPC1-containing late endosomes are not an obligate part of that pathway.

An intact plasma membrane to endoplasmic reticulum cholesterol transport pathway was also indicated in studies of cholesterol balance in the NPCnih mouse model of NPC disease. Xie et al. (31) found that cholesterol delivered via HDL to the adrenal was used as normal for cholesterol esterification and steroid hormone production, and that HDL-cholesterol delivered to the liver could apparently be secreted into the bile or converted to bile acids as normal in NPCnih mice. This activity of cholesterol delivered via HDL contrasted with cholesterol delivered via LDL or chylomicrons, which was sequestered within a metabolically inactive pool (31).

We can think of no apparent reason for LDL cholesterol to be released by non-NPC1-containing late endosomes/lysosomes and rapidly transported to the plasma membrane followed by quick retrieval and delivery to NPC1-containing organelles, as proposed by Cruz et al. (7) (Fig. 1B). If this rapid transit does take place, it is not clear how those cholesterol molecules would be quantitatively retrieved. Does the retrieval mechanism distinguish between the newly delivered cholesterol molecules and the other plasma membrane pool(s), or are they delivered to a plasma membrane domain that does not diffuse laterally? One possibility is that the cholesterol detected by Cruz et al. (7) is actually an internal pool that is capable of being diverted to the plasma membrane by CD depletion of plasma membrane cholesterol content.

Our hypothesis is that membrane lipid and receptor-bound cargo such as LDL is internalized and delivered to the endocytic recycling compartment (32) (Fig. 1A). LDL is released from its receptor and sorted into early endosomes. LDL-cholesterol ester hydrolysis likely begins when the LDL particle is in transit through the endocytic pathway. In multivesicular late endosomes, NPC1 facilitates the rapid transport of lipids, whether they arrived as constituents of the lipoprotein particles or of the vesicle membranes. NPC1-containing vesicles have been observed to bud from late endosomes, presumably carrying cholesterol and other cargo, and fuse with perinuclear and peripheral membranous compartments, although not the plasma membrane (20). Cholesterol may be delivered via NPC1 to the endocytic recycling compartment (32) from which it recycles to the plasma membrane. When NPC1 is dysfunctional, cholesterol accumulates in terminal organelles because its acquisition by vesicular transport and lipoprotein uptake is greater than its dispersal by vesicular trafficking alone. We conclude that plasma membrane cholesterol can reach the endoplasmic reticulum by NPC1-dependent and NPC1-independent pathways. The NPC1-dependent pathway involves the
movement of membrane lipids by vesicle trafficking through endosomes to the NPC1-containing late endosomes, where it is dispersed by NPC1. The NPC1-independent pathway is responsible for rapid cycling of cholesterol to the cell interior and back and represents the pathway that is defective in mutant 3-6.

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