Flux of Fatty Acids through NPC1 Lysosomes*

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Niemann-Pick type C (NPC) is an autosomal recessive lipid storage disorder characterized by lysosomal accumulation of cholesterol and gangliosides resulting from a defect in intracellular lipid trafficking. The NPC1 gene encodes a 1278-amino acid integral membrane protein involved in the sub-cellular trafficking of lipids. The exact biological function of NPC1 remains unclear. Recent evidence suggests that NPC1 is a eukaryotic member of the RND permease family of transport proteins, which when expressed in bacteria is capable of transporting fatty acids. The goal of this project was to assess the role of NPC1 in the transport of fatty acids in primary human fibroblasts using normal fibroblasts and fibroblasts from patients with three lysosomal storage diseases: NPC, mucolipidosis IV, and Sandhoff disease. If NPC1 is a fatty acid transporter, we expect to find fatty acid accumulation only in NPC fibroblasts. We used three experimental approaches to assess the role of NPC1 as a fatty acid transporter. First, we evaluated the accumulation versus metabolism of low density lipoprotein-derived oleic acid. Second, we assessed the amount of free fatty acid present after growth in lipoprotein-containing media. Third, we assessed the cellular accumulation of acriflavine, a fluorescent substrate for a number of resistance-nodulation-cell division permease transporters. Our results indicate that fatty acid flux through NPC1-deficient lysosomes is normal.

Niemann Pick C (NPC) is a neurodegenerative autosomal recessive lysosomal storage disease characterized by the accumulation of cholesterol and other lipids both in the periphery and in the brain. NPC is caused by mutations in either the NPC1 (95% of clinical cases) or NPC2 (5% of clinical cases) genes; the phenotype resulting from mutations in either gene is identical (1). Symptoms of NPC can present at various points in development, and they include seizures, dystonias, ataxia, and a hallmark vertical gaze palsy (1). Currently, there is no effective therapy for NPC. A key step in the development of therapeutics for this disease is likely to be a better understanding of the function of the NPC1 protein.

NPC1 is a 1278-amino acid membrane protein found in late endosomes and lysosomes (2). It contains 13 transmembrane domains, a sterol sensing domain, an NPC domain containing a leucine zipper, and a C-terminal lysosomal targeting sequence (3). Mutations leading to NPC disease occur throughout the protein, and the majority of patients are compound heterozygotes (4, 5). The effects of mutations in NPC1 have been well studied in fibroblasts, where mutations lead to defective lipid trafficking and lysosomal accumulation of cholesterol and glycosphingolipids. Despite knowledge of the physical characteristics of the protein and its effects on cellular lipid transport, the exact function of the NPC1 protein remains elusive. It is clear that NPC1 is involved in the trafficking of lipids in the endosomal/lysosomal system; however, it is not clear how NPC1 exerts this effect. The first indication of a specific function that could be ascribed to NPC1 came from bioinformatics analysis, which showed that NPC1 is structurally similar to the resistance-nodulation-cell division (RND) family of prokaryotic permeases (6, 7). RND permeases serve as proton-dependent efflux pumps, which extrude hydrophobic drugs, heavy metals, lipooligosaccharides, and antibiotics (6). When human NPC1 was expressed in Escherichia coli, it was found to mediate the transfer of an RND permease substrate, acriflavine, as well as a fatty acid, oleic acid, but not cholesterol, or cholesterol oleate (7). NPC1-mediated fatty acid flux made some physiological sense, because fatty acids are released by late endosomal and lysosomal digestion of lipoproteins and membrane constituents; however, no studies have been conducted in mammalian cells to confirm that NPC1 transports fatty acids. The goal of this study was to address the hypothesis that endogenous NPC1 functions as a fatty acid transporter in human fibroblasts. This study was conducted in normal and NPC human skin fibroblasts. If NPC1 functions as a fatty acid transporter in normal cells, then we would expect fatty acids to accumulate in late endosomes and lysosomes of NPC cells. We also examined fibroblasts from patients with other lysosomal storage diseases to distinguish between lysosomal fatty acid accumulation specific for NPC1 dysfunction versus secondary accumulation due to lipid accumulation. No other lysosomal storage disease has an identical storage profile to NPC; however, two had similar attributes. One attribute of NPC that sets it apart from the other lysosomal storage diseases is that NPC results from a mutation in a protein involved in trafficking, whereas most other lysosomal storage diseases result from a mutation in a metabolic enzyme. Therefore, mucolipidosis IV (MLIV) was chosen as a control fibroblast because it shares this attribute with NPC1. The protein mutated in MLIV, mucolipin, is involved in intracellular trafficking of endocytosed lipids (8). A second attribute of NPC that we considered is the fact that
neuronal abnormalities in NPC closely resemble those of primary gangliosidoses. Thus we chose a primary gangliosidosis, Sandhoff disease, as our second control cell.

We used three approaches to test the hypothesis that NPC1 is a fatty acid transporter. First, we evaluated the accumulation of LDL-derived oleic acid in normal and NPC1 fibroblasts. Second, the amount of free fatty acid present in the four fibroblast cultures after growth in lipoprotein-containing media was assessed. Third, we assessed the accumulation of acriflavine, a fluorescent substrate for a number of RND permease transporters, in normal, NPC, MLIV, and Sandhoff fibroblasts. Our results indicate that fatty acid flux through NPC1-deficient lysosomes is normal.

**EXPERIMENTAL PROCEDURES**

**Materials**—The acyl-CoA/cholesterol acyltransferase (ACAT) inhibitor CI-976 was obtained from Pfizer. [9,10-3H]Triolein (52.6 Ci/mmol) and [4-14C]cholesteryl oleate (56 mCi/mmol) were obtained from American Radiolabeled Chemicals. Tissue culture reagents were from Mediatech Cellgro or Fisher. Tetramethylrhodamine dextran (TMRD, 70,000) was obtained from Molecular Probes. Other chemicals were from Sigma unless otherwise indicated. Nunc Lab-Tek Chambered Cover Glasses were obtained from Fisher Scientific.

**Cultured Cells, Preparation of LDL, Lipoprotein-deficient Serum, Media, and [3H/CO-LDL**—Normal (GM05659D), NPC1 (GM3123A), MLIV (GM02408), and Sandhoff (GM11707) fibroblasts were purchased from Coriell Institute for Medical Research. Low density lipoprotein (LDL) was prepared by ultracentrifugation (9). Lipoprotein-deficient serum was prepared as described, omitting the thrombin incubation (9). The following media were prepared: Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, 2 mM glutamine, 100 units/ml penicillin (D-10% FCS); D-10% LPDS; DMEM 10% FCS; and DMEM 10% high-glucose (A) media containing 100 units/ml insulin, 100 units/ml glutamine, 2 mM ascorbic acid, and 25 g of lecithin, and 4,200 dpm of [14C]cholesteryl oleate were added to each sample during the extraction. Labeled cellular and media lipids and media were analyzed by TLC using the Bligh-Dyer method (13). 

**Reconstitution of [3H-T]/LDL**—Reconstitution of LDL with [3H]triol-ein ([3H-T]/LDL) was prepared by reconstituting LDL with [3H]triol-ein as described previously (11). Briefly, 1.9 mg of LDL was frozen and lyophilized in a starch tube. LDL core lipids were extracted twice with 5 ml of cold heptane. 100 µCi of [9,10-3H]triol-ein and 6 mg of cold triol-ein in 200 µl of heptane were added to the LDL. The solvent was evaporated, and the sample was resuspended in 500 µl of Tris buffer at pH 7.3, and incubated overnight at 4 ºC. The sample was then centrifuged at 105,000 g for 30 min. The supernatant was removed, and 100 µl of Tris buffer was added. The sample was incubated at 4 ºC overnight. Cells were pulsed with [3H]oleate, 10 µg of triol-ein, and 2300 dpm of [14C]cholesteryl oleate was added to each sample. Labeled cellular lipids were analyzed by TLC developed with heptane::ethyl ether::glacial acetic acid (90:30:1). Radioactivity was measured by liquid scintillation counting using ReadySafe.

**Microscopy of Acriflavine/Filipin-labeled Cells**—Cells were plated in 4-well coverglass slides in D-10% FCS and grown to 60–80% confluency. On the day of the experiment, cells were incubated with 10 µg/ml acriflavine for 30 min, rinsed with media, and refed D-10% FCS. At the indicated time points, slides were rinsed three times with media after which phenol-free DMEM was added to each well. Slides were viewed immediately using an Axiovert inverted fluorescence microscope (Zeiss, Germany). Photographs were taken using Hamamatsu ORca cooled-charged-coupled device camera and evaluated using Metamorph 5.0 software (Universal Imaging Corp., Downingtown, PA).

**Cholesterol Esterification**—Cells were plated in 6-well plates at a density of 30,000 cells/well in D-10% FCS. Cells were washed with Hanks' buffered saline solution and refed D-10% LPDS 48 h prior to the start of the experiment. On the day of the experiment, cells were pulsed with [3H]acetate for 6 h. Cells were pulsed with 2 µg/ml [3H]acetate for 4 h, and then rinsed with media, and [3H]acetate was added to each sample. Cells were then incubated for 24 h, and then the media were collected and the cells were trypsinized. A 200-µl aliquot of each 1-ml cell sample was removed for protein determination (12). Lipids from the media and cells were extracted using the Bligh-Dyer method (13). 10 µl of recovery standard containing 100 µg of cholesteryl oleate, 50 µg of triol-ein, 100 µg of oleic acid, 25 µg of lecinthin, and 4,200 dpm of [14C]cholesteryl oleate was added to each sample during the extraction. Labeled cellular and media lipids were analyzed by TLC developed with hexane::ethyl ether::glacial acetic acid (130:30:2). Radioactivity was measured by liquid scintillation counting using ReadySafe.

**Gas Chromatography**—For each fibroblast culture, a confluent 75-cm² flask was trypsinized, and the cells seeded into six 150-mm dishes in D-10% FCS. Cells were cultured until confluent, with removal of D-10% FCS twice a week. On the day of harvest, cells were washed with phosphate-buffered saline, scraped, and pelleted in three-dish aliquots. Cell pellets were resuspended in 1.6 ml of phosphate-buffered saline and subjected to a modified Bligh and Dyer extraction (13). Briefly, to each cell sample, we added 2 ml of methanol, 1 ml of chloroform, 10 µg of nonadecanoic acid (Matreya, Inc.), 1 ml of chloroform, and 1 ml of 0.9% sodium chloride, with vortexing after each addition. The pH was adjusted to 3 with 3 N HCl, after which the samples were vortexed and subjected to centrifugation (2000 × g, 10 min). The bottom organic phases were transferred to a new tube, and the top aqueous phases were re-extracted with chloroform/methanol (7:4:1). Bottom organic phases for each sample were pooled and dried under nitrogen. Samples were subjected to TLC on plastic-backed silica gel 60 plates developed with hexane:ethyl ether:formic acid (80:20:2). The Rₚ of free fatty acids was determined by iodine staining of flanking standard lanes; sections of the TLC plates were cut out, placed in a test tube with 1.5 ml of 1% sulfuric acid in methanol, and heated for 10 min at 50 ºC (14). Two milliliters of 5% sodium chloride was added, and the fatty acid methyl esters were extracted with hexane, and the hexane extracts were back-washed with water and dried under nitrogen. Free fatty acid methyl esters were quantified on a Hewlett-Packard 5890 gas chromatograph using a Supelcowax 10 capillary column (30 m, 0.25-mm internal diameter, Supelco, Inc.). The oven temperature was maintained at 150 °C for 2 min, ramped to 180 °C/min to 200 °C, and held for 4 min, then ramped again at 5 °C/min to 240 °C and held for 3 min, and then finally ramped at 10 °C/min to 270 °C, where it was held for 5 min. The injector and flame ionization detector were maintained at 260 °C and 280 °C, respectively. Carrier gas flow rate was maintained at a constant 0.8 ml/min throughout. Peaks were identified by comparing retention times to known fatty acid methyl esters (Matreya, Inc.).

**RESULTS**

Oleic Acid Derived from LDL Does Not Accumulate in NPC1 Cells—In normal cells, LDL is endocytosed and transported to endocytic vesicles where its core lipids, such as cholesteryl linoleate, are hydrolyzed to release cholesterol and linoleic acid. In NPC1 cells, LDL-derived cholesterol accumulates to high levels in endosomes and lysosomes. If NPC1 is a fatty acid transporter, in normal cells, LDL is endocytosed and transported to endocytic vesicles where its core lipids, such as cholesteryl linoleate, are hydrolyzed to release cholesterol and linoleic acid. In NPC1 cells, LDL-derived cholesterol accumulates to high levels in endosomes and lysosomes. If NPC1 is a fatty acid transporter, in normal cells, LDL is endocytosed and transported to endocytic vesicles.
transporter, we would expect fatty acids to be sequestered in storage compartments along with the cholesterol, sphingomyelin, lysobisphosphatidic acid, neutral and acidic glycosphingolipids, and phospholipids that are found in NPC cells. To evaluate the flux of LDL-derived oleic acid, normal and NPC cells were incubated with either \(^{1}H\)-LDL, in which the core lipids of LDL were replaced with \(^{1}H\)triolein or with \(^{1}H\)CO-LDL, in which the core lipids contained cholesteryl \(^{1}H\)oleate. In both experiments, hydrolysis of the \(^{3}H\)-labeled core lipids by acid lipase in endosomes will lead to release of \(^{1}H\)oleic acid (15). \(^{3}H\)Oleic acid sequestered within the late endosome/lysosome compartment should remain as cellular free fatty acid, whereas that which is transported from late endosomes and lysosomes may be incorporated into cellular lipids or effluxed from the cells. We have examined the amount of \(^{1}H\)oleic acid that remains free, is incorporated into cellular lipids, or is released from the cells into the medium.

Fig. 1A shows cellular \(^{3}H\)lipids in normal and NPC cells after 4 h of pulse with \(^{1}H\)-LDL and no chase incubation. The cells had similar levels of \(^{3}H\)triolein (white bars) indicating equivalent LDL uptake. Very little free \(^{3}H\)oleic acid (gray bars) was present in either cell type; instead, equivalent amounts had been incorporated into \(^{3}H\)phospholipids (shaded bars). The oleic acid was not incorporated into cholesterol esters (black bars) as a result of the presence of the ACAT inhibitor. There were no significant differences between normal and NPC values.

After a 24-h chase incubation, the \(^{3}H\)triolein had largely been hydrolyzed in both normal and NPC cells (Fig. 1B). Still the level of free \(^{1}H\)oleic acid (gray bars) was close to zero in both cell types. \(^{3}H\)Oleic acid released by \(^{3}H\)triolein hydrolysis had been incorporated into phospholipids or released from the cells in amounts that were not significantly different in normal and NPC cells.

Fig. 1C shows the distribution of cellular and medium \(^{3}H\)lipids after 24 h of chase, expressed as a percentage of the total cell \(^{3}H\)lipids in panel A.
0-h chase. The key point is that the distribution is equivalent in normal and NPC cells and media, and that only 1% of the \(^{3}H\)oleate remained as cellular fatty acid. The bulk of the \(^{3}H\)oleate was found as unhydrolyzed \(^{3}H\)triolein, or had been hydrolyzed and re-esterified to \(^{3}H\)triolein and \(^{3}H\)phospholipids. Approximately 25% of free \(^{3}H\)oleate was released into the media.

Fig. 2 shows that incubating the cells with \(^{3}H\)CO-LDL instead of \(^{3}H\)-TLDL leads to a similar distribution of \(^{3}H\)oleic acid. After 4 h of incubation and no chase (Fig. 2A), most of the \(^{3}H\)oleate was in unhydrolyzed cholesteryl \(^{3}H\)oleate (black bars). Some has been incorporated into other cellular lipids. There was no buildup of free \(^{3}H\)oleic acid (gray bars) in either cell type. After 24 h of chase (Fig. 2B), the amount of cholesteryl \(^{3}H\)oleate had decreased. Some of the released \(^{3}H\)oleic acid had been incorporated into phospholipids (shaded bars). There was also a release of unhydrolyzed cholesteryl \(^{3}H\)oleate, free \(^{3}H\)oleic acid, and \(^{3}H\)phospholipids into the media. There was no significant difference in the levels of free \(^{3}H\)oleic acid inside the normal and NPC cells (gray bars).

It had been proposed that NPC1 functions as a fatty acid transporter (7). If this is the case then \(^{3}H\)oleic acid derived from LDL should accumulate in NPC late endosomes and lysosomes. It should not be incorporated into other lipids or effluxed from the cell as efficiently as in normal cells. Figs. 1 and 2 indicate that oleic acid is released from NPC endosomes and lysosomes, incorporated into other lipids, and effluxed from the cells at similar percentages to normal cells.

**There Is No Detectable Accumulation of Free Fatty Acid in NPC Fibroblasts**—Fatty acids are released in the cell's endocytic compartment, not only due to hydrolysis of endocytosed lipoproteins, but also due to autophagy of cellular organelles, and digestion of membrane constituents. As stated above, if NPC1 facilitates fatty acid efflux from the late endosome/lysosome compartment, then NPC fibroblasts should have a buildup of free fatty acids, sequestered away from acyl-CoA-synthetase and other metabolizing enzymes, that is detectable by mass measurements.

To determine whether NPC fibroblasts have a higher level of free fatty acids than normal fibroblasts, lipids were extracted from normal, NPC, MLIV, and Sandhoff fibroblasts cultured in D-10% FCS. Each sample contained ~1.5 mg of cell protein. Free fatty acids were isolated and analyzed by gas chromatography. Levels were normalized for nonadecanoic acid, which was added during the extraction procedure. We found that the relative levels of fatty acids in NPC, MLIV, and Sandhoff fibroblasts were 69, 63, and 75% of that in normal fibroblasts, respectively (average of two experiments). If lipoprotein-derived fatty acids were sequestered preferentially in NPC fibroblasts, we would expect to find higher levels of linoleic acid in NPC cells; however, the fatty acid distribution was equivalent in the four cell samples (Table I).

**Acriflavine Does Not Accumulate Specifically in NPC1 Cells**—NPC1 was first identified as a eukaryotic member of the RND permease family based on its structural homology with other RND family members such as E. coli AcrB and Pseudomonas aeruginosa MexD (6). Functional evidence that NPC1 might be an RND permease came from acriflavine loading experiments. When fibroblasts are incubated with acriflavine, the cationic fluorescent dye binds to nucleic acids; it also accumulates in perinuclear vesicles. Davies et al. (7) identified the punctate vesicles as endosomes and lysosomes by colocalization with LysoTracker. Upon washout, they found that the dye was more quickly extruded from the normal lysosomes than NPC1 lysosomes (7). Is this sequestration due to NPC1 dysfunction, or is it secondary to the presence of storage material in the compartments? To test this, acriflavine accumulation was assessed in normal, NPC, MLIV, and Sandhoff fibroblasts by fluorescence microscopy. Fig. 3 shows that punctate acriflavine accumulation was not specific to NPC cells. After a 30-min incubation with acriflavine and no washout time, all cells contained nuclear fluorescence and some punctate fluorescence; however, the punctate fluorescence in normal cells (panel A) was less than that in the NPC (panel B), Sandhoff (panel C) and MLIV (panel D) cells. The greatest punctate fluorescence was seen in the MLIV cells. After 16 h of washout, all cells had similar punctate staining (panels E–H).

These results indicate that accumulation of acriflavine is not specific to NPC cells. All three lysosomal storage disease fibroblasts have immediate and lasting punctate staining.

**MLV and Sandhoff Fibroblasts Do Not Have an NPC Phenotype**—NPC, MLIV, and Sandhoff fibroblasts showed similar punctate acriflavine accumulation (Fig. 3). To determine whether the acriflavine accumulation was due to NPC1 inhibition secondary to lipid storage in MLIV and Sandhoff lysosomes, we performed the classic diagnostic test for NPC activity, LDL stimulation of cholesterol esterification. Fig. 4 shows that ACAT-catalyzed cholesterol esterification was low when cells were cultured in the absence of LDL and was increased in normal fibroblasts given LDL but not in NPC1 cells. Both MLIV and Sandhoff fibroblasts showed normal LDL stimulation of cholesterol esterification. From this we concluded that the acriflavine accumulation in these lysosomal storage disease fibroblasts is independent of NPC1 activity.

**Acriflavine Does Not Accumulate in the NPC1 Cholesterol Storage Compartment**—The acriflavine that accumulates in perinuclear vesicles in NPC cells was proposed to be in endo-
somes and lysosomes due to co-localization with LysoTracker (7). We investigated the acriflavine compartment further by examining its co-localization with a fluid phase marker and with filipin fluorescence.

NPC1 fibroblasts were incubated with acriflavine, washed, and then incubated with dextran for 15 min. Cells were washed, cultured for an additional 2 h, and then viewed immediately (A–D) or after a 16-h washout (E–H). There was no NPC1-specific sequestration of acriflavine. The results are representative of three independent experiments.

**Fig. 3. Acriflavine accumulation in normal, NPC, MLIV and Sandhoff fibroblasts.** Cells were plated and incubated with 10 μg/ml acriflavine for 30 min as described under “Experimental Procedures.” Normal (A and E), NPC (B and F), Sandhoff (C and G), and MLIV (D and H) cells were viewed immediately (A–D) or after a 16-h washout (E–H). There was no NPC1-specific sequestration of acriflavine. The results are representative of three independent experiments.

**Fig. 4. LDL-stimulation of cholesterol esterification is normal in MLIV and Sandhoff fibroblasts.** Cells were cultured, incubated with the indicated amount of LDL, and then pulsed with [3H]oleate as described under “Experimental Procedures.” Cholesteryl [3H]oleate formation was determined as described under “Experimental Procedures” and is expressed as nanomoles/mg/h. The results are representative of two independent experiments.

**Flux of Fatty Acids through NPC1 Lysosomes**

NPC1 fibroblasts were incubated with acriflavine, washed, and then incubated with dextran for 15 min. Cells were washed, cultured for an additional 2 h, and then viewed immediately without fixation. We found that the acriflavine (Fig. 5A) and dextran (Fig. 5B) were not in the same compartments (Fig. 5C). This indicated that acriflavine was not sequestered in endocytic compartments. The same result was found when cells were incubated with dextran for 15 min to 2 h, indicating that acriflavine is not in early or late endocytic compartments.

Next, NPC cells were incubated with acriflavine and then stained with filipin to identify the cholesterol storage compartments. We found that the acriflavine (Fig. 5D) and filipin staining (Fig. 5E) were not in the same vesicles (Fig. 5F). This result indicated that acriflavine was not sequestered in the NPC1-containing cholesterol storage compartment.
Lastly, we incubated the NPC1 cells with dextran and then stained the cells with filipin to determine if the fluid phase marker (Fig. 5G) reached the cholesterol storage compartment (Fig. 5H). The results in Fig. 5I show that the dextran and filipin were found in the same vesicles. The results presented in Fig. 5 indicate that acriflavine is not sequestered in the NPC1-containing lysosomes.

**DISCUSSION**

In this study we used three different experimental approaches to address the hypothesis that NPC1 is a fatty acid transporter. It is generally accepted that NPC1 facilitates the transport of lipid cargo from late endosomes and lysosomes. Storage compartments isolated from NPC tissues show accumulation of unesterified cholesterol, sphingomyelin, phospholipids, glycolipids, and gangliosides (1). Lipid trafficking studies in cultured fibroblasts show delayed movement of cholesterol. It is not clear how NPC1 exerts this effect on lipid movement or whether lipid storage is a direct or indirect consequence of NPC1 dysfunction. One recent proposal is that NPC1 functions to clear late endosomes and lysosomes of fatty acids that are released by acid lipase digestion of lipoproteins (7).

Fatty acids are generated in the endosomal/lysosomal system due to hydrolysis of lipoproteins and also due to autophagy of cell organelles and degradation of membrane constituents. In cultured cells, the bulk of the fatty acids released from lysosomes is effluxed into the media, whereas the remaining fatty acids are incorporated into triglycerides, phospholipids, and cholesterol esters (16). If NPC1 were responsible for the movement of fatty acids out of lysosomes, there should be a buildup of free fatty acids that are trapped in lysosomes as well as a decrease in the fatty acids incorporated into triglycerides, phospholipids, and cholesteryl esters or released into the media. To test this hypothesis, we chose NPC fibroblasts GM3123 with a classic phenotype: no LDL-stimulated cholesterol esterification (17), and greatly delayed movement of LDL-derived cholesterol from lysosomes to plasma membrane (18).

In our first experimental approach, the fate of LDL-derived fatty acids in NPC cells was evaluated. We found that LDL-derived fatty acids were incorporated into cellular lipids in similar amounts in normal and NPC cells. Additionally, similar amounts of the fatty acids were released into the media from both cell types. These results indicate that mutations in NPC1 did not prevent LDL-derived fatty acids from leaving the late endosomes and lysosomes.

Our second approach looked directly at the mass of free fatty acids in normal and NPC cells. We found that LDL-derived fatty acids were incorporated into cellular lipids in similar amounts in normal and NPC cells. Additionally, similar amounts of the fatty acids were released into the media from both cell types. These results indicate that mutations in NPC1 did not prevent LDL-derived fatty acids from leaving the late endosomes and lysosomes.

Our third approach looked at the accumulation of an RND permease substrate, acriflavine, in the four cell types. Acriflavine...
vine had previously been shown to accumulate in perinuclear vesicles in NPC GM3123 fibroblasts (7). We wished to determine if the sequestration resulted specifically from NPC1 dysfunction. Most lysosomal storage diseases accumulate multiple substrates. Some are primary and result from a blockage in a metabolic pathway, whereas others appear to be secondary. Lysosomal sequestration of acriflavine could occur because it is a substrate for NPC1 transporter activity, or it may accumulate secondarily either due to lipid accumulation or a trafficking defect. To test these possibilities, we examined NPC1 fibroblasts alongside two other lysosomal storage disease fibroblasts. We chose MLIV because, like NPC, it derives from mutation in a protein involved in endocytic trafficking (8). We also chose Sandhoff fibroblasts, a GM2 gangliosidosis with a lipid accumulation similar to NPC. Our data show that the lysosomal storage disease fibroblasts initially sequester more acriflavine than normal; however, that equalizes with time. There was little difference in the acriflavine accumulation in NPC, Sandhoff, and MLIV fibroblasts, although MLIV cells consistently have the brightest fluorescence. These data indicate that acriflavine does not accumulate specifically in NPC cells.

How do our results differ from those of previous work? Our results in fibroblasts indicate that dysfunctional NPC1 does not lead to aberrant fatty acid efflux from late endosomes and lysosomes and reduced incorporation into other lipids; however, prior experiments in macrophages indicated that either NPC1 mutation or U18666A treatment results in a decreased incorporation of LDL-derived oleic acid into phospholipids (19). Our results are consistent with other fibroblast studies indicating that progesterone and imipramine, which act like U18666A to block the NPC-mediated pathway, do not prevent the release of fatty acids from lysosomes (16). However, it was noted in this latter study that progesterone, but not imipramine, stimulated incorporation of oleic acid into triacylglycerol at the expense of incorporation into phospholipids.

It is possible that the discrepancy in the role of NPC1 in fatty acid incorporation into phospholipids is due in part to the different cell types. Another possibility is that there is a difference in fatty acid utilization based on its mode of entry into the cell. A previous study indicated that there is a difference in oleic acid incorporation into different phospholipids based on whether the oleic acid was derived from LDL-tri olein or LDL-cholesterol oleate (20); however, we found no difference in the utilization of oleic acid by normal or NPC fibroblasts when we used LDL-cholesterol [3H]oleate instead of LDL-[3H]tri olein.

Previous work implicating NPC1 as a fatty acid transporter relied on three lines of evidence. First, the structural similarity between NPC1 and RND permeases suggested that NPC1 was a member of the RND family of transport proteins (6). This similarity is striking, and it seems likely that NPC1 is a member of this family of transporters. Nevertheless, members of this family transport many different substrates, and the structure does not suggest which substrate(s) NPC1 might transport. Our results do not dispute the possibility that NPC1 is a eukaryotic member of the RND permease family but do indicate that acriflavine is not an NPC1 substrate.

The second line of evidence is based on studies in a model system of E. coli-expressing human NPC1. The experiments with this model, in which E. coli have a leaky outer membrane, indicate that NPC1 transports oleic acid but not cholesterol. E. coli express a number of endogenous RND permeases that work in concert with membrane fusion proteins and outer membrane factors, and to facilitate movement of substrates across both membranes and the periplasmic space (21). Normally, NPC1 is located in the late endosome membrane, and putative substrates of NPC1 would only need to cross one membrane. In the modified E. coli environment, which is different from the normal environments of NPC1 as well as endogenous E. coli RND permeases, one cannot be certain that substrates found in this model are in fact endogenous substrates for NPC1. It is also possible that NPC1 is able to transport fatty acids in mammalian cells, but that this function is not the only method by which cells mobilize fatty acids from late endosomes and lysosomes.

The third line of evidence suggesting that NPC1 is a fatty acid transporter is based on accumulation of acriflavine, a cationic dye, in lysosomes of NPC fibroblasts (7). Our data in the same fibroblasts suggest that acriflavine accumulation is not specific for NPC1 mutations and that acriflavine is not accumulating in the same compartment as cholesterol in NPC cells.

Taken together, the results of our three experimental approaches indicate that NPC1 dysfunction does not result in a build up of fatty acids in human fibroblasts. The results do not dispute the idea that NPC1 is a member of the RND family of proteins, only that NPC1 is not solely responsible for the efflux of fatty acids from late endosomes and lysosomes. Therefore, we hypothesize that any NPC1 fatty acid efflux activity is unlikely to play a role in NPC disease pathology and that NPC1 is an RND permease with an unidentified endogenous substrate.

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