Trafficking defects in endogenously synthesized cholesterol in fibroblasts, macrophages, hepatocytes, and glial cells from Niemann-Pick type C1 mice

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Abstract **Niemann-Pick type C1 disease (NPC1) is an inherited neurovisceral lipid storage disorder, hallmarked by the intracellular accumulation of unesterified cholesterol and glycolipids in endocytic organelles. Cells acquire cholesterol through exogenous uptake and endogenous biosynthesis. NPC1 participation in the trafficking of LDL-derived cholesterol has been well studied; however, its role in the trafficking of endogenously synthesized cholesterol (endoCHOL) has received much less attention. Previously, using mutant Chinese hamster ovary cells, we showed that endoCHOL moves from the endoplasmic reticulum (ER) to the plasma membrane (PM) independent of NPC1. After arriving at the PM, it moves between the PM and internal compartments. The movement of endoCHOL from internal membranes back to the PM and the ER for esterification was shown to be defective in NPC1 cells. To test the generality of these findings, we have examined the trafficking of endoCHOL in four different physiologically relevant cell types isolated from wild-type, heterozygous, and homozygous BALB/c NPC1^{NIH} mice. The results show that all NPC1 homozygous cell types (embryonic fibroblasts, peritoneal macrophages, hepatocytes, and cerebellar glial cells) exhibit partial trafficking defects, with macrophages and glial cells most prominently affected. Our findings suggest that endoCHOL may contribute significantly to the overall cholesterol accumulation observed in selective tissues affected by Niemann-Pick type C disease.**—Reid, P. C., S. Sugii, and T-Y. Chang. **Trafficking defects in endogenously synthesized cholesterol in fibroblasts, macrophages, hepatocytes, and glial cells from Niemann-Pick type C1 mice.** ***J. Lipid Res.*** **2003.** 44: **1010–1019.**

Supplementary key words intracellular cholesterol trafficking • NPC disease • cholesterol biosynthesis • acyl-CoA:cholesterol acyltransferase • membrane recycling

Niemann-Pick type C (NPC) disease is a fatal autosomal recessive neurovisceral disorder characterized clinically by progressive neurodegeneration in the central nervous system (CNS) and hepatosplenomegaly. Biochemically, it is characterized by the intracellular accumulation of unesterified cholesterol and glycolipids within the endosomal/lysosomal system in cells of the liver, spleen, brain, etc. (1, 2). The disease can be caused by mutations in one of two genetically distinct loci, NPC1 and NPC2 (3, 4). The *Npc1* gene encodes a 1,278 amino acid multipass transmembrane protein that contains a putative sterol-sensing domain (5, 6). The *Npc2* gene encodes a soluble protein known as HE1, a lysosomal protein that binds cholesterol with high affinity and that can be secreted into the growth medium (7, 8). Mutations in *Npc1* account for 95% of all cases of NPC disease, whereas mutations at NPC2 account for the remaining 5% (5, 6). The NPC1 protein is involved in the tubulovesicular trafficking of cholesterol from the late endosome/lysosome to the plasma membrane (PM), and/or to the endoplasmic reticulum (ER) (9–14). These trafficking events may involve the *trans*-Golgi network/Golgi, and appear to be mediated by Rab proteins (9, 11, 15–17). Various glycolipids, including gangliosides, also accumulate in NPC1 cells; the role of NPC1 in causing the accumulation of glycolipids is under active investigation (18–20). In addition to NPC1 and NPC2, the late endosomal protein MLN 64, an integral membrane protein that contains the cholesterol binding domain StAR, has also been shown to be intimately involved in cholesterol trafficking (21).

Mammalian cells acquire cholesterol via exogenous uptake, mainly from LDL via the LDL receptor pathway. The involvement of NPC1 in distributing LDL-derived cholesterol to the PM and/or to the ER for esterification [by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT)] has been well-recognized [as reviewed in refs. (1, 2, 13)]. In

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Abbreviations: CD, cyclodextrin; CHO, Chinese hamster ovary; CNS, central nervous system; endoCHOL, endogenously synthesized cholesterol; ER, endoplasmic reticulum; MEF, mouse embryonic fibroblast; MPM, mouse peritoneal macrophage; NPC1, Niemann-Pick type C1; PM, plasma membrane.

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addition to exogenous uptake, cells also acquire cholesterol through endogenous biosynthesis de novo. The involvement of NPC1 in trafficking of endogenously synthesized cholesterol (endoCHOL) has received little attention. Because the major source of cholesterol in the CNS is provided through de novo endogenous cholesterol synthesis (22, 23), understanding the involvement of NPC1 in the trafficking of endoCHOL remains paramount to elucidating the etiology of NPC disease in the CNS. In cells, cholesterol is synthesized endogenously in the ER, and the majority of this newly synthesized cholesterol quickly moves to the PM within a few minutes, arriving at the cholesterol-rich domain caveolae (24–29). This initial movement of newly synthesized cholesterol to the PM is independent of NPC1 (30, 31). To pursue the post-PM fate of endoCHOL, this laboratory used a Chinese hamster ovary (CHO) cell mutant defective in the NPC1 protein (CT43) and its parental cell line (25RA) as the model system (31–33). To label the newly synthesized cholesterol, we incubated cells with [^{3}H]acetate, a precursor for cholesterol biosynthesis. To monitor the fate of labeled cholesterol, we performed chase experiments. The results confirmed that the movement of newly synthesized cholesterol from the ER to the PM is independent of NPC1. After initially reaching the PM, significant equilibration of endoCHOL between the PM and the internal membranes takes place within 8 h. In 25RA cells, endoCHOL internalizes from the PM to internal membranes, than recycles back to the PM; endoCHOL also moves to the ER to be esterified by ACAT. When cyclodextrin (CD), a water-soluble molecule that binds to cholesterol with high affinity, was added to the growth medium, the majority of [^{3}H]cholesterol resided in the PM and was largely susceptible to extraction by CD. In contrast, in CT43 cells, a significant portion of the [^{3}H]cholesterol accumulated in the late endosome/lysosome became less available for esterification in the ER and to extraction by CD (31). These studies, along with findings from other investigators (34), suggest that irrespective of the origin of cholesterol, the lack of a functional NPC1 protein invariably leads to intracellular cholesterol accumulation, mainly in the late endosome/lysosome. The pathways by which LDL-derived or newly synthesized cholesterol enters the late endocytic pathway may or may not be the same; the cellular nature of these pathways is under investigation in various laboratories.

Both the 25RA cells and the CT43 cells contain the same gain-of-function mutation in the protein called SCAP, the SREBP cleavage-activating protein that is involved in the transcription control of many sterol-sensitive genes (33, 35). It is thus possible that the findings made in 25RA and CT43 cells may not be applicable to other cell types. To test the generality of our findings, in the current work, we examined the role of NPC1 in the intracellular trafficking of endoCHOL in four different cell types isolated from the wild-type, the heterozygous, and the homozygous BALB/c NPC1^{NIH} mice. The cell types include mouse embryonic fibroblasts (MEFs), mouse peritoneal macrophages (MPMs), primary hepatocytes, and cerebellar glial cells. The murine model for the NPC disease, the BALB/c NPC1^{NIH} mouse, has a well-defined mutation in the NPC1 gene, and exhibits a phenotype very similar to that of the human NPC disease (5, 36–40).

MATERIALS AND METHODS

Materials

[^{3}H]acetate (20 Ci/mmol) and [^{3}H]cholesterol (60–90 Ci/mmol) were from American Radiolabeled Chemicals. 2-Hydroxypropyl-β-cyclodextrin, egg phosphatidylcholine, oleic acid, FBS, and trypsin were from Sigma. Medium RPMI-1640, stock solutions of penicillin/streptomycin, trypsin-EDTA, and HBSS were from GibcoBRL. DMEM plus sodium pyruvate and high glucose were from Cellgro. Collagen type I-coated 6-well plates were from Becton Dickinson's Biocoat. Tissue culture flasks or dishes were from Costar or Falcon. Nylon mesh (35 μm) for hepatocyte isolation was from Small Parts, Inc. (Miami Lakes, FL). Organic solvents were from Fisher.

Animal breeding

The NPC1 mutant mice (BALB/c NPC1^{NIH} mice) were discovered at the National Center for Toxicological Research, Little Rock, AR (41), and were generously provided by Peter Pentchev at the National Institutes of Health. NPC1^{NIH} homozygotes are reproductively incompetent because of their neurological impairment and short life span. Heterozygous BALB/c NPC1^{NIH} mice were used to generate the homozygous NPC1 mice and the wild-type (control) mice used in these studies. The genotypes of mice were determined from genomic DNAs isolated from tail snips, using a previously described PCR method (5, 42).

All experimental protocols were approved by the Institutional Animal Care and Research Advisory Committee at Dartmouth Medical School and conducted in accordance with the US Public Health Service Guide for the Care and Use of Laboratory Animals.

Cell isolation

Isolation of MEFs was performed according to a previously described procedure, with minor modifications (43). Briefly, at E17, mouse embryos were taken out of the uterus of pregnant females from genotype-confirmed heterozygous breeding pairs by cesarean section. For each embryo, the tail and a portion of a limb were removed and prepared for DNA extraction and genotyping. After dissection and incubation in 0.05% trypsin solution, the softened tissue was disrupted by repeated pipetting. Cell debris was separated, and the supernatants containing dissociated cells were plated in 25 cm^2 flasks, and grown for five successive passages before cells were used for experiments. Isolation of mouse resident (nonstimulated) peritoneal macrophages was performed according to the procedure previously described (44), from 7- to 8-week-old genotype-confirmed mice via peritoneal lavage. Isolation of mouse primary hepatocytes was performed via a two-stage liver perfusion protocol according to the procedure previously described (45, 46), from 7- to 8-week-old genotype-confirmed mice. Isolation of glial populations was performed according to the procedure previously described (47), with minor modifications. Briefly, cerebellums from 3- to 5-day-old genotype-confirmed animals were isolated and digested with collagenase (at 37°C for 30 min) and then with trypsin (at 37°C for 2 min) as described; the resultant cell homogenates were plated into 75 cm^2 flasks. To purify the cell population, the glial populations were grown for five successive passages before they were used for experiments. At this point the glial populations were comprised of at least 95% astrocytes, with microglia, oligodendroglia, and their precursor cells comprising less than 5% of the total cell population (47).

Cell culturing

MEFs (between passages 5–15) were maintained in DMEM containing 10% FBS in 75 cm^2 flasks. For experiments, they were seeded into 6-well plates. Freshly isolated macrophages were seeded at 1×10^6 cells into 6-well plates or 25 cm^2 flasks, in RPMI-1640 containing 10% FBS for experiments. After plating, they were allowed to adhere for 2 h; the nonadherent cells were washed away, and fresh media were added. Freshly isolated hepatocytes were seeded at 1×10^6 cells into collagen type I-coated 6-well plates in DMEM containing 10% FBS supplemented with 0.1 μM insulin and 0.1 μM dexamethasone. Hepatocytes were allowed to adhere for 4 to 6 h; nonadherent cells were washed away, and fresh media were added. Cell viability, tested via trypan-blue exclusion, was greater than 95%. Glial cells were maintained in 75 cm^2 flasks in DMEM containing 10% FBS, seeded into 6-well plates or 25 cm^2 flasks for experiments. All cell types were maintained in a humidified incubator at 37°C with 10% CO_2. Media with 10% FBS is referred to as Media A. The delipidated media used in various experiments, referred to as Medium D, consists of the same base medium used for each cell type, with the 10% FBS replaced with 5% delipidated FBS and 35 μM oleic acid. The delipidated FBS was prepared as described previously (48). All media contained penicillin/streptomycin.

Cholesterol-trafficking assays

On day 1, cells were seeded into 6-well plates and cultured in 2 ml Medium A/well for 24 h. On day 2, cells were washed once with prewarmed PBS (for MEF/MPM) or HBSS (for hepatocytes), and then fed with 2 ml/well of Medium D/well for 48 h. On day 4, cells were washed once with PBS/HBSS, and then subjected to various cholesterol trafficking assays as follows.

Assay A. This assay examines the efflux of endoCHOL to CD within 1 h after the newly synthesized cholesterol arrives at the PM. The cells were labeled with 20 μCi [^{3}H]acetate/well for 1 h at 37°C in Medium D, washed four times, and incubated in Medium D containing 4% CD for 0–30 min. At each time point, the media, along with two PBS washes, were collected. The cells were harvested in 0.2 N NaOH; HCl and phosphate buffer were then added to neutralize the cell lysates. The media and cell lysates were separately extracted with chloroform-methanol (2:1; v/v); the radiolabeled lipids were separated via thin-layer chromatography (TLC) and measured in a liquid scintillation counter as described previously (31).

Assay B. This assay examines the efflux of endoCHOL to CD after significant equilibration of endoCHOL between the PM and the internal membranes takes place. The cells were labeled with 20 μCi [^{3}H]acetate/well for 8 h at 37°C and chased in Medium D for 0 h or 16 h at 37°C. They were washed and then treated with Medium D containing 4% CD for 10 min at 37°C before being harvested and analyzed as described above. Here we utilized a short CD incubation period because we have found that short incubation periods with CD (≤10 min) remove cholesterol predominantly from the PM without inducing the efflux of cholesterol from internal membranes (results to be published from this laboratory).

Assay C. This assay examines the esterification of endoCHOL after a longer labeling period (8 h). The cells were labeled with [^{3}H]acetate in the same manner as described in Assay B. After labeling, cells were washed four times with buffer, and were either harvested in NaOH immediately (for the 0 h chase time point), or chased in Medium D for 16 h then harvested in NaOH (for the 16 h-chase time point). Labeled cholesteryl esters were extracted and analyzed as described previously (31). The percent of cholesterol efflux and percent of cholesterol esterification were calculated as previously described (31). Previous results showed that when 35 μM oleic acid was included in the medium, more than 98% of the ^{3}H present in the labeled cholesteryl ester derived from [^{3}H]acetate resided in the cholesterol moiety rather than the fatty acid moiety.

Assay D. This assay examines the esterification of cell surface-labeled cholesterol. The cells were labeled with [^{3}H]cholesterol containing liposome (49) for 30 min at 37°C in Medium D. Afterwards, samples were chased for 2 h or for 24 h as indicated, then processed to determine the radiolabeled cholesterol and cholesteryl ester as previously described (50). To determine the relative sterol biosynthesis rates, cells were cultured in Medium D for 48 h and then labeled with 30 μCi [^{3}H]acetate for 1 h at 37°C. Cells were washed four times with PBS/HBSS and then harvested with 1M KOH. Samples underwent saponification; the radioactive cholesterol bands were quantified after separation via TLC as previously described (51).

Percoll gradient analysis

Percoll gradient analysis was performed as previously described (20), with minor modifications. Briefly, cells cultured in one 150 mm dish were washed three times with cold PBS, scraped, and centrifuged at 4°C to collect the cells. The cell pellets were resuspended in buffer A (250 mM sucrose, 20 mM HEPES, and 1 mM EDTA, pH 7.3) and homogenized on ice with a Dura-Grind stainless-steel homogenizer. Postnuclear supernatants (800 μl) were layered onto 9 ml of 11% (v/v) Percoll in buffer A and centrifuged at 20,000 *g* for 40 min at 4°C using a Beckman model 50Ti rotor. Ten fractions were collected from the top. Control experiments showed that >80% of the PM marker (Na^+/K^+-ATPase α-1) was concentrated in fractions 1 and 2, whereas >80% of the late endosomal/lysosomal marker (LAMP-1) and the lysosomal marker (LAMP-2) were concentrated in fractions 9 and 10, consistent with results previously reported (50).

Statistical analysis

Statistical comparisons were made using a two-tailed, unpaired Student's *t*-test. The difference between two sets of values was considered significant when the *P* value was less than 0.01.

RESULTS

Earlier work has shown that when CHO cells are pulse-labeled with radiolabeled acetate at 14°C, the majority of the newly synthesized cholesterol remains in the cell interior as unesterified cholesterol. Upon warming the cells to 37°C, the labeled cholesterol moves to the PM within minutes (52). To monitor the arrival of newly synthesized cholesterol at the PM, we developed a pulse-chase protocol by pulse-labeling CHO cells with [^{3}H]acetate, then chasing cells in growth media containing CD and measuring CD-extractable cholesterol. Our results show that the movement of newly synthesized cholesterol to the PM is independent of NPC1 (31). In the current work, we attempted to apply the same protocol to mouse fibroblasts, macrophages, and hepatocytes, and found that when incubated with [^{3}H]acetate at 14°C for several hours, these cells were unable to synthesize a large amount of [^{3}H]sterol (results not shown). We therefore had to modify the protocol by incubating the cells with [^{3}H]acetate at 37°C for 1 h, and then chased the cells in the presence of CD for 0 to 30

min at 37°C (Assay A). This assay monitors the efflux of endoCHOL to CD within 1 h after endoCHOL arrives at the PM. The results (**Fig. 1A–C**) show that in all three cell types tested (MEFs, MPMs, and hepatocytes), no significant difference in cholesterol efflux to CD is observed between the $NPC^{+/+}$, $NPC^{+/-}$, and $NPC^{-/-}$ cells, confirming that the movement of newly synthesized cholesterol to the PM is independent of NPC1 in these cell types. In MEFs and MPMs, the cholesterol efflux in the $NPC^{-/-}$ and $NPC^{+/-}$ cells is slightly slower than that in the $NPC^{+/+}$ cells; however, the difference observed did not reach statistical significance.

In CHO cells, we have previously performed pulse-chase experiments and have shown that after arrival at the PM, the endoCHOL eventually moves back to the cell interior; significant equilibration of endoCHOL between the PM and internal membranes occurred in 8 h (31). On the basis of these findings, in the current study, we incubated the cells with [^{3}H]acetate at 37°C for 8 h, then chased the cells in unlabeled medium for either 0 h or 16 h, after which CD was added in growth media for 10 min at 37°C (Assay B). This assay monitors the availability of endoCHOL to CD-mediated efflux at the PM after equilibration of endoCHOL between the PM and internal membranes has occurred. Our results (**Fig. 2A–C**) show that in MEFs without any chase period, ~15% to 20% of

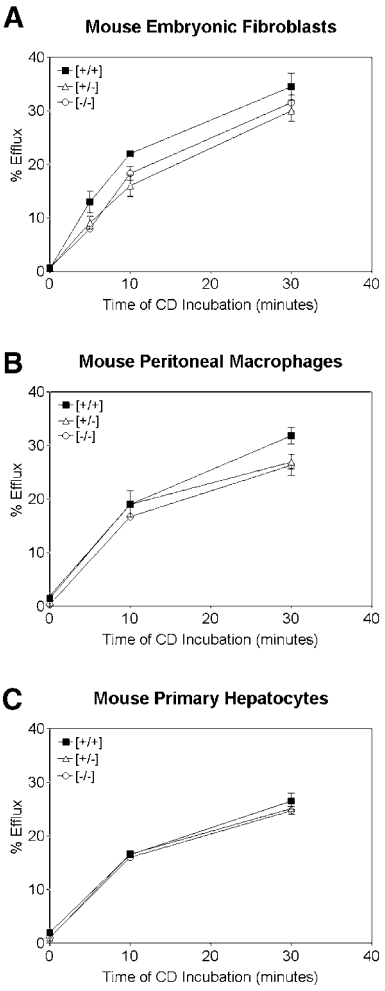

Fig. 1. Efflux of endogenously synthesized cholesterol (endoCHOL) to cyclodextrin (CD) after a short labeling period (1 h). Mouse embryonic fibroblasts (MEFs) (A), mouse peritoneal macrophages (MPMs) (B), and primary hepatocytes (C) were subjected to cholesterol trafficking Assay A as described in Materials and Methods. Each data point represents the mean and SEM of values from triplicate wells. Each cell population is representative of cells independently isolated from two embryos or two animals of the same genotype. The results are representative of two independent experiments. Symbols used in this and other figures: ($^{+/+}$), WT; ($^{+/-}$), heterozygous in Niemann-Pick type C1 (NPC1) mutation; ($^{-/-}$), homozygous in NPC1 mutation. Sterol synthesis rates (expressed as dpm/μg protein/h) in $NPC^{+/+}$, $NPC^{+/-}$, and $NPC^{-/-}$ cells were as follows: for MEFs, 5.25 ± 0.32, 5.38 ± 0.33, and 2.33 ± 0.25, respectively; for MPMs, 26 ± 0.86, 14.28 ± 0.38, and 15.83 ± 0.92, respectively; and for hepatocytes, 3.51 ± 0.62, 3.68 ± 0.46, and 3.82 ± 0.74, respectively.

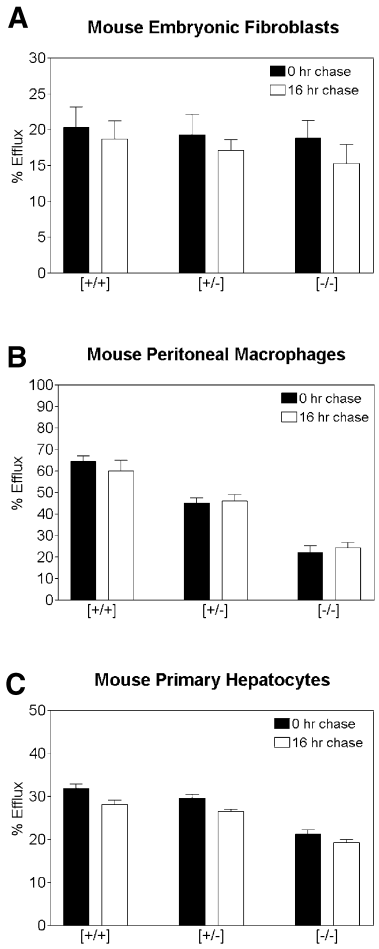

Fig. 2. Efflux to CD after a long labeling period (8 h). MEFs (A), MPMs (B), or primary hepatocytes (C) were subjected to cholesterol trafficking Assay B as described in Materials and Methods. Each data point represents the mean and SEM of values from triplicate wells, utilizing cells independently isolated from three animals of the same genotype to represent each cell population. The results are representative of three independent experiments. Statistical analysis was performed as described in Materials and Methods. There was no statistically significant difference in values for $^{+/+}$, $^{+/-}$, and $^{-/-}$ MEFs, whereas differences in values for $^{+/+}$, $^{+/-}$, and $^{-/-}$ MPMs and hepatocytes were statistically significant.

the labeled cholesterol is extractable by CD. The cholesterol efflux in NPC$^{-/-}$ MEFs is essentially the same as that of NPC$^{+/+}$ MEFs and NPC$^{+/-}$ MEFs. After a 16 h chase, the cholesterol efflux in the NPC$^{-/-}$ MEFs is slightly lower than that of NPC$^{+/+}$ or NPC$^{+/-}$ MEFs; however, this difference was not statistically significant. In contrast, in NPC$^{+/+}$ MPMs with no chase or with a 16 h chase, ~59% to 64% of the labeled cholesterol is extractable by CD; the efflux values significantly decrease (by 25% to 30%) in NPC$^{+/-}$ MPMs, and decrease even further (by 65% to 70%) in NPC$^{-/-}$ MPMs. In NPC$^{+/+}$ hepatocytes with no chase or with a 16 h chase, ~28% to 32% of the labeled cholesterol is extractable by CD; the efflux values slightly decrease (by 5%) in NPC$^{+/-}$ hepatocytes, although the difference was not statistically significant. The efflux values decrease further (by 30% to 32%) in NPC$^{-/-}$ hepatocytes, and the difference observed was statistically significant.

We have previously shown in parental 25RA CHO cells that 8 h or longer after arrival at the PM, a certain portion of the endoCHOL moved back to the ER and was esterified by the ER resident enzyme ACAT1 (31). This post-PM esterification was shown to be defective in NPC1-deficient CT43 CHO cells (31). To monitor the post-PM esterification of endoCHOL in MEFs, MPMs, and hepatocytes, we incubated the cells with [^{3}H]acetate at 37°C for 8 h, then chased the cells in unlabeled medium for either 0 h (closed bars) or 16 h (open bars). Labeled cellular lipids were extracted and subjected to TLC analysis to quantify the [^{3}H]cholesteryl esters formed (Assay C). The results (**Fig. 3A–C**) show that in NPC$^{+/+}$ MEFs with no chase, ~9% of the [^{3}H]cholesterol is esterified; the percent of esterification is slightly reduced in both NPC$^{+/-}$ MEFs (by 12% to 15%) and NPC$^{-/-}$ MEFs (by 13% to 18%). These differences are not statistically significant. After a 16 h chase, the percent of esterification decreases to ~7% in NPC$^{+/+}$ MEFs; the values found in NPC$^{+/-}$ and NPC$^{-/-}$ MEFs are essentially the same. In NPC$^{+/+}$ MPMs with no chase, ~17% of [^{3}H]cholesterol is esterified. With a 16 h chase, the percent of esterification decreases to ~13%. The percent of esterification values significantly decrease in NPC$^{+/-}$ MPMs (by 45% to 47%) and decrease further in NPC$^{-/-}$ MPMs (by 70%). In NPC$^{+/+}$ hepatocytes with no chase, ~11% of [^{3}H]cholesterol is esterified; after a 16 h chase, the value decreases to ~8%. The percent of esterification values significantly decrease in NPC$^{+/-}$ hepatocytes (by 10% to 22%) and decrease further in NPC$^{-/-}$ hepatocytes (by 23% to 35%). These findings collectively support the functional involvement of NPC1 in the post-PM trafficking of endoCHOL from the PM to the ER, and suggest that endogenous cholesterol could be contributing to the cholesterol accumulation characteristic of NPC disease.

In light of the recent reports suggesting the increasingly important role that glial cells may play in neuronal cell maintenance (53–55), we isolated cerebellar glial cells from NPC$^{+/+}$, NPC $^{+/-}$, and NPC $^{-/-}$ mice, and used Assays A–C to monitor the intracellular trafficking of endoCHOL. The results of using Assay A (**Fig. 4A**) show that

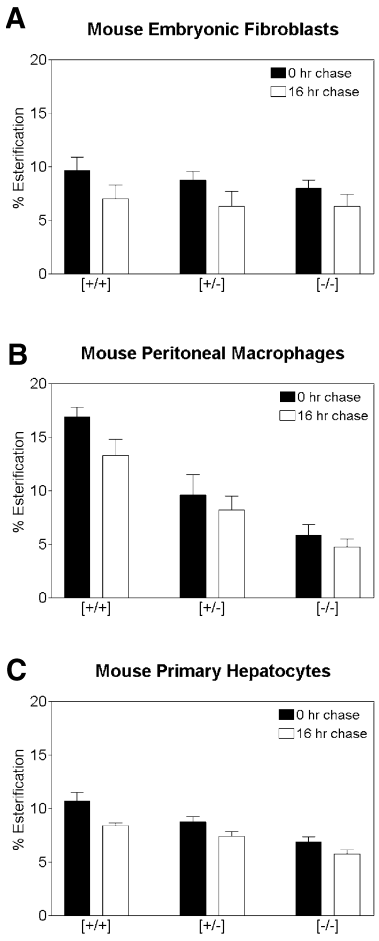

Fig. 3. Esterification of endoCHOL after a long labeling period (8 h). MEFs (A), MPMs (B), and primary hepatocytes (C) were subjected to cholesterol trafficking Assay C as described in Materials and Methods. Each data point represents the mean and SEM of values from triplicate wells, utilizing cells independently isolated from three animals of the same genotype to represent each cell population. Results are representative of three independent experiments. There was no statistically significant difference between $^{+/+}$, $^{+/-}$, and $^{-/-}$ MEFs, whereas differences observed between $^{+/+}$, $^{+/-}$, and $^{-/-}$ MPMs and hepatocytes were statistically significant.

the efflux to CD of endoCHOL within 1 h after its arrival at the PM is essentially the same in NPC$^{+/+}$, NPC $^{+/-}$, and NPC $^{-/-}$ cells; ~15% to 20% of the labeled cholesterol is extractable by CD within 10 min. Utilizing Assay B (Fig. 4B), we show that after allowing ample time for endoCHOL to internalize to the cell interior, its availability to CD-mediated efflux is severely defective in NPC$^{-/-}$ glial cells. In the NPC$^{+/+}$ cells, ~37% to 42% of the labeled cholesterol is extractable by CD within 10 min. The values decrease by 8% to 12% in the NPC$^{+/-}$ cells, and decrease further (by 46%) in the NPC$^{-/-}$ cells; in both cases, the differences seen are statistically significant. The results of Assay C (Fig. 4C) show that the percent of esterification of endoCHOL in NPC$^{+/+}$, NPC $^{+/-}$, and NPC $^{-/-}$ glial cells was very low, ranging between 4% and 6%, with no statistically significant difference observed among cells with different genotypes.

Thus far we have shown that the trafficking of newly

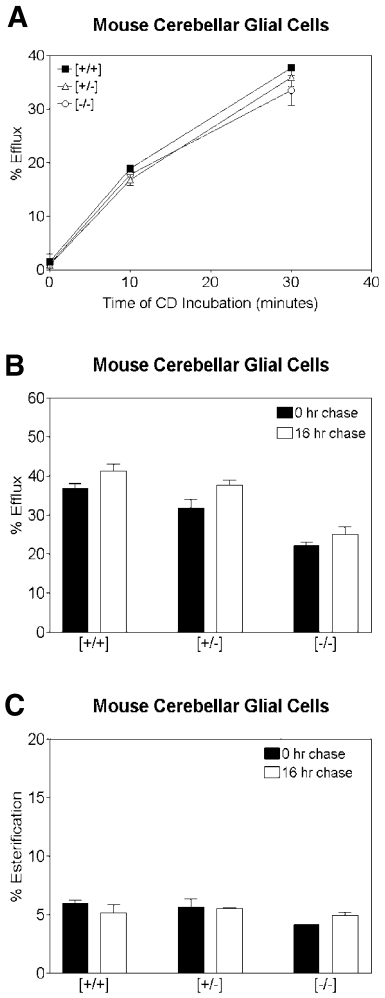

Fig. 4. Efflux of endoCHOL after short labeling period (2 h) or long labeling period (8 h), and esterification of endoCHOL in glial cells. Glial cells were subjected to cholesterol trafficking Assays A (A), B (B), or C (C) as described in Materials and Methods. Each data point represents the mean and SEM of values from triplicate wells, utilizing cells independently isolated from three animals of the same genotype to represent each cell population. The results are representative of three independent experiments. In B, the differences observed between $^{+/+}$, $^{+/-}$, and $^{-/-}$ glial cells were statistically significant. Sterol synthesis rates (expressed as dpm/μg protein/h) in NPC$^{+/+}$, NPC$^{+/-}$, and NPC$^{-/-}$ glial cells were 18.21 ± 2.12, 18.01 ± 1.78, and 19.75 ± 2.65, respectively.

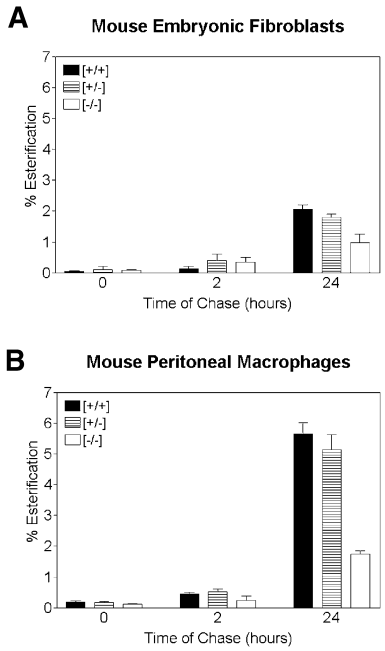

Fig. 5. Esterification of cell surface-labeled cholesterol. MEFs (A) and MPMs (B) were subjected to cholesterol trafficking Assay D. Each data point represents the mean and SEM of triplicate wells. Each cell population is representative of cells independently isolated from two embryos or two animals of the same genotype. The results are representative of two independent experiments. For the 24 h time point, there was no statistically significant difference between $^{+/+}$ and $^{+/-}$ MEFs and MPMs, whereas the differences observed between $^{+/+}$ and $^{-/-}$ MEFs and MPMs were statistically significant.

synthesized cholesterol is severely perturbed in MPMs, yet MEFs exhibit only a mild defect. Using CHO cells and human fibroblast cells, we had previously shown that the percent of esterification of cell surface-labeled cholesterol (representing the bulk of PM cholesterol) was defective in NPC1 cells (50). To test the generality of this finding, we labeled MEFs or MPMs with liposome-containing [^{3}H]cholesterol. Cells were subsequently harvested at zero time, or chased for 2 h or 24 h as indicated, then harvested to determine the radiolabeled cholesterol and cholesteryl ester (Assay D). The results (**Figs. 5A**, **B**) show that for both cell types, no significant esterification is observed at zero time or after a 2 h chase. After a 24 h chase, in MEFs, ~2% of the labeled cholesterol is esterified in NPC$^{+/+}$ cells. The value is essentially the same in NPC $^{+/-}$ cells, but significantly decreases (by 45%) in NPC$^{-/-}$ cells. In MPMs, ~5.6% of the labeled cholesterol is esterified in NPC$^{+/+}$ cells. This value is essentially the same in the NPC$^{+/-}$ cells, but significantly decreases (by 65%) in the NPC$^{-/-}$ cells.

In our NPC1-deficient CHO cells (CT43 cells), after a long chase period (24 h), a significant amount of endoCHOL was found to accumulate in the internal membrane fraction, as demonstrated by subcellular fractionation analysis using Percoll gradient centrifugation (31). To test the generality of this finding, we labeled MEFs or MPMs with [^{3}H]acetate for 24 h. Cells were subsequently harvested and processed for Percoll gradient centrifugation analysis, and the percent of distribution of radiolabeled cholesterol in each fraction was determined. In both NPC$^{+/+}$ and NPC$^{-/-}$ MEFs (**Fig. 6A**), the majority of labeled cholesterol is located in the lighter fractions (fractions 2–4), which are rich in PM and early endosomes (31). We observed no major differences between the NPC$^{+/+}$ and NPC$^{-/-}$ MEFs in endoCHOL distribution profiles, except a slight increase of labeled cholesterol in both the very buoyant fraction (fraction 1) and the heavy fraction (fraction 10) in NPC$^{-/-}$ MEFs. In striking contrast, we observed significant differences between the NPC$^{+/+}$ and NPC$^{-/-}$ MPMs in endoCHOL distribution profiles. In NPC$^{+/+}$ macrophages (Fig. 6B), the majority

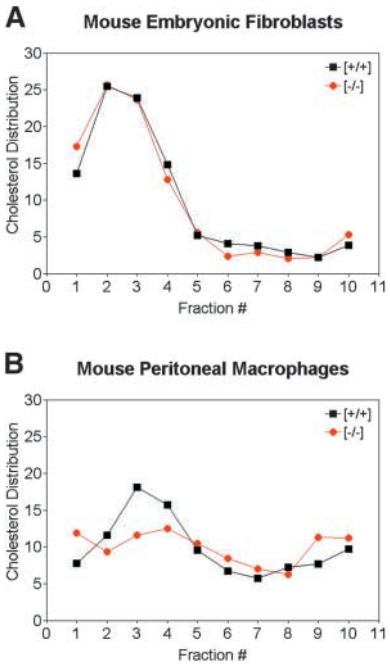

Fig. 6. Subcellular fractionation analysis of ^{3}H-labeled cholesterol by Percoll gradient centrifugation. MEFs (A) and MPMs (B) were labeled with [^{3}H]acetate for 24 h at 37°C. Cells were homogenized, and the postnuclear supernatants were subjected to 11% Percoll gradient centrifugation analysis as described in Materials and Methods and previously (50). Data shown are representative of two independent experiments.

of labeled cholesterol is located in the lighter fractions (fractions 2–4) as well as in the denser fractions (fractions 8–10). In NPC$^{-/-}$ macrophages, significantly less labeled cholesterol is found in fractions 2–4, whereas significantly more label is found in fractions 9 and 10, which are rich in late endosome/lysosomes (31). In addition, significantly more label was also found in the very buoyant fraction (fraction 1) in NPC$^{-/-}$ macrophages. This fraction may contain the abnormal, buoyant lysosome that was previously shown to be present in cells with the NPC1 mutation (56, 57). EndoCHOL distribution profiles for heterozygous MEFs and MPMs were essentially the same as those of wild-type MEFs and MPMs, and were omitted from Fig. 6 for simplicity.

DISCUSSION

Previously, we have shown that mutant CHO cells (CT43), which contain a well-defined mutation in the *Npc1* gene (50), are severely defective in the post-PM esterification and efflux of endoCHOL, compared with the parental 25RA cells (31). Both the CT43 and 25RA cells contain a gain-of-function mutation in the protein SCAP. SCAP is a key protein that mediates the sterol-dependent transcriptional regulation of a variety of sterol-specific genes (33, 35). Because of the SCAP mutation, the endogenous cholesterol biosynthesis in 25RA and CT43 cells is resistant to down-regulation by sterol present in the growth medium. Therefore, it is important to test the validity of information derived from studying 25RA and CT43 cells in other relevant cell populations.

In the current manuscript, we have utilized pulse-chase methods to examine the role of NPC1 in the trafficking of endoCHOL in four different cell types isolated from NPC$^{+/+}$, NPC$^{+/-}$, and NPC$^{-/-}$ mice. Our results demonstrate that the trafficking of endoCHOL to the PM is normal in each of the NPC1 cell types examined, while the subsequent post-PM trafficking of endoCHOL to the ER (as judged by ACAT esterification), and the recycling of endoCHOL back to the PM (as judged by efflux to CD) were partially defective. The extent of defect is cell type dependent, with MPMs the most severely affected, glial cells and hepatocytes moderately affected, and MEFs only mildly affected. Subcellular fractionation analysis demonstrated that the accumulation of endoCHOL in fractions rich in late endosome/lysosome and in buoyant lysosome was much more prominent in NPC$^{-/-}$ MPMs than in NPC$^{-/-}$ MEFs. Thus, the trafficking defects in NPC1 cells observed could be attributed to the sluggish release of endoCHOL from the late endosome/lysosome. Overall, these results support and extend our previous studies using the CHO cells 25RA and CT43 (31). It has been reported that the liver cholesterol concentration increases almost 10-fold in the NPC$^{-/-}$ BALB/c NPC^{NIH} mouse (37). Our current results suggest that endoCHOL likely contributes to the abnormal cholesterol accumulation observed in the liver and in other selective tissues affected by NPC disease. Interestingly, our finding that the involvement of NPC1 in the trafficking of endoCHOL is cell type dependent correlates with observations at the in vivo level: studies in human NPC1 patients and in the BALB/c NPC1^{NIH} mouse model have shown that various cell populations exhibit varying levels of lipid storage. Among the cell types examined in the current study, macrophages have the largest amounts, glial cells and hepatocytes intermediate amounts, and fibroblasts the least amount of the stored material (2). Since the trafficking defects in various NPC1 cell types examined are only partial, these results imply that trafficking of endoCHOL independent of NPC1 exists in various cells; such event(s) may be particularly more prominent in fibroblasts. This interpretation is consistent with the work of Underwood and colleagues, who showed that in NPC1 mutants, a certain portion of LDL-derived cholesterol could be esterified in an NPC1-independent manner (58). The finding that the involvement of NPC1 in the trafficking of endoCHOL is cell type dependent is not surprising. Other investigators have shown that quantitative differences in the regulation of cholesterol metabolism exist in a cell type-dependent manner. For example, Tabas and colleagues (59) and Havekes and colleagues (60) showed that, in contrast to the human fibroblast cells, mouse macrophages and hepatocyte-like HepG2 cells exhibited a partial resistance phenotype in terms of sterol-dependent down-regulation of the LDL receptor activity.

Regarding the heterozygosity of the NPC1 mutation in affecting CHOL trafficking, the results presented in this

work show that the partial defect was readily demonstrable in the NPC$^{+/-}$ macrophages but not as readily demonstrable in the NPC$^{+/-}$ glial cells or hepatocytes. Earlier, Chen and colleagues (61) and Tall and colleagues (62) showed that the defect in apoA-I-mediated cholesterol efflux could be readily demonstrated in macrophages isolated from the NPC$^{+/-}$ mice. Thus, it appears that heterozygosity in the NPC1 mutation may also affect intracellular cholesterol trafficking in a cell type-dependent manner. Why are macrophages more sensitive to the NPC1 mutation than other cell types, and why should the extent of defect in endoCHOL be cell type specific? It is possible that cells engaged in utilizing PM for various purposes, such as phagocytosis or exocytosis, may have a higher cholesterol-recycling rate between the PM and internal membranes, and may depend more on NPC1 to maintain a high cholesterol-recycling rate. Cells that have a lower cholesterol-recycling rate may not depend on NPC1 as much for such activity. The cell type-dependent defect observed in the current work may also be explained in part by the following scenario: the cells used in our current study were maintained in FBS-containing medium, then incubated in lipid-free medium for 2 days before the assay began. By using a very sensitive intracellular cholesterol stain, BC-θ, we recently showed that mouse NPC1 MEFs grown under this condition still contained a significant amount of cholesterol accumulated in the late endosome/lysosome (63). Similar results were obtained in the NPC1 MPMs, hepatocytes, and glial cells (P. C. Reid, S. Sugii, and T-Y. Chang, unpublished observations). It is thus possible that in addition to cholesterol, other lipid material (such as sphingomyelin and/or gangliosides, etc.) may also be present in the late endosome/lysosome of various NPC1 cells. This material may interact with endoCHOL arriving at the late endosome/lysosome and influence its accumulation in a cell type-specific manner. The two possible mechanisms described above are not mutually exclusive; they may act synergistically to cause the overall lipid accumulation in the late endosome/lysosome system.

NPC is a neurodegenerative disorder. The major source of cholesterol in the CNS is provided through de novo cholesterol synthesis. It has been suggested that astrocytes may play a larger role in the pathology of NPC disease than has been previously suspected (53–55). In the current manuscript, we show that in glial cells, the post-PM recycling of endoCHOL is significantly defective in NPC$^{-/-}$ cells. Interestingly, we found esterification rates in all genotypes to be low, suggesting that in glial cells, NPC1 appears to be primarily involved in cholesterol recycling between internal compartment(s) and the PM, and less involved in delivering cholesterol to the ER for esterification. Previously, Suresh and colleagues showed that in NPC$^{-/-}$ glial cell populations, the secretion of apoD was defective, whereas the secretion of apoE was normal (64). Recently, it has been shown that glial-secreted cholesterol, associated with lipoprotein complexes, is the critical factor required for functional and efficient neuronal synaptogenesis (53). Taken together, these findings suggest that defects in cholesterol trafficking in glial cells may result in the defective secretion of these critical lipoprotein-cholesterol complexes, and may contribute to the neurodegeneration observed in NPC disease. It has been suggested that genetic or age-related defects in the synthesis, transport, or uptake of cholesterol in the CNS may directly impair the development and plasticity of the synaptic circuitry (53). Such impairment would likely contribute to the early axonal atrophy and subsequent neurodegeneration characteristic of NPC disease (65, 66). Therefore, we believe that in addition to neurons (42, 67, 68), cholesterol trafficking in glial cells may be defective in vivo, and this deficiency would likely represent an important contributing factor in the etiology of NPC disease. Whether the cholesterol accumulated in neurons originates from newly synthesized cholesterol from neurons themselves, or is provided by astrocytes remains to be determined. The current study involves the use of the cell culture system in vitro. It remains to be determined whether a defect in the trafficking of lipoprotein-bound cholesterol, and/or endoCHOL, and/or other lipid material is the primary cause for the overall lipid accumulation observed in various tissues affected by the NPC1 mutation in vivo.

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