Dynamics of lysosomal cholesterol in Niemann-Pick type C and normal human fibroblasts

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Abstract The dynamics of endolysosomal cholesterol were investigated in Niemann-Pick type C (NPC) cells and in human fibroblasts treated with class 2 amphiphiles to mimic NPC cells. We showed through new approaches that the massive pools of endolysosomal cholesterol in these cells are not trapped but, rather, circulate to the cell surface at about the normal rate. This flux spared NPC and amphiphile-treated cells from disruption by the extraction of their plasma membrane cholesterol with cyclodextrin. Nocodazole, a microtubule-depolymerizing agent, reversed the resistance of NPC and U18666A-treated cells to cholesterol depletion, apparently by reducing the flux of endolysosomal cholesterol to the plasma membrane. Neither nocodazole nor bafilomycin A1 (an inhibitor of the vacuolar proton pump) acted in the same way as the NPC mutation or class 2 amphiphiles: both agents decreased plasma membrane cholesterol at the expense of the endolysosomal pool and both blocked the actions of the amphiphile, U18666A. Finally, the resistance of NPC cells to lysis by amphotericin B was shown not to reflect a reduction in plasma membrane cholesterol arising from a block in lysosomal cholesterol export but rather the diversion of the amphotericin B to cholesterol-rich endolysosomes. We conclude that the large pool of endolysosomal cholesterol in NPC and amphiphile-treated fibroblasts is dynamic and that its turnover, as in normal cells, is dependent on microtubules.—Lange, Y., J. Ye, M. Rigney, and T. L. Steck. **Dynamics of lysosomal cholesterol in Niemann-pick type C and normal human fibroblasts.** ***J. Lipid Res.*** **2002.** 43: **198–204.**

Supplementary key words cholesterol traffic • endocytosis • endosomes • homeostasis • lysosomes

The circulation of cholesterol between late endosomes/lysosomes (LE/L) and the cell surface is of increasing interest because of its relationship to endomembrane traffic (1, 2). These compartments receive cholesterol both from ingested plasma lipoproteins and from the endocytic plasma membrane bilayer; the internalized cholesterol is promptly returned to the cell surface (3). A vesicular shuttle is likely to be involved; however, the pathways traversed by the cholesterol are obscure (4, 5).

Cholesterol accumulates to massive levels in the LE/L of Niemann-Pick type C (NPC) cells because of the absence of an integral endomembrane vesicle protein, NPC1 (6). It has been widely inferred that there is a block in the export of ingested lipoprotein cholesterol from the LE/L in these cells (5). However, recent evidence suggests that cholesterol is not trapped in intracellular compartments and that the accumulated endolysosomal cholesterol is derived predominantly from the plasma membrane (3, 7). Fibroblasts treated with a variety of drugs called class 2 amphiphiles show similar endolysosomal cholesterol accumulation (3, 8, 9), suggesting that these agents act upon NPC1 or its pathway (7).

Much of the current work on endolysosomal cholesterol in these cells relies on its staining by the specific fluorescent probe, filipin. This approach, however powerful, does not readily provide quantitation of the rates of transfer between the compartments in question. We have therefore explored new ways to assess the movement of endolysosomal cholesterol to the plasma membrane in NPC and amphiphile-treated cells. Our basic strategy was to extract the cell surface cholesterol with cyclodextrin and follow its replenishment from intracellular compartments by functional and biochemical analyses.

MATERIALS AND METHODS

Materials

[1α,2α-^{3}H]cholesterol was from Amersham Pharmacia Biotech (Piscataway, NJ). Lipoprotein-deficient serum (LPDS), filipin, and amphotericin B were from Sigma (St. Louis, MO). Hydroxypropyl-β-cyclodextrin (HPCD) and methyl-β-cyclodextrin (MBCD) were from Research Plus, Inc. (Bayonne, NJ). Nocoda-

Abbreviations: CD, cyclodextrin; ER, endoplasmic reticulum; HPCD, hydroxypropyl-β-cyclodextrin; LE/L, late endosomes/lysosomes; LPDS, lipoprotein-deficient serum; MBCD, methyl-β-cyclodextrin; NPC, Niemann-Pick type C.

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zole was from Calbiochem (San Diego, CA). U18666A was provided by Pharmacia and Upjohn Co. (Kalamazoo, MI).

Cell culture and treatments

Normal human foreskin fibroblasts were obtained as described (10). Human NPC fibroblast lines 93.41 and 93.59 were generously provided by Peter Pentchev (NIH). Both lines lack detectable NPC1 protein (P. Pentchev, personal communication). The two lines behaved similarly in this study, except that 93.59 had a lower intracellular cholesterol level and showed less dramatic resistance to cholesterol extraction. Therefore, we only present experiments done with line 93.41. Cells were cultured in DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. For cholesterol depletion experiments, the FBS was replaced by 5% LPDS. Controls for drug treatments utilized solvent at <1% of volume.

Cholesterol distribution

The distribution of cholesterol mass between the cell surface and interior was measured as described recently (7). Briefly, the cells were labeled with a pulse of exogenous [^{3}H]cholesterol and then extracted with MBCD. Cholesterol distribution was calculated from the cholesterol mass and label in the extract and residual cells. Cholesterol and protein masses were determined as described (3). The P values in all paired t-tests of data from which inferences were drawn were 0.05 or less.

RESULTS

Resistance to cyclodextrin toxicity of NPC and amphiphile-treated cells

A variety of cyclodextrins have been shown to extract a significant fraction of cell surface cholesterol and thereby undermine cell integrity (11). In our system, ~25–50% of plasma membrane cholesterol was extracted from normal and NPC fibroblasts in 1 or 2 h by 1–2% HPCD and, even more efficiently, by MBCD (Fig. 3) (7, 11). We reasoned that if the large pool of endolysosomal cholesterol in NPC and amphiphile-treated cells were dynamic, it would replenish the extracted plasma membrane cholesterol and offset cyclodextrin injury.

Cyclodextrin extraction caused minimal changes in the appearance of NPC cells compared with the conspicuous distortion of controls. After mild treatments, the control cells typically became contracted (shriveled) with refractile zones, whereas NPC cells were unchanged (**Fig. 1**). Following vigorous extraction (e.g., 1–2% MBCD for 4 h), control fibroblasts did not recover during an overnight incubation without cyclodextrin. In contrast, NPC cells and fibroblasts pretreated with the class 2 amphiphile, U18666A, either retained their normal appearance throughout or underwent an initial small morphological change that reversed completely during the overnight chase. The presence of mevinolin in several of these experiments ruled out biosynthesis as a source of plasma membrane cholesterol replenishment.

Damage was also assessed by following the release of lactate dehydrogenase, a cytosolic protein, following extraction of cells with cyclodextrin. As seen in **Fig. 2**, a few hours of incubation with 1% MBCD caused leakage from normal cells but not from NPC cells or from fibroblasts that had been pretreated with the class 2 amphiphile, U18666A.

Transfer of endolysosomal cholesterol to the plasma membrane

We quantitated the replenishment of plasma membrane cholesterol following extraction of NPC cells with cyclodextrin. NPC cells have a near-normal plasma membrane cholesterol level, ~35 μg/mg protein (Figs. 3, 4, 6, and 7) (7). A brief extraction decreased this pool by ~50% without significant loss from the intracellular pools (**Fig. 3**, open bar). During a subsequent chase (Fig. 3, hatched bar), comparable amounts of cholesterol were lost from the intracellular compartments and gained by the plasma membrane.

Effect of nocodazole on endolysosomal cholesterol

We investigated the role of microtubules in the export of cholesterol from endocytic compartments (12). First, the microtubule disruptor, nocodazole, was shown to suppress the loss of endolysosomal cholesterol from NPC cells during a 4 h extraction of the cell surface with MBCD (**Fig. 4**). (The extraction of plasma membrane cholesterol

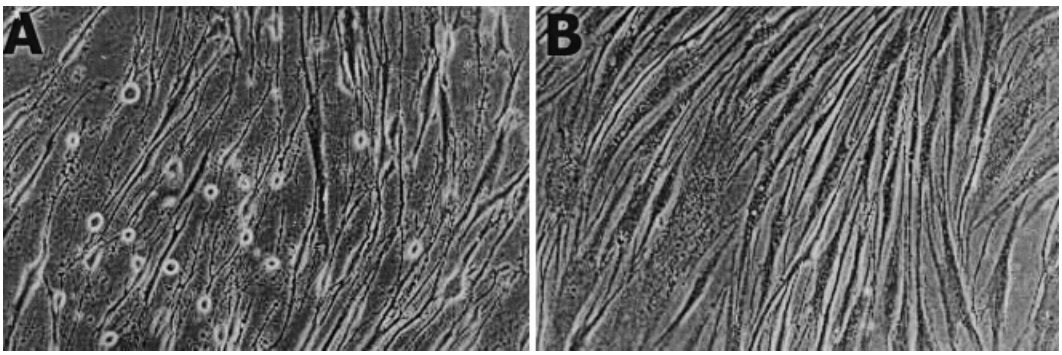

Fig. 1. Effect of cholesterol extraction on the morphology of normal and NPC fibroblasts. Culture flasks were incubated overnight in medium containing 5% LPDS plus 25 μM mevinolin and 2% hydroxypropyl-β-cyclodextrin (HPCD). The cells were rinsed, fixed with 1% glutaraldehyde for 10 min at room temperature, and examined by phase contrast microscopy. A: Normal fibroblasts; B: NPC fibroblasts. Unextracted control and NPC cells had a normal fibroblast morphology indistinguishable from that seen in B; no change was observed over time in unextracted cells.

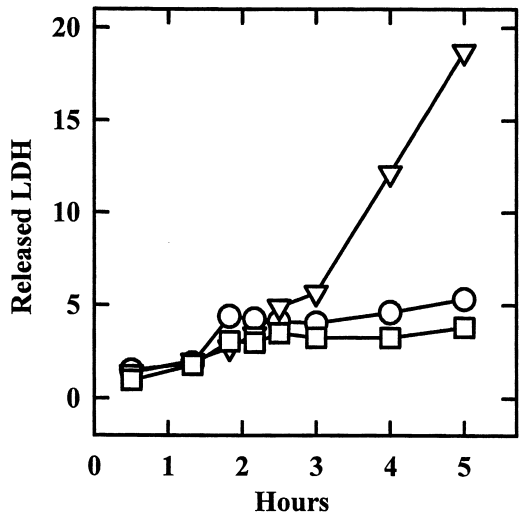

Fig. 2. Effect of cholesterol extraction on the release of a cytoplasmic protein from normal and U18666A-treated fibroblasts. Culture flasks of human fibroblasts were preincubated for 2 days in growth medium with (open square) or without (inverted open triangle) 5 μM U18666A. NPC fibroblasts (open circle) were grown in parallel. The medium was replaced with PBS containing 1% MBCD and the incubation continued at 37°C. At the times indicated, aliquots were spun and the supernatants assayed for lactate dehydrogenase (LDH) activity. Values are in arbitrary units; approximately 30% of cellular LDH was released from normal fibroblasts in such experiments. The release of LDH from unextracted fibroblasts after 5 h was typically ~10% that of extracted cells. Shown is an experiment representative of three.

was not significantly reduced by nocodazole.) As predicted from this finding, microscopic assay showed that nocodazole also increased the susceptibility of NPC and U18666A-treated cells to the toxic effects of cyclodextrin extraction, but not to the level of control fibroblasts (not shown).

The inhibition of cholesterol loss from the LE/L by nocodazole could have resulted from reduced egress, increased delivery to the LE/L, or both. In particular, the return of endocytic plasma membrane cholesterol to the cell surface might be blocked by nocodazole in an early (upstream) compartment, increasing its movement downstream to endolysosomes by default (1, 2, 7). To examine this possibility, we labeled the surfaces of NPC cells with [^{3}H]cholesterol and, after a chase period, determined the fraction of radioactivity that had moved to the endolysosomal compartment (**Fig. 5**). In three such experiments, nocodazole did not significantly alter the labeling of the buoyant, endolysosomal fraction (expressed either in total dpm or dpm/μmol cholesterol). The specific activity in the lysosomal fraction was 50 ± 3% that in the plasma membrane fraction after the 29 h chase in both the presence and absence of nocodazole. The apparent transfer rate agrees well with the first-order rate constant previously observed for the isotopic equilibration of cholesterol between these donor and acceptor compartments (3). Because the rate of delivery of plasma membrane cholesterol to the LE/L was not altered, we infer that nocodazole inhibits the transport of intracellular cholesterol to the cell surface.

These findings raise the possibility that nocodazole also blocks the egress of endocytic cholesterol in normal fibroblasts and causes its intracellular accumulation (12). In a test of this hypothesis, we showed that an overnight incubation of normal fibroblasts with nocodazole caused a doubling of intracellular cholesterol and a commensurate decrease in plasma membrane cholesterol of ~15% (Fig. 6). Total cell cholesterol was not changed by this treatment; namely, there was 32 ± 1 μg cholesterol/mg protein in the control cells compared with 32 ± 3 (n = 3) in the treated cells. The accumulation of cholesterol in the LE/L

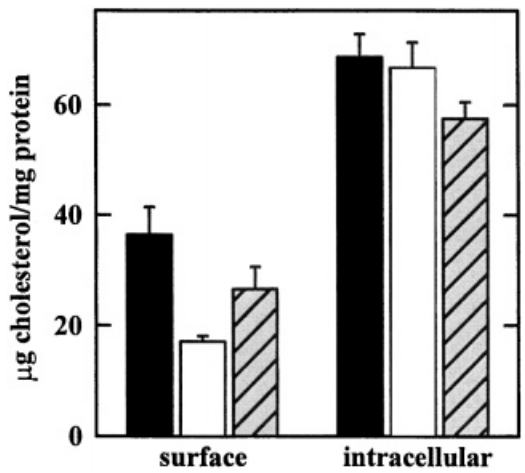

Fig. 3. Distribution of cholesterol in Niemann-Pick Type C (NPC) cells following plasma membrane depletion. Replicate flasks were incubated for 45 min at 37°C in PBS containing 0% or 2% methyl-β-cyclodextrin (MBCD) and then chased for 2 h in medium containing 5% lipoprotein-deficient serum (LPDS) but lacking MBCD. Total, plasma membrane, and intracellular cholesterol were then determined. Minus MBCD; no chase (solid bar). Plus MBCD; no chase (open bar). Plus MBCD; plus chase (hatched bar).

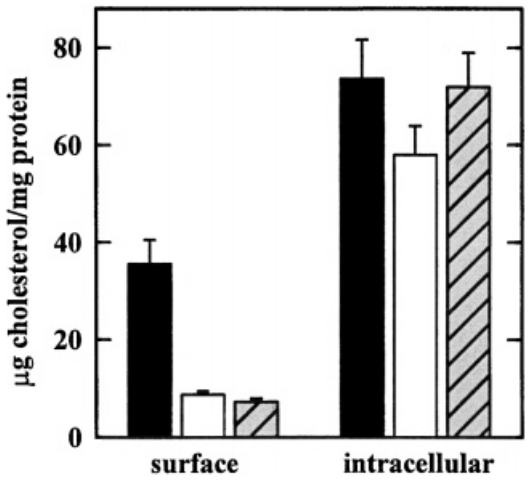

Fig. 4. Effect of nocodazole on the distribution of cholesterol following extraction of NPC fibroblasts. Replicate flasks were incubated for 4 h at 37°C in PBS ± 1% MBCD and ± 50 μM nocodazole. Total, plasma membrane and intracellular cholesterol were then determined. Control (minus MBCD and minus nocodazole) (solid bar). Plus MBCD and minus nocodazole (open bar). Plus MBCD and nocodazole (hatched bar). Values are means and standard deviations from four determinations.

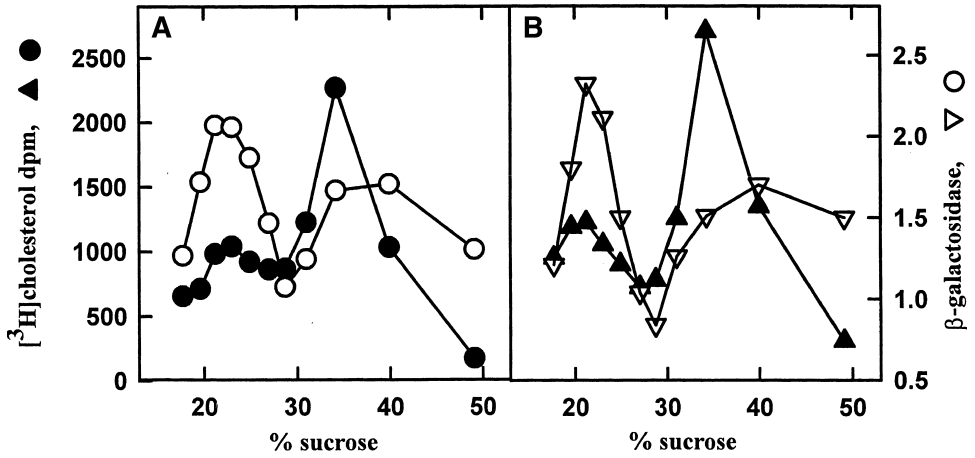

Fig. 5. Effect of nocodazole on transfer of cholesterol from the plasma membrane to endolysosomes in NPC fibroblasts. Cells in replicate flasks were surface-labeled for 10 min at room temperature with a pulse of [^{3}H]cholesterol in ~0.05% HPCD (final concentration), washed and chased at 37°C for 29 h in fresh medium containing 5% LPDS alone (A) or plus 67 μM nocodazole/ml (B). The cells were homogenized and the homogenates analyzed on equilibrium sucrose density gradients for [^{3}H]cholesterol (closed triangle, closed circle) and β-galactosidase activity (open inverted triangle, open circle), as described (3). The buoyant fractions contain the endolysosomes, while the dense peak corresponds to plasma membranes. At zero time, all of the label was confined to the latter fraction (plasma membrane) (3). Control experiments showed that nocodazole did not alter the distribution of [^{3}H]cholesterol in the gradient.

of nocodazole-treated cells occurred both in the presence and the absence of plasma lipoproteins, suggesting that the LE/L cholesterol was derived at least in part from the plasma membrane. Finally, whereas nocodazole treatment reduced plasma membrane cholesterol in normal fibroblasts (**Fig. 6**), this did not occur in NPC cells (not shown).

Endolysosomal acidification and cholesterol distribution

It has been suggested that the lipid accumulation in the LE/L of NPC and amphiphile-treated cells derives from a compromised pH-dependent function (13, 14). Supporting this idea is the observation that bafilomycin A1, a specific inhibitor of the vacuolar H^+-ATPase proton pump, induces cholesterol accumulation in macrophage lysosomes (15). The effect of this agent on the subcellular distribution of cholesterol was therefore investigated. First, we documented that 2 μM bafilomycin A1 eliminated the accumulation of the pH indicator, acridine orange, in fibroblast cytoplasmic vacuoles (not shown). We then demonstrated that an overnight incubation of fibroblasts with bafilomycin A1 doubled their intracellular cholesterol while decreasing their plasma membrane cholesterol by ~30% (**Fig. 7**). The total cell cholesterol was thus not significantly altered in these cells. Percoll gradient fractionation showed that such bafilomycin A1

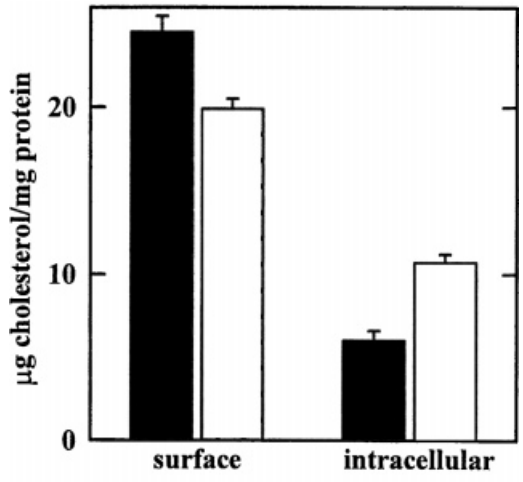

Fig. 6. Effect of nocodazole on the distribution of cholesterol in fibroblasts. Replicate flasks of human fibroblasts were incubated at 37°C for 18 h in growth medium without (solid bar) or with (open bar) 50 μM nocodazole. The distribution of cell cholesterol was then determined. Values are means and standard deviations from four determinations.

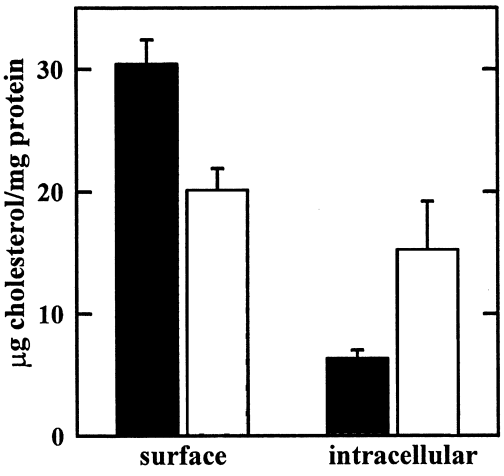

Fig. 7. Effect of bafilomycin A1 on the distribution of cholesterol in fibroblasts. Replicate flasks were incubated at 37°C for 18 h in growth medium without (solid bar) or with (open bar) 2.2 μM bafilomycin A1. The distribution of cell cholesterol was then determined. Data are means and standard deviations from five experiments.

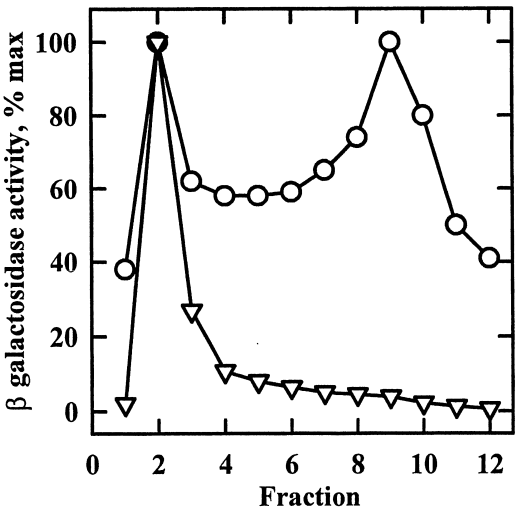

Fig. 8. Effect of bafilomycin A1 on the buoyancy of lysosomes. Replicate flasks of fibroblasts were incubated at 37°C for 17 h in growth medium with (open circle) or without (inverted open triangle) 2 μM bafilomycin A1. Cell homogenates were fractionated on Percoll gradients and assayed for the distribution of β-galactosidase (23). Fraction 1 is the most dense. Representative of three experiments.

TABLE 1. Effect of nocodazole and bafilomycin A1 on cholesterol accretion in normal fibroblasts treated with U18666A

Addition	Cell Cholesterol	Change in Cell Cholesterol
	μg cholesterol/mg protein	
None	41.4	0
U18666A	51.3	+9.9
Nocodazole	39.9	−1.5
U18666A+nocodazole	41.3	0
None	37.6	0
U18666A	60.0	+22.4
Bafilomycin	38.1	+0.5
U18666A+bafilomycin	33.9	−3.7

Replicate flasks were incubated in growth medium ±5 μM U18666A and/or 67 μM nocodazole or 2 μM bafilomycin A1 for 18 h. The cells were then assayed for cholesterol and protein. Values are the means of duplicate determinations in two representative experiments.

treatment shifted a fraction of the lysosomal marker, β-galactosidase, to a buoyant position (**Fig. 8**), as is characteristic of cholesterol-filled LE/L (3). These effects occurred in both the presence and absence of plasma lipoproteins, suggesting that the accumulated endocytosed cholesterol can be provided by the endocytic plasma membrane bilayer.

We also showed that, unlike nocodazole, bafilomycin A1 did not reduce the resistance of NPC cells to disruption by cyclodextrin extraction in assays like that shown in Fig. 1. This observation raised the possibility that bafilomycin A1 was, in fact, acting as a class 2 amphiphile.

Does nocodazole or bafilomycin A1 act as a class 2 amphiphile?

Class 2 agents not only cause the buildup of intracellular cholesterol by diverting plasma membrane cholesterol to LE/L, but also prompt a compensatory accumulation that keeps plasma membrane cholesterol close to normal (7). However, this was not the case with either nocodazole or bafilomycin A1 (Figs. 6 and 7). Rather, plasma membrane cholesterol decreased commensurately with the increase in intracellular cholesterol. To further explore this question, we tested the effect of these agents on the action of the prototypic class 2 amphiphile, U18666A. As shown in **Table 1**, nocodazole and bafilomycin A1 not only failed to stimulate the accretion of cell cholesterol but blocked the ability of U18666A to do so. The same outcome was observed using cells starved of lipoproteins overnight prior to treatment with the agents, demonstrating that the cholesterol involved was not derived from ingested plasma lipoproteins but from cellular stores (namely, the plasma membrane).

Mechanism of resistance of NPC cells to amphotericin B

Cells lacking NPC1 have been selected by virtue of their resistance to amphotericin B, a sterol-directed membranolytic agent (16–18). It has been inferred that this phenotype reflects a reduction in the level of plasma membrane cholesterol in NPC cells resulting from a block in the export of ingested lipoprotein cholesterol from lysosomes. However, NPC cells have a normal level of plasma membrane cholesterol and robust lysosomal cholesterol export (Fig. 3) (7).

In considering alternative mechanisms for the amphotericin B resistance phenotype, we tested the hypothesis that the large pool of cholesterol in the endolysosomal compartment can take up and sequester enough of the lytic agent to protect the plasma membrane. We showed by fluorescence microscopy that amphotericin B accumulated in NPC cells in multiple cytoplasmic vacuoles, taken to be LE/L, whereas the plasma membrane was poorly stained (**Fig. 9A**). This pattern is similar to that seen with the popular endolysosomal cholesterol probe, filipin (Fig. 9B).

DISCUSSION

Because of conflicting views in the literature (3–7), we sought new information concerning the dynamics of endolysosomal cholesterol in NPC cells and fibroblasts treated with class 2 amphiphiles. We obtained functional evidence for the brisk return of intracellular cholesterol to the cell surface by observing that these cells resisted disruption caused by the extraction of plasma membrane cholesterol (Figs. 1 and 2). We also determined the net rate of cholesterol transfer from intracellular to plasma membrane pools following surface extraction of NPC cells; it was 13% and 15% of plasma membrane cholesterol per hour in Fig. 3 and 4, respectively. This net transport rate is similar to that reported previously for both normal and NPC cells using a different method (3); the absolute exit rate would, of course, be even higher. The replenishment of plasma membrane cholesterol was too extensive and rapid to have come from either the hydrolysis of the small cholesterol ester pool (~10% of total cholesterol) or from biosynthesis, especially in the presence of mevinolin. Furthermore, this restoration of plasma membrane cholesterol did not occur in normal fibroblasts.

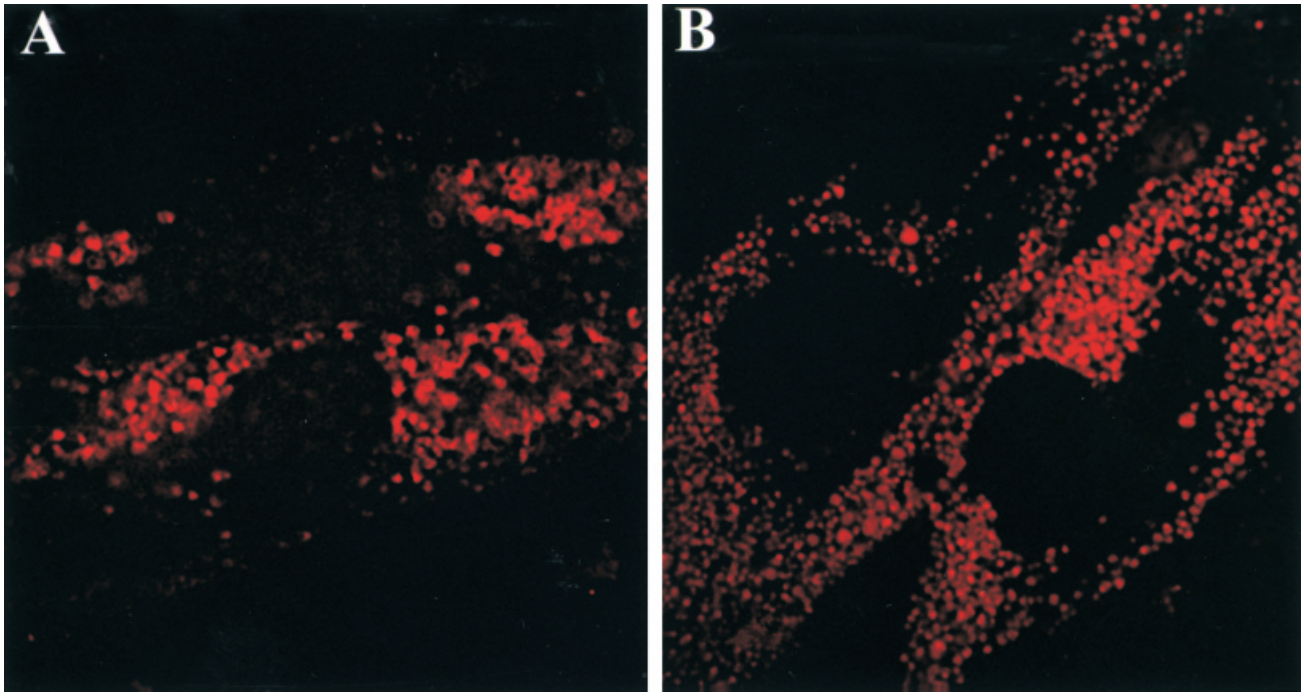

Fig. 9. Distribution of amphotericin B and filipin in NPC fibroblasts. Monolayers of NPC cells were grown on glass coverslips, fixed for 15 min with 3% formaldehyde at room temperature, rinsed, and incubated for 10 min with 50 mM NH_4Cl. The cells were rinsed and incubated for 20 min in the dark at room temperature in PBS containing 300 μg/ml amphotericin B (A) or 125 μg/ml filipin (B). Cells were viewed in an Olympus IX inverted fluorescence microscope using a Photometrix cooled CCD camera (CH350/LCCD) driven by Delta Vision software from Applied Precision Inc. (Seattle, WA) as described (24).

We therefore infer that the phenotype in NPC and amphiphile-treated cells reflects a dynamic misallocation of cell cholesterol to LE/L rather than a block in endolysosomal transport.

Nocodazole and bafilomycin A1 also cause LE/L cholesterol accumulation (Figs. 6–8) (12, 15). However, several lines of evidence suggest that these agents do not have the same action as the absence of NPC1 or the presence of class 2 amphiphiles. First, replenishment of the plasma membrane in cyclodextrin-extracted NPC and amphiphile-treated cells was inhibited by nocodazole (Fig. 4). Second, in morphological assays, nocodazole reversed the resistance of NPC and amphiphile-treated cells to cyclodextrin extraction (not shown). Third, both nocodazole and bafilomycin A1 increase LE/L cholesterol at the expense of the plasma membrane pool, whereas U18666A and the absence of NPC1 maintain normal plasma membrane cholesterol while increasing the LE/L pool (Fig. 3) (3, 7). Fourth, both nocodazole and bafilomycin A1 blocked the ability of U18666A to increase cell cholesterol (Table 1). It could be that in nocodazole- and bafilomycin A1-treated cells, unlike NPC and amphiphile-treated cells, the putative homeostatic sensor is not informed that plasma membrane cholesterol levels have fallen (19).

Our data are also at odds with the supposition (16–18) that plasma membrane cholesterol is reduced in NPC cells and fibroblasts treated with class 2 amphiphiles. Figures 3, 4, 6, and 7 (7) present quantitative evidence for normal plasma membrane cholesterol in these cells. Figures 1 and 2 provide functional support for this conclusion, since the affected cells resist the toxic effect of cell surface cholesterol extraction. [In contrast, cells treated with nocodazole and, especially, bafilomycin A1, have reduced plasma membrane cholesterol levels (Figs. 6 and 7) and are notably fragile to routine handling (not shown).] Furthermore, the basis for the supposition of reduced plasma membrane cholesterol was the resistance of these cells to amphotericin B (16–18). However, Fig. 9 raises the possibility that the large LE/L cholesterol pool in these cells competes with the plasma membrane for amphotericin B, thereby mitigating its injury to the plasma membrane.

Our results support the following hypotheses regarding intracellular cholesterol dynamics. Cholesterol is rapidly delivered to the LE/L from both ingested plasma lipoproteins and endocytic cell surface bilayer, and rapidly returns to the plasma membrane. The ratio of the rates of influx to efflux might, in the simplest case, set the level of the dynamic pool of cholesterol in the LE/L. This has been estimated to be ~6% of the cell total in normal human fibroblasts (3) and ~10-fold higher in NPC cells (7). The half-time for the turnover of LE/L cholesterol is ~1 h in normal human fibroblasts and ~30 h in NPC cells and cells treated with class 2 amphiphiles (3).

The accumulation of cholesterol in LE/L presumably occurs when the rate of delivery exceeds the rate of export. A small difference between these rates could lead to the observed deposits in the LE/L of cells lacking func-

tional NPC1 protein and in cells treated with class 2 amphiphiles even though both of these cell systems return endolysosomal cholesterol to the plasma membrane at close to the normal absolute rate. Because ~5% of plasma membrane cholesterol in normal fibroblasts passes through the lysosomes per hour (3), a partial block in its exit could easily account for the observed rate of endolysosomal accumulation in NPC and amphiphile-treated cells; namely, 1–2% per hour (7). The affected site need not be a distal endocytic compartment. For example, reduced recycling from upstream endosomes (20) might cause excess cholesterol to move to the lysosomes by default, overloading their export capacity and fostering the accumulation of a large yet dynamic pool. Such a mechanism would not only account for the behavior of NPC and amphiphile-treated cells, where prelysosomal defects have been postulated (4), but also for the actions of nocodazole and bafilomycin A1 described above. In particular, the latter agents might interfere with the return of endocytic cholesterol to the cell surface, given that prelysosomal compartments utilize both microtubules and vacuolar acidification (21, 22). A detailed understanding of the action on intracellular cholesterol of each of the perturbants discussed herein is still lacking, as is a road map for the return of endocytic cholesterol to the plasma membrane.

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