Characterization of the Niemann-Pick C pathway in alveolar type II cells and lamellar bodies of the lung

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Lysosomes regulate cellular cholesterol concentration and trafficking through the Niemann-Pick C (NPC) pathway, which consists of two cholesterol-binding proteins: NPC1, a large 1,278-amino acid transmembrane protein, and NPC2, a smaller 132-amino acid soluble lysosomal protein (6, 37, 38, 41). Evidence from studies using gene-targeted mice indicates that NPC1 and NPC2 are members of a common pathway needed for lysosomal cholesterol transport (53). One current model for the functioning of the NPC pathway predicts that cholesteryl ester from internalized LDL is hydrolyzed in the lysosomal lumen by lysosomal acid lipase (LAL) to release fatty acid and free cholesterol (33). The free cholesterol is bound by NPC2, with the 3β-hydroxyl portion of the cholesterol molecule facing out of the binding pocket (63). Then cholesterol is exchanged in a “hydrophobic handoff” between NPC2 and the NH2-terminal domain of NPC1, with the isooctyl moiety of the lipid exposed to the surface of the protein (28, 56). Finally, NPC1 exports the free cholesterol to the plasma membrane or the endoplasmic reticulum (ER) via an unknown mechanism, possibly involving oxysterol-binding protein-related protein 5 (17).

A possible link between the NPC pathway and surfactant cholesterol content was suggested by studies of NPC disease, a rare hereditary lysosomal storage disorder marked by the accumulation of free cholesterol and other lipids in cells of a variety of organs, including brain, liver, and lung (29, 46, 62). The majority (95%) of cases of NPC disease are caused by a mutation in NPC1, while the remaining cases (5%) are due to mutations in NPC2 (6, 38, 41). Although NPC disease is generally associated with neuronal degeneration, lung pathology, such as pulmonary alveolar proteinosis, foamy macrophage infiltration, and emphysema, has been reported in patients with both subtypes (19, 24, 40, 42, 43, 52). Griese et al. (24) analyzed the surfactant content of the bronchoalveolar lavage (BAL) fluid from a patient with NPC2 insufficiency. In addition to marked lung morphological abnormalities, this patient suffered from alveolar proteinosis. Although total lipid levels in the surfactant were elevated, the lipid composition of the surfactant demonstrated disproportionate enrichment in cholesterol. Cholesterol lipid content of the BAL made up ~50% of the total lipid species, up from the normal level of 10% (wt/vol) (24).

Lamellar bodies have been referred to as specialized lysosomes or “lysosome-related organelles” because of the similarities in the protein content and the acidic environment of the two organelles (60). Given that there are shared attributes between lamellar bodies and lysosomes, that lysosomes process LDL cholesterol through NPC1 and NPC2, and that pneumocytes bind and take up LDL, with LDL cholesterol recovered in lamellar bodies (25), we hypothesized that lamel-
lar bodies regulate the cholesterol content of surfactant through the NPC pathway. In the present study, we define the localization of NPC1, NPC2, and LAL proteins in lamellar bodies of isolated type II pneumocytes and provide evidence for a role of the NPC pathway in the regulation of lamellar body cholesterol content.

**MATERIALS AND METHODS**

Chemicals were obtained from Fisher Scientific (Pittsburgh, PA) unless otherwise noted. Lung tissue, isolated type II pneumocytes, and isolated lamellar bodies. All animal protocols adhered to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of Pennsylvania Animal Care and Use Committee. Pathogen-free Sprague-Dawley rats or C57BL/6 mice were used. Rodents were anesthetized with pentobarbital sodium, the trachea was cannulated, and the lungs were ventilated while they were cleared of blood by perfusion through the pulmonary artery. The cleared lungs were used for type II cell isolation, lavaged for alveolar macrophages and surfactant, or homogenized for lung tissue samples. Alveolar macrophages were isolated from rat lung lavage by centrifugation. Type II cells were isolated from rat cells by elutriation digestion (10, 16) or from mice using dispase, as described previously (4, 58). Macrophages were removed from the type II cell preparation by panning on IgG-coated dishes. Nonadherent cells were pelleted and resuspended in MEM supplemented with 10% FCS and plated on 35-mm dishes (Costar, Cambridge, MA) or on coverslips in 12-well plates. After overnight incubation, >95% of attached cells were type II pneumocytes. Isolated type II cells were grown in MEM (GIBCO, Carlsbad, CA) supplemented with 10% FCS (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin (GIBCO).

Lamellar bodies were isolated as previously described (8, 18). The phospholipid-to-protein ratio was ~6:1 (wt/wt). Contamination of the lamellar body preparation by ER was minimal (Fig. 1F). Immunoblotting was performed as described previously (31).

**Antibodies.** Primary antibodies used for immunocytochemistry were as follows: polyclonal rabbit anti-NPC1 diluted 1:200 (Novus Biologicals, Littleton, CO); rabbit anti-NPC2 diluted 1:100 and rabbit anti-β-actin diluted 1:5,000 (Sigma); monoclonal anti-NPC2 (H-10) diluted 1:500 and goat anti-cathepsin D (C-20) diluted 1:50 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-ATP-binding cassette transporter A3 (ABC-A3; mAb 3C9) diluted 1:400 (65); rabbit anti-NPC1 diluted 1:100 (D01P, Abnova, Walnut Creek, CA); and rabbit anti-calnexin diluted 1:1,000 (Stressgen, Victoria, BC, Canada). For Western blot analysis, anti-NPC1 (1:1,000 dilution) or monoclonal anti-NPC2 (1:200 dilution) was used.

For immunocytochemistry, secondary antibodies were goat anti-mouse Alexa 488, goat anti-rabbit Alexa 594, or donkey anti-goat Alexa 594 (Molecular Probes, Eugene, OR), all diluted 1:200. For Western blot analysis, secondary antibodies were goat anti-rabbit IgG horseradish peroxidase-conjugated (Upstate, Millipore, Billerica, MA), and sheep anti-mouse IgG horseradish peroxidase-conjugated antibody (Amersham, Piscataway, NJ), both diluted 1:3,000.

**Cellular cholesterol quantitation.** Total cholesterol was determined using an Amplex Red cholesterol assay (A12216, Molecular Probes, Invitrogen, San Diego, CA) following the manufacturer’s instructions. The protein concentration for each sample was determined by the assay of Lowry et al. (39). Results are presented as micrograms of...
cholesterol per milligram of cell protein or calculated as percentage of control. All values represent the average of at least three independent experiments.

**Immunofluorescence confocal microscopy.** Cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. The permeabilization step was sometimes omitted when the cells were stained for NPC1 to produce a better signal. Cells were quenched with 0.3% glycine and incubated in blocking solution containing 2% BSA and 5% normal goat serum (goat serum was omitted when cells were stained with goat primary antibody). Cells were incubated in primary antibody for 2 h at room temperature for NPC2 or overnight at 4°C for NPC1, with the addition of anti-ABCA3 (1:250 dilution) for double staining. Cells were incubated with secondary antibodies for 1 h at room temperature. When sequential secondary antibody staining was required, cells were incubated with donkey anti-goat Alexa 594, fixed for 2 min with 4% paraformaldehyde, and incubated with a goat anti-rabbit Alexa 488 secondary antibody. To control for cross-reactivity, primary antibodies were omitted. Cells grown on coverslips were washed twice with distilled H2O and mounted with mounting medium (Vector Laboratories, Burlingame, CA).

For filipin staining, cells were labeled according to the manufacturer’s instructions (Filipin III, Cayman Chemical, Ann Arbor, MI). Cells were visualized using a Zeiss LSM 510 Meta microscope coupled to a Chameleon Ultra mode-locked femtosecond pulse laser (set at 720 nm for 2-photon excitation of filipin) and He-Ne and Ar lasers. All images were acquired using Plan Apochromatic ×63 1.4 numerical aperture oil- and water-immersion objectives and ZEN 2009 software (Zeiss). Colocalization and z-stack analysis were performed using ZEN 2009 software and obtained from three independent experiments, each consisting of at least six different image fields from two dishes per condition.

**Image analysis.** Filipin quantitation of whole cell and late endosome/lysosome-like storage organelle (LSO) was performed as previously described (13, 48). Briefly, images were converted to gray scale and quantitated to calculate whole cell filipin values using the ImageJ built-in measure function. Next, the same images were set to a higher threshold to measure only regions that were intensely stained with filipin to calculate LSO values (48).

**Glycosidase treatment.** The method for peptide N-glycosidase (PNGase) treatment was adapted from a protocol reported elsewhere (56). Protein lysates from lung tissue and type II pneumocytes were suspended in denaturing buffer supplied by the manufacturer to a final volume of 10 μl. The solution was incubated at room temperature for 30 min and then brought to a final volume of 20 μl with G7 reaction buffer and NP-40 according to the manufacturer’s instructions. PNGase F (500 U) (New England Biolabs, Ipswich, MA) was added, and reactions were incubated at 37°C for 1 h. Samples were then subjected to SDS-PAGE and immunoblotted with NPC1 antibody.

**Statistical analysis.** Values are means ± SE. Experimental conditions were compared using a Student’s t-test or ANOVA in SigmaStat (SPSS, San Jose, CA). Results were considered statistically significant at P < 0.05.

**RESULTS**

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proteins in these cells and in the surfactant storage organelles, lamellar bodies. We isolated alveolar epithelial type II cells from rat and mouse lungs and, using NPC antibodies, performed Western blot analysis of lung cell and tissue samples. Type II cell protein extracts from either rodent species showed a band corresponding to the glycosylated form of NPC1 of \( \sim 170 - 190 \) kDa (Fig. 1A, lanes 1 and 6) (50). In lysates of whole rat lung tissue (Fig. 1A, lane 2), we identified two bands, one corresponding to the glycosylated form of NPC1 and a smaller, 120- to 140-kDa band, similar to the predicted mass of the 142-kDa nonglycosylated NPC1 protein, as previously reported (6, 22, 59). Next, lamellar bodies were isolated from total rat lung. Surprisingly, the expression of NPC1 in lamellar bodies appeared enriched over total lung extracts and isolated type II cells at equal protein loading (Fig. 1A, lane 3). The lamellar body preparations contained approximately equal amounts of both forms of NPC1, glycosylated (43 ± 2% of total) and deglycosylated (57 ± 2% of total, \( n = 7 \)), with the ratio remaining consistent between preparations. Rat alveolar macrophages isolated from BAL contained NPC1 (Fig. 1A, lane 4), predominantly the higher-molecular-mass form, as previously reported for human macrophages (49). As expected, NPC1 was not detectable in 30 \( \mu \)g of lavage fluid protein (Fig. 1A, lane 5).

Next, we probed for NPC2 in preparations isolated from the rat lung. Rat alveolar epithelial type II cells (Fig. 1B, lane 1) and whole rat lung extracts (Fig. 1B, lane 2) contained a 16- to 22-kDa NPC2-positive band corresponding to the predicted molecular mass of NPC2 (41). In isolated lamellar bodies, the NPC2 band was enhanced compared with type II cells and lung tissue when total protein loaded on the gel was held constant (Fig. 1B, lane 3). Alveolar macrophages were particularly enriched with NPC2 compared with the other lung samples. Lavage fluid contained a distinct band for NPC2 (Fig. 1B, lane 5), corroborating previous findings that NPC2 is present in the lavage (36).

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**Fig. 3.** Immunocytochemical localization of NPC2 in alveolar type II cells. A: isolated rat alveolar type II cells after 24 h in culture were double-labeled with antibody against ABCA3 (green) and NPC2 (red) and imaged using confocal microscopy. A cluster of cells is shown. A region containing lamellar bodies (box) is selected for enlargement. Single cell panels for ABCA3 (green), NPC2 (red), and ABCA3 and NPC2 (Merge) and merged image with the light micrograph (Merge w/ light) show that NPC2 decorates lamellar body and nonlamellar body regions. B: a type II cell labeled with ABCA3 (green) and NPC2 (red) with a single lamellar body highlighted (box). Arrows point to NPC2, which appears yellow (yellow arrow) or partially yellow (white arrows) because of its close approximation to the limiting membrane. Enlarged panels show projections from a \( z \)-stack focused on a lamellar body along the top view (\( x-y \)), side view (\( x-z \)), and axial view (\( y-z \)). Green ring indicates ABCA3 on the boundary of a lamellar body; red dot indicates NPC2 within the lamellar body. Scale bars, 5 \( \mu \)m.
Figure 1C quantitates in arbitrary units the amounts of NPC1 and NPC2 protein in the rat lung samples over several experiments. At equal protein loading, NPC1 levels were slightly lower in lung tissue than type II cells, while levels of NPC2 were comparable between type II cells and the lung. Lamellar bodies were greatly enriched in NPC1 and NPC2 compared with type II cells or the rat lung tissue from which they were isolated. Alveolar macrophages had high levels of both NPC proteins.

To establish that the 120- to 140-kDa NPC1 band represented the deglycosylated form of NPC1, type II cell lysates were treated with the glycosidase enzyme PNGase F. Endoglycosidase treatment of type II cells containing the upper 170- to 190-kDa NPC1 band resulted in the removal of N-linked carbohydrates and the generation of the lower-molecular-mass NPC1 band (Fig. 1D), similar to previous reports (56). Since lamellar bodies are a major component of type II cells and both bands were present in lamellar bodies, it was surprising that the lower-molecular-mass species (Fig. 1E, lane 3), unlike lamellar bodies isolated from lung tissue (Fig. 1E, lane 4). Thus, the lower-molecular-mass form of NPC1 in the lamellar bodies from lung was present, but to a lesser extent, in freshly isolated type II cells and disappeared after cell isolation and culture for 24 h. Disappearance of the nonglycosylated NPC1 band may be a result of the transdifferentiation process that occurs in type II cells after isolation and culture, characterized by changes in morphology and gene expression (2, 15). The reason for the presence of abund ant levels of the nonglycosylated form of NPC1 in the lung lamellar bodies was not clear. Although a possible explanation could be contamination with ER, which would contain newly synthesized nonglycosylated NPC1, this was unlikely, as there was little calnexin, a protein marker for ER, in the preparation (Fig. 1F).

Localization of NPC1 and NPC2 in alveolar type II cells. Intracellular localization of the NPC proteins in type II alveolar epithelial cells was visualized using immunocytochemical techniques. Lamellar bodies were identified using the monoclonal antibody 3C9 raised against ABCA3, located in the limiting membrane of this organelle (64, 65). In isolated type II cells, a double label of ABCA3 and NPC1 revealed NPC1 labeled in a punctate pattern in regions overlapping the borders of lamellar bodies and in punctate vesicles in regions of the cytosol (Fig. 2). NPC1 label was heterogeneous, with various amounts of red NPC1 adjacent to green ABCA3 on lamellar bodies. This staining pattern suggested the presence of distinct domains of NPC1 in the limiting membrane of lamellar bodies, which was confirmed by the alternating pattern of red and green label apparent in the z-stack projections shown in Fig. 2, axial view (y-z), for example. Colabeling of type II cells with...
ABCA3 and NPC2 revealed that some NPC2 was in the lumen of lamellar bodies (Fig. 3A, Merge), a conclusion confirmed by a z-stack projection of a single lamellar body (Fig. 3B, x–y, x–z, and y–z). Some NPC2 appeared yellow or partially yellow, probably because of its close approximation to the limiting membrane, as was previously seen in colabeling studies with ABCA3 and two other soluble proteins in the lumen of lamellar bodies, peroxiredoxin 6 (61) and surfactant protein A (21). The protein also appeared distributed throughout the cell in punctate dots (Fig. 3A).

To identify the NPC1/NPC2-positive small vesicles that were not lamellar bodies, type II cells were labeled with antibody against cathepsin D, a marker of late endosome/lysosome organelles (3) but not found in lamellar bodies (5, 14, 51, 57). As expected, cathepsin D did not colocalize with large lamellar body-like organelles that display rings of NPC1 signal in double-label experiments (Fig. 4A); however, cathepsin D did colocalize with many small NPC1-positive (Fig. 4A, Merge) and NPC2-positive (Fig. 4B) vesicles. In addition, there were distinct NPC1 or NPC2 punctate vesicles that were not cathepsin D-positive, possibly representing a transport organelle (32).

To determine whether NPC1 and NPC2 were in the same organelle, cells were double-labeled with NPC1 and NPC2 antibody. The micrographs showed localization of the two proteins with large lamellar body-like structures (Fig. 5A) and in smaller vesicles not apparently associated with lamellar bodies (Fig. 5B); however, some vesicles contained NPC1 or NPC2 alone. Taken together, these results suggest that a subset of NPC1 and NPC2 proteins was associated with lamellar bodies, another subset of both proteins was present in the lysosomal organelle, and a third subset was alone in other types of unidentified vesicles.

LAL is another component of the NPC cholesterol trafficking pathway. When internalized LDL is delivered to acidic compartments, the enzyme hydrolyzes LDL cholesteryl ester to free cholesterol and fatty acid, thereby providing the cargo (cholesterol) for the NPC pathway (23). It is known to be present in late endosomes/lysosomes and colocalizes with many small cathepsin D-positive vesicles (Fig. 6). To determine whether LAL was present in lamellar bodies, we used immunocytochemical and Western blot approaches. First, type II cells were double-labeled with antibody against LAL and ABCA3. By immunocytochemistry, LAL was found to asso-

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**Fig. 5.** Immunocytochemical colocalization of NPC1 and NPC2 in alveolar epithelial type II cells. A and B: isolated rat alveolar epithelial type II cells after 24 h in culture were double-labeled with an antibody against NPC1 (red) and NPC2 (green) and imaged using confocal microscopy. A region of the cell (box) is selected for enlargement. Enlarged region is shown for NPC1, NPC2, and merged images. White arrows in A indicate perilamellar body labeling of NPC1 (thick white arrows) and NPC2 (thin white arrows) that are adjacent to each other. Arrowheads in A indicate signals that will be yellow (colocalized) in the merged image. Arrows in B indicate colocalized vesicles that are not lamellar bodies. Scale bars, 5 μm.
ciate with the majority of ABCA3-positive lamellar bodies (Fig. 7A) and was located in the lumen, as shown by z-stack analysis. To corroborate these findings, we performed Western blot analysis on isolated lamellar bodies. LAL was found in lamellar bodies isolated from type II cells that were in culture for 24 h (Fig. 7B, lane 1) and in lamellar bodies isolated directly from lung tissue (Fig. 7B, lane 2). For comparison, whole type II cells that were in culture for 24 h (Fig. 7B, lane 3) are shown. These results show that LAL was abundant in type II cells and that a portion of this was present in lamellar bodies.

Effect of blocking the NPC pathway with U18666A. With all the components of the NPC cholesterol trafficking pathway present in lamellar bodies, we hypothesized that the pathway was playing a role in the regulation of the cholesterol content of this organelle. To test this hypothesis, we used the class 2 amphiphil U18666A (UA) to block cellular cholesterol trafficking. The ability of UA to induce cellular accumulation of cholesterol in lysosomes is well documented in many cell types (7). The response of type II cells to increasing concentrations of UA in the media from 0 to 1 μg/ml with 10% FCS was determined. After 5 h of incubation, there was no change in cholesterol content (data not shown), but after 18 h, there was a dose-dependent increase in cholesterol accumulation (Fig. 8A). There was no further change after 24 h of incubation with 0.5 μg/ml UA. The cholesterol content was 175.1 ± 13.0% of control at 18 h and 178.5 ± 20.0% at 24 h (n = 3, difference not significant). Figure 8B demonstrates that the presence of serum (10% FCS) was necessary for increased cholesterol levels with UA treatment, as, in the absence of serum, the cholesterol content of the cells remained unchanged.

To visualize the intracellular compartments of type II cells that were affected because of UA-induced accumulation of cholesterol, cells were labeled with filipin, a highly fluorescent polyene macrolide antibiotic that specifically binds free cholesterol. As seen in Fig. 9, the untreated control cells demonstrated a weak and diffuse filipin staining. In contrast, after 18 h of treatment, most cholesterol was found in intracellular aggregates (Fig. 9A). Within a single treated cell, some lamellar body-like structures exhibited intense filipin staining (Fig. 9A, arrows), while others were lacking in filipin staining (Fig. 9A, arrowheads). Fluorescent signal intensity generated by filipin staining of type II cells was measured by conversion of the microscopic data to gray scale and quantitation using ImageJ. The whole cell filipin staining showed that UA treatment increased cholesterol 2.3-fold relative to untreated type II cells (Fig. 9B). Quantitation of filipin fluorescence of only brightly fluorescing LSO showed cholesterol accumulation 14.5 ± 3.4 fold higher in UA-treated than control cells (Fig. 9B).

To confirm that the large filipin-containing organelles in type II cells were lamellar bodies, cells were incubated with UA for 18 h and double-stained with anti-ABCA3 antibody and filipin. The confocal images showed that, in the UA-exposed cells, filipin was located within ABCA3-positive lamellar bodies (Fig. 10). The amount of filipin staining in lamellar bodies was variable, ranging from completely filling the lumen of the organelle to barely visible. This pattern indicated that the lamellar bodies were not uniform in their response to UA (Fig. 10, panels 5’–8’).

Relationship of lamellar body cholesterol and NPC expression. To determine whether the presence of NPC protein on the lamellar body correlated with cholesterol accumulation, type II cells were treated with UA and 10% FCS and triple-labeled with ABCA3 antibody, filipin, and NPC1 or NPC2 antibody. Some ABCA3-positive lamellar bodies that were also positive for NPC1 (Fig. 11A, boxes 1 and 2) or NPC2 (Fig. 11B, boxes 1 and 2) exhibited obvious filipin labeling (Fig. 11, A and B, box 1), while others exhibited little to no filipin labeling (Fig. 11, A and B, box 2). Therefore, NPC protein presence on lamellar bodies did not appear to be the only factor important for the extent of cholesterol accumulation in these organelles upon UA exposure.

Fig. 6. Lysosomal acid lipase (LAL) colocalization with cathepsin D. Isolated rat alveolar type II cells were fixed and double-labeled with antibody against LAL (green) and cathepsin D (red) and imaged using confocal microscopy. A single type II cell, outlined by a dashed line, is shown as a double-labeled image. A region of the cell (box) is selected for enlargement. Enlarged panels for LAL (green), cathepsin D (red), LAL and cathepsin D (Merge), and merged image with the light micrograph (Merge w/light) are shown. Colocalized signal is represented by a yellow color. Scale bar, 5 μm.
DISCUSSION

The NPC pathway regulates the intracellular trafficking of cholesterol from lysosomes in many cell types. Thus a possible role for the protein components NPC1 and NPC2 in the control of cholesterol in primary alveolar epithelial type II cells was explored using biochemical and imaging techniques. Both proteins were expressed in type II cells in small vesicles identified as lysosomes by cathepsin D labeling, as found for other cell types. Surprisingly, NPC1 and NPC2 also were associated with lamellar bodies, specialized organelles found in type II cells that serve to store surfactant. Three-dimensional projections of a single lamellar body confirmed NPC1 expression along the entirety of the limiting membrane of lamellar bodies, localized in alternating adjacent domains with ABCA3, while NPC2 and another component of the NPC pathway, LAL, were found within the lumen of the organelle. Furthermore, inhibition of NPC1 function resulted in cholesterol accumulation within lamellar bodies. Our results are compatible with an important role for the NPC pathway in regulating lamellar body cholesterol composition.

A polyclonal antibody against NPC1 recognized two bands representing a glycosylated and a nonglycosylated form of the protein in rat lung tissue and lamellar bodies. PNGase-catalyzed deglycosylation of the higher-molecular-mass band re-
sulted in the production of the lower-molecular-mass form of NPC1, confirming the glycosylation status of this protein. Both forms of NPC1 were present in lamellar bodies, reflecting the situation in whole lung tissue and, presumably, in type II cells of the lung in situ, as they are the cells in the lung that contain lamellar bodies. Interestingly, enzymatic removal of the type II cells from the lung resulted in a partial loss of the nonglycosylated form, and culture of the cells for 24 h resulted in a near-complete absence of this form of NPC1 from the cells, leaving only the glycosylated NPC1 form. However, the NPC1 function of cells in culture was retained, since blockage of the NPC pathway with drug resulted in cholesterol accumulation in lamellar bodies. The variation in glycosylation status of NPC1 implies that there is a difference in function dependent on glycan modification, although such a role for glycosylation has not been established. Other studies that mutated four potential glycosylation sites in the NH2 terminus of NPC1 demonstrated that the lack of a single N-glycosylation site did not affect the ability of NPC1 to remove cholesterol from lysosomes (59). Mutation of all four sites resulted in less biological activity, but this may have been due to low expression of this protein form (59). NPC1 has confirmed glycosylation sites on at least two locations, Asp134 and Asp524 (9). According to a model of NPC1 topology, both asparagine residues are present in loops facing the luminal side of the endosome/lysosome (12). Such orientation would allow for access of luminal endoglycosidases.

Fig. 8. Cholesterol accumulation in type II cells treated with compound U18666A (UA). A: type II cells were treated with UA (0–1 μg/ml) for 18 h, harvested, and analyzed for cholesterol content. Values are means ± SE of triplicate determinations from a single experiment and are representative of the 3 performed. *P < 0.05 vs. control (0 μg/ml UA). B: type II cells incubated with (10% FCS) or without (None) FCS for 18 h in the presence or absence of 0.5 μg/ml UA. Values are means ± SE (n = 4–6). *P < 0.05 vs. Con (no UA).

Fig. 9. Cholesterol accumulation in type II cells visualized with filipin after treatment with UA. A: type II cells untreated (Control) or treated with 0.5 μg/ml UA for 18 h in 10% FCS were fixed and labeled with filipin. Filipin alone (Filipin, gray) and the corresponding light micrograph (Light) are shown. Outline of cells in all images is marked by dashed lines. A single UA-treated cell (box) is selected for enlargement. Enlarged panels (UA [enlarged]) for filipin (gray) and light micrographs (Light) are shown. Arrows indicate a lamellar body-like structure that is positive for filipin; arrowheads indicate a lamellar body-like structure that has relatively little filipin label. Scale bars, 20 μm. B: quantitation of filipin staining in type II cells. Pixel intensity was quantified in ImageJ as arbitrary units for whole cells and late endosome/lysosome-like storage organelle (LSO). In each experiment, 3–8 fields per treatment were quantitated. Top: whole cell. Values are means ± SE (n = 5 fields from a representative experiment of the 4 performed). UA stimulation of filipin staining was statistically significant from control (Ctrl) for each experiment and ranged from 2.2- to 8.1-fold over control for the 4 experiments. *P < 0.05 vs. Ctrl. Bottom: LSO. Images were set to a higher threshold to measure only regions that were intensely stained with filipin to calculate LSO values. Values are means ± SE relative to control (n = 5 experiments). *P < 0.05 vs. Ctrl.
to these sites for cleavage of high-mannose residues. Endoglycosidases have not been reported in lamellar bodies but have been identified in lysosomes and, when mutated, result in lysosomal storage disorders (27).

The luminal environment of lamellar bodies in the lung may permit endoglycosidase cleavage of NPC1, while that of cultured type II cells may not. It was feasible that the presence of nonglycosylated NPC1 was due to contamination of the lamellar body preparation with ER and, therefore, represents NPC1 that has not yet passed through the Golgi. This possibility was unlikely, as the amount of the ER marker protein calnexin in our lamellar body preparation was very low. Thus the presence of the nonglycosylated NPC1 protein form in lung lamellar bodies raises the possibility that this form has a specific role in these organelles. While unusual, lamellar bodies are not unique in having two forms of NPC1, as these forms also have been described in feline fibroblasts and monkey brain homogenates (22, 44).

NPC2 was notably enriched in lamellar bodies compared with type II cells and lung tissue on an equal protein basis. Immunocytochemical characterization showed that NPC2 was present in many lamellar bodies. NPC2 also was found in lung lavage fluid, corroborating previous reports (36) and indicating that NPC2 is released from lung tissue into the alveolar space. Whether NPC2 is secreted with surfactant from lamellar bodies or from the lysosomes of type II cells remains to be determined.

The NPC pathway likely was functioning in cellular compartments other than lamellar bodies, such as lysosomes, as described for various cell types. In type II cells, NPC1, NPC2, and LAL were found to colocalize with cathepsin D-positive vesicles, consistent with a late endosomal/lysosomal localization. This represents a separate, nonlamellar body compartment, since cathepsin D is not found in lamellar bodies, a conclusion based on our immunocytochemical data and the work of others (5, 14, 51, 57).

NPC proteins were not only present in lamellar bodies, but they were also functional. The hallmark of NPC1 and NPC2 mutant cells is formation of enlarged cholesterol-rich organelles. UA is a well-accepted inhibitor of cholesterol egress from late endosomes/lysosomes, resulting in cholesterol accumulation by blockage of the NPC pathway, an effect observed in a variety of cell types (7, 32, 34, 35). Exposure of type II cells to UA resulted in cholesterol-rich aggregates in lamellar bodies and a dose-dependent increase in total cholesterol. UA is thought to mimic NPC deficiency by inhibiting the proteins in the pathway or by altering membrane lipid structures (7, 32, 35). In addition, UA has been shown to inhibit the rapid vectorial movement of NPC1 vesicles that transport material to and from the cell periphery in a Chinese hamster ovary cell line (32). All these mechanisms could be used by type II cells to control cholesterol content. What is the necessity for a cholesterol removal mechanism in lamellar bodies? Two possibilities arise from a consideration of the process of lamellar body formation. Lamellar body biogenesis has been proposed to occur through fusion of multivesicular bodies, organelles that contain a number of small vesicles. In addition, the multive-
sicular bodies are members of the LDL receptor-mediated endocytosis pathway and serve to deliver lipoproteins to lysosomes and, presumably, to lamellar bodies. Thus multivesicular bodies provide two sources of cholesterol, small-vesicle membrane lipids and LDL. The cholesterol-to-phospholipid ratio of membrane lipids is usually 60:40, while in surfactant the ratio is 10:90 (11, 45). Because of this relative enrichment of cholesterol over phospholipids in most membranes, an excess of cholesterol would need to be removed from the lamellar body, potentially via the NPC pathway. However, if this were the situation, blocking the NPC pathway should result in cholesterol accumulation whether or not serum was present in the medium, which was not the case. Type II cells did not accumulate cholesterol within the cells or the lamellar bodies in the absence of exogenous FCS. Thus the most likely scenario is that the cholesterol that accumulates in lamellar bodies arises from uptake of LDL-derived cholesterol, a conclusion supported by the findings of others that labeled lipoprotein cholesterol is recovered in type II cell lamellar bodies (25).

It was unexpected that cholesterol accumulation in lamellar bodies was not uniform upon UA treatment. It is tempting to speculate that LDL cholesterol is only being delivered to organelles undergoing maturation. Mature lamellar bodies

Fig. 11. Cholesterol accumulation in NPC1- and NPC2-positive lamellar bodies after treatment with compound UA. A: rat type II cells were treated with UA and triple-labeled with ABCA3 (green), filipin (gray or blue), and NPC1 (red). In merged (Merge) micrograph, filipin is shown in blue. An enlarged image of an NPC1-positive/filipin-high lamellar body is shown in box 1, and an NPC-1-positive/filipin-low lamellar body is shown in box 2. B: type II cells treated with UA and triple-labeled with ABCA3 (green), filipin (gray), and NPC2 (red). In merged (Merge) micrograph, filipin is shown in blue. An enlarged image of an NPC2-positive/filipin-high lamellar body is shown in box 1, and a NPC-2-positive/filipin-lamellar body is shown in box 2. Scale bars, 5 μm.
would no longer fuse with multivesicular bodies containing LDL cholesterol and, thus, would not accumulate cholesterol when the NPC pathway is blocked. Evidence of biochemically distinct lamellar body subpopulations has been shown through heterogeneous expression of the small GTP-binding proteins Rab3D (54) and Rab38 (66). It also is feasible that the concentration of UA may not be the same in all lamellar bodies or that NPC2 and LAL, as water-soluble proteins, may traffic between organelles and may not be available in sufficient quantities at all times. However, the presence of NPC1 and NPC2 on a lamellar body did not predict the extent of cholesterol accumulation when the NPC pathway was blocked with UA exposure. Whether this is also the case for lysosomes has not been investigated.

Historically, lamellar bodies have been considered principally as a storage organelle for surfactant, and not a particularly biologically active compartment, although acid hydrolases and phospholipases are a normal component of lamellar bodies (20, 26). It is presumed that the appropriate lipids and proteins are delivered to the lamellar body and held until they are secreted into the alveolar space. The NPC pathway is a mechanism for the trafficking of lipoprotein cholesterol through lysosomes to other cellular compartments. With the demonstration that there is an active NPC pathway in lamellar bodies, these lysosome-like organelles may be more dynamic than originally thought. Our work and the work of others provide evidence that lamellar bodies contain all the components found to be necessary for lysosomal cholesterol transport, including an acidic compartment (8), LAL, NPC2, and NPC1, and thus have the capacity to function as lysosomes in exporting free cholesterol to the cell from LDL cholesterol esters within the lumen. Lamellar bodies, on the other hand, may have the additional role of retaining free cholesterol within the organelle for use with surfactant lipids. Studies of NPC disease and resulting alveolar proteinosis support a role for the latter function. In the absence of a functional NPC pathway, these patients demonstrate a marked enrichment of surfactant cholesterol (24). The association of NPC1 and NPC2 with lamellar bodies offers a potential mechanism. In the absence of a functional NPC pathway in alveolar epithelial cells, the increase in cholesterol content of lamellar bodies could conceivably lead to changes in cholesterol composition of surfactant secreted by these cells.

Our working model for the NPC pathway is as follows. First, LDL-derived cholesteryl ester is taken up via receptor-mediated endocytosis on the basolateral surface and delivered to multivesicular bodies, which fuse with lysosomes or lamellar bodies. Our data indicate that lamellar bodies contain LAL, an enzyme responsible for cholesteryl ester hydrolysis to free cholesterol and fatty acid. The released free cholesterol may remain in the phospholipid-rich environment of the lamellar body or may be released via the NPC pathway for redistribution throughout the cell. A lysosomal compartment that is independently capable of cholesteryl ester cleavage and redistribution of free cholesterol may also exist in type II cells. Upon inhibition of the NPC pathway with UA, free cholesterol accumulates in lamellar bodies.

In summary, the present study was designed to define the role of NPC1 and NPC2 in alveolar type II cells of the lung. This is the first report that NPC1 and NPC2 are associated with the lamellar bodies of type II cells and function to regulate cholesterol content within this organelle. Interference with the NPC pathway by a pharmacological inhibitor resulted in accumulation of cholesterol in these large vesicles. These studies are consistent with the possibility that disruption of NPC function may result in a pathophysiological condition of the pulmonary system. Thus lamellar bodies are proving to be organelles used not only for storage of surfactant, but also for biologically active processes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

B.R.R., J.-Q.T., K.J.Y., and S.H. performed the experiments; B.R.R., S.H., and S.R.B. analyzed the data; B.R.R. and S.R.B. interpreted the results of the experiments; B.R.R. and S.R.B. prepared the figures; B.R.R. and S.R.B. drafted the manuscript; B.R.R. and S.R.B. edited and revised the manuscript; S.R.B. is responsible for conception and design of the research; S.R.B. approved the final version of the manuscript.

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