Characterization of Rabbit Lung Lysosomes and Their Role in Surfactant Dipalmitoylphosphatidylcholine Catabolism

Evelyn D. Rider †, Kent E. Pinkerton §, and Alan H. Jobe ‡

From the †Harbor-UCLA Medical Center, Department of Pediatrics, Torrance, California, 90609, and the §University of California, School of Veterinary Medicine, Department of Anatomy, Davis, California 95616

Although alveolar surfactant is rapidly catabolized in adult rabbit lungs, the pathways have not been characterized. Pathways of surfactant secretion and recycling involve lamellar bodies and multivesicular bodies, organelles shown to be related to lysosomes by cytochemistry and autoradiography. Since lysosomes are central to intracellular catabolic events, it is possible that lysosomes are involved in intrapulmonary surfactant catabolism. Lysosomes relatively free of contaminating organelles (as determined morphologically and by marker enzymes for mitochondria, endoplasmic reticulum, peroxisomes, and plasma membranes) were obtained from post-lavage lung homogenates of 1-kg rabbits by differential centrifugation in buffered sucrose and gradient separation in percoll (density, 1.075–1.165). The role of lung lysosomes in catabolism of dipalmitoylphosphatidylcholine (DPC) was then studied in rabbits killed 4, 12, and 24 h following intratracheal injection of [3H]DPC and [14C]dihexadecyl phosphatidylcholine (DPC-ether). While equal amounts of label were in the lamellar body containing fractions at 4 h, nearly 6-fold more DPC-ether label than DPC label was recovered in the lysosomal fractions. By 24 h, there was 15-fold more DPC-ether in the lysosomes. This is the first report of successful isolation of lysosomes relatively free of other organelles from rabbit lungs. The tracer studies indicate that DPC and DPC-ether follow similar intracellular processing after alveolar uptake. The subsequent accumulation of the ether analog in the lysosomal fractions supports a role for these organelles in surfactant DPC catabolism.

Dipalmitoylphosphatidylcholine (DPC) ¹ is the major phospholipid component of lung surfactant. As recovered by alveolar lavage procedures, it represents about 60% of the surfactant lipid by weight (1). This predominance has made DPC a useful tool for in vivo investigations of surfactant phospholipid metabolic pathways, particularly alveolar uptake, recycling, and secretion. Resolution of surfactant phospholipid catabolic pathways using [3H]DPC has been more difficult because DPC is rapidly degraded in adult animal lungs such that about 75% of airspace surfactant DPC is lost from the lungs within 24 h of administration (2). Dihexadecyl phosphatidylcholine (DPC-ether), a poorly hydrolyzed diether analog of DPC resistant to degradation by phospholipases A₃ (3) and A₂ (4), was previously shown to have similar alveolar uptake and recycling kinetics as synthetic DPC and surfactant-derived DPC despite moderate but nontoxic accumulation in the lung tissue (6). The resistance to phospholipase A₂ additionally makes DPC-ether insensitive to the pathways involved with platelet-activating factor metabolism (5). This analog should be useful in defining intracellular processing of surfactant DPC within the lung.

The intracellular site of surfactant degradation, likewise, has not been defined. At the subcellular level in type II cells, surfactant DPC secretion and recycling involve lamellar bodies and multivesicular bodies, respectively. These organelles have been shown to be related to lysosomes by cytochemistry (7, 8) and autoradiography (9). Since lysosomes are central to general catabolic activities within cells, it seems reasonable that intrapulmonary intracellular degradation of surfactant phospholipids may involve the lysosomal pathway. Focused investigations of lung lysosomes, however, have been hampered by technical difficulties in isolating lysosomes free of other contaminating organelles (10). This study reports isolation of relatively pure fractions of lung lysosomes utilizing differential and density gradient centrifugation techniques. Intracellular surfactant phospholipid catabolism was then investigated using radiolabeled synthetic DPC and DPC-ether to determine whether lung lysosomes have a role in intrapulmonary surfactant DPC degradation.

EXPERIMENTAL PROCEDURES

Materials

All substrates and chemicals for the enzyme assays were obtained from Sigma. Percoll was from Pharmacia LKB Biotechnology Inc. Synthetic DPC and DPC-ether were prepared as before (6, 11) from the respective precursors, dipalmitoyl dimethyl phosphatidylethanolamine and dihexadecyl dimethyl phosphatidylethanolamine, using a methylation process in the presence of potassium carbonate and 18-Crown-6 (Aldrich Chemical Co.) in benzene. Dipalmitoyl dimethyl phosphatidylethanolamine was methylated with [mett-1H]iodide (14.4 Ci/mol, Amersham) to yield DPC (700 Ci/mmol) while dihexadecyl dimethyl phosphatidylethanolamine was methylated with [mett-1H]iodide (56.6 Ci/mol, ICN Radiochemicals, Irvine, CA) to yield DPC-ether (20 Ci/mmol). Efficiencies of radiolabel conversions were 25 and 83% for ¹H and ¹⁴C, respectively. Pure synthetic DPC or DPC-ether was separated by thin-layer chromatography using chloroform/methanol/acetic acid/water (65/25/8/4, v/v). Pure unlabeled lipids were run in a parallel lane as standards of identity. All precursor lipids and pure standards were from Serdary Research Laboratories (Port Huron, MI). Each synthetic lipid contained one radiolabeled methyl group on the choline head group. Prior to preparation of liposome suspensions for intratracheal injections the purity of the labeled lipids was confirmed by repeat thin layer chromatography.
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Isolation of Lung Lysosomes—Young male rabbits weighing 1.0 ± 0.1 kg were killed using intravenous pentobarbital (200 mg/kg) containing heparin (1 unit heparin/mg pentobarbital) followed by exsanguination. Tracheotomy was performed, and a tracheal tube was secured for alveolar lavage. The chest was then opened, the inferior vena cava clamped, and the lungs perfused via the main pulmonary artery with cold Tris-buffered sucrose solution (0.25 M sucrose containing 1 mM EDTA and pH adjusted to 7.0 with Tris base). A total of 400 ml was perfused while the lungs were simultaneously gently inflated through the tracheal tube to full distension with air at a rate of 10–15 inflations per min. This maneuver greatly enhanced the cleaning of blood from the lung. The lungs were then lavaged with five aliquots of 100 ml of 0.9% NaCl as previously described and alveolar surfactant (12). The total recovered volume from the five aliquots was, on average, 344 ± 21 ml for each animal. After the lavage, the lungs were excised and the large conducting airways stripped from the lung parenchyma.

A cell and nuclei free organelle suspension was prepared by a series of differential centrifugations as modified from the method described by Symons and Jonas for rat liver (13). The lung parenchyma was chopped to 0.5–1 mm pieces with scissors and homogenized in a Ten Broeck tissue homogenizer (10 strokes with a Dounce homogenizer “B” pestle followed by six strokes with the Ten Broeck pestle). The homogenate (1 ml 2.5 M sucrose, 0.2 M Tris, 0.05 M NaCl, pH 7.0) was centrifuged, suspended to an average protein concentration of 20 mg/ml, was centrifuged for 10 min at 750 × g. All centrifugations were carried out at 4 °C in a Sorvall SS-34 rotor (Du Pont-New England Nuclear). The nuclear pellet was discarded, the centrifugation was repeated, and the resulting supernatant was centrifuged for 10 min at 35000 × g. The supernatant was discarded, the pellet resuspended to 35 ml in Hepes-buffered sucrose (0.25 M sucrose, 20 mM Hepes, pH 7.0), and again centrifuged for 10 min at 20000 × g. The resulting organelle pellet was resuspended in the Hepes-buffered sucrose to an average protein concentration of 10–15 mg/ml. The organelle suspension was mixed with isoeptic sucrose (1 ml of 2.5 M sucrose, 200 mM Hepes, pH 7.0 added to 9 ml of percoll) at a proportion of 45/55 (v/v, organelle suspension/sucrose percoll). A shallow gradient of this mixture was generated by centrifugation at 35000 × g for 90 min. The resulting gradient was divided into 10 equal volume fractions. The density of each fraction was measured prior to dilution with 10 volumes of Hepes-buffered sucrose and centrifugation at 27000 × g to remove excess percoll. The resulting pellet from each fraction was resuspended in 1 ml of Hepes-buffered sucrose and stored at −70 °C for further analysis. Each gradient fraction assayed was subjected to the same series of biochemical and enzymatic characterization. In the radiolabeled tracer tracers and studies of alveolar lavages, post-lavage lung homogenates, organelle pellets, and supernatants from each centrifugation step were similarly extracted to determine disposition of the lung-associated labeled lipid. Lipid phosphorous was quantitated from these extracts using the method of Bartlett (15). In samples from animals receiving radiolabeled phospholipids, an aliquot was taken for measurement of radioactivity in the sedimentation fluid. Protein was measured spectrophotometrically using bovine serum albumin as standard (16).

Subcellular Marker Enzyme Analyses—Lysosomal aminosulfatase B (EC 3.1.6.1) activity was measured spectrophotometrically using para-nitro-phenylsulfate as the substrate (17). Mitochondrial succinate dehydrogenase (EC 1.3.99.1) was assayed with 2-(para-nitrophenyl)-3-(para-nitrophenyl)-5-phenyltetrazolium chloride as the acceptor (18). Plasma membrane-associated 5’-nucleotidase (EC 3.1.3.5) activity was determined by the release of inorganic phosphate from adenosine monophosphate (19). Endoplasmic reticulum-associated glucose 6-phosphatase (EC 3.1.3.7) activity was assayed using 2-(para-nitrophenyl)-4-hydroxy-3-methoxynaphthalene-6-phosphate as the substrate (20). For both the 5’-nucleotidase and glucose-6-phosphatase assays, sodium-potassium tartrate (12 mM) was added in the assay medium to inhibit nonspecific acid phosphatases (21). Inorganic phosphate release was measured by the method of Chen et al. (EC 3.1.1.6) was used as the peroxisome marker and assayed as previously described (23, 24). All assays were performed in substrate excess.

Electron Microscopy—Separately pooled aliquots of fractions 3 to 5 and of fractions 8 to 9 from one gradient preparation were pelleted by centrifugation, fixed in 2% glutaraldehyde/2% tannic acid in 0.085 M sodium cacodylate buffer containing 3 mM EDTA and post-fixed in 1% osmium tetroxide and 2% uranyl acetate according to Young et al. (25). The specimens were then dehydrated in a graded series of acetone and propylene oxide solutions at 4 °C prior to embedding in araldite. Thin sections (70–90 nm) were then stained in 4% aqueous uranyl acetate and lead citrate.

Recovery of Radiolabeled Tracer Lipids from Gradient Fractions

Preparation of Intratracheal Injection Solutions—Liposome suspensions containing [3H]DPC and [14C]DPC-ether were prepared as previously described and associated with trace amounts of unlabeled natural surfactant in 0.45% NaCl (6). The unlabeled surfactant was purified from pooled lung washes of adult rabbit lungs by a series of centrifugations through sucrose (26). The liposomes prepared in this manner were shown to behave in a fashion similar to radiolabeled surfactant (27). The final liposome suspension contained 1.5 μCi of [3H]DPC, 0.15 μCi of [14C]DPC-ether, and 0.3 μmol of total phospholipid in each 3-ml injection volume.

Animal Procedures—Male New Zealand White rabbits each weighing 1.15 ± 0.05 kg were anesthetized with diethyl ether and injected intratracheally with the liposome suspension containing the radiolabeled lipid tracers using an 8-Fr flexible bronchoscope (2). The animals rapidly recovered, and animals were killed in groups of four to six with intravenous pentobarbital at each of four time points. The lungs were processed, and resulting gradients were characterized as described above.

Data Analysis—All enzyme activity data were expressed as specific enzyme activity per mg of protein in the assay sample. For the initial gradient characterization, values were given as group means ± S.E. for measurements made in fractions from three separate gradient preparations. Radiolabel recovery data, given as means ± S.E., were expressed as radioactivity per μmol of lipid phosphorous. Differences in label recovery among the groups were evaluated by analysis of variance followed by the Student Newman Keuls test. Significance was defined as p < 0.05.

RESULTS

Characterization of the Gradient Fractions—The density profile and the protein and lipid phosphorous contents of the fractions collected from three separate gradients are shown in Fig. 1. The percoll gradient generated a shallow gradient with a density range of 1.075 ± 0.005 to 1.165 ± 0.002 g/ml. The distribution profiles of protein and lipid phosphorous along the gradient were nearly identical. Each gradient preparation had two visibly distinct bands, a thick, creamy proteinaceous phase and a less prominent, thin band near the bottom of the tube corresponding to the lysosome-rich fractions.

Distribution of Marker Enzyme Activities—The specific activities of selected marker enzymes for various subcellular organelles along the gradient fractions are presented in Fig. 2. Specific activity of the lysosomal marker enzyme, ariyul-
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**Morphologic Characterization of Selected Gradient Fractions**—Samples of fractions 3 to 5, where lysosomal enzyme activity was concentrated, and fractions 8 to 9, where lamellar bodies would be expected to separate by density gradient centrifugation, were analyzed by electron microscopy (9, 12). Representative low power electron micrographs of these fractions at the same magnification are shown in Fig. 3. The appearance of the processed fractions near the top of the gradient was heterogeneous, with intact lamellar bodies and mitochondria identifiable (Fig. 3A). Consistent with the enzyme activity distribution data, structures appearing morphologically like lysosomes were also found. In addition, membrane-bound multivesicular bodies were noted. In contrast, the lysosome-enriched fractions near the bottom of the gradient (Fig. 3B) appeared more homogeneous, containing dense membrane-bound vacuolar structures consistent with lysosomes. No organelles resembling mitochondria or lamellar bodies were seen in preparations from these lower fractions.

**Recoveries of Radiolabeled DPC and DPC-ether**—Trace amounts of radiolabeled DPC and DPC-ether associated as liposomes with tracer natural surfactant were given to other animals to investigate the possible role of lung lysosomes in the catabolism of surfactant DPC. Each 3-ml injection contained 0.3 μmol of lipid phosphorous, an amount less than 10% of the endogenous alveolar saturated phosphatidylcholine pool in this size rabbit (2, 6). Results of biochemical and enzymatic analyses of the fractions from these gradients were the same as those described above. Table II is a summary of the label recoveries in the total alveolar washes, macrophages, and post-lavage lung homogenates. The recoveries in lung homogenates were somewhat lower than those observed pre-

**TABLE I**

Enzyme specific activities

Specific activities are given as means ± S.E. for three separate gradient preparations. Units of enzyme activity are defined as OD/mg protein for lysosomal arylsulfatase B (●) and mitochondrial succinate dehydrogenase (○). Catalase (△) was measured as defined by Badhui (23) in units/mg protein. B, activities of plasma membrane-associated 5'-nucleotidase (○) and endoplasmic reticulum glucose-6-phosphatase (●) are given as micromoles of inorganic phosphorous released per mg of protein in the presence of sodium tartrate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Lung homogenate</th>
<th>Lysosome (fraction 4)</th>
<th>Enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arylsulfatase B</td>
<td>2.4 ± 0.4</td>
<td>122.3 ± 8.9</td>
<td>65</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>3.1 ± 0.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.43 ± 0.04</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>1.3 ± 0.1</td>
<td>0.04 ± 0.02</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>9.8 ± 2.8</td>
<td>0.16 ± 0.16</td>
<td>0.04 ± 0.04</td>
</tr>
</tbody>
</table>

**Fig. 2.** Distribution of marker enzyme activities. The specific activities are given as means ± S.E. of fractions from three separate gradients. A, units of enzyme activity are defined as OD/mg protein for lysosomal arylsulfatase B (●) and mitochondrial succinate dehydrogenase (○). Catalase (△) was measured as defined by Baudhui (23) in units/mg protein. B, activities of plasma membrane-associated 5'-nucleotidase (○) and endoplasmic reticulum glucose-6-phosphatase (●) are given as micromoles of inorganic phosphorous released per mg of protein in the presence of sodium tartrate.

**Fig. 3.** Electron micrographs of the lamellar body containing upper fractions and lysosome-enriched lower fractions. Scale bar = 1 μm. A, the lamellar body containing fractions near the top of the gradient appeared heterogeneous with intact lamellar bodies, mitochondria (open arrows), simple vesicular structures, and membrane-bound multivesicular bodies identifiable. Structures appearing morphologically like lysosomes were also noted (solid arrows). B, the lysosome-enriched fractions in the more dense, lower portion of the gradient appeared homogeneous, containing primarily dense membrane-bound vacuolar structures consistent with lysosomes. No mitochondria or lamellar bodies were identified in these fractions.

fatase B, was highest in fractions 2 to 6. These fractions were relatively free of other subcellular organelles, including mitochondria, peroxisomes, and endoplasmic reticulum, as indicated by the absence of significant activity of marker enzymes for these organelles. Activity of the marker enzyme for plasma membrane, 5'-nucleotidase, was detected throughout the gradient but was minimal in the fractions containing the highest lysosomal enzyme activity. Lysosomes in fraction 4 were purified approximately 65-fold from the lung homogenate, as indicated by the specific activity of arylsulfatase B (Table I).

**Fig. 3.** Electron micrographs of the lamellar body containing upper fractions and lysosome-enriched lower fractions. Scale bar = 1 μm. A, the lamellar body containing fractions near the top of the gradient appeared heterogeneous with intact lamellar bodies, mitochondria (open arrows), simple vesicular structures, and membrane-bound multivesicular bodies identifiable. Structures appearing morphologically like lysosomes were also noted (solid arrows). B, the lysosome-enriched fractions in the more dense, lower portion of the gradient appeared homogeneous, containing primarily dense membrane-bound vacuolar structures consistent with lysosomes. No mitochondria or lamellar bodies were identified in these fractions.
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TABLE II
Radiolabel recovery in lipid extracts

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Gradients analyzed</th>
<th>[3H]DPC</th>
<th>[14C]DPC-ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>3</td>
<td>23.1 ± 3.7</td>
<td>35.1 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Alveolar wash</td>
<td>17.1 ± 7.2</td>
<td>27.4 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>Macrophage</td>
<td>34.5 ± 4.0</td>
<td>40.4 ± 5.2</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>7.9 ± 0.9</td>
<td>16.9 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Alveolar wash</td>
<td>3.7 ± 1.7</td>
<td>9.1 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Macrophage</td>
<td>27.7 ± 1.5</td>
<td>43.4 ± 2.4</td>
</tr>
<tr>
<td>24</td>
<td>4</td>
<td>6.7 ± 1.7</td>
<td>20.7 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Alveolar wash</td>
<td>1.5 ± 1.3</td>
<td>13.7 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>Macrophage</td>
<td>19.1 ± 3.3</td>
<td>36.3 ± 6.1</td>
</tr>
</tbody>
</table>

* Alveolar wash values include macrophage contribution.

* Macrophage values given as the average of two observations at each time point.

TABLE III
Disposition of lung-associated labeled lipids

Data are given as means ± S.E., expressed as a percentage of the label recovered in the lung homogenate.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>[3H]DPC</th>
<th>[14C]DPC-ether</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Lung homogenate</td>
<td>33.7 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>Celluar debris</td>
<td>41.7 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>Discarded supernatants</td>
<td>14.8 ± 2.1</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung homogenate</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Celluar debris</td>
<td>54.1 ± 8.6</td>
</tr>
<tr>
<td></td>
<td>Discarded supernatants</td>
<td>30.0 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>Organelle pellet</td>
<td>14.0 ± 1.5</td>
</tr>
<tr>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lung homogenate</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Celluar debris</td>
<td>51.8 ± 5.5</td>
</tr>
<tr>
<td></td>
<td>Discarded supernatants</td>
<td>34.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>Organelle pellet</td>
<td>14.6 ± 1.3</td>
</tr>
</tbody>
</table>

* Cellular debris represent the discarded pellets from the two 750 × g centrifugations performed prior to obtaining the organelle pellet which was loaded on the percoll gradient.

Previously (6); however, these lung homogenates were obtained by a distinctly different process. The apparent accumulation of ether-derived label in the total alveolar compartment represented retention within the macrophages due to lack of degradation. When the label recoveries in the alveolar washes were corrected for macrophage contribution, no significant differences between the ester derived and ether derived labels were seen.

Table III is a summary of the disposition of the lung-associated labeled lipids. To achieve our initial intent of recovering relatively pure lysosome preparations with minimal contamination from other organelles, yield was compromised, as evident by the greater than 70-85% losses during processing. The total amount of label recovered in the final organelle pellets used for gradient separation were similar at approximately 15% of the amount recovered in the corresponding lung homogenates. In pilot experiments, we found that this percentage could be increased by nearly 3-fold when the cellular debris pellet obtained from the initial 750 × g centrifugation was rehomogenized two more times (data not shown). This indicated that cell lysis and release of intact intracellular organelles were incomplete following the first homogenization. The residual cellular debris from the rehomogenizations became progressively more gelatinous and difficult to separate from the solubilized organelles. This process lengthened the total processing time from animal sacrifice to organelle separation in order to minimize ongoing degradation in isolated organelles. However, consistency of the isolation procedure was evident in the similar percentage recoveries in organelle pellets over the 24-h study period.

The patterns of label recoveries in lipid extracts of the gradient fractions from each of the various time points studied are summarized in Fig. 4. At 0 time, essentially no label was found in any of the gradient fractions (results not shown). While similar amounts of label were found in the upper fractions containing the lamellar bodies and other cellular organelles at 4 h (Fig. 4A), a nearly 6-fold accumulation of the ether label was noted in the lysosome-enriched fractions. Progressive accumulation of the [14C]DPC-ether label occurred in the lysosomal fractions with time as presented in Fig. 4, B and C, such that by 24 h, there was a 15-fold accumulation relative to the [3H]DPC. The ratios of the [14C] DPC-ether label to the [3H]DPC label in each gradient fraction at the different time points studied are shown in Fig. 4D. Despite the striking retention of the ether analog within the lysosomal fractions, the ratio of the two lipids remained at unity near the top of the gradient where lamellar bodies and other organelles were noted on morphologic evaluation.

The partitioning of label recovery into water-soluble metabolites along the gradient is shown in Fig. 5. Label in the aqueous phase as a percentage of the total label (lipid phase + aqueous phase) contained in each fraction are presented for the lysosome-enriched and the more heterogeneous upper areas of the gradient. A greater percentage of total label was recovered as water-soluble metabolites in the denser fractions, near the bottom of the gradient as compared to the lighter fractions containing other organelles toward the top of the gradient. The pattern of label partitioning did not significantly change over the 24-h study period for either the 3H or 14C-derived label. Consistent with relatively greater resistance to phospholipase and nonenzymatic acid hydrolysis of the ether analog, at 12 and 24 h, significantly less of the 14C label was recovered in the aqueous phase of the lysosome-enriched fractions as compared to the 3H label derived from DPC. In contrast, less than 3% of the total label in the fractions near the top of the gradient were recovered in water-soluble metabolites for both 3H and 14C. While the specific identities of the labeled aqueous metabolites were not determined in this study, the presence of label in the choline headgroup of the phospholipids would make these choline-containing water-soluble metabolites such as glycerophosphocholine, phosphocholine, and free choline.

DISCUSSION

There is considerable knowledge of surfactant compositional and functional complexity. However, an understanding of the basic mechanisms involved in surfactant catabolism remains limited. In the era of eoxogenous surfactant replacement therapy, understanding of the catabolic phenomena is important to the optimal application of this therapeutic regimen. The cellular heterogeneity of the lung and the very rapid degradation of DPC have hampered attempts to resolve the catabolic process while investigation of intracellular events were made difficult by technical limitations in separation of organelles, such as the lysosomes, which may have a role in catabolism. The present study was designed primarily to
develop a technique of isolating lung lysosomes relatively free of other organelles. Using radiolabeled phospholipids associated with trace amounts of natural surfactant, we also investigated the possible role of lysosomes in intracellular surfactant phospholipid processing and degradation. Rabbits were used because of the knowledge about surfactant metabolism available in the literature for this species. Young adult rabbits weighing about 1 kg were used because this size rabbit has surfactant metabolism similar to larger adult animals and the lungs of these young animals are not obviously infected, a problem often encountered in older rabbits.

The fractionation scheme utilized in the present study generated the very shallow density gradient required in order to adequately separate lysosomes from other subcellular organelles of very similar densities. An inherent problem with fractionation schemes for organelle separation is relative purity. Results of the marker enzyme analyses indicated that the fractions with the peak activity for the lysosomal marker enzyme, arylsulfatase B, were relatively free of other contaminating organelles. The mid-peak density of 1.12 was consistent with what had been reported for liver lysosomes (13) and fibroblast lysosomes (28). The integrity of the lysosome isolations were further supported by the findings on electron microscopic examination of selected fractions. No similar isolation process in lung tissue had been attempted to our knowledge. Previous attempts using other separation techniques to generate shallow enough gradients for adequate separation of lysosomes have not been attempted in the lung (13, 28–31). Surrogate gradients yielded lysosome-enriched fractions with significant contamination by other organelles, including mitochondria, endoplasmic reticulum, plasma membranes, and peroxisomes (10). The density of the upper gradient fractions (1.075–1.090) where lamellar bodies were found on morphologic examination was somewhat higher than what had been observed when isolation was carried out in buffered sucrose gradients (10).

DPC-ether was used as a probe in this system to help localize the intracellular processing and degradation of DPC. Radiolabeled DPC served as a marker for movement of the naturally occurring surfactant phospholipid through the subcellular compartments. Previous observations (6, 34) indicated that these two lipids were virtually indistinguishable in the alveolar space in that both had similar alveolar clearance kinetics, turnover times, and reutilization efficiencies despite accumulation of the ether analog within the lung tissue. This accumulation was nontoxic to the animal and presumably occurred due to inadequate degradation, primarily as a result of resistance to phospholipases A\(_1\) and A\(_2\) imparted by the ether bonds in place of the ester bonds of DPC. It was additionally shown that, similar to DPC, the lung was the primary site of degradation for the DPC-ether (6). These characteristics made this analog an extremely useful tool for the possible localization of intracellular surfactant DPC catabolic processes.

The tracer studies indicated that both DPC and DPC-ether followed similar initial intracellular routing. However, as early as 4 h following intratracheal injection, moderate accumulation of the ether label relative to the DPC label was found in the presumably degradative lysosome-enriched fractions. The relative accumulation was progressive over time, consistent with ineffective degradation of the ether analog with ongoing breakdown of the DPC. The lack of impact of this relative accumulation on the ratio of DPC to DPC-ether label in the upper fractions containing the lamellar bodies indicated that phospholipid within the catabolic lysosomal compartment was unable to contribute directly to the recycling process. These results were consistent with earlier findings in whole animal studies whereby accumulation of the ether analog within the lung tissue did not alter recoveries of the material from the alveolar space when macrophage contribution was accounted for (6).

Consistent with the lysosome-enriched fractions containing the degradative compartment, the recovery of labeled water-soluble choline metabolites as a fraction of total label (in lipid and aqueous phases of the extracts) was highest in the lower gradient fractions which contained the lysosomes. We did not further characterize the water-soluble metabolites in the present study. Work by other investigators using rat type II cells in primary culture and from isolated perfused whole lung models have shown that the water-soluble choline metabolites include glycerophosphocholine, phosphorylcholine, and free choline (32, 33). Contribution from newly biosynthesized cytidine 5′-diphosphate-choline, another water-soluble choline intermediate was not likely to be significant in the lower
Lung Lysosomes and Surfactant Catabolism

Fig. 5. Recoveries of $^3$H]DPC (A) and $^{14}$C]DPC-ether (B)-derived water-soluble choline metabolites in the indicated gradient fractions over time. Values, given in means ± S.E., are expressed as label in the aqueous phase as a percentage of the total label lipid phase of aqueous phase contained in each gradient fraction. A significantly greater percentage of total label was recovered in the aqueous phase in the denser lysosome-enriched fractions (numbers 3–5) as compared to the lighter fractions (numbers 8–10) near the top of the gradient containing a mixture of organelles, indicating that lysosomal activities found in the upper fractions containing the lamellar bodies despite accumulation of surfactant phospholipids are likely to be different from the "heavier" organelles. Although these lamellar body-containing fractions do not represent a traditional lamellar body preparation, this further supported the earlier concept that once within the catabolic compartment, phospholipids re-entered the secretory pathway primarily through reincorporation of degradation products into newly synthesized DPC via the endoplasmic reticulum and Golgi apparatus. Moreover, the incorporation of labeled degradation products (choline and lysophosphatidylcholine) into newly synthesized DPC was relatively low since there was no relative increase in the amount of DPC label in the upper fractions containing the greatest concentration of endoplasmic reticulum as indicated by marker enzyme analyses. The very small recovery of aqueous degradative metabolites in the upper fractions enriched with endoplasmic reticulum and mitochondria suggest that relatively little degradative processing of DPC to yield these products occurred within these organelles. Alternatively, it is possible that the water-soluble metabolites may be cleared rapidly from these compartments, a possibility not addressed in this study.

Fig. 6 is a schematic diagram of possible intracellular trafficking of surfactant phospholipids following uptake from the alveolar space which would be consistent with previous observations in whole animal studies and with the results of these experiments. This diagram is a modification of a previously proposed model which was adapted from the concepts of several investigators (7, 8, 34–36). Alveolar surfactant phospholipids are internalized via endocytosis and form multivesicular endosomes. A fixed fraction of these multivesicular bodies is directed to enter either the recycling pathway leading to resorption via the lamellar bodies or the catabolic pathway involving the lysosomes, which eventually lead to degradation.

There was no direct communication of the catabolic compartment with the recycling pathways since there were no differences in the amount of ether-associated label in the upper fractions containing lamellar bodies despite accumulation of surfactant phospholipids. Alveolar surfactant phospholipids are internalized via endocytosis and form multivesicular endosomes. A fixed fraction of these multivesicular bodies is directed to enter either the recycling pathway leading to resorption via the lamellar bodies or the catabolic pathway involving the lysosomes, which eventually lead to degradation.

At later time points, label deposition within lamellar bodies intracellular organelles occurred in a time-dependent fashion. The localization of the labeled surfactant within different intracellular organelles occurred in a time-dependent fashion. At later time points, label deposition within lamellar bodies and acid phosphatase-containing organelles, such as dense multivesicular bodies and homogenous vesicles consistent with lysosomes, was observed. These observations indicate that lysosomal organelles have a role in intracellular processing of surfactant phospholipids to yield metabolites that are distinct from those found in the secretory pathway.

In summary, these results indicate that lysosomes play a role in the intracellular processing of surfactant phospholipids, and their role is consistent with the findings of the present study.
of the ether label in the lysosomal fractions and the recovery of water-soluble metabolites in these fractions indicate that these catabolic organelles have a role in intrapulmonary intracellular surfactant DPC degradation. The primary objective of this study to develop a method of isolating pure isolates of lung lysosomes resulted in significant compromise of yield. The magnitude of the role of lysosomes in surfactant DPC degradation needs to be studied further in preparations where losses to processing can be minimized. The label studies further support the distinct compartmentalization and separation of the recycling and catabolic processes for surfactant DPC within the lung.

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REFERENCES


Fig. 6. Possible pathways of intracellular surfactant phosphatidylcholine processing following alveolar uptake. See text for details. lyso-PC, lysophosphatidylcholine.